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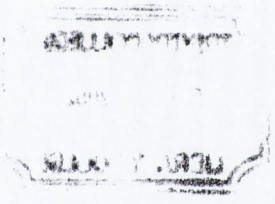
The Exploitation of Soil Organic Phosphate by Grassland Plants

Nyree-Dawn Carroll



Thesis submitted in fulfilment for the degree of Ph.D.

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SUMMARY

This study was concerned with the availability of soil organic phosphate (Po) to plants. To address this issue semi-natural Irish grassland soils and plants were used. Greenhouse experiments were carried out in which soil was amended with inositol hexaphosphate (IHP), a prominent soil Po compound. To maintain a natural growth environment for the plant, the indigenous microbial population of the soil was retained.

Several factors, vesicular arbuscular mycorrhizae, soil bacterial and phosphatase enzymes, which can influence P nutrition of plants were examined. In addition the influence of soil N levels on the Po use of plants was determined. Nuclear magnetic resonance was used to examine the range of Po compounds in this soil. Traditional wet chemical analysis was used to measure soil phosphate levels and plant growth was followed by biomass and P accumulation data. Among the methods employed in the course of this work were quantification of soil bacteria and root infection by VAM fungi. Assays for determination of the activity of a range of soil phosphatase enzymes were performed.

IHP was an available source of P for plants that could be accessed by VAM hyphae for transport to plants. The Po concentration in the soil did not affect the extent of infection of roots by VAM fungi and these fungi were beneficial to plant growth at low soil P concentrations. Streptomycin sensitive bacteria in soil were not involved in the hydrolysis of Po for plant use. Root acidic monoesterase was indicated as being the most important phosphatase enzyme for IHP breakdown to orthophosphate. Addition of N to the soil increased the potential for plant growth and the need for Po breakdown by the root acidic monoesterase. A group of Po compounds (monoester P, diester P, inorganic orthophosphate, pyrophosphate and teichoic acid) was detected in soil extracts by the technique of NMR.

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Abbreviations

ANOVA	analysis of variance
C	carbon
DW	dry weight
F	variance ratio
H_2SO_4	sulphuric acid
HCL	hydrochloric acid
KOH	potassium hydroxide
M	molar
N	nitrogen
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
ns	not significant
P	phosphorus
p	statistical significance
Pi	inorganic phosphate
Po	organic phosphate
%RLC	percentage root length colonisation
PP1/PP2	Phoenix Park site 1/2
SE	standard Error
Tp	total phosphate
VAM	vesicular arbuscular mycorrhizae

CHAPTER 1: GENERAL INTRODUCTION

1.1 Background to the study

Soil phosphate includes the well-researched inorganic phosphate compounds such as iron and aluminium phosphates, and organic phosphate (Po) compounds, about which less is known. Despite the prominence of organic phosphate in soils, the majority of work on soil phosphate and plant nutrition has been carried out on inorganic phosphate. This is because of the large-scale use of inorganic fertilisers to increase crop productivity. Considerable information is available on the nature and behaviour of inorganic P in soil. It was assumed until recently that any contribution from organic phosphate to phosphate for plant growth was small in temperate regions, because Po compounds had long turnover times and were less labile. However, work by Power, Jeffrey and Tunney (1995) suggested that in highly productive grasslands the Po pool is considerable in size, complex in nature and totally underestimated by soil testing for available soil phosphate.

The work presented in this thesis was undertaken to examine the organic phosphate compounds present in an Irish grassland soil and to determine their contribution to the active labile P pool, which supports plant growth. Irish grassland soil and plants were chosen to investigate this question, because grasslands are easily accessible and are of a scale that are readily adapted to experimental approaches of the plant soil system. Furthermore studies of grasslands in Leinster (Byrne, 1996) and in the Burren have provided valuable background data on the chemistry and floristics of approximately 30 biodiverse grassland sites. Soil and seeds were obtained from two semi-natural grassland sites for a series of experimental approaches. These sites are complementary to the productive agricultural Irish grasslands characterised by Power, Jeffrey and Tunney (1995), where residual P from many years of agricultural fertiliser application had led to high total P concentrations.

The Leinster grassland study was a search for biodiversity in plagioclimax grasslands and the environmental characteristics supporting a diverse polydominant sward. Soil phosphate availability was shown to be a powerful variable controlling production and species composition from all the characteristics investigated. Responses to phosphate addition were obtained in grasslands in Upper Teesdale, by Jeffrey and Pigott (1973). However in the Burren the climax *Sesleria*-dominated swards were not sensitive to P addition. This suggested that the affects of additional P supply on grassland productivity varied from being critical to being relatively unimportant but not in a readily predicted way. Further work was necessary to examine the role of additional soil phosphate in plant nutrition under various conditions and to compile a set of rules governing the soil P pool.

This research is at the interface between plant biology, ecology and agriculture but the main focus is fundamental environmental biology and research into processes of nutrient cycling. The work in this thesis will provide information on the role of organic phosphate in supplying P to the plant available P pool in semi-natural and natural systems. However the grassland model used here should be readily adaptable as a working hypothesis of other systems, such as agricultural and forestry systems. Work was commenced on fertile agricultural grasslands by Power, Jeffrey and Tunney (1995) but the information gained in this work is relevant to lower intensity agriculture with lower nutrient inputs. Agricultural and horticultural use of landspread slurries and composted materials will certainly increase. The rate of use is usually calculated on crude total nutrient content. More sophisticated utilisation could depend on knowledge of P fractions in such materials and soils and their behaviour.

1.2 Experimental approach

The work in this thesis was based on greenhouse experiments using natural soil collected from two grassland sites, un-amended with any other artificial growth media such as vermiculite or sand. This was in contrast to many of the current studies on soil phosphate, which employ agricultural soil, artificial media such as sand or sand –soil mixtures or sterile agar media. The plants used for this project were grown from seed collected from plants growing on the sites naturally. In experiments dealing with the relationship between vesicular arbuscular mycorrhiza (VAM), organic phosphate and plant growth, there was no sterilisation of soil and re-inoculation with designated species. VAM indigenous to the site and already present in the soil were used.

In order to determine the availability of organic phosphate to plants and its affect on plant growth, inositol hexaphosphate was added to the soil in varying amounts. This particular Po compound was chosen, as inositol phosphates are the most prominent group of Po compounds present in soil (Stevenson and Cole, 1999; confirmed in Chapter 7 section 7.3.1). To determine the phosphate supply phosphate bioavailability estimations have been established, many based on chemical extractions of soil. These may have some bearing on the theory of phosphate supply but they do not mimic the processes involved in the P cycle. In this work therefore the affect of organic phosphate on P growth has been determined directly by examining plant growth.

1.3 Site descriptions

The soils used in the course of the experiments described in this thesis were collected from two sites, both of which are in the Phoenix Park, which is located to the west of Dublin city centre. These sites are referred to as the PP1 and PP2 sites. Both are grassland sites but are of contrasting P status - the PP1 site has a lower total P concentration than the PP2 site.

These sites were also included in a study by McNamee (2001) who examined the phosphorus fractionation in Irish grassland soils. Byrne (1996) studied the ecology, classification and management of semi-natural grasslands with particular reference to eastern Ireland. Included in this study were the grasslands of the Phoenix Park, which were surveyed to classify the grasslands within the park in terms of their floristic composition in conjunction with edaphic and environmental variables. This study by Byrne (1996) presents valuable background information concerning the PP1 and PP2 sites.

The PP1 site is located in the south site of the Park near the Chapelizod Gate, on top of a steep south-facing slope, on flat terrain (Plate 1.1). The soil of this semi-natural grassland site is a well drained fine textured clay loam soil. There are no records of fertiliser addition to this site or mowing by scythe or otherwise (Byrne, 1996). It is one of the areas in the park that has been allocated as an unmanaged site and is accessible for grazing by deer. Byrne (1996) classified the PP1 grassland vegetation as belonging to the *Centaureo-Cynosuretum* sub-association *galietosum,* corresponding to MG5b sub community grassland as defined by Rodwell (1992). Table 1.1 presented a list of grass and herb species found on the PP1 site by Byrne (1996).

Table 1.1: Grass and herb species found growing on the PP1 site by Byrne (1996).

Grass Species	**Dicotyledonous Herbs**
Agrostis capillaris	*Cerastium fontanum*
Anthoxanthum odoratum	*Galium verum*
Arrhenatherum elatius	*Lotus corniculatus*
Briza media	*Plantago lanceolata*
Cynosurus cristatus	*Potentilla reptans*
Dactylis glomerata	*Sanguisorba minor*
Festuca pratensis	*Trifolium pratense*
Festuca rubra	*Trifolium repens*
Holcus lanatus	
Lolium perenne	
Poa pratensis	
Trisetum flavescens	

The PP2 site was part of the Ashtown Farm Estate located in the Phoenix Park (Plate 1.2). It was located in one of a series of meadows which was fertilised by dung for at least 200 years (McNamee, 2001). This grassland site is regularly cut during the year. The soil of this semi-natural grassland site is imperfectly drained. The grasslands of the Ashtown Estate were classified by Byrne (1996), as a *Lolio-Cynosuretum* grassland otherwise known as the MG6 grassland as defined by Rodwell (1992). Among the species found in this PP2 site by Byrne (1996) are *Agrostis capillaris, Holcus lanatus* and *Dactylis glomerata..*

The basic soil characteristics for the PP1 and PP2 sites were presented in Table 1.2. Methods for the determination of the values presented in Table 1.2 were given in Appendix 1.

Plate 1. 1: The PP1 site in the Phoenix Park, Dublin

Plate 1. 2: The PP2 site in the Phoenix Park, Dublin

Table 1.2: basic soil characteristics for the soils from the PP1 and PP2 sites.

Soil Characteristic		PP1 site	PP2 site
Total P	(mg P/kg DW soil)	855	1586
	(mg P/l soil)	584.79	1085.2
Inorganic P	(mg P/kg DW soil)	328	704
	(mg P/l soil)	224.55	481.54
Organic P	(mg P/kg DW soil)	527	882
	(mg P/l soil)	360.30	603.67
% Organic P		61	56
pH		6.7	7.2
% organic matter		13.1	14.2
Bulk density (g/ml)		0.68	0.69
% sand		85.9	83.9
% silt		9.7	9.7
% clay		4.4	6.4

1.4 Species descriptions

Four species that were common and prominent in both the PP1 and PP2 sites were chosen for use during the course of this thesis. These species are *Agrostis stolonifera, Dactylis glomerata, Holcus lanatus* and *Plantago lanceolata.* Nomenclature of plants followed Webb, Parnell and Dooge (1996).

Agrostis stolonifera

Agrostis stolonifera is a short (200 mm), fast growing, stoloniferous, patch forming perennial grass. It is a shallow rooting grass and is often associated with vesicular arbuscular mycorrhiza. It is favoured by soil pH values in the range 5.5 to 8.0. *Agrostis stolonifera* is winter green and flowers from July to August, setting seed from August to October.

It is a competitive ruderal, and this species is favoured in relatively productive and moderately disturbed conditions. It has been recorded in fertile soils in a wide range of habitats but is particularly abundant on road verges, paths, meadows and pastures. *Agrostis stolonifera* forms extensive clonal patches by means of long stolons.

Detached shoots readily root to form a new plant and this mode of regeneration appears to be important in disturbed habitats. This diversity of regenerative strategies aids the success of *Agrostis stolonifera* as a colonist of new sites and a survivor in disturbed ones. (Grime *et al.*, 1996)

Holcus lanatus

Holcus lanatus is a tufted softly hairy perennial, growing to an average height of <350 mm, the roots of which are associated with vesicular arbuscular mycorrhiza. It is winter green but shows little winter growth. It flowers mainly in June and July with seed shed in July and August. Seed production from the plants second year onwards is often prolific. Seedling establishment is the main source of population expansion.

Holcus lanatus is recorded at least as seedlings in every type of habitat. It is however most abundant in meadows and pastures but is also prominent on waste ground, grass verges, and paths. It is most frequent on soils of intermediate pH status (5.0 – 6.0) but is relatively common over the pH range 4.5 – 8.0. This species has an extremely wide-ranging distribution but is concentrated on vegetation associated with moderately disturbed and relatively fertile conditions.

Holcus lanatus shows lax tillering and so is not tolerant of close grazing. It achieves maximum vigour in moist habitats and has restricted tolerance of shaded habitats. It is also sensitive to winter damage and above ground biomass is reduced after severe winters. Despite this *Holcus lanatus* is an exceptionally versatile grass rivalled only by *Agrostis stolonifera* in its capacity to exploit widely different soil conditions. (Grime *et al.*, 1996)

Dactylis glomerata

Dactylis glomerata is a potentially robust, winter green, densely tufted perennial grass reaching < 400 mm. The roots are associated with vesicular arbuscular mycorrhiza. It flowers from May to July and sets seed from July to October.

Dactylis glomerata occurs in most dryland habitats but most frequently as a constituent of meadows, pastures, rough grassland and road verges. It is found on all but the most acidic soils but most commonly with a pH range of 5.0 – 8.0. Distribution is very wide ranging, but is centred on relatively productive, moderately disturbed vegetation. It exhibits some tolerance to drought.

Dactylis glomerata regenerates almost exclusively by seed in the spring, although it has a limited capacity for lateral tussocking. Seeds may persist near the soil surface for 2 to 3 years but *Dactylis glomerata* does not form a persistent seed bank. This feature appears to reduce the resilience of the species in heavily grazed and trampled pastures. (Grime *et al.*, 1996)

Plantago lanceolata

Plantago lanceolata is a frost tolerant, winter green, rosette forming perennial herb capable of flowering in its first year. Its short ovate leaves, usually <150mm, form a prostrate rosette in exposed sites and the root system is deep and with vesicular arbuscular mycorrhiza. It flowers from April until August but particularly in June and July. Seed is ripe 2 to 3 weeks after fertilisation but some seeds may over-winter in the seed head.

Plantago lanceolata is abundant in meadows, base-rich pastures, rock outcrops and waysides. It is particularly common on soils in the pH range 5.0 – 8.0 and is rarely recorded in soils with pH <4.5. It has a wide range of distributions but is most frequent in vegetation associated with reduced soil fertility and moderate disturbance. *Plantago lanceolata* is described as one of the world's 12 most successful colonising species and as one of the world's most successful weeds.

The production of buds from the stem affords a method of vegetative regeneration in some established colonies. *Plantago lanceolata* is also capable of regeneration from root fragments but it is uncertain to what extent this is important in the field. This species has a capacity for vegetative spread and flexibility in regeneration by seed. Some seed is shed in the summer and some may over winter in the parent plant. It also forms a buried seed bank. (Grime *et al.*, 1996)

1.5 Origin of soil phosphate

Soil phosphate (Tp) comprises inorganic phosphate (Pi) and organic phosphate (Po) and is quantitatively expressed as phosphate phosphorus. Total P content of soil is related to the content of parent materials from which the soils are derived and the parent materials vary in their P content. A significant relationship between the organic P content and the total P content of soils has been found. Approximately 58.5% of the variation in the total organic P content of surface soils world-wide can be accounted for by total P content (Harrison, 1987). The range for phosphate in soils is between 10 and 3000 mg P / kg soil. The proportion of total soil P present in organic forms varies considerably and ranges from 30 to 65% (Harrison, 1987). Recent work by Daly, Jeffrey and Tunney (2001), in which ninety grassland soils of variable type and P status collected throughout Ireland were surveyed, indicated total P concentrations ranging from 193 to 1553 mg P / l soil and organic P concentrations ranging from 31 to 982 mg P / soil. Organic P in the soil decreases with depth (Stevenson and Cole, 1999).

Factors that affect the organic phosphorus content of soils include: parent material, climate, drainage, soil pH and cultivation (Stevenson and Cole, 1999). Soils derived from granite tend to contain lower amounts of organic phosphorus than soils derived from basalt or basic igneous materials (Stevenson and Cole, 1999). Poorly drained soils contain less organic phosphorus than well drained soils and organic phosphorus content generally increases as soil pH decreases (Dalal, 1977). When soils are first placed under cultivation the content of organic P usually declines because of greater rates of Po mineralisation (Dalal, 1977). Upward transport of P by plants and retention in the surface soil as a result of plant litter decomposition and low mobility of phosphates, may alter the vertical distribution of P in the soil profile (Stevenson and Cole, 1999).

The native P in soils was derived from the apatite present in soil forming parent materials. During centuries of weathering and soil development the P in the apatite is liberated. This liberated P is absorbed by plants and transferred to all other biota in an ecosystem and is recycled. It is incorporated into the organic matter of soils and sediments and re-deposited as sparingly soluble mineral forms such as Ca-, Fe- and Al-phosphates and the occluded P of Fe- and Al- oxides (Stevenson and Cole, 1999).

1.6 Types of soil phosphate

Both inorganic and organic phosphate are present in soil and neither are present to the exclusion of the other.

Inorganic phosphate is present in ionic form (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$) in solution and also in mineral form. The commonest form of mineral inorganic phosphate is apatite, which has the general formula $Ca_{10}X_2(PO_4)_6$ where X is OH^- or F^- giving hydroxyapatite or fluorapatite respectively (Addiscott *et al.*, 2000). Pi is also found in combination with Al and Fe oxides and hydroxides, clay minerals, solid carbonates and soil organic matter (Tate, 1984).

The form of phosphate ion in solution varies according to pH. In dilute solution, phosphoric acid dissociates as follows:

$$H_3PO_4 \underset{+H^+}{\overset{-H^+}{\leftrightarrow}} H_2PO_4^- \underset{+H^+}{\overset{-H^+}{\leftrightarrow}} HPO_4^{2-} \underset{+H^+}{\overset{-H^+}{\leftrightarrow}} PO_4^{3-}$$

In the pH range of most soils (5 - 8) the amounts of the un-dissociated H_3PO_4 and trivalent PO_4^{3-} are negligible and essentially all of the phosphate consumed by plants is in the $H_2PO_4^-$ and HPO_4^{2-} forms (Stevenson and Cole, 1999). Fig 1.1 illustrates the affect of pH on the solubility of Al, Fe and Ca inorganic phosphate compounds.

Organic P constitutes 30 to 65% of soil phosphate. Inositol phosphates can make up to 50% of the identifiable Po (Harrison, 1987). Usually no more than one third of the organic P in soils can be identified in known compounds. A large proportion of Po remains unidentified because of the stable nature of Po-soil complexes that prevent extraction of these compounds. The Po composition of the soil is quantitatively different from that of living organisms. This indicates that specific processes lead to selective stabilisation of various Po compounds in soils. (Frossard *et al.*, 1995)

The Po content of soils is typically measured using the ignition method of Saunders and Williams (1995). This method is based on oxidation of Po to Pi by ignition. Ignited and un-ignited soil samples are extracted with a dilute acid to quantify Tp and Pi respectively. Total Po is obtained as the difference between Pi in ignited and unignited samples following extraction.

Principal forms of organic P in soil are the inositol phosphates, phospholipids and nucleic acids or their degradation products. Other organic P compounds present in trace quantities include sugar phosphates, phosphoproteins, phosphonates and teichoic acid. The percentage of the forms of organic P detected in soils is detailed in Table 1.3.

The recovery of soil organic P in the three principal forms follows the order: inositol phosphates >> phospholipids > nucleic acid. The same types of organic P compounds are found in plants but the order is reversed. The higher abundance of inositol phosphates in soils in contrast to

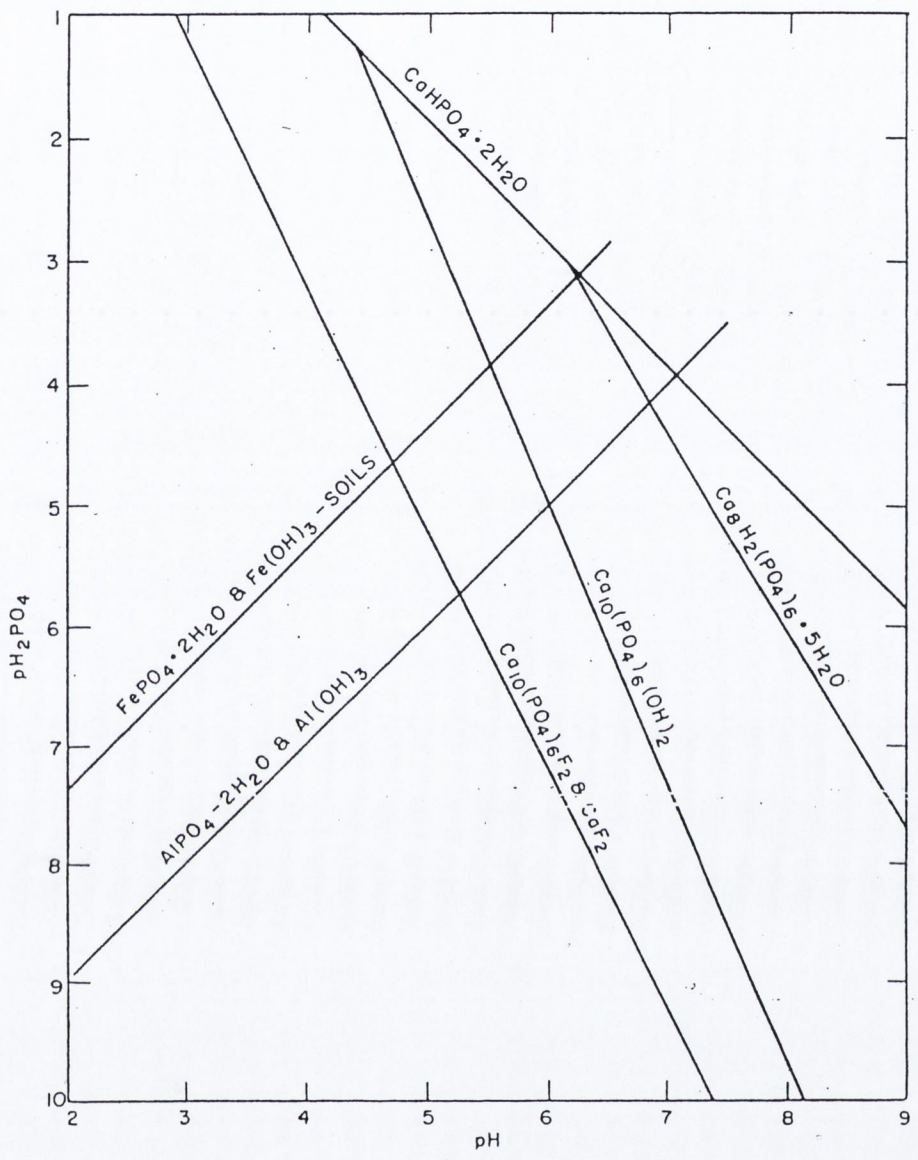


Fig 1.1 solubility diagram for Fe, Al and Ca inorganic phosphate compounds at increasing pH (Barber, 1995)

plants may be due to their tendency to form complexes of low solubility with polyvalent cations such as Fe and Al in acidic soils and Ca in calcareous soils. These insoluble complexes are undoubtedly less available to microorganisms and degradative enzymes than are the more soluble forms. (Stevenson and Cole, 1999)

Table 1.3: Organic P compounds present in soil and the ranges for percentage of organic P detected in the various forms. (Stevenson and Cole, 1999)

Po compound	**% of Po**
Inositol phosphates	10 – 50
Phospholipids	1.0 – 5.0
Nucleic Acids	0.2 – 2.5
Phosphoproteins	trace
Metabolic phosphates (eg. ATP)	trace

Inositol phosphates

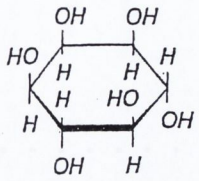
The term monoester phosphate is used to describe compounds with the general structure ROH_2PO_3 (Addiscott *et al.*, 2000). Inositol phosphates are included in these compounds.

Inositol phosphates are esters of hexahydrohexahydroxy cyclohexane, commonly referred to as inositol. Inositol is a hexane ring on which each carbon atom carries a hydrogen atom and a hydroxyl group. Nine positional stereoisomers of inositol are possible, depending on arrangements of H and OH groups. The best known form is *myo*-inositol (Fig 1.2, a) which is widely found in nature. A variety of esters of inositol are possible, the most common being the hexaphosphate or phytic acid (Fig 1.2, b). For many years the inositol hexaphosphates in soil were thought to be derived from the phytin of higher plants but recent work indicates that they are probably of microbial origin (Stevenson and Cole, 1999). Other naturally occurring stereoisomers of inositol, which have been isolated from soils, *are D-chiro* and *L-chiro*-inositol and *scyllo*-inositol (Fig 1.3).

The occurrence in soil of the hexaphosphate of *myo*-inositol and *D*- and *L-chiro*-inositol as well as *neo*-inositol and *scyllo*-inositol has been demonstrated (Cosgrove, 1969). In most cases *myo*- and *scyllo*-inositol phosphates make up approximately 90% of the total inositol bound phosphate, the remainder being *D-chiro*-inositol phosphates and *neo*-inositol phosphates (Cosgrove, 1980). Cosgrove (1969) suggested that *D-chiro*, *scyllo*- and *neo*-inositol hexaphosphates are synthesised by soil microorganisms by a mechanism which does not involve epimerization reactions. Only *myo*-inositol hexaphosphate has been detected in plant material (Cosgrove, 1969).

Penta-phosphates of *myo*-, *chiro*- and *scyllo*-inositol have been shown to be present in soil. The ratio of hexa- to penta- is very variable (Dalal, 1977). Lower esters are also present but the percentage of inositol phosphates as the mono- di- tri- tetra- penta- and hexaphosphate varies

(a) (b)



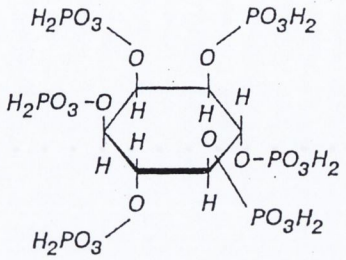
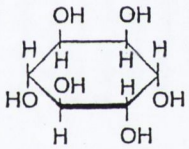
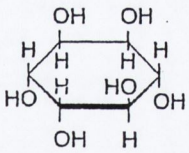


Fig 1.2: (a) myo- inositol (b) inositol hexaphosphate also known as phytic acid

(a)



(b)



(c)

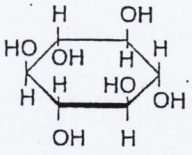


Fig 1.3: (a) D-chiro-inositol (b) L-chiro-inositol (c) scyllo-inositol

widely from one soil to another. The quantities of lower esters are much less than the penta- and hexaphosphate fraction possibly because of their lower stability in soil. (Dalal, 1977)

Diesters have the general structure (RO)(R'O)-HPO_3 but this simplified structure covers a wide range of compounds that include phospholipids and fragments of RNA (Addiscott *et al.*, 2000).

Phospholipids

Phospholipids represent a group of biologically important organic compounds that include the glycerophosphatides such as phosphatidyl inositol, phosphatidyl choline or lecithin (Fig 1.4), phosphatidyl serine (Fig 1.5) and phosphatidyl ethanolamine (Fig 1.6) (RC=O is a long chain fatty acyl group). The total quantity of phospholipids in soil is small, approximately 0.6 to 3.0% of the soil organic P. The phospholipids in soil are undoubtedly of microbial origin. Phosphatidyl choline (~40%) appears to be the predominant soil phospholipid followed by phosphatidyl ethanolamine (~30%) (Dalal, 1977). The fact that phospholipids comprise the major part of total organic phosphorus in plant tissue but only a small part in soil organic phosphorus shows that their synthesis and degradation may be fairly rapid in soil. (Dalal, 1977)

Nucleic Acids

Nucleic acids are found in all living cells and they are synthesised by soil microorganisms during degradation of plant and animal residues. Two types are known, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), each consisting of a chain of nucleotides. Estimates indicate that no more than 3% of the organic P in soil occurs as nucleic acids or their derivatives. (Stevenson and Cole, 1999) Only a small proportion of soil organic phosphorus exists as nucleic acids or their derivatives in spite of the fact that these are probably added to the soil through decomposing microbial, plant and animal remains in greater amounts than other phosphate esters. It appears that nucleic acids added to the soil are either rapidly degraded or re-synthesised and combined with other soil constituents. The evidence available so far shows that nucleic acids can be rapidly mineralised in soil and incorporated into microbial biomass. (Dalal, 1977)

1.7 Plant P uptake

Phosphorus as phosphate is an essential constituent of all living organisms. Symptoms of plant P deficiency include stunted growth, delayed maturity and yellowing of the lower leaves which wither and drop off. An abnormally dark green, often shading to red and purple pigmentation, may be observed because of excess anthocyanin accumulation. Phosphorus is needed for favourable seed formation, root development and strength of straw in cereal crops and crop maturity. (Stevenson and Cole, 1999)

Phosphorus is taken up by plants mainly as the negatively charged primary and secondary orthophosphate ions ($H_2PO_4^-$ and HPO_4^{2-}) which are present in the soil solution (Stevenson and

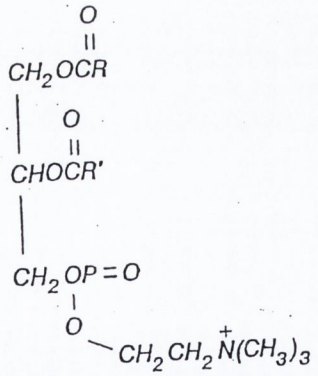


Fig 1.4: phosphatidyl choline or lecithin

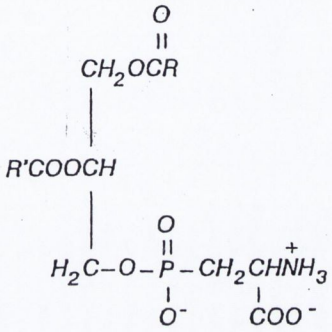


Fig 1.5: phosphatidyl serine

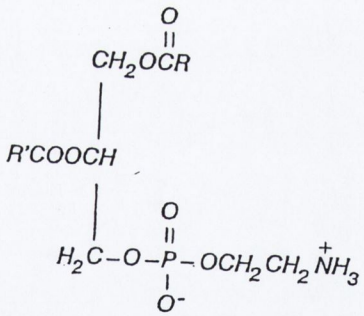


Fig 1.6: phosphatidyl ethanolamine

Cole, 1999). As ions can only exist in solution a water layer between soil particles containing available P and active roots is absolutely necessary to allow the transfer of soil P from soil particles towards the roots (Fardeau, 1996). This uptake occurs through the membrane transport systems of plant roots in an active energy dependant process that occurs preferentially in young suberised roots, but also along most suberised root surfaces (Frossard *et al.*, 1995). The process can result in concentrations of phosphates of 10^{-3} M within plant root cells and xylem tissue, from concentrations ranging between 10^{-5} and 10^{-8} M in the soil solution. Uptake takes place against a steep electrochemical gradient and is mediated by a H^{+} co-transport (Frossard *et al.*, 1995).

Movement of phosphate to plant roots by mass flow is believed to account for only a small portion of the phosphate consumed by plants (Stevenson and Cole, 1999) approximately 1-4% (Schilling *et al.*, 1998). The rate of phosphate transport to the root surface by mass flow of soil water cannot satisfy the demand for phosphate at the rhizoplane that results from the root absorbing power. Diffusion is the main mechanism for P transport (Schilling *et al.*, 1998). The concentration of phosphate in the rhizoplane solution is rapidly depleted through active uptake, but the diffusion of phosphate to the depleted zone around the root is slow. This is because in most soils the equilibrium condition between solution phosphate and exchangeable soil surface phosphate is often 1000 fold in favour of the soil surface. The diffusion coefficient of phosphate through soil becomes a major factor limiting the rate of plant P uptake. (Frossard *et al.*, 1995)

A mycorrhizal association is a symbiotic relationship between the root system of higher plants and a particular fungus. A vesicular arbuscular mycorrhizal (VAM) fungus is characterised by the penetration of hyphae into deep regions of the cortex of the root, with formation of arbuscules and vesicles. The hyphae of these VAM fungi can extend beyond the root surface and into the soil for substantial distances (up to 70mm) thereby increasing the soil volume from which plant roots can obtain nutrients. VAM have shown to be particularly important in the greater uptake of nutrients such as P by plants. VAM fungi extend the zone from which plants can absorb P and so help to alleviate the difficulty in obtaining P because of the limiting rate of diffusion of P through the soil. VAM can also store P as polyphosphates, which can then be passed on to the plant as required. A full discussion of VAM fungi and Po nutrition of plants is presented in Chapter 2.

Inside the plant the phosphorus anions are rapidly metabolised into organic compounds in which the phosphate is esterified with OH groups of sugars or alcohols, or bound as a pyrophosphate bond to another phosphate group. These move freely in both the xylem and phloem transport systems. Movement can also occur in inorganic form (Archer, 1988). Dominant P containing compounds are the nucleic acids, phospholipids and phosphorylated polysaccharides. Phytin, the Ca-Mg salt of inositol hexaphosphate is also found in plant material. Vital compounds for

metabolism includes ATP that functions in energy storage and transfer. (Stevenson and Cole, 1999) This contains high energy pyrophosphate bonds which on hydrolysis release this energy to drive other plant processes including active uptake of plant nutrients (Archer, 1988). Phosphopyridine nucleotides serve as electron carriers (Stevenson and Cole, 1999).

1.8 The P cycle

The P cycle is a dynamic system involving soil, plants and microorganisms. Major processes include uptake of soil P by plants, recycling through the return of plant and animal residues, and abiotic and biological transformations of P. The quantity of P present in the soil solution represents only a small fraction of plant needs and the remainder must be obtained from the solid phase by a combination of the abiotic and biotic processes.

Precipitation-dissolution and sorption-desorption reactions control the abiotic transfer of P between the solid phase and soil solution. Precipitation reactions result in the formation of calcium, aluminium and iron phosphates in soil while the opposing step of dissolution, requires a source of H+ which can originate from the soil itself or from roots or microbes. Sorption-desorption reactions result from the sorption of P on soil minerals and organic compounds and their subsequent desorption controlled by the concentration of P in solution. (Frossard *et al*, 1995)

The main biological processes controlling the transformations of P between inorganic and organic forms are mineralisation and immobilisation of P. These reactions are principally microbial in origin, occur simultaneously in soil and account for the short-term changes in the P cycle. The microbial conversion of organic P to more available inorganic forms is termed mineralisation. These inorganic P fractions may also be converted biologically into unavailable organic forms by microbial immobilisation. It is the net affect of factors controlling the rates of mineralisation and immobilisation that determine biological changes in P distribution. (Dalal, 1977) The turnover or flux of P through the soil microbial biomass is widely acknowledged to be an important factor that determines the availability and utilisation of organic P in the soil-plant system. Despite extensive investigation, our understanding of organic P dynamics in soil and the precise role of soil microbial biomass are limited. (Frossard *et al.*, 2000)

The availability and transfer of P to plants depends not only on the total P content of the soil but on other factors such as solubility product relationships between soil minerals and soil solution, mineralisation and immobilisation, grazing by predators and decomposition of plant and animal debris. Since plant P requirements exceed the quantities of P dissolved in soil solution, solution P must be replenished and maintained by some buffering mechanism. Soil solution P buffering is usually treated as an abiotic equilibrium among precipitated and sorbed solid phase P and solution inorganic P. This approach ignores P contained in soil biomass and other organic forms

(Seeling and Zasoski, 1993). All reactions that contribute to the P cycle must be considered when examining P availability.

In natural ecosystems plant nutrients return to soils at or near the place where they were previously taken as bioavailable soil P by plants. In such ecosystems the P cycle can be considered as closed and the rate of P cycling depends on biogeochemical and soil properties and on climatic conditions. (Fardeau, 1996)

A diagrammatic representation of the phosphate cycle is presented in Fig 1.7. Unlike the C, N, and S cycles the P cycle does not have a gaseous component and therefore movement of P to and from the atmosphere is of minor importance due to small amounts that are circulated as atmospheric particulates (Stevenson and Cole, 1999). Phosphate is taken up by the root system of plants, which are often associated with vesicular arbuscular mycorrhiza. This P is rapidly esterified in the plant tissue. Invertebrate and vertebrate predators return P to the soil as both produce orthophosphate rich urine. The phosphorus in plant litter is attacked by fungi and is physically moved into the soil in fungal hyphae, which are then attacked by bacteria providing a continuing source of organic phosphorus. The more soluble fraction of this phosphorus is immobilised by new microbial tissue or converted into more resistant compounds forming soil humus. The microbial biomass may take up P as a result of the addition of organic matter or in response to changes in physiology caused by moisture fluctuations or other disturbances. (Frossard *et al.*, 2000) The orthophosphate produced by mineralisation of plant and animal remains by decomposers may also go into soil solution where it may be taken up by plants adsorbed by soil colloids and fixed into unavailable inorganic form or again appropriated by microorganisms (Dalal, 1977). Vertebrates accumulate massive skeletons of hydroxy-apatite, which appear to be dispersed by predators and decomposers either by fragmentation or dissolution in gastric fluids (Jeffrey, 1988). The orthophosphate pool is in equilibrium with the large pool of inorganic P present in the soil, usually in the form of Fe, Al and Ca phosphates. Any fertiliser P added, usually in the form of inorganic P, will contribute to this pool. A soil organic P pool is also present. Usually this accessed by plants through enzymatic hydrolysis of stable P esters to form orthophosphate for plant uptake. Fire may lead to a short cut in the cycle. (Jeffrey, 1988).

The term microbial biomass has evolved as a collective term for the bacteria, fungi and small soil animals that between them affect the turnover of soil organic matter. Phosphate is a constituent of phospholipids, DNA and RNA in these organisms and is also involved in their metabolic energy transfers (Addiscott *et al.*, 2000). It has been estimated that from 1 to 2% of the soil total P occurs in microbial biomass (Stevenson and Cole, 1999) and from 3 to 24% of the Po in temperate arable or pasture soils (Frossard *et al.*, 1995).

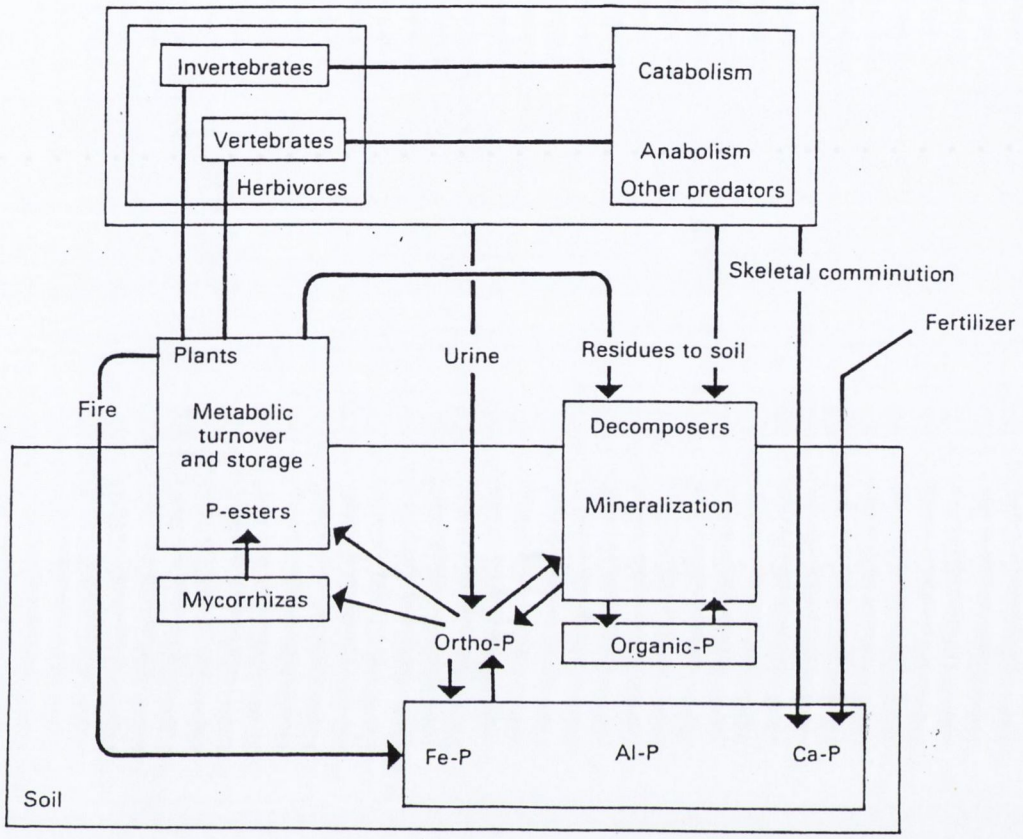


Fig 1.7: The phosphate cycle (reproduced from Jeffrey, 1988)

All living cells, including those in the soils microbial biomass use the ADP-ATP energy shuttle to link energy supplying and energy requiring reactions in their metabolism. The turnover time of the terminal phosphate group on an ATP molecule is measured in fractions of a second. This suggests that phosphate concentrations could change rapidly during periods of intense microbial activity (Addiscott *et al.*, 2000).

Soil organic phosphate contributes to the phosphorus nutrition of plants after being mineralised into inorganic phosphate. The mineralisation of organic phosphorus in soil is largely due to phosphatase enzymes that catalyse the hydrolysis of esterases of orthophosphoric acid (Feder, 1973). These phosphatase enzymes originate from plant roots, mycorrhizal and saprotrophic fungi, bacteria and soil fauna. A full discussion of phosphatase enzymes is presented in Chapter 4. Since the processes of mineralisation and immobilisation are associated with microbial activity, factors affecting the population dynamics of microorganisms in soil also control the balance between these two processes. These factors include temperature, aeration, pH and moisture of soils.

Mineralisation of organic phosphorus increases with increasing temperature. The temperature affect has been linked to the build up of Po during winter and decline in Po during spring. In general if aeration becomes poor the rate of decomposition of organic matter becomes smaller. As soil pH increases microbial activity increases. The affect of moisture levels on microbial activity may be less marked than that of temperature as detailed studies on this affect are few (Dalal, 1977). Wetting and drying cycles greatly stimulate the mineralisation of organic matter (Birch, 1961).

The different categories of organic phosphate have different susceptibilities to mineralisation. Diester phosphates are more readily mineralised than monoester phosphate. Monoesters such as inositol phosphate are probably protected from mineralisation by the strength with which they are sorbed by the mineral components in the soil (Addiscott *et al.*, 2000).

1.9 Thesis structure and organisation

This thesis is divided into a further six chapters. All chapters contain an introduction to their subject, which includes a literature review on the topic and the aim of the experiment in question. Methods particular to a subject described in an individual chapter are described in the methods section of that chapter. Methods common to several chapters are described in the first experimental chapter, Chapter 2. The results section presents the data in graphical or tabular form. Statistical analysis of the data is presented and formal statements of statistical significance for the data are included in this section. The discussion section discusses the significant results in the context of their place in the overall experiment and what they actually mean with respect

to plant growth and the behaviour of Po in soil. Finally the summary presents the significant findings of the chapter briefly and in condensed form.

Chapter 2 deals with the role of vesicular arbuscular mycorrhiza (VAM) in the use of organic phosphate by plants. Aspects of the symbiotic relationship between plants and the mycorrhizal fungi which infect their roots is examined in relation to Po content of the soil and plant uptake of P and growth.

Chapter 3, following on from chapter 2, examines the significant of the other major component of soil microorganisms – bacteria, on the use of organic phosphate by plants. As with the VAM, the bacteria are indigenous to the soil and are present in populations occurring naturally.

Chapter 4 investigates soil and root phosphatase activity and its relationship with soil organic phosphate, particularly inositol hexaphosphate and affects of any changing of activity on plant growth.

The interaction between organic phosphorus and nitrogen is examined in Chapter 5. The affect of increased soil nitrogen on plant growth with increased levels of soil organic P present is determined.

Chapter 6 describes the use of nuclear magnetic resonance (NMR) to identify Po species present in the soil, and determine if this technique is suitable for determining changes in levels of these individual compounds due to plant P uptake.

Chapter 7 concludes this work and provides a summary of the main points and the information gained. It also suggests ideas for future experiments that could continue the work from this project.

CHAPTER 2: THE ROLE OF VESICULAR ARBUSCULAR MYCORRHIZAE IN THE USE OF ORGANIC PHOSPHATE BY PLANTS

2.1 Introduction

2.1.1 Objectives of experiment

The objectives of the experiments described in this chapter were:

- to examine the role of vesicular arbuscular mycorrhizal (VAM) fungi in the use of soil organic phosphate, at various concentrations, for plant growth.
- to examine the affect of increasing concentrations of soil organic phosphate on the percentage root length colonisation by VAM fungi and subsequent affects on plant growth.
- to examine the contribution made by VAM fungi to soil and root acidic monoesterases.
- to examine the ability of VAM fungi to obtain organic P for plant use outside the root zone and inaccessible to the roots of a plant.

The approach used for the experiments described in this chapter was different to that of previous workers due to the combination of factors: (1) a natural soil collected from the PP1 site was used (for site descriptions refer to Chapter 1, Section 1.3), (2) the indigenous VAM population of the soil was used instead of inoculation with a specific species of VAM, and (3) plants of *Dactylis glomerata*, *Agrostis stolonifera* and *Plantago lanceolata* were grown from seeds collected from plants growing on the PP1 site.

Inositol hexaphosphate (IHP) was chosen as the organic P compound to add to the soil as this compound is one of the principal forms of soil organic phosphate (Stevenson and Cole, 1999). Soil from the PP1 site was chosen as the control soil on which to base the treatments. IHP was added to the soil at increasing concentrations, 100, 200 and 400 mg P/ l soil. An inorganic P treatment, based on superphosphate addition, was included at 100mg P/l soil in order to compare the performance of plants grown under equal concentrations of Po and Pi.

Two experiments were presented in this chapter.

Experiment 1 - Benlate addition experiment

In this experiment the infection of roots by VAM fungi was reduced, using the fungicide Benlate. The affects of reduced VAM infection on plant growth and soil P, and any interaction with changing soil organic phosphorus can be observed. The 'growth bag' method was also used in order to examine the contribution of roots and hyphae to plant P uptake. As different plant species respond to mycorrhizal infections to different extent (Sanderss, Koide and Shumway 1995) the experiment was repeated with three species, two of the most common grass species on the PP1 site *Dactylis glomerata* and *Agrostis stolonifera*, and a herb *Plantago lanceolata*.

Experiment 2 - Hyphal exploration experiment

In this experiment the 'growth bag' method was used again. PP1 soil was within the bag, in the root compartment and PP1 soil enriched with Po was in the surrounding section, the hyphal compartment. This experiment was designed to examine the efficiency of the VAM hyphae to explore soil outside the reach of roots and obtain any Po present for plant growth. *Plantago lanceolata* was used in this experiment.

2.1.2 Experimental approaches employed

2.1.2.1 The 'growth bag' method

As mycorrhizal infection may change root morphology and physiology (Kothari, Marschner and George, 1990), the direct mycorrhizal contribution to P acquisition by plants cannot be sufficiently characterised by comparing P uptake of mycorrhizal and non-mycorrhizal plants (Gnekow and Marschner, 1989). The most appropriate way to distinguish between contributions of roots and of mycorrhizal hyphae to P acquisition is the spatial separation of zones containing root and hyphae (Kothari, Marschner and Romheld, 1991). This is done most frequently using a container system and 30 μm nylon mesh to separate a root compartment from a hyphal compartment. Only hyphae can pass through the mesh as the passage of roots is blocked. Various modifications of this approach have been used in numerous studies of hyphal nutrient transport (Schweiger and Jakobsen, 1998; Kothari *et al.,* 1990; Li *et al.*, 1991, Li *et al.*, 1997).

For this experiment such a system was designed (Fig 2.1). A cylindrical bag was made from 30 μm nylon mesh and placed in a pot twice its volume. The soil within the bag is for the growth of roots and hyphae, forming the root compartment. The soil surrounding the bag in the pot forms the hyphal compartment and can be accessed only by hyphae.

2.1.2.2 Use of a Fungicide

Experiments designed to study the interaction between VAM fungi and plants usually involve comparison of infected and un-infected individuals. This is often achieved through soil sterilisation and re-inoculation. However this results in highly atypical growth conditions. Soil sterilisation is achieved through either γ-irradiation or steaming, which often result in increases in soil toxins and changes in soil chemical and physical properties. Even the use of chemical steriliants results in almost total destruction of the soil biota and disruption of nutrient cycles. Total sterilisation eliminates VAM but also bacteria and soil fauna, both of which interact with VAM fungi and so complicate results. (Fitter and Nicholas, 1988) Re-inoculation, although it may produce large growth responses, cannot be used to measure the contribution to plant growth of the indigenous VAM fungi (Jakobsen, 1994).

A fungicide that would specifically eliminate VAM fungi, with little or no affect on the remaining biota would be an ideal alternative to sterilisation for use in greenhouse and

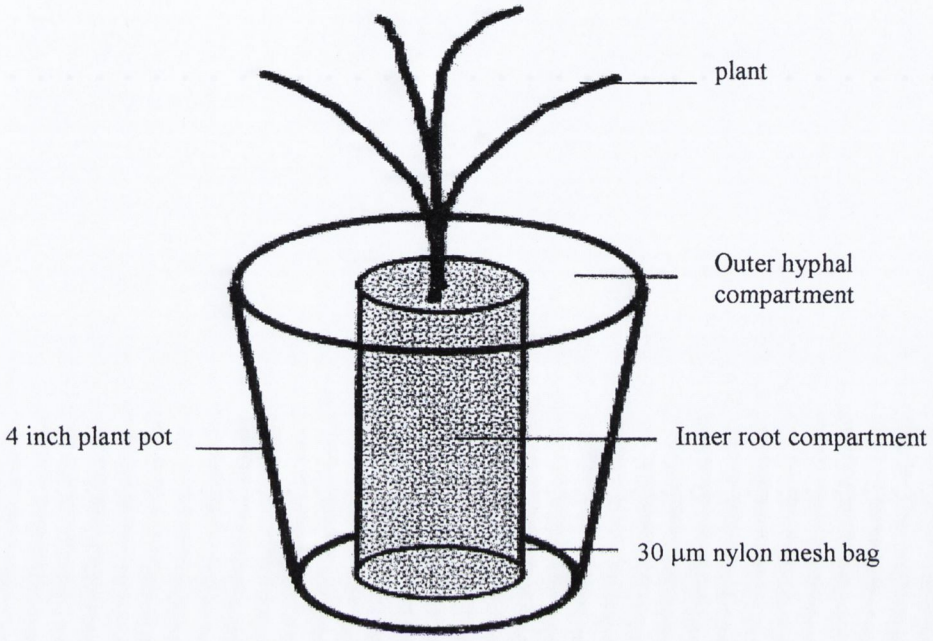


Fig 2.1 The 'growth bag' method used to separate the root and hyphal zones. The soil inside the bag is the root zone. The soil outside the bag is the hyphal zone that is inaccessible to roots.

fieldwork (Paul *et al.*, 1989). In the absence of such a product, fungicides are available that can control hyphal growth of VAM fungi with less disruption to the soil system. One of the most widely used of such fungicides is benomyl [(1-butyl-carbamoyl)-2-(benzimidazole) carbamic acid, methyl ester)], which is widely sold as Benlate (Fitter and Nicholas, 1988). This fungicide is known to reduce mycorrhizal development (Menge, 1982), and is used widely to control VAM infection in mycorrhizal experiments (Fitter and Nicholas, 1988; Merryweather and Fitter, 1996; Habte, 1997; Sukarno et al., 1993). Benlate is thought to inhibit hyphal growth (Carr and Hinkley, 1985) but does not kill spores or hyphae in excised root pieces (Fitter and Nicholas, 1988).

It has also been shown that the fungicide does not have a direct affect on the growth of a wide range of plant species (Carey *et al.*, 1992; Newsham *et al.*, 1994; Merryweather and Fitter, 1996) including *Plantago lanceolata*, *Dactylis glomerata*, *Agrostis capillaris* (Paul *et al.*, 1989). No adverse affects on non-target soil microorganisms (De Bertoldi, *et al.*, 1997; Vyas, 1998; Habte, 1997) have been found. Benlate has a low phytotoxicity (Paul *et al.*, 1989; Sukarno *et al.*, 1993; Newsham *et al.*, 1994) and does not alter soil P availability (Fitter and Nicholas 1988; Merryweather and Fitter, 1996).

In order to obtain non-mycorrhizal plants benlate was used in this experiment at a rate of 0.89 g/kg soil (equivalent to 0.61 g/l soil). This dosage is in line with the dosage used by Hale and Saunders who used 0.55 g/l soil in order to reduce VAM presence in their work. Doses used by other workers include 1.125 g/l soil (Merryweather and Fitter, 1996), 1.5 g/l soil (Fitter and Nicholas, 1988), 1 g/l soil (Koide *et al.*, 1988) and 2.2 g/l soil (Carey *et al.*, 1992). This benlate approach achieves a more natural environment for the analysis of VAM and plant growth than can be achieved with methods of soil sterilisation.

2.1.3 Vesicular arbuscular mycorrhiza

Mycorrhizas are mutualistic symbioses, which involve associations between a fungal partner and plant roots (Isaac, 1992). The vesicular-arbuscular mycorrhiza (VAM) are the most common form and widely occurring of all the mycorrhizal associations (Issac, 1992). They are formed in the roots of an enormously wide variety of host plants including angiosperms, gymnosperms and pteridophytes (Smith and Read, 1997). The fungi involved are aseptate and obligately symbiotic, are in the order Glomales (Smith and Read, 1997) and are widespread in the temperate lowlands (Read, 1991). It seems likely that the fungi had their origins between 353 and 462 million years ago and that the symbiosis is similarly ancient and was probably important in the colonisation of land by vascular plants (Smith and Read, 1997).

The range of potential host plants for VAM fungi is extremely wide. Gerdemann (1968) stated, "it is so ubiquitous that it is easier to list plant families in which it is not known to occur than to

compile a list of families in which it has been found". Trappe (1987) from a survey of published literature has produced a compilation of the incidence of all types of mycorrhizas within the angiosperms. Records of VAM are to be found in all the orders from which plants have been examined, however only approximately 3% of species have been examined. Consequently it can be said that 95% of the present-day species of plants belong to families that are characteristically mycorrhizal. However it cannot be said that 95% of the world's species are mycorrhizal. (Smith and Read, 1997).

The name vesicular-arbuscular is derived from the characteristic structures, the arbuscules that are formed within the cortical cells by the invading fungus, and the vesicles that occur between or within them. Approximately 80% of the species described form both arbuscules and vesicles. The remainder do not form vesicles and are often called arbuscular mycorrhiza (Smith and Read, 1997). A VA mycorrhiza has three important components: the root itself, the fungal structures within the cells of the root and an extraradical mycelium that radiates out into the soil. These hyphae bifurcate into finer and finer hyphae with each branch order until the 5^{th} or 6^{th} order when the hyphae may be no more than 2 μm diameter (Allen, 1996). Hyphae spread through the soil ramifying several centimetres from the infected root surface (Issac, 1992).

In order for a fungus to penetrate a root it must loosen the walls to grow intercellularly and then forcibly penetrate cell walls (Allen, 1996). In the outer cortex, coils of hyphae are found within the cells. In the outer to middle cortical layers of the root the fungus forms terminal swellings, the vesicles. These are often large and thick walled, rich in lipids and probably used as storage organs. Within the cells of the inner cortical layers, nearer to the central steele, the invading fungus branches dichotomously and forms finely divided projections that penetrate into the host cells, causing invagination of the host membrane. These are the arbuscules. As they provide a large increase in the surface area of contact between the fungus and the plant they are thought to be the regions of nutrient transfer between plant and fungus (Issac, 1992). Fig 2.2 depicts the infection of a plant root by a VAM fungus.

There is little evidence for specificity between particular fungi and host plant. That is a VA mycorrhizal fungus isolated from one species of host plant will colonise any other species that has been shown to be capable of forming VA mycorrhizas (Smith and Read, 1997).

There are three sources of inoculum from which colonisation of roots by VA mycorrhiza can arise: spores, infected root fragments and hyphae, collectively termed propagules. The large spores have thick resistant walls and are possibly long-term survival structures. In many habitats the hyphal network present in the soil and root fragments are probably the main method by which plants become colonised even when significant spore populations are also present (Smith and Read, 1997). Assessment of the affect of storage, on the vitality of mycorrhizal fungi inside

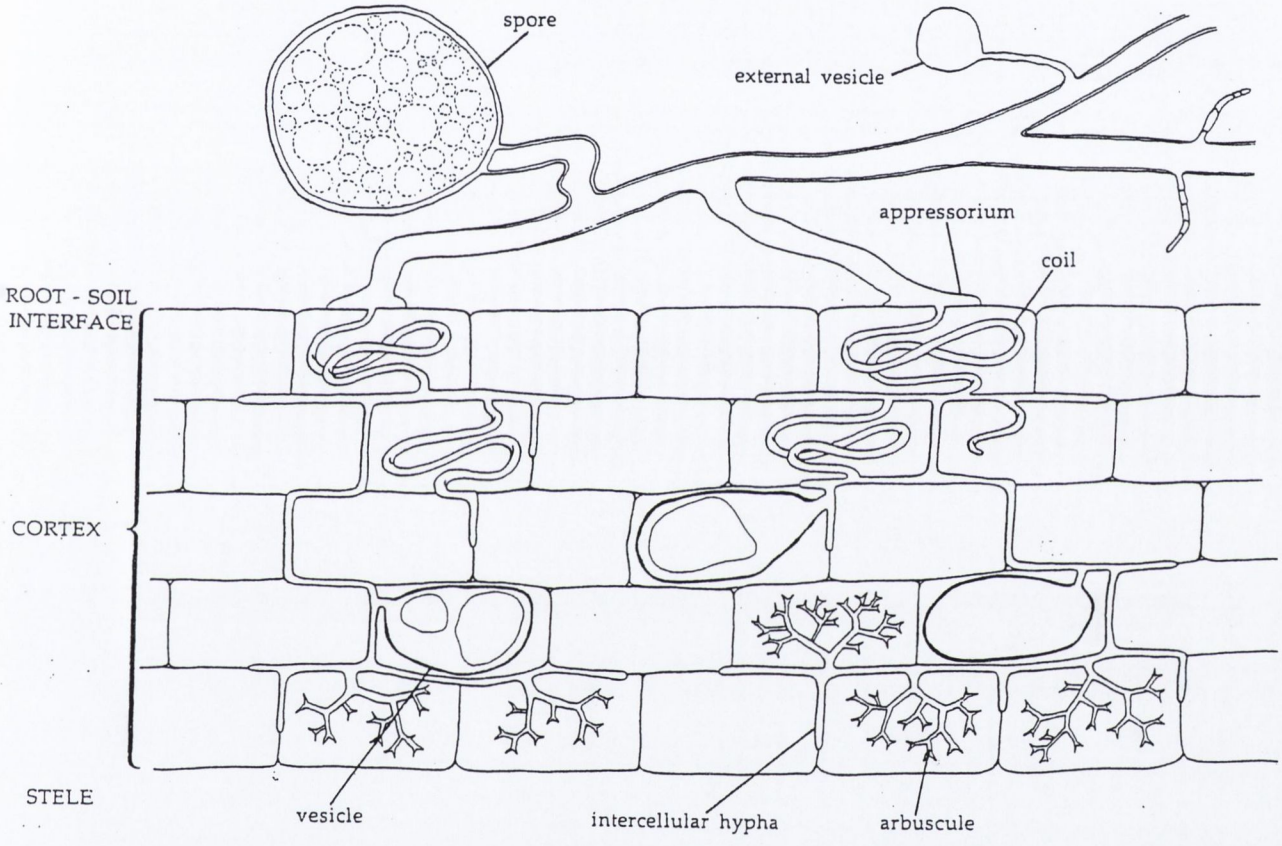


Fig 2.2 : the relationship between the fungus and the host plant root in a vesicular arbuscular mycorrhizal relationship. (Isaac, 1992).

plant roots was recently carried out. In roots refrigerated at 5°C the vitality of internal hyphae and arbuscules remained constant for nearly three weeks. This gives an insight into how fresh root material should be when undertaking plant mycorrhizal fungus studies (Staddon and Fitter, 2001).

2.1.4 Vesicular arbuscular mycorrhiza and phosphate uptake

Root infection with VAM fungi can increase efficiency of nutrient absorption and, in turn enhance growth of mycorrhizal plants, particularly at low availability of phosphorus in the soil (Kothari *et al.*, 1990). Phosphorus uptake by the roots of mycorrhizal plants has received particular attention because of the marked affects on plant growth (Ayling *et al.*, 1997). Phosphate ions are supplied to the plant root by diffusion. The rate of diffusion of phosphate ions in soil is low and will vary with, for example, P content of the soil and buffering capacity. Slow diffusion of phosphate ions in the soil solution and, their rapid absorption by roots results in the development of a depletion zone around them. Uptake of P is therefore limited by the rate of diffusion of P ions into these depletion zones, rather than by the rate of transport across membranes into the roots.

There are several possible explanations for the efficiency of P uptake by mycorrhizal roots.

1. Mycorrhizal fungi can extend extraradical hyphae into the soil uncolonised by roots and so outside the depletion zone, absorb P from the soil solution and translocate it to the root. The smaller diameter of hyphae compared to the roots allows them to penetrate soil pores of smaller diameter than roots increasing the volume of accessible soil. The absorbing surface of roots is in affect increased by mycorrhizal hyphae.
2. The hyphae as they are thinner and well spread out, are more affective than roots in competing with free living soil microorganisms for recently solubilized Pi.
3. The kinetics of P uptake into the hyphae may differ from that of roots, with a higher affinity of uptake leading to more affective absorption from low concentration in soil solution.
4. Mycorrhizal roots can use sources of P in soil that are not available to roots. This could involve increasing rates of solubilization of Pi of low solubility or hydrolysis of Po and, would depend on localised alterations in pH, production of organic anions as chelating agents, and the production of surface or soluble phosphatases. Results are conflicting (Smith and Read, 1997).

The high efficiency of VAM hyphae in P uptake is not any only caused by their small diameter and large surface area, but also by the accumulation of P, in the form of polyphosphates, in their vacuoles. This capacity for storage of polyphosphate means that uptake is not limited by current metabolic demand. These polyphosphates are hydrolysed in the arbuscules and transported as Pi to the host plant cells (Harrison, 1995).

In VAM plants uptake of phosphorus is normally from the same labile pool from which roots, and thus also non–mycorrhizal plants take up phosphorus (Bolan, 1991). The greatest benefit for a plant when infected by a VAM fungus, is achieved with supply of the least soluble source of phosphorus eg. crystalline iron phosphate (Tarafdar and Marschner, 1994). Organic phosphate (Po) does not seem to provide a major pool directly available to plants with VA mycorrhizas (Smith and Read, 1997). Phosphate mineralised from P sources appears to be more readily available to mycorrhizal than non-mycorrhizal plants, but there is no good evidence that mycorrhizal fungi are actually involved in the mineralisation process. (Smith and Read, 1997).

2.1.5 Previous research

The majority of previous studies relating to VAM and plant growth have focused on the influence of Pi, in the form of fertilisers, on plant growth and VAM (Li *et al.*, 1991; Jasper and Davy, 1993; Mendoza and Gigli, 1995; Frey and Ellis, 1996; Nurlaeny *et al.*, 1996; Thompsom 1996; Schreiner *et al.*, 1997; Khaliq and Sanders, 1997; Thingstrup, *et al.*, 2000; Jakobsen *et al.*, 2001; Dekkers and van der Werff, 2001).

A few studies look at VAM and Po, occasionally in the form of phytate, but use inoculation with one strain of specially selected VAM and artificial agar-culture medium (Allen *et al.*, 1981) or artificial and sterilised sand-like medium (Jayachandran *et al.*, 1992; Tarafdar and Marschner, 1995).

Organic P in soil may be utilised by plants after mineralisation and subsequent release of Pi. This mineralisation may occur through hydrolytic cleavage of Po by extracellular phosphatase of root or microbial origin (Joner *et al.*, 1995). VAM increase the uptake of this Pi by plants through extraradical hyphae. However few workers have considered the utilisation of Po by VAM plants, through increased phosphatase release from AM roots or fungal hyphae (Smith and Read, 1997). Subsequent reports on the role of VAM in production of extracellular phosphatases and mobilisation of Po are conflicting (Tarafdar and Marschner, 1994; Joner *et al.*, 1995). Some workers conclude that extracellular phosphatases are not produced by extraradical hyphae of VAM symbiosis (Dodd *et al.*, 1987; Joner *et al.*, 1995). The influence of VAM hyphae on phosphatase activity in root free soil seems to be indirect through interactions with other soil microorganisms (Joner and Jakobsen, 1995). Others however claim results demonstrate the production of phosphatase by mycorrhizal hyphae and their contribution towards plant P uptake (Tarafdar and Marschner, 1994). A recent paper (Koide and Kabir, 2000) showed evidence for hydrolysis by mycorrhizal hyphae of Po in the forms of BCIP (toludine salt of 5-bromo-4-chloro-3-indolyl phosphate) and PDP (tetrasodium salt of phenol-phthalein diphosphate). Utilisation of phytate by the fungus was also demonstrated but enzymatic hydrolysis for uptake was not shown. This work was carried out in sterile culture medium.

Previous studies of extracellular phosphatase in the rhizosphere of VAM plants have shown increased activity (Dodd *et al.*, 1978; Tarafdar and Marschner, 1994) no difference (Dodd *et al.*, 1987; Joner *et al.*, 1995) and decreased activity (Joner and Jakobsen, 1995) associated with mycorrhizal colonisation. The work carried out on VAM and influence of VAM on phosphatase has been carried out in sterile artificial medium with inoculation by various selected strains of VAM (Dodd *et al.*, 1987; Jayachandran *et al.*, 1992; Tarafdar and Marschner, 1994; Joner *et al.*, 1995; Joner and Jakobsen, 1995; Joner and Johansen, 2000) or in nutrient medium in petri dishes (Koide and Kabir, 2000). Inter-fungal differences seem common (Dodd *et al.*, 1987; Joner and Jahansen, 2000). Ideally the study of mechanisms of VAM action would be undertaken in sterile culture containing only plant and fungus. However these conditions do not mimic those found in the field (Allen, 1996).

2.2 METHODS

2.2.1 Collection and storage of soil and seeds

Soil was collected from the PP1 site (for site description refer to Chapter 1 section 1.3) approximately two weeks before the beginning of the experiment. Soil was collected as sods of earth from the top 10 cm of the soil profile. The top 10 cm were chosen as the depth for sampling because it was within the rooting depth for the species used in this experiment and microorganisms are most abundant in this region. The presence of soil microorganisms decreases with increasing distance from the rooting zone. Total soil organic phosphate also decreases with depth down the soil profile and the majority of soil phosphatase activity is in the top 10 cm of the soil profile (Harrison, 1987). It is within the depth range commonly sampled by other workers as reviewed by Harrison (1987) and follows the recommendations by Archer (1988) for soil sampling. Above ground plant material was removed from the sods and the soil was stored in plastic bags at 4 °C until required.

Seeds were collected from the PP1 site, from plants growing on the site and picked at random. The seeds were dried in a desiccator containing silica gel at room temperature and stored in the freezer at –18 °C until required. (Seeds were collected during the late summer to early autumn of the first year of this project, 1997, and were used to grow plants for all the experiments described in this thesis.)

2.2.2 Preparation of soil

Within a week of collection and immediately before the setting up of the experimental pots, the soil was removed from the cold room, the sods were broken up and stones removed. Large coarse roots (>3 mm) were removed, remaining roots were cut into small pieces and mixed with the soil. The soil was then 4 mm sieved before use.

In order to carry out the experiment in as natural a soil environment as possible for a pot experiment, it was necessary to preserve soil bacterial and fungal populations and enzyme activity.

Large roots were removed as they may interfere with the root growth of the experimental species and make recovery of the root material difficult when harvesting. However smaller roots were returned to the soil as they are a source of inoculum for vesicular arbuscular mycorrhizal fungi (Staddon and Fitter, 2001). The vitality of mycorrhizal fungi inside plant roots remains constant for three weeks when refrigerated at 5°C (Staddon and Fitter, 2001). Leaving roots to decay in soil has been shown to have no affect on the growth of *Holcus lanatus* over a three-month period (McLellan *et al.*, 1995)

If soils are to be stored, in order to preserve the microbial biomass and activity, the ISO (1993) and the OECD (1995) have recommended a temperature of +4 ± 2 °C for a maximum of three

months (Stenberg *et al.*, 1998). Soil collected for this experiment was stored at +4 °C for a maximum of two weeks. The storage of the soil for a maximum of two weeks at 4 °C has been shown to have no affect on phosphatase activity as phosphatase activity remains stable over 28 days in moist soils stored at 4 °C. (Kramer and Green, 2000).

2.2.3 Seed Germination and growth conditions

All seeds were germinated in seed trays containing soil from the PP1 site. After 5/6 days seedlings of uniform size were selected and transplanted into the growth bags in the experimental pots (4-inch plastic plant pots). The 'growth bag' method consisted of a cylindrical bag, made from 30 μm nylon mesh (mesh supplied by Plastok, England), and placed in a pot twice its volume (volume of pot = 510 cm^3, volume of bag = 255 cm^3). The soil inside the bag was for the growth of roots and hyphae, forming the root compartment. This was referred to as the inner soil. The soil surrounding the bag in the pot formed the hyphal compartment and could be accessed only by hyphae. This was referred to as the outer soil.

All pots were placed in a heated greenhouse maintained at a constant 15 °C in a random arrangement. An automatic watering system using capillary matting was in place.

2.2.4 Experimental layout

2.2.4.1 Experiment 1 – Benlate addition experiment

This experiment was based on the PP1 site soil and had a control (no P addition) and 3 P treatments. The P treatments were: P added at 2 rates 100 and 400 mg P/l soil in the form of Po, P added at 1 rate 100 mg P/l soil in the form of Pi. This was carried out for soil treated and untreated with the fungicide benlate. The growth bags were used in this experiment. Soil inside and outside the growth bag received the same P treatment. Where benlate treatment was applied, soil inside and outside the growth received benlate. Each control and P treatment had 3 replicate pots, giving a total of 24 pots. This experiment was carried out concurrently for the species *Dactylis glomerata* and *Plantago lanceolata.* (Table 2.1, a) The 100 mg P / l soil Pi treatment was included to examine in order to compare the affects of a Po treatment and a Pi treatment at equivalent concentrations.

Table 2.1(a): Experimental layout for the benlate addition experiment using the species *Dactylis glomerata* and *Plantago lanceolata.* There were 3 replicate pots per treatment. This layout was replicated for soil treated with benlate at a rate of 0.89 g benlate/kg soil

P Treatments	Conc P added	Source of P
Control (PP1 soil)	no P addition	
100Po (PP1 soil)	100 mg P/l soil	IHP
400Po (PP1 soil)	400 mg P/l soil	IHP
100Pi (PP1 soil)	100 mg P/l soil	Pi fertiliser

The benlate addition experiment was also carried out using the species *Agrostis stolonifera.* However a 200 mg P/l soil Po treatment was included to provide an intermediate treatment to the low 100 mg P/l soil and the high 400 mg P/l soil treatments. Each control and P treatment had 3 replicate pots, giving a total of 30 pots. (Table 2.1 b)

Table 2.1 (b): Experimental layout for the benlate addition experiment using the species *Agrostis stolonifera.* There were 3 replicate pots per treatment. This layout was replicated for soil treated with benlate at a rate of 0.89 g benlate / kg soil

P Treatments	Conc P added	Source of P
Control (PP1 soil)	no P addition	
100Po (PP1 soil)	100 mg P/l soil	IHP
200Po (PP1 soil)	200 mg P/l soil	IHP
400Po (PP1 soil)	400 mg P/l soil	IHP
100Pi (PP1 soil)	100 mg P/l soil	Pi fertiliser

For experiments described in Table 2.1 a and b:

- Organic phosphate was added in the form of phytic acid (myo-inositol hexakis [dihydrogen phosphate], Sigma Chemicals) which was added to the soil as a powder and was thoroughly mixed with soil before the soil was put into the 'growth bags' and plant pots.
- Inorganic phosphate was added in the form of Super Phosphate fertiliser (A.J. Bower Fertilisers) which was added to the soil as a powder and was thoroughly mixed with soil before the soil was put into the 'growth bags' and plant pots.
- Benlate (Du Pont Chemicals) was added to the soil at the rate of 0.89 g benlate/kg soil, as a solution (0.89 g benlate in 28 ml distilled water) and was thoroughly mixed with the soil before the soil was put into the 'growth bags' and plant pots. Where soil was not treated with benlate 28 ml of water per kg soil was added to the soil to ensure all treatment received the same levels of moisture.
- Each pot was filled with 400 g soil (200 g in 'growth bag', 200 g in surrounding area) of the appropriate treatment soil. One seedling was transplanted to each pot in the growth bag.

2.2.4.2 Experiment 2 – Hyphal exploration experiment

This experiment was based on the PP1 site soil and had a control (no P addition) and 1 P treatment, 60 mg P/l soil in the form of Po (abbreviated to 60Po). In the case of the P treatment pots only the soil outside the growth bag was amended with 60 mg P/l soil in the form of Po. Soil inside the bag was PP1 soil with no P addition. For the control, soil inside and outside the growth bag was PP1 soil with no P amendments. This control and P treatment were carried out for soil treated and untreated with the fungicide benlate. Where benlate was added to soil, this was to soil inside and outside the growth bag.

Each control and P treatment had 3 replicate pots, giving a total of 12 pots. This experiment was carried out for the species *Plantago lanceolata.* (Table 2.2). A P concentration of 60 mg P / l

soil was used in this experiment to examine how efficient VAM hyphae were in such a low P concentration soil in obtaining P for plant growth, and to determine if VAM presence was affected by a low soil P concentration such as this.

Table 2.2: Experimental layout for the hyphal exploration experiment using the *Plantago lanceolata*. There were 3 replicate pots per treatment. X indicates no addition, YES indicates addition of 60Po and / or benlate. Where benlate was added this was at a rate of 0.89 g benlate / kg soil.

	Soil Inside Growth Bag		**Soil Outside Growth Bag**	
Treatment	**60Po**	**+Benlate**	**60Po**	**+Benlate**
Control (PP1)	X	X	X	X
Control + Benlate (PP1)	X	YES	X	YES
60Po (PP1)	X	X	YES	X
60Po + Benlate (PP1)	X	YES	YES	YES

Organic phosphate and benlate were added to the soil as described for experiment 1. Each pot was filled with 400 g soil (200 g in 'growth bag', 200 g in surrounding area) of the appropriate treatment soil. One seedling was placed in each pot in the growth bag.

2.2.5 Harvesting

Experiments were harvested 9 weeks after transplanting seedlings into treatment pots. On harvesting each pot, soil from inner root compartment and outer hyphal compartment was put into separate labelled plastic bags and stored at 4°C until analysis. All soil analyses: soil phosphatase activity, soil total, inorganic and organic P, and soil hyphal content were carried out for inner and outer soil for each replicate pot.

Above ground plant material was excised from the roots. Above ground material was dried for dry weight measurements and total P analysis. Roots were stored in distilled water until root phosphatase analysis was carried out. The root material remaining after the root monoesterase analysis was stored in FAA (formaldehyde: acetic acid: alcohol) until % root length colonisation by vesicular arbuscular mycorrhizae was analysis was carried out.

2.2.6 % Root length colonisation by vesicular arbuscular mycorrhizae

In order to quantify the % root length colonisation by vesicular arbuscular mycorrhizae (VAM) the VAM material in the root was stained. The method of staining was a hot staining method described by Kormanik and McGraw (1982).

Roots which had been stored in FAA since harvest in order to preserve the VAM structures within the root were washed with distilled water and blotted dry. Approximately 0.06 g of root material was put into a test tubes and 10% KOH (BDH chemicals) added to cover the root material. The tubes were placed in a water bath at 90°C for 30 minutes. Roots were drained and rinsed with distilled water. The root material was transferred to clean test tubes containing 1% HCL (BDH chemicals) and left for one hour. The 1% HCL was poured off and 0.01% acid

fuchsin dissolved in destaining solution (lactic acid, glycerol and distilled water in a ratio of 14:1:1) was added to the test tubes. These test tubes were placed in a water bath at 90 °C for 25 minutes. Roots were then drained and washed with distilled water. The root material was transferred to clean test tubes containing destaining solution and left for 16 hours. The root material was then mounted in destaining solution on clean microscope slides and a cover slip placed on them.

Quantification of the % root length colonisation by VAM was carried out using the method of McGonigle *et al.*, (1990). The slide was placed on the stage of a compound microscope equipped with a cross-lines eyepiece graticule. The slide was scanned methodically, one axis of the graticule aligned with the long axis of each root encountered. For a count of the % of root length colonised by VAM (%RLC) the presence or absence of an arbuscular endophyte touched by the graticule axis that crossed the root, each time a root was encountered was scored. The presence or absence of an arbuscular endophyte at 100 intersections per slide was counted. Counts were converted to percentages as follows:

$$\%RLC = 100 \times \frac{\text{Number of intersections with arbuscular mycorrhizal presence}}{\text{Total number of intersections counted}}$$

The presence of an arbuscular endophyte at an intersection was indicated by hyphae, an arbuscule, vesicle or entry point of hyphae into a root.

2.2.7 Quantification of external hyphae in the soil

In order to quantify the VAM hyphae in the soil external to the root, the hyphal material was extracted from the soil and stained. The method of extraction and staining was described by Brundrett *et al.* (1994).

30 g of fresh soil was added to 500 ml of distilled water in a beaker and stirred on a magnetic plate at high speed for 2 minutes. 100 ml of the mixture was immediately poured into a 100 ml tall beaker and stirred at approximately 50% of the speed for 30 seconds. The mixture was then counter-stirred using a flat stirring rod for 30 seconds. Using a clean syringe 5 ml was extracted from the mixture and filtered through a 0.45 μm nitro-cellulose membrane filter on a Millipore filter apparatus. A few drops of 0.01% acid fuchsin dissolved in destaining solution (lactic acid, glycerol and distilled water in a ratio of 14:1:1) were dropped on the filter while on the filter apparatus under vacuum. The membrane was transferred to a clean slide and left with any remaining acid fuchsin for 1 hour. The membrane was then replaced on the filter apparatus and rinsed with distilled water while under vacuum. The filter was then placed on a clean slide and mounted in destain under a coverslip. Quantification of the length of soil hyphae was carried out using the method of Brundett *et al.* (1994).

2.2.8 Total, inorganic and organic P levels in soil

Total P was measured by the ignition method using 1 M H_2SO_4 (BDH chemicals) as the extracting solution. 2 g of dried soil was weighed out into a crucible and ignited at 550°C for 1 hour in a furnace (Thermolyne furnace Type 6000). The ignited sample was transferred to a 100 ml conical flask and 50 ml of 1 M H_2SO_4 was added. The mixture was heated at 90 °C for one hour. The cooled solutions were diluted with 50 ml of distilled water, filtered through Whatman no. 1 filter paper and made up to 100 ml in a volumetric flask. The solution was then analysed for P as described in section 2.2.10.

Inorganic P was measured using the method described above with the exception of the ignition step. The two grams of dried soil was transferred directly into the 100 ml conical flask containing 50 ml H_2SO_4 and heated at 90°C for one hour. Organic P was measured as the difference between total P and inorganic P.

2.2.9 General Experimental procedures

Analar grade reagents were used. Stock solutions of phosphorus standards (1000 ppm P) were prepared from oven dried KH_2PO_4 (Sigma Chemicals) and kept refrigerated at 4°C. Working standards (100 ppm P) were prepared weekly from which a range of standard solutions (0.2-0.4 mg/l solution) was prepared. Reagents were kept refrigerated at 4°C, and unstable solutions were prepared fresh on the day of analysis.

All standards and reagents were prepared using volumetric flasks. All glassware was soaked in 10% HCL (BDH Chemicals) and washed with distilled water and oven dried. Pipetting was carried out using Gilson automatic pipette. Dispensing was carried out using grade A graduated cylinders. Silica gel desiccators were employed for storage of dry material at room temperature.

2.2.10 Colorimetric determination of P

Phosphorus levels in samples were measured using a mixed reagent method, a modified version of that described by Eisenreich, Bannerman and Armstrong, (1975).

Stock solutions were prepared as follows:

- Antimony: .37 g $K(SbO)C_4H_4O_6.1/2H_2O$ was dissolved in distilled water and made up to 500 ml with distilled water. Any precipitate formed was removed by filtration. This reagent was stored in the fridge.
- Molybdate: 10.839 g of $Na_2MoO_4.2H_2O$ was dissolved in approximately 500 ml of distilled water and diluted to 1 litre.
- 3.6 N H_2SO_4: 100ml of concentrated H_2SO_4 were added to 500ml of distilled water. After cooling the solution was diluted to 1 litre.

The mixed reagent was prepared by measuring 23.5 ml 3.6 N H_2SO_4 into a 100 ml volumetric flask. 25 ml of antimony and 25 ml of molybdate stock solutions were added. 0.2 g ascorbic

acid was added and dissolved. The solution was then made up to 100 ml with distilled water. Where mixed reagent was being used for analysing plant material samples which had been digested in concentrated H_2SO_4, 13.5 ml 3.6 N H_2SO_4 was used. The completion of the mixed reagent remains as described.

1 ml of mixed reagent was pipetted into test tubes containing 5 ml of sample (or a suitable dilution of sample) or 5 ml standard solution. Absorbance was read on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer) at 882 nm after approximately 15 minutes. A standard curve was obtained using a range of standards (0.2, 0.4, 0.6, 0.8 and 1.0 mg P/l) and the concentration of P in samples read from this standard curve.

2.2.11 Plant dry weights

Following harvest root and shoot material was placed in labelled envelopes and dried in an oven at 80°C for 48 hours. The material was then cooled in a desiccator and weighed to 4 decimal places.

2.2.12 Plant material digest for total P

Shoot material was cut into small pieces using a scissors. 0.25 g of shoot material was then weighed into a digest tube. 0.5 g catalyst mixture (100 g anhydrous sodium sulphate mixed with 3 g powdered copper sulphate, BDH Chemicals) and 5 ml concentrated H_2SO_4 were added. Tubes were placed in a hot block at 370°C for 2 hours 15 minutes. Following cooling approximately 50 ml distilled water was added to the digest tubes and the samples were filtered through Whatman no. 1 filter paper into 100 ml volumetric flasks. Flasks were made up to volume with distilled water. The solution was then analysed for P as described in 2.2.10.

2.2.13 Assay for soil acidic phosphomonoesterase activity

As described in Chapter 4, section 4.2.6.1

3.2.14 Assay for root acidic phosphomonoesterase activity assay

As described in Chapter 4, section 4.2.6.2

2.2.15 Statistical procedures

All statistical analysis was carried out using the statistics package Data Desk, version 6.0. All data was checked for normality before statistical analysis. Where necessary data was transformed to obtain a normal distribution. Soil monoesterase, phosphate and external soil VAM hyphae data was analysed using a three way ANOVA with P treatment, benlate treatment and location of the soil (i.e. inner or outer soil) as the three factors. Plant dry weights, P concentrations and contents, %RLC and root monoesterase data were analysed using a two way ANOVA with P treatment and benlate treatment as the two factors.

2.3 Results

Plate 2.1 illustrates the infection of a *Dactylis glomerata* root by a vesicular arbuscular mycorrhizal fungus. In order to visualise the fungus within the root, the root was hot stained with acid fuchsin, following the method of Kormanik and McGraw (1982). Visible are arbuscules within the cells, vesicles and intercellular hyphae. Plate 2.2 presents an overview of vesicular arbuscular mycorrhizae structures within an *Agrostis stolonifera* root. Plate 2.3 illustrates the entry point of a vesicular arbuscular mycorrhizal hypha into a *Dactylis glomerata* root. The appressorium, the swelling in the hypha where it has penetrated the root, is visible.

Where results for control plants were not included in some graphs this was because there was insufficient plant material available for these tests. Where there was a limited supply of plant material this material was used for the root acidic monoesterase assay.

Section A: Results from experiment 1 – The Benlate Addition Experiment

In the description of the results that follows, P treatment referred to the addition of organic phosphate to the soil at the 2 concentrations employed and the addition of inorganic phosphate to the soil at the single concentration employed. The two organic phosphate concentrations used were 100 and 400 mg P/l soil abbreviated to 100Po and 400Po for the purpose of graphs and the results and discussion section. The single inorganic phosphate concentration used was 100 mg P/l soil abbreviated to 100Pi. The control treatment is PP1 soil with no phosphate addition. Benlate treatment referred to the addition of the fungicide benlate to the soil.

The term 'location' referred to either inner root compartment soil or the outer hyphal compartment soil, which are abbreviated to inner and outer soil respectively. Start soil referred to the soil as prepared at the start of the experiment, with the relevant P and benlate treatment, but before contact with any plants. Post harvest soil refers to soil from which the plants have been harvested after their 12-week growth period.

2.3.1 *Dactylis glomerata*

Plate 2.4 presents *Dactylis glomerata* growing in the control and P treatment soils with no benlate treatment. Plate 2.5 presents *Dactylis glomerata* growing in the control and P treatment soils with benlate treatment. The control and 100Po treatment plants grown in benlate treated soils were smaller than those grown in soils with no benlate treatment. In the case of the 400Po and 100Pi treatment plants the plants grown in the benlate treated soils were larger than those grown in soils with no benlate treatment. (Plates 2.4 and 2.5).

2.3.1.1 Soil phosphate

The soil total P (Tp), inorganic P (Pi) and organic P (Po) concentration data were presented in Fig 2.3 (a, b), 2.4 (a, b) and 2.5 (a, b) respectively. Three way ANOVAs were carried out to

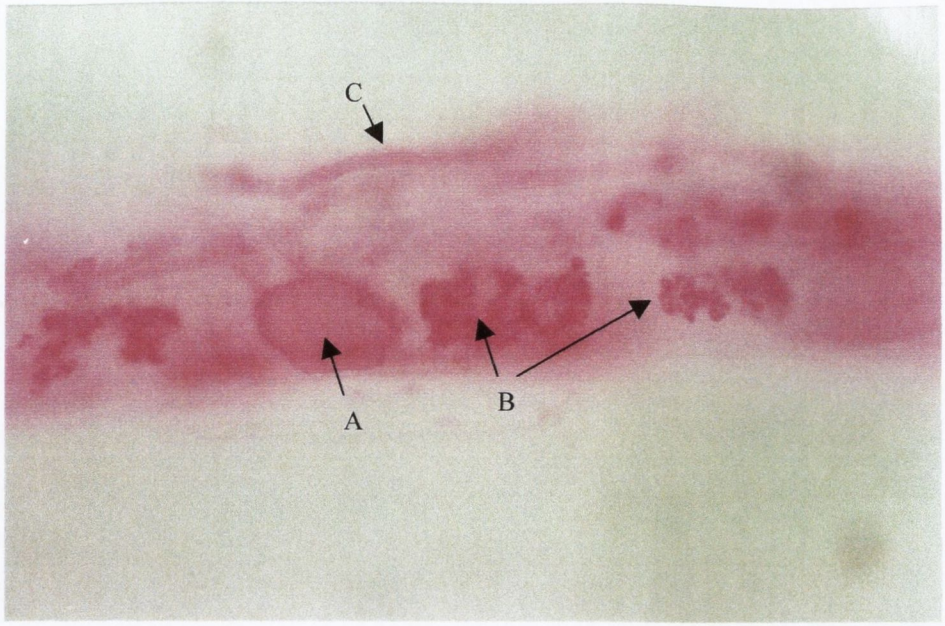


Plate 2. 1: A section of a *Dactylis glomerata* root infected by a vesicular arbuscular mycorrhizal fungus. The structures are identified as follows: A – vesicle, B – arbuscule, C – intercellular hypha

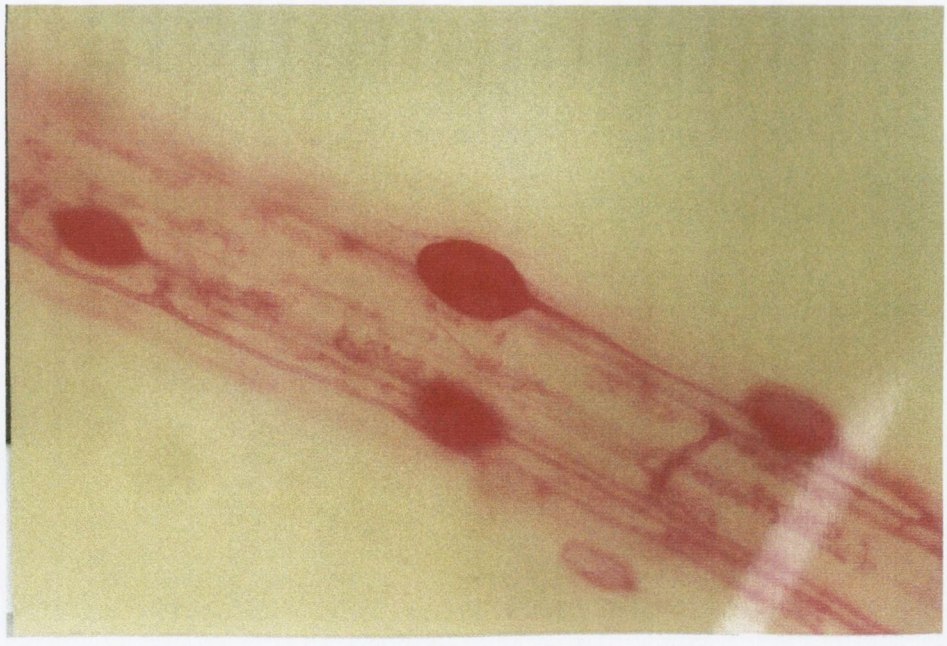


Plate 2. 2: An overview of the vesicular arbuscular mycorrhizal structures within a *Agrostis stolonifera* root showing arbuscules, vesicles and hyphae.

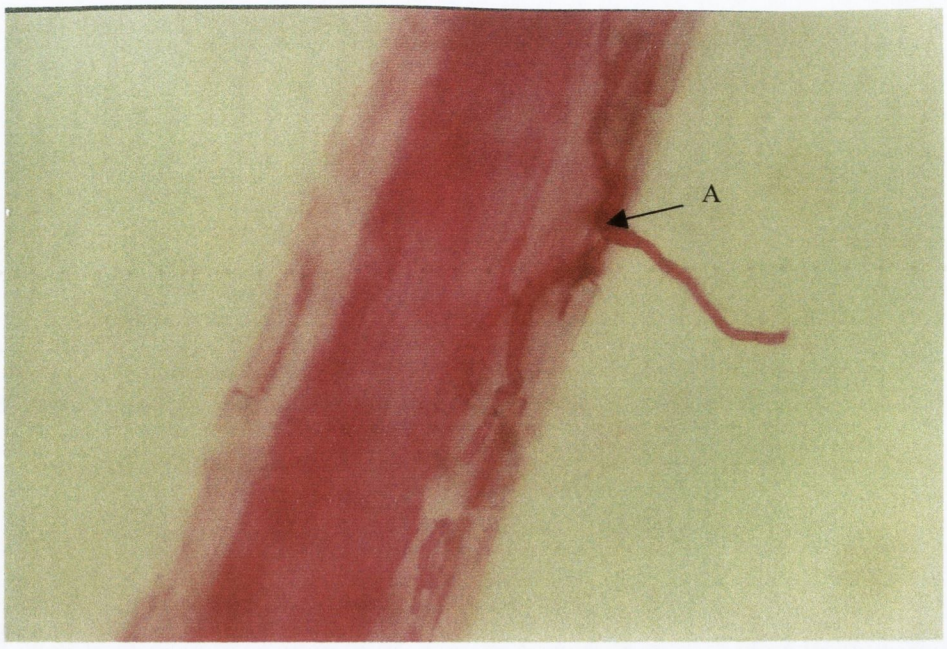


Plate 2. 3: The entry point of a vesicular arbuscular mycorrhizal hypha into a *Dactylis glomerata* root. The swelling, known as the appressorium, where the hypha has penetrated the root is indicated as A.

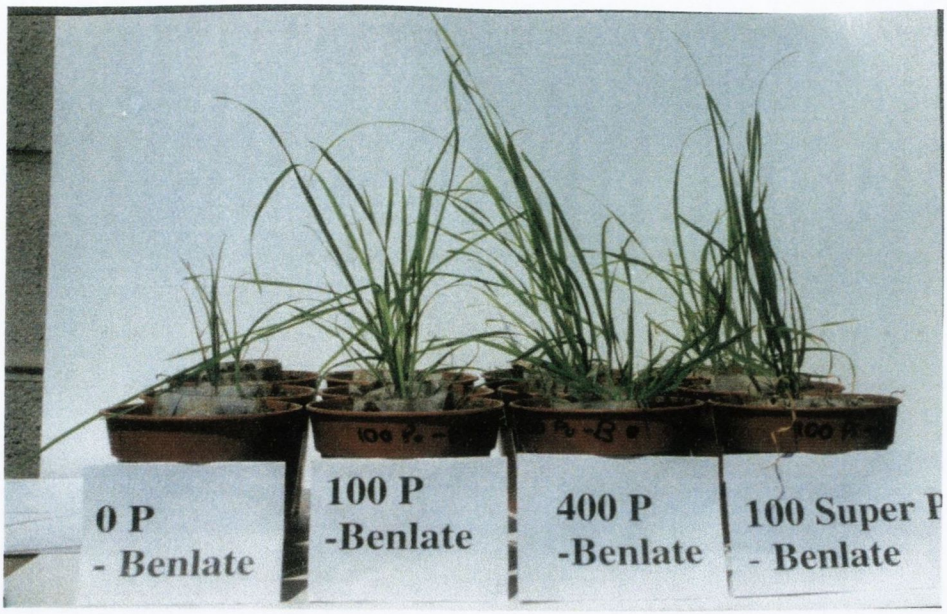


Plate 2. 4:*Dactylis glomerata*, growing in the control (0P) and P treatment soils with no benlate treatment on the day of harvest.

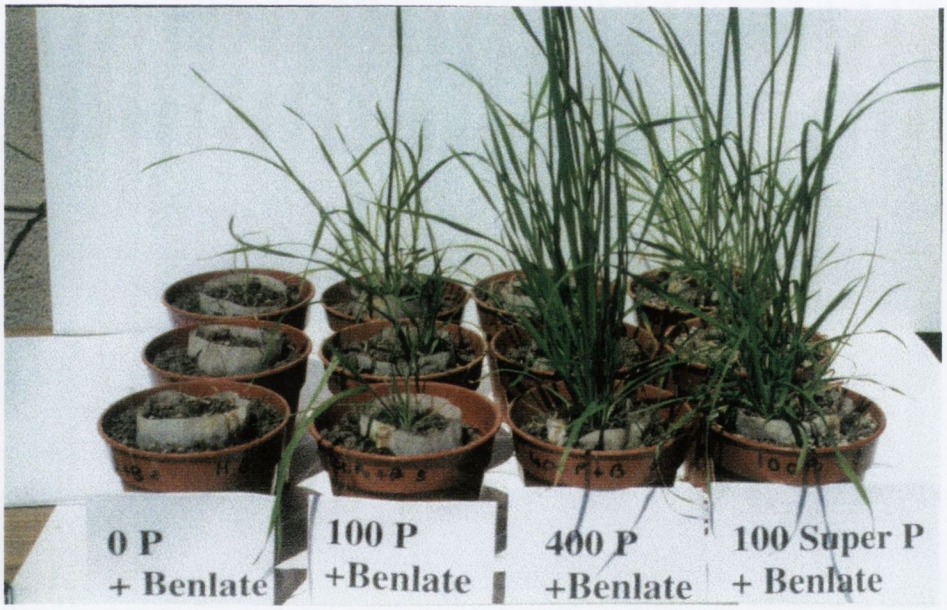


Plate 2. 5:*Dactylis glomerata*, growing in the control (0P) and P treatment soils with benlate treatment on the day of harvest.

determine the affect of P treatment and benlate treatment and location of soil on soil Tp, Pi and Po concentration. These ANOVAs were presented in Appendix 2, Tables 1, 2, and 3.

For Tp and Po there was a highly significant P affect (Tp F ratio 62.25***, Po F ratio 19.84***). There was also a significant P - location of soil interaction (Tp F ratio 5.21**, Po F ratio 5.17**) For Pi only P treatment had a significant affect (F ratio 23.60***). All other treatments and interactions were not significant. (Appendix 2, Tables 1, 2 and 3).

Total P

Fig 2.3 (a) presented soil Tp concentration data for soil untreated with benlate. Fig 2.3 (b) presented soil Tp concentration data for soil treated with benlate.

From Fig 2.3 (a, b) there was an expected increase in start soil Tp levels with increasing levels of P added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Tp concentrations. There was a significant decrease in Tp level for the 400Po treatment outer soil with benlate addition (Fig 2.3 b). There was significant increase in Tp level for the 100Po and 100Pi treatment outer soil with no benlate addition (Fig 2.3 a).

There was no significant affect of benlate treatment on the soil Tp concentration of the postharvest soils (Appendix 2, Table 1 and Fig 2.3 a, b).

From Fig 2.3 (a, b) for the post harvest soil there was an increase in total P concentration in the soil with increasing levels of P addition for all soils. This significant affect of P treatment on Tp concentration was examined using a Scheffe post hoc test (Table 2.3).

Table 2.3: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Tp concentration for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	***
control < 400Po	***
control < 100Pi	***
400Po > 100Po	***
400Po > 100Pi	***
100Pi - 100Po	ns

The significant interaction between P treatment and the location of soil was examined using a Scheffe post hoc test (pooled –Benlate/+Benlate means) (Table 2.4). All levels of Po addition resulted in an increase in soil Tp for the inner and outer soils compared to the control.

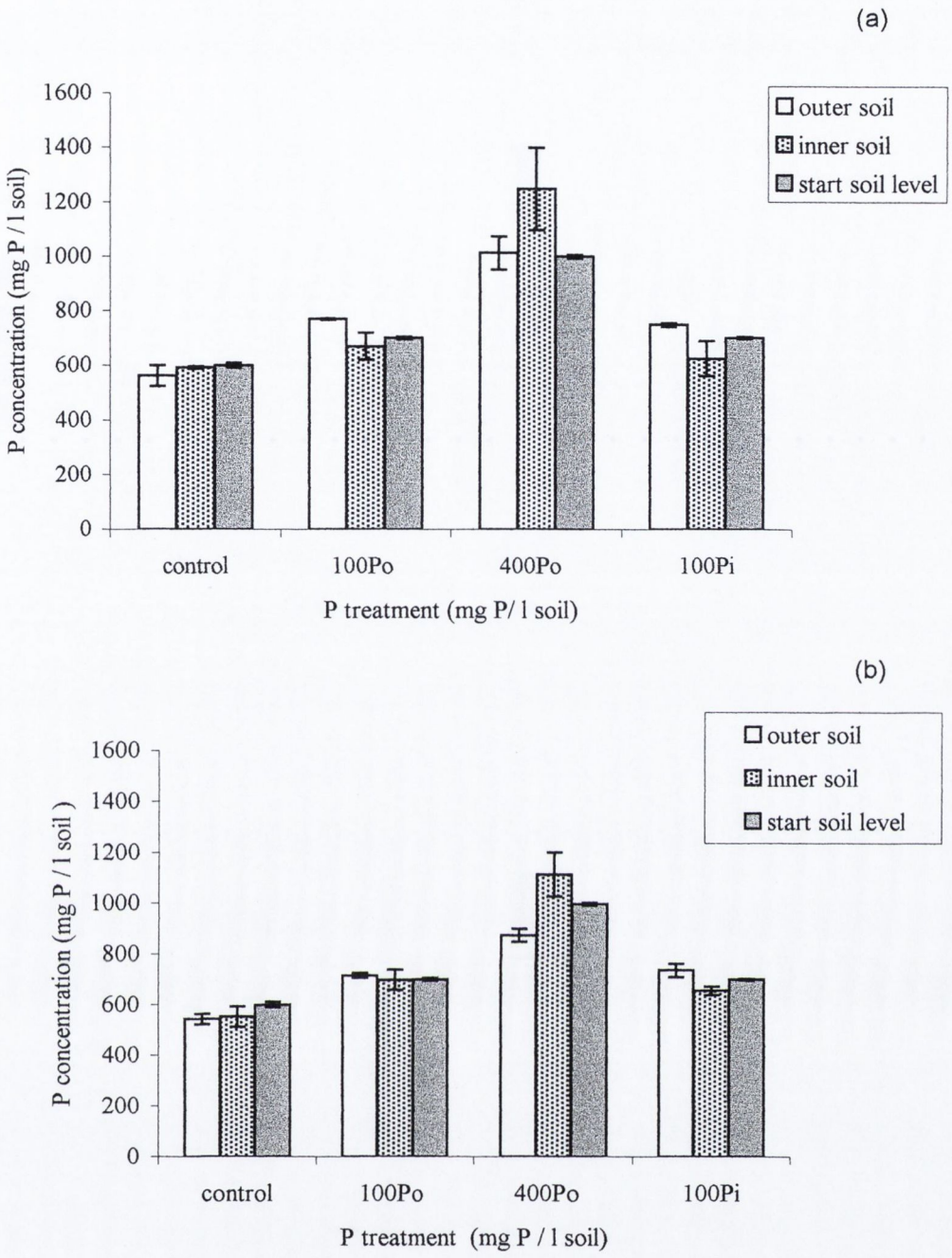


Fig 2.3: soil total phosphate concentration for the control and P treatment soils of the *Dactylis glomerata* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

Table 2.4: probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors location of soil and P treatment, that affected soil Tp concentration for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
Inner, control - Outer, control	ns
Inner, 100Po - Outer, 100Po	ns
Inner, 400Po - Outer, 400Po	ns
Inner, 100Pi - Outer, 100Pi	ns
Inner, control < Inner, 100Po	*
Inner, control < Inner, 400Po	***
Inner, control - Inner, 100Pi	ns
Outer, control < Outer, 100Po	***
Outer, control < Outer, 400Po	***
Outer, control < Outer, 100Pi	***

Inorganic P

Fig 2.4 (a) presented soil Pi concentration data for soil untreated with benlate. Fig 2.4 (b) presented soil Tp concentration data for soil treated with benlate.

From Fig 2.4 (a, b) the only significant change in start soil Pi was in the 100Pi treatment. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and postharvest soil Pi concentrations. There was a significant increase in Pi level for the 100Po and 400Po treatment inner soil and the 100Pi treatment outer soil with benlate addition (Fig 2.4 b). There were significant increases in Pi level for all treatments, inner and outer soil with no benlate addition (Fig 2.4 a).

There was no significant affect of benlate treatment on the soil Pi concentration of the postharvest soils (Appendix 2, Table 2 and Fig 2.4 a, b) .

From Fig 2.4 (a, b) for the post harvest soil there was an increase in Pi concentration for all soils. This significant affect of P treatment on Pi concentration was examined using a Scheffe post hoc test. (Table 2.6).

Table 2.5: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Pi concentration for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control < 400Po	***
control < 100Pi	***
400Po > 100Po	***
400Po - 100Pi	ns
100Pi > 100Po	**

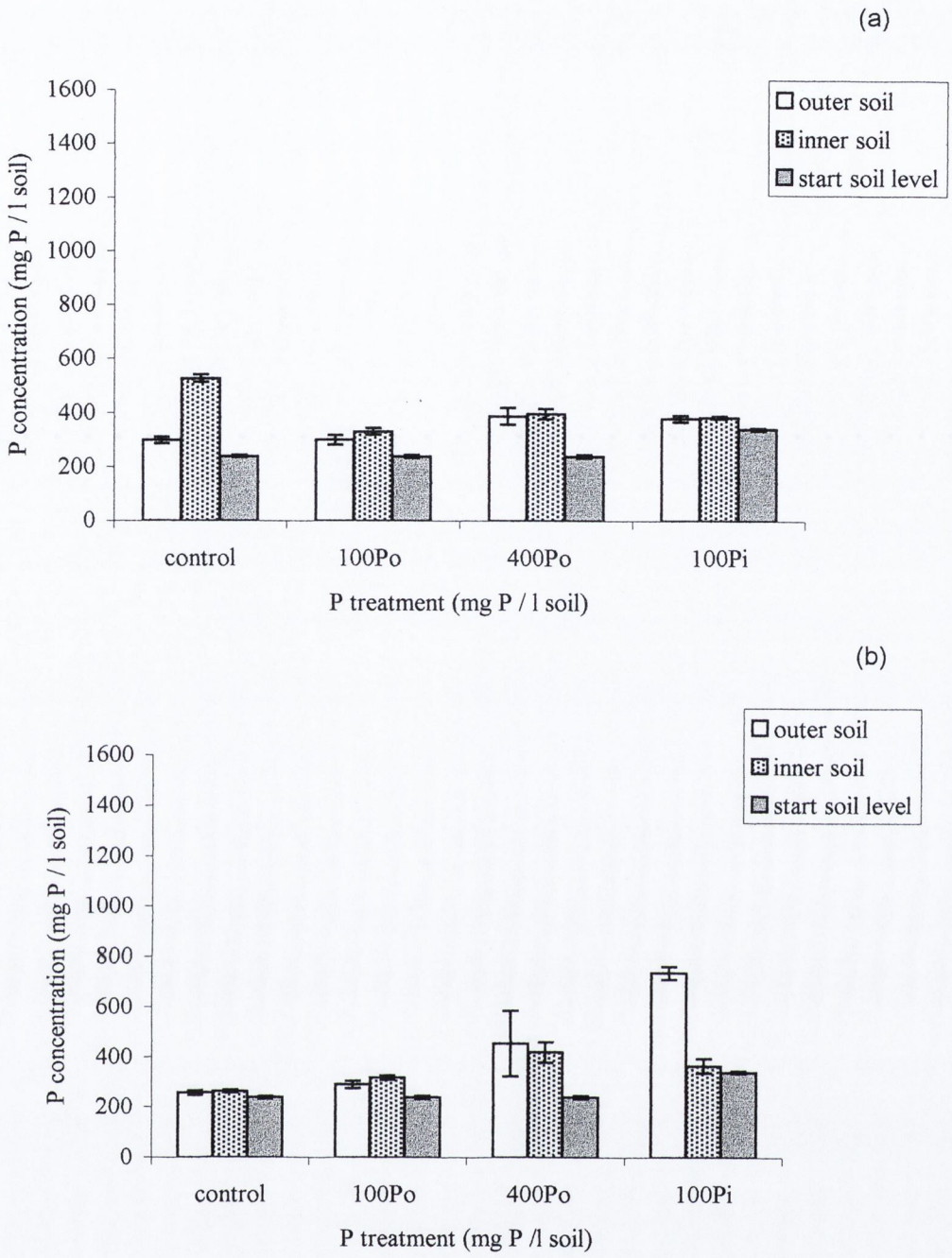


Fig 2.4: soil inorganic phosphate concentration for the control and P treatment soils of the *Dactylis glomerata* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

Organic P

Fig 2.5 (a) presented soil Po concentration data for soil untreated with benlate. Fig 2.5 (b) presented soil Po concentration data for soil treated with benlate.

From Fig 2.5 (a, b) there was an increase in start soil Po levels with increasing levels of P added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and postharvest soil Po concentrations. There was a significant decrease in Po level for the control, 100Pi and 400Po treatment outer soils with benlate addition (Fig 2.5 b). There was significant decrease in Po level for the control inner soil and 400Po treatment outer soil with no benlate addition (Fig 2.5 a).

There was no significant affect of benlate treatment on the soil Po concentration of the postharvest soils (Appendix 2 Table 3 and Fig 2.5 a, b).

From Fig 2.5 (a, b) for the post harvest soil there was an increase in Po concentration with increasing levels of P addition for all soils This significant affect of P treatment on Po concentration was examined using a Scheffe post hoc test. (Table 2.6).

Table 2.6: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Po concentration for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	*
control < 400Po	***
control - 100Pi	ns
400Po > 100Po	**
400Po > 100Pi	***
100Pi - 100Po	ns

There was a significant interaction between P treatment and the location of soil (Appendix 2, Table 3) that was examined using a Scheffe post hoc test (pooled –Benlate/+Benlate means) (Table 2.7). There were minor differences in the statistical significance and magnitude of the increases in soil Po concentration caused by Po addition, between inner and outer soils.

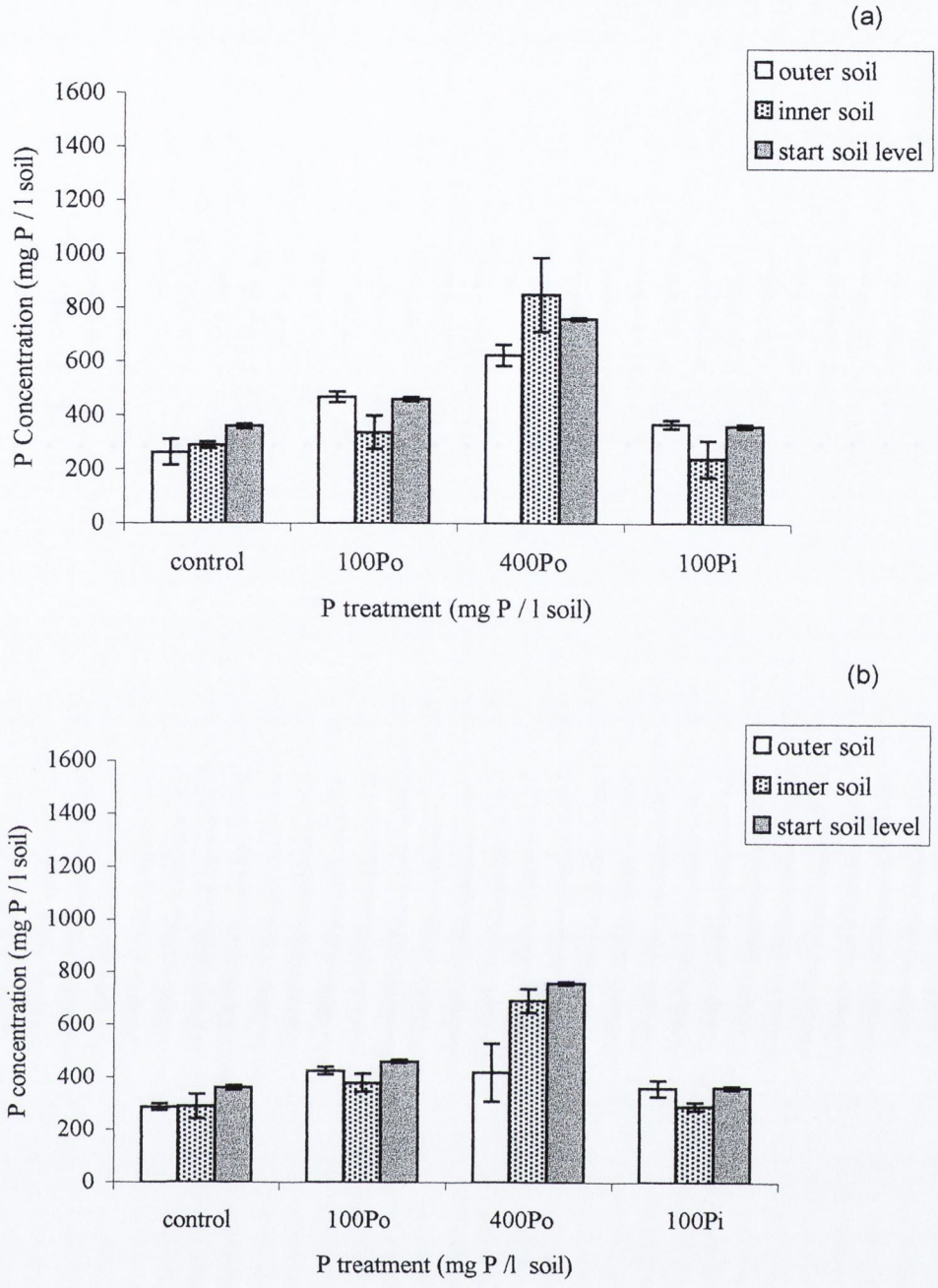


Fig 2.5: soil organic phosphate concentration for the control and P treatment soils of the *Dactylis glomerata* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

Table 2.7: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors location of soil and P treatment, that affected soil Po concentration for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
Inner, control - Outer, control	ns
Inner, 100Po - Outer, 100Po	ns
Inner, 400Po - Outer, 400Po	ns
Inner, 400Pi - Outer, 100Pi	ns
Inner, control - Inner, 100Po	ns
Inner, control < Inner, 400Po	***
Inner, control - Inner, 100Pi	ns
Outer, control < Outer, 100Po	*
Outer, control < Outer, 400Po	**
Outer, control - Outer, 100Pi	ns

2.3.1.2 Colonisation of Roots by VAM

The colonisation of roots by vesicular arbuscular mycorrhizal (VAM) fungi was measured by % root length colonisation (%RLC). The %RLC data was presented in Fig 2.6. A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on %RLC. This ANOVA was presented in Appendix 2, Table 4.

Figure 2.6 presented %RLC data *Dactylis g.* plants grown in benlate treated and untreated soil. From Fig 2.6 there was a decrease in %RLC, for all P treatments, in plants grown in benlate treated soil from the %RLC colonisation present in non-benlate treated plants. This affect of benlate treatment on %RLC was highly significant (F ratio 20.15***) (Appendix 2, Table 4).

Percentage RLC was affected by addition of P in both benlate treated plants and non-benlate treated plants (Fig 2.6, Appendix 2, Table 4). After an initial drop in %RLC from the control plants to the 100Po treatment plants there was an increase in %RLC from the 100Po treatment to the 400Po, which had a %RLC similar to that present in the control. There was then an increase in %RLC from the 400Po treatment to the 100Pi treatment (Fig 2.6). This significant affect of P treatment (F ratio 3.64*) was examined using a Scheffe post hoc test. (Table 2.8).

Table 2.8: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on %RLC for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control - 400Po	ns
control - 100Pi	ns
400Po - 100Po	ns
400Po - 100Pi	ns
100Pi > 100Po	*

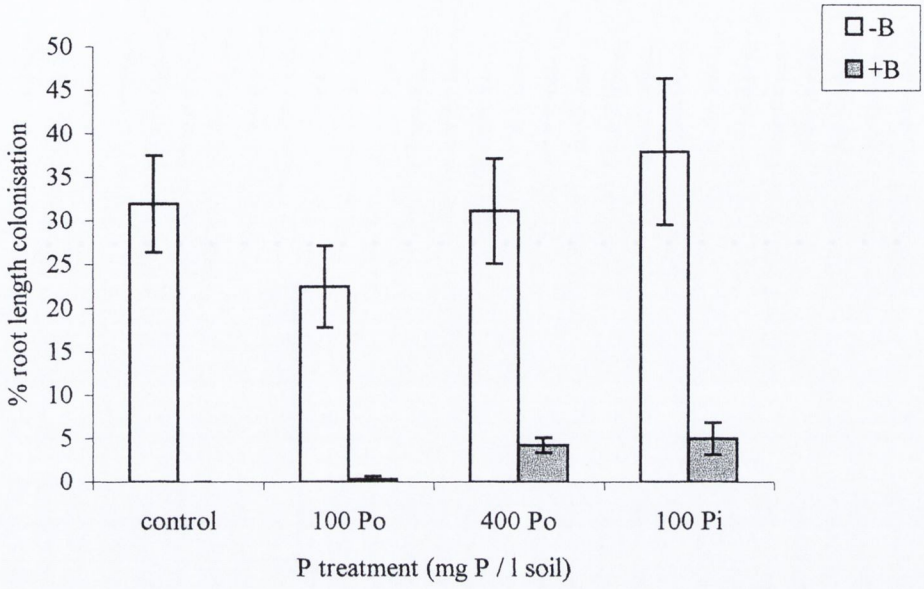


Fig 2.6: % root length colonisation for the control and P treatment plants, of the *Dactylis glomerata* experiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

2.3.1.3 Soil and root monoesterase activity

The phosphatase activity results are presented as follows:

soil acidic monoesterase data in Fig 2.7 (a, b) and the root acidic monoesterase data in Fig 2.8. A three way ANOVA was carried out to determine the affect of P treatment, benlate treatment and location of soil on soil acidic monoesterase activity. A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on root acidic monoesterase activity. These ANOVAs were presented in Appendix 2 Tables 5 and 6 respectively.

There was a significant affect of P treatment on soil acidic monoesterase activity (F ratio 3.53*) (Appendix 2, Table 5) and root acidic monoesterase activity (F ratio 6.16**) (Appendix 2, Table 6). All other treatments and interactions were not significant (Appendix 2, Tables 5 and 6).

Soil acidic monoesterase

Fig 2.7 (a) presented soil acidic monoesterase activity data for soil not treated with benlate. From Fig 2.7 (a), for the outer compartment soil, there was a drop in monoesterase activity from that present in the control soil to that in the 100Po treatment soil. The level then increased in the 400Po treatment soil. A drop in monoesterase activity was detected for the 100Pi treatment soil. For the inner compartment soil (Fig 2.7 a) the level of activity was similar for the control and 100Po treatment soil. The activity then increased in the 400Po treatment soil with a further slight increase in the 100Pi treatment soil.

Fig 2.7 (b) presented soil acidic monoesterase activity data for soil treated with benlate. From Fig 2.7 (b), for outer compartment soil, there was an increase in soil phosphatase activity from that present in the control soil to that detected in the 100Po and 400Po treatment soils, both of which showed similar levels of phosphatase activity. There was then a further increase in activity to that present in the 100Pi treatment soil. A similar pattern was repeated for inner compartment soil (Fig 2.7, b). The phosphatase level in increased from that present in the control soil to that found in the 100Po treatment soil. There was then a drop to the activity in the 400Po soil to a level similar to that present in the control soil. The level then increased to its highest value for the 100Pi treatment soil.

There was no significant affect of location of soil on the monoesterase activity level (Appendix 2 Table 5).

The significant affect of P treatment on soil acidic monoesterase activity (Appendix 2, Table 5) was examined using a Scheffe post hoc test Table 2.9.

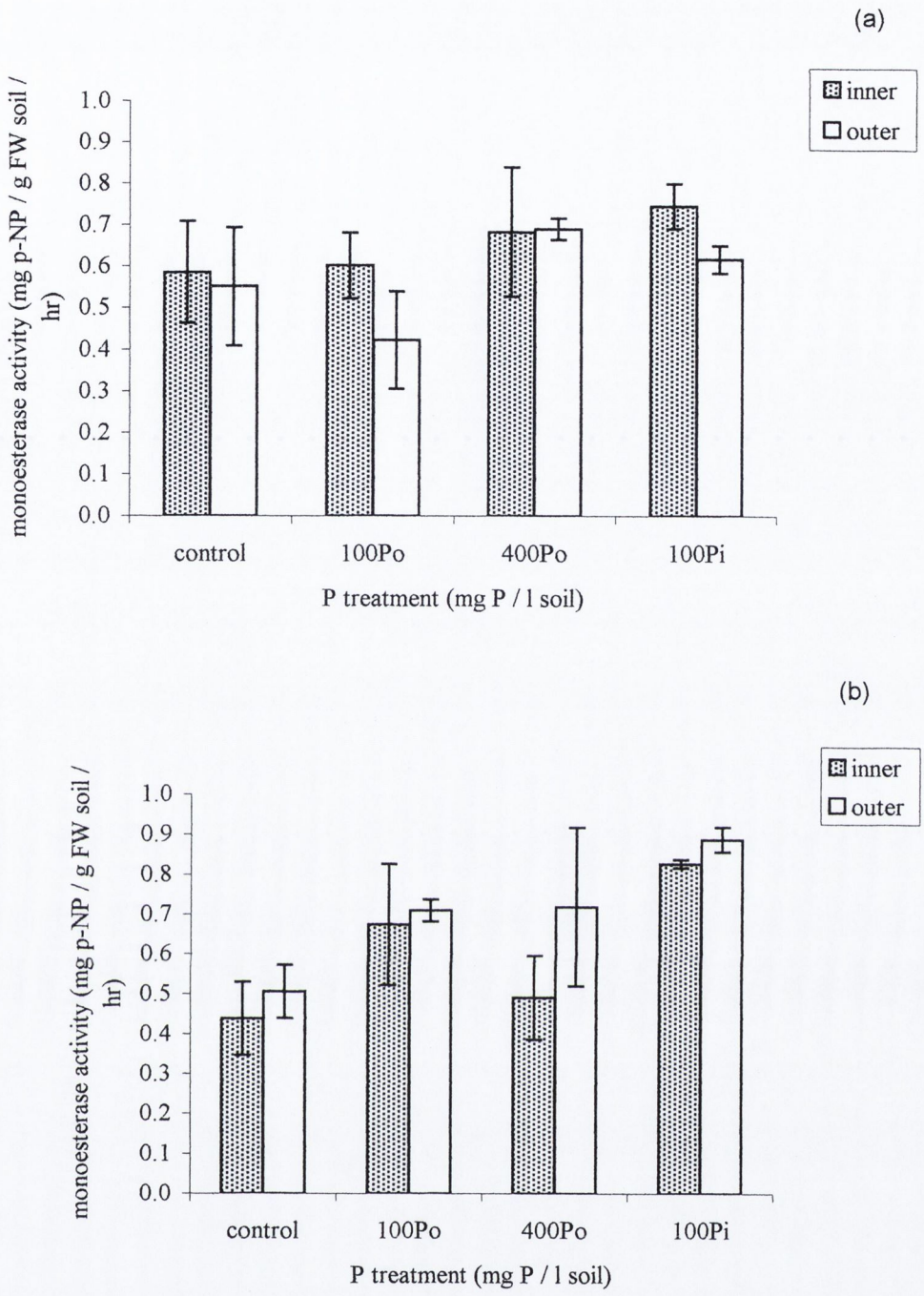


Fig 2.7: soil acidic monoesterase activity for the control and P treatment soils for the Dactylis glomerata experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

Table 2.9: probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on soil acidic monoesterase activity for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control - 400Po	ns
control < 100Pi	*
400Po - 100Po	ns
400Po - 100Pi	ns
100Pi - 100Po	ns

Root acidic monoesterase

Fig 2.8 presented root acidic monoesterase activity data for plants grown in soil treated and untreated with benlate. From Fig 2.8, for all plants, there was a dramatic decrease in root acidic monoesterase activity from that of the control to the 100Po, 400Po and 100Pi treatment plants. The activity detected for the 100Po, 400Po and 100Pi plants was similar. This significant affect of P treatment on root acidic monoesterase activity was examined using a Scheffe post hoc test (Table 2.10).

Table 2.10: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root acidic monoesterase activity for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control > 100Po	*
control > 400Po	*
control > 100Pi	*
400Po - 100Po	ns
400Po - 100Pi	ns
100Pi - 100Po	ns

2.3.1.4 Shoot Dry Weight, P concentration and content

The shoot dry weight data, shoot P concentration data and shoot P content data was presented in Figs 2.9, 2.10 and 2.11 respectively. Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot dry weight, P concentration and P content. These ANOVAs were presented in Appendix 2, Tables 7, 8, 9.

There was a significant P affect on the shoot dry weight (F ratio 8.46***), shoot P concentration (F ratio 3.35*) and shoot P content (F ratio 5.62**) (Appendix 2, Tables 7, 8, 9). All other treatments and interactions were not significant (Appendix 2, Tables 7, 8, 9).

Shoot Dry Weight

Fig 2.9 presented shoot dry weight data for all plants. From Fig 2.9, for plants grown in soil not treated with benlate, there was a similar increase in shoot dry weight as a result of all P treatments. For plants grown in benlate treated soil there was an increase in dry weight as a result of the 100Po treatment, with further steep increases for the 400Po and 100Pi treatments.

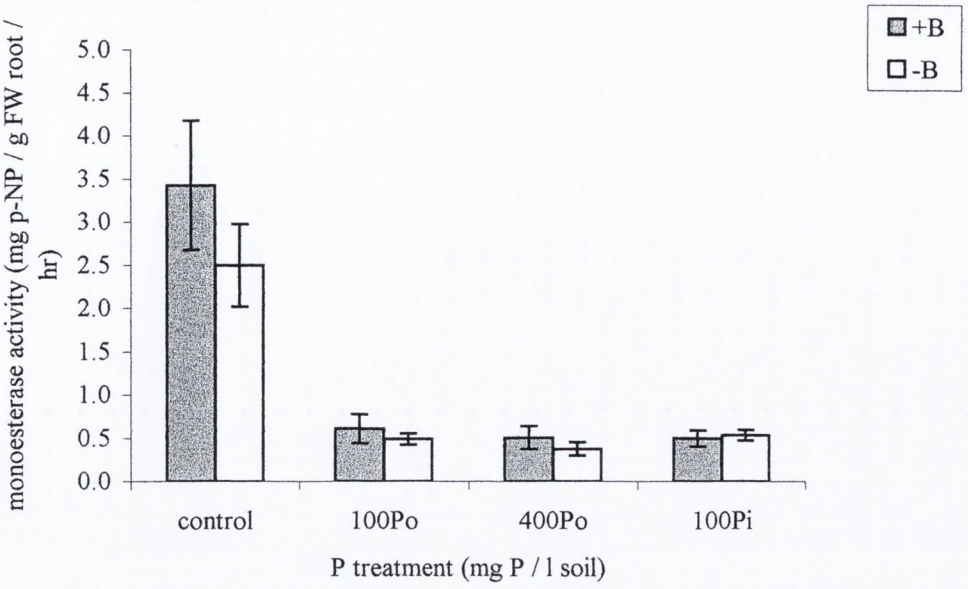


Fig 2.8: root acidic monoesterase activity for the control and P treatment plants, for the *Dactylis glomerata* experiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

This significant affect of P treatment on shoot dry weight was examined using a Scheffe post hoc test (Table 2.11).

Table 2.11: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot dry weights for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control < 400Po	**
control < 100Pi	**
400Po - 100Po	ns
400Po - 100Pi	ns
100Pi - 100Po	ns

Shoot P concentration

Fig 2.10 presented shoot P concentration data for all plants. From Fig 2.10, for plants grown in soil untreated with benlate, the shoot P concentration increased from that of the control plants, to 100Po treatment plants. There was a further increase to the 400Po and 100Pi treatment plants, which were similar in weight. This trend was repeated for plants grown in benlate treated soil (Fig 2.10). This significant affect of P treatment on shoot P concentration was examined using a Scheffe post hoc test (Table 2.12).

Table 2.12: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P concentrations for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	*
control < 400Po	*
control < 100Pi	**
400Po - 100Po	ns
400Po - 100Pi	ns
100Pi - 100Po	ns

Shoot P content

Fig 2.11 presented shoot P content data for all plants. From Fig 2.11, for plants grown in soil treated with benlate, the shoot P content increased from that of the control plants, to 100Po treatment plants. There was a further increase to the 400Po and 100Pi treatment plants. For plants grown in untreated soil there was a similar increase in shoot P content as a result of all P treatments (Fig 2.11). This significant affect of P treatment on shoot P content was examined using a Scheffe post hoc test (Table 2.13).

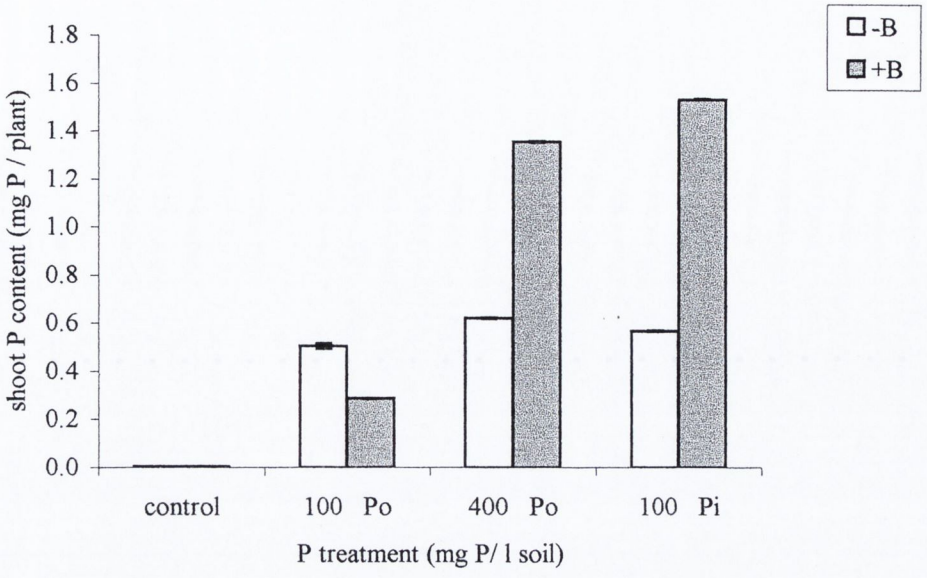


Fig 2.11: shoot P content for the control and P treatment plants of the *Dactylis glomerata* experiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

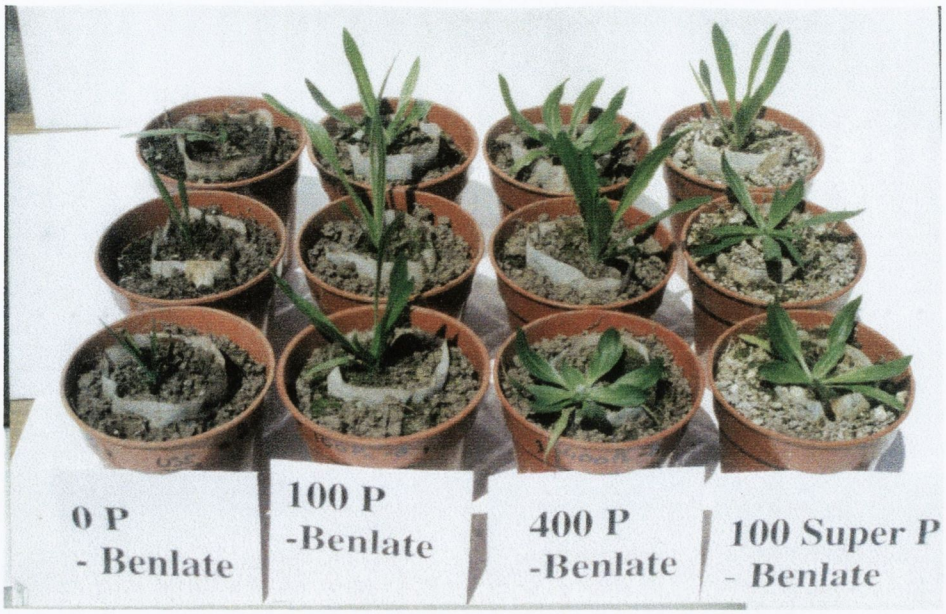


Plate 2. 6: *Plantago lanceolata*, growing in the control (0P) and P treatment soils with no benlate treatment on the day of harvest.

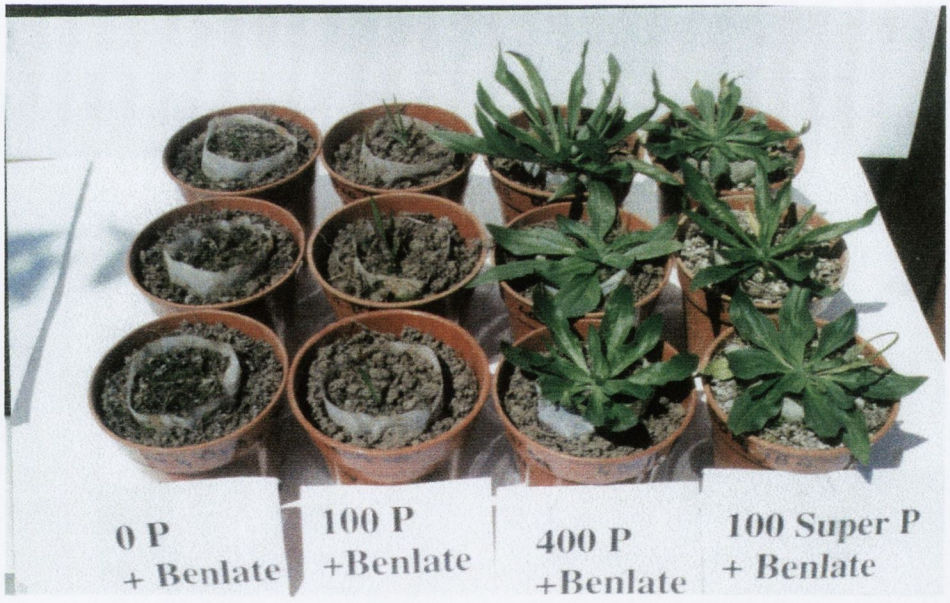


Plate 2. 7: *Plantago lanceolata*, growing in the control (0P) and P treatment soils with benlate treatment on the day of harvest.

Table 2.13: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P content for the *Dactylis g.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	*
control < 400Po	*
control < 100Pi	**
400Po - 100Po	ns
400Po - 100Pi	ns
100Pi - 100Po	ns

2.3.2 *Plantago lanceolata*

Plate 2.6 presented *Plantago lanceolata* growing in the control and P treatment soils with no benlate treatment. Plate 2.7 presented *Plantago lanceolata* growing in the control and P treatment soils with benlate treatment. The control and 100Po treatment plants grown in benlate treated soils were smaller than those grown in soils with no benlate treatment. In the case of the 400Po and 100Pi treatment plants, the plants grown in the benlate treated soils were larger than those grown in soils with no benlate treatment. (Plates 2.6 and 2.7).

2.3.2.1 Soil phosphate

The soil total P (Tp), inorganic P (Pi) and organic P (Po) concentration data were presented in Fig 2.12 (a, b), 2.13 (a, b) and 2.14 (a, b) respectively. Three way ANOVAs were carried out to determine the affect of P treatment and benlate treatment and location of soil on soil Tp, Pi and Po concentration. These ANOVAs were presented in Appendix 2, Tables 10, 11 and 12.

For Tp, Pi and Po there was a highly significant P affect (Tp F ratio 70.19***, Pi F ratio 18.99***, Po F Ratio 32.12***). All other P treatments and interactions were not significant (Appendix 2, Tables 10 , 11 and 12).

Total P

Fig 2.12 (a) presented soil Tp concentration data for soil untreated with benlate. Fig 2.12 (b) presented soil Tp concentration data for soil treated with benlate.

From Fig 2.12 (a, b) there was an expected increase in start soil Tp levels with increasing levels of P added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Tp concentrations. There was a significant decrease in Tp level for the control inner and outer soil with benlate addition (Fig 2.12 b). There was significant decrease in Tp level for the control inner and outer soil and the 100Pi treatment outer soil with no benlate addition (Fig 2.12 a).

There was no significant affect of benlate treatment on the soil Tp concentration of the postharvest soils (Appendix 2, Table 10 and Fig 2.12 a, b).

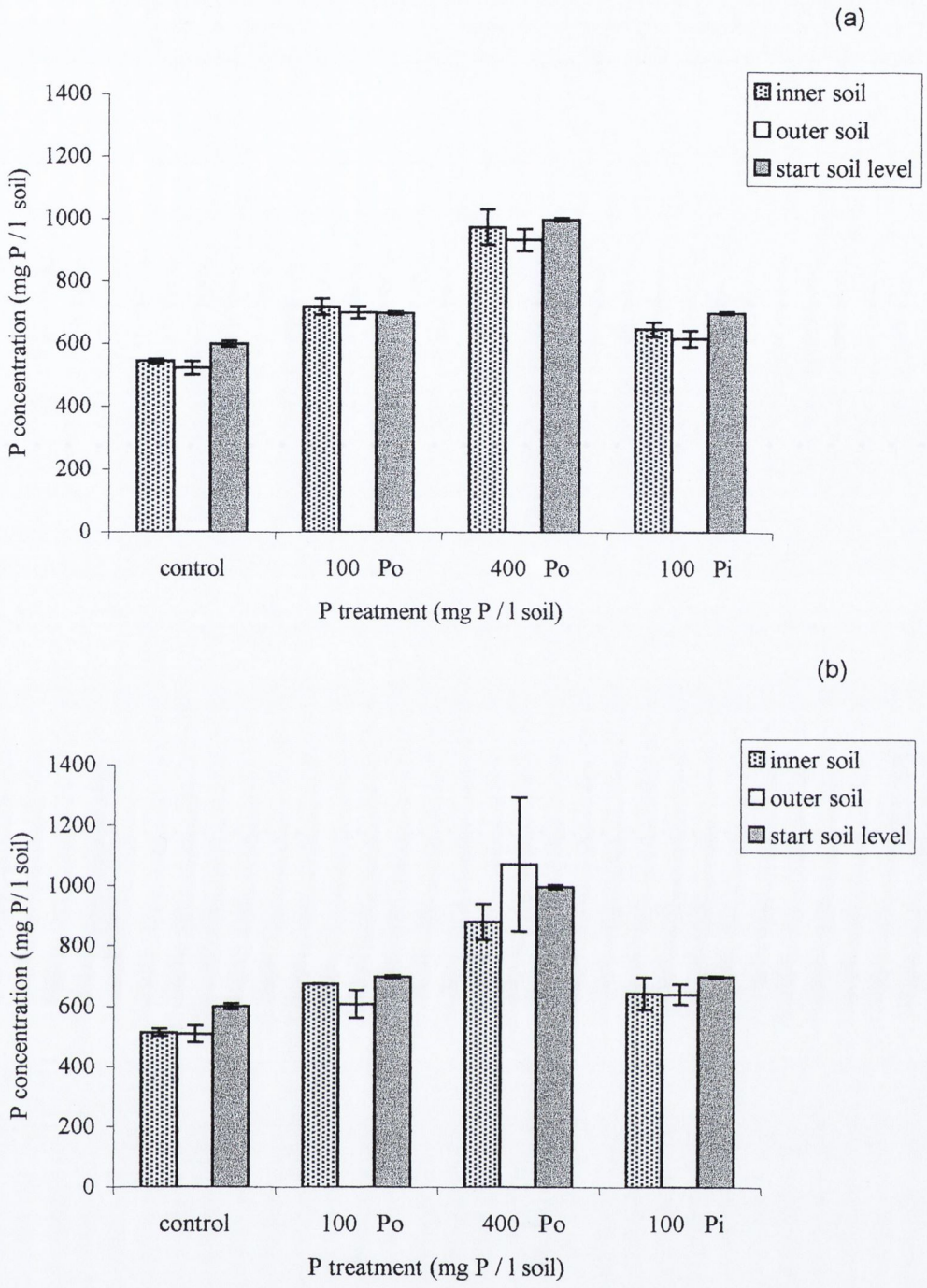


Fig 2.12: soil total phosphate concentration for the control and P treatment soils of the *Plantago lanceolata* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

From Fig 2.12 (a, b) for the post harvest soil there was an increase in total P concentration with increasing levels of P addition for all soils. This significant affect of P treatment on Tp concentration was examined using a Scheffe post hoc test (Table 2.14).

Table 2.14: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Tp concentration for the *Plantago l.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	***
control < 400Po	***
control < 100Pi	***
400Po > 100Po	***
400Po > 100Pi	***
100Pi - 100Po	ns

Inorganic P

Fig 2.13 (a) presented soil Pi concentration data for soil untreated with benlate. Fig 2.13 (b) presented soil Pi concentration data for soil treated with benlate.

From Fig 2.13 (a, b) the only significant change in start soil Pi concentration was in the 100Pi treatment. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Pi concentrations. There was a significant increase in Pi level for the 100Po inner soil, the 400Po treatment inner and outer soil and the 100Pi treatment outer soil with benlate addition (Fig 2.13 b). There were significant increases in Pi level for all treatments, inner and outer soil with no benlate addition (Fig 2.13 a).

There was no significant affect of benlate treatment on the soil Pi concentration of the postharvest soils (Appendix 2, Table 11 and Fig 2.13 a, b).

From Fig 2.13 (a, b) for the post harvest soil there was an increase in Pi concentration in the soil both the soil treated and untreated with benlate. This significant affect of P treatment on Pi concentration was examined using a Scheffe post hoc test (Table 2.15).

Table 2.15: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Pi concentration for the *Plantago l.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control < 400Po	***
control < 100Pi	***
400Po < 100Po	**
400Po - 100Pi	ns
100Pi > 100Po	***

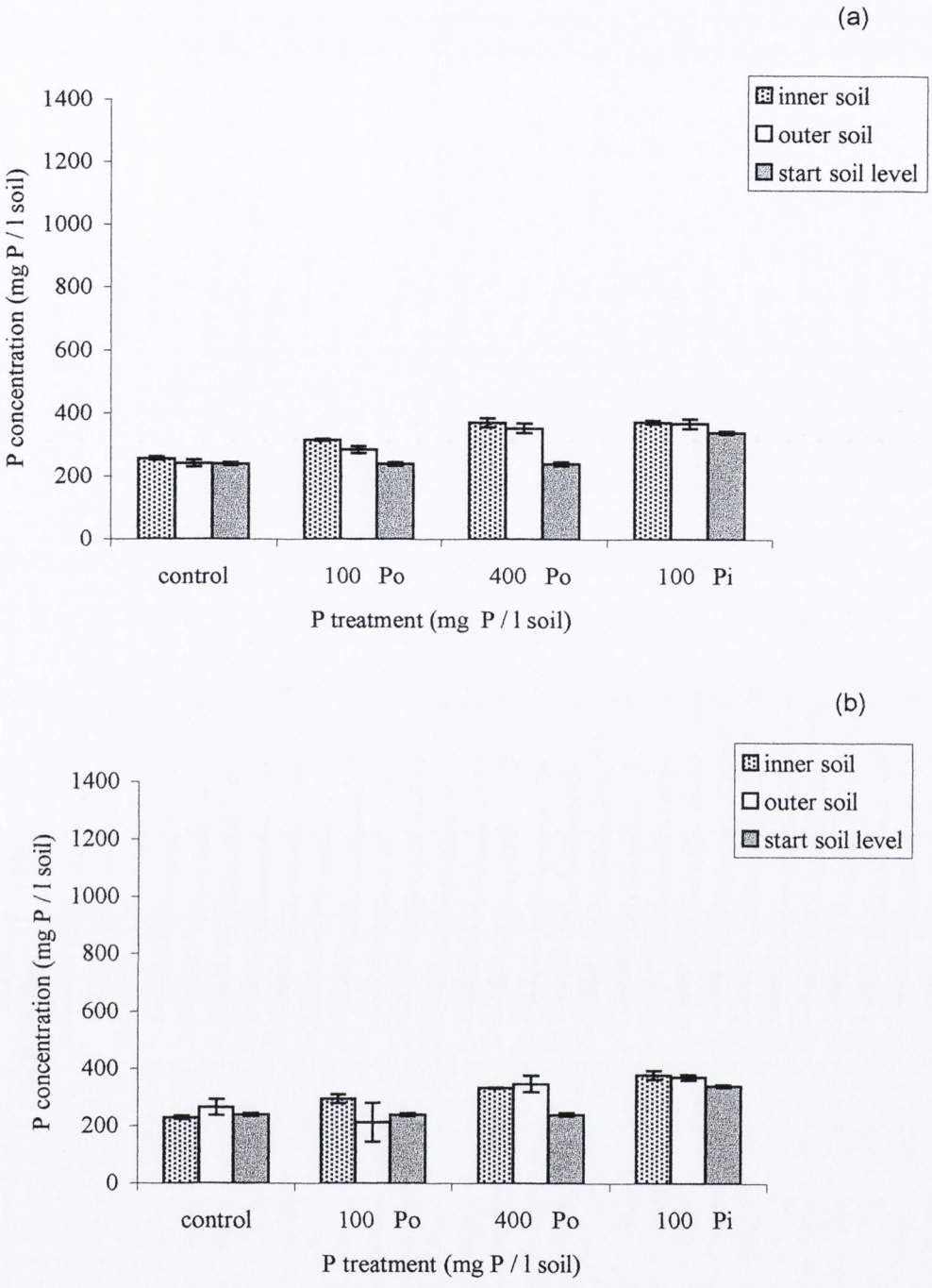


Fig 2.13: soil inorganic phosphate concentration for the control and P treatment soils of the *Plantago lanceolata* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

Organic P

Fig 2.14 (a) presented soil Po concentration data for soil untreated with benlate. Fig 2.14 (b) presented soil Po concentration data for soil treated with benlate.

From Fig 2.14 (a, b) there was an increase in start soil Po levels with increasing levels of P added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Po concentrations. There was a significant decrease in Po level for the control, 100Po and 400Po treatment inner soils and the 100Pi treatment outer soil with benlate addition (Fig 2.14 b). There was significant decrease in Po level for the control, 400Po and 100Pi treatment inner and outer soils with no benlate addition (Fig 2.14 a).

There was no significant affect of benlate treatment on the soil Po concentration of the postharvest soils (Appendix 2, Table 12 and Fig 2.14 a, b).

From Fig 2.14 (a, b) for the post harvest soil there was an increase in Po concentration in the soil with increasing levels of P addition for all soils. This significant affect of P treatment on Po concentration was examined using a Scheffe post hoc test (Table 2.16).

Table 2.16: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Po concentration for the *Plantago l.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	**
control < 400Po	***
control - 100Pi	ns
400Po > 100Po	***
400Po > 100Pi	***
100Pi < 100Po	**

2.3.2.2 Colonisation of Roots by VAM

The colonisation of roots by vesicular arbuscular mycorrhizal (VAM) fungi was measured by % root length colonisation (%RLC). The %RLC data was presented in Fig 2.15. A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on %RLC. This ANOVA was presented in Appendix 2, Table 13.

For %RLC there was a highly significant benlate affect (F ratio 25.99***) and a significant P affect (F ratio 3.68*).

Figure 2.15 presented %RLC data for all *Plantago l.* plants. From Fig 2.15 there was a decrease in %RLC, for all P treatments, in plants grown in benlate treated soil from the %RLC colonisation present in non-benlate treated plants. This affect of benlate treatment on %RLC was statistically significant (Appendix 2, Table 13).

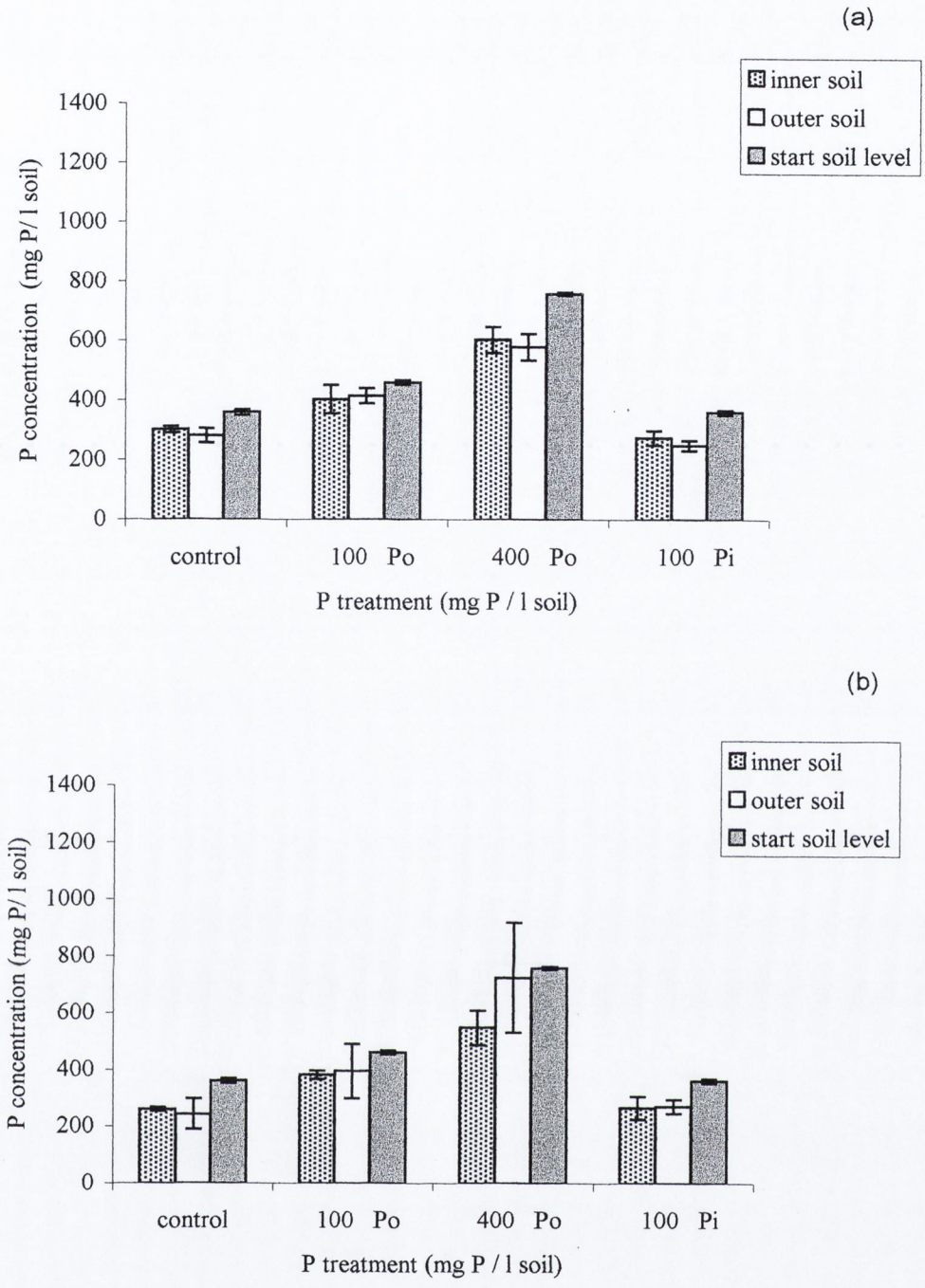


Fig 2.14: soil organic phosphate concentration for the control and P treatment soils of the *Plantago lanceolata* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

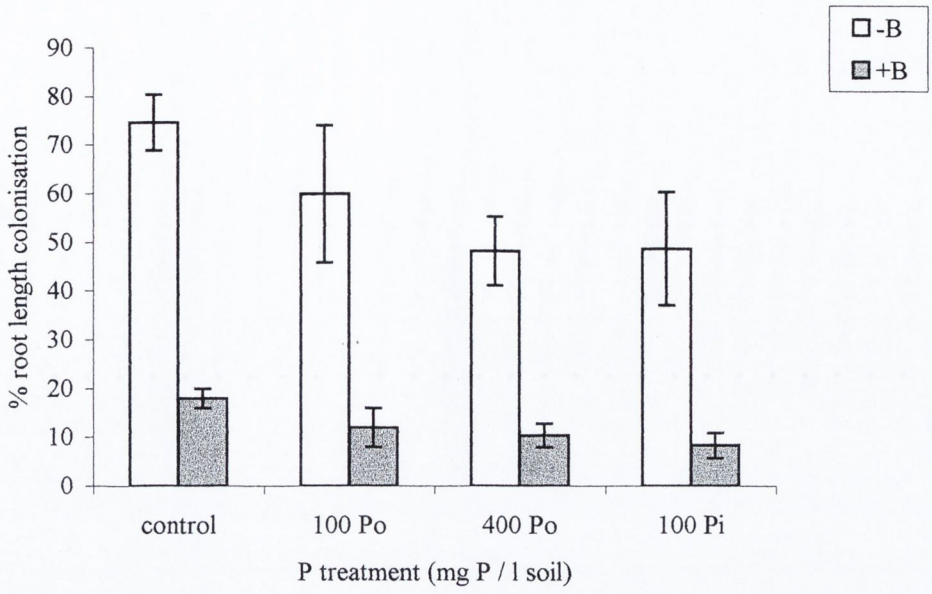


Fig 2.15: % root length colonisation for the control and P treamtment plants of the *Plantago lanceolata* experiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

There was a trend of decreasing %RLC from that present in the control plants with increasing P treatment for benlate treated and untreated plants (Fig 2.15). This significant affect of P treatment on %RLC was examined using a Scheffe post hoc test (Table 2.17),

Table 2.17: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on %RLC for the *Plantago l.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control > 400Po	*
control > 100Pi	*
400Po - 100Po	ns
400Po - 100Pi	ns
100Pi - 100Po	ns

2.3.2.3 Soil and root monoesterase activity

The phosphatase activity results are presented as follows:

soil acidic monoesterase data in Fig 2.16 (a, b) and the root acidic monoesterase data in Fig 2.17. A three way ANOVA was carried out to determine the affect of P treatment, benlate treatment and location of soil on soil acidic monoesterase activity. A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on root acidic monoesterase activity. These ANOVAs were presented in Appendix 2, Tables 14 and 15 respectively.

There was a statistically significant affect of P treatment on soil acidic monoesterase activity (F ratio 10.53*). There was a statistically significant affect of P treatment (F ratio 73.12***) and benlate treatment (F ratio 10.28**) on root acidic monoesterase activity. There was also a significant P - benlate interaction (F ratio 14.76***) for root acidic monoesterase activity. All other treatments and interactions for soil and root activity were not significant (Appendix 2, Table 14 and 15).

Soil acidic monoesterase

Fig 2.16 (a) presented soil acidic monoesterase activity data for soil not treated with benlate. From Fig 2.16 (a), for outer and inner soil, there was an increase in soil monoesterase activity as a result of the 100Po treatment with a further increase to the 400Po treatment soil. There was then a slight decrease in the activity with the 100Pi treatment soil to a level similar to that found in the 100Po treatment soil.

Fig 2.16 (b) presented soil acidic monoesterase activity data for soil treated with benlate. The same pattern of changing soil monoesterase activity with P treatments described for the soil untreated with benlate is again repeated for soil treated with benlate for both inner and outer soil.

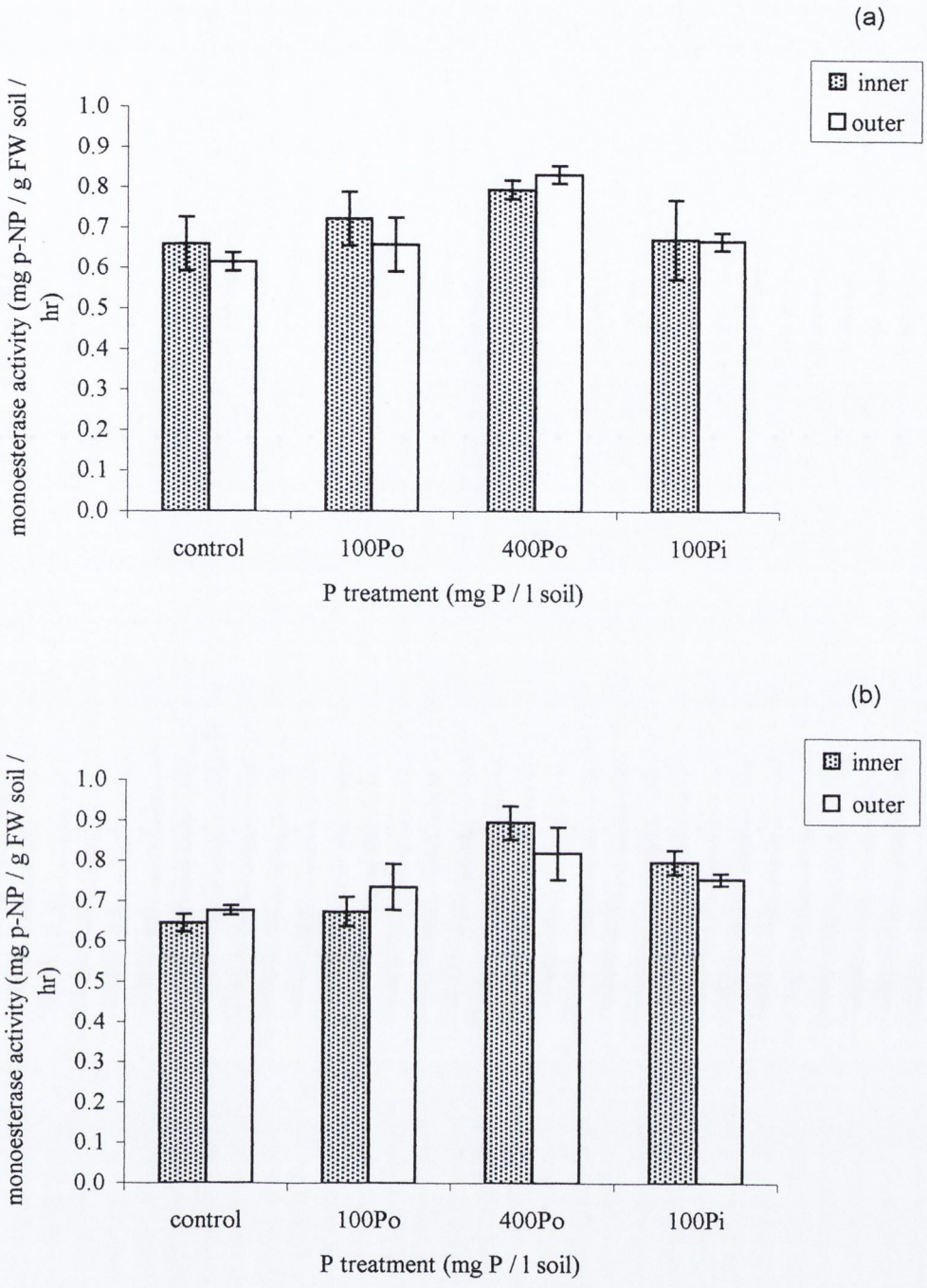


Fig 2.16: soil acidic monoesterase activity for the control and P treatment soils for the *Plantago lanceolata* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

There was no significant affect of location of soil on monoesterase activity levels (Appendix 2, Table 14).

The significant affect of P treatment on soil acidic monoesterase activity was examined using a Scheffe post hoc test (Table 2.18).

Table 2.18: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on soil acidic monoesterase activity for the *Plantago l.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control < 400Po	***
control - 100Pi	ns
400Po > 100Po	**
400Po > 100Pi	*
100Pi - 100Po	ns

Root acidic monoesterase

Fig 2.17 presented root acidic monoesterase activity data for plants grown in soil treated and untreated with benlate. From Fig 2.17, there was a dramatic decrease in root acidic monoesterase activity as a result of the Po and Pi treatments. The activity detected for the 100Po, 400Po and 100Pi plants was similar. This was the case for plants grown in benlate treated and untreated soil. This significant affect of P treatment on root acidic monoesterase activity was examined using a Scheffe post hoc test (Table 2.19).

Table 2.19: probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root acidic monoesterase activity for the *Plantago l.*. experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control > 100Po	***
control > 400Po	***
control > 100Pi	***
400Po < 100Po	***
400Po - 100Pi	ns
100Pi < 100Po	***

The was a statistically significant affect of benlate treatment on the root acidic monoesterase activity (Appendix 2, Table 15). Plants grown in benlate treated soil had a higher the root acidic monoesterase activity than plants grown in soil with no benlate treatment (Fig 2.17).

The significant interaction between P and benlate treatment was examined using a Scheffe post hoc test (Table 2.20).

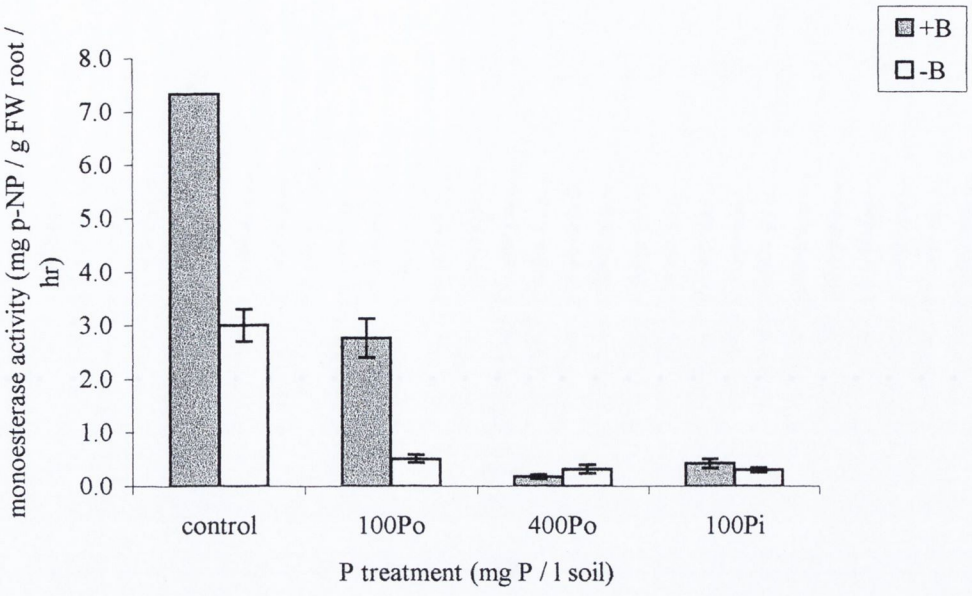


Fig 2.17: root acidic monoesterase activity for the control and P treatment plants, for the *Plantago lanceolata* experiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

Table 2.20: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected root acidic monoesterase activity for the *Plantago l.* experiment. Soil without and with benlate treatment was represented as –B and +B respectively.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-B, control - +B, control	ns
-B, 100Po < +B, 100Po	***
-B, 400Po - +B, 400Po	ns
-B, 100Pi - +B, 100Pi	ns
-B, control > -B, 100Po	***
-B, control > -B, 400Po	***
-B, control > -B, 100Pi	***
+B, control - +B, 100Po	ns
+B, control > +B, 400Po	***
+B, control > +B, 100Pi	***

From Table 2.20 the Po and Pi treatments resulted in a significant decrease in root acidic monoesterase activity in soil with no benlate treatment. In soil with benlate treatment however only the 400Po and 100Pi treatments resulted in a significant decrease in root acidic monoesterase activity. Benlate treatment resulted in a significant increase in root acidic monoesterase activity for plants grown in the 100Po treatment soil but not those grown in control, 400Po or 100Pi treatment soils (Table 2.20).

2.3.2.4 Shoot Dry Weight, P concentration and content

The shoot dry weight data was presented in Fig 2.18. The shoot P concentration data was presented in Fig 2.19. The shoot P content data was presented in Fig 2.20. Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot dry weight, P concentration and P content. These ANOVAs were presented in Appendix 2, Tables 16, 17 and 18.

There was a highly significant P affect on the shoot dry weight (F ratio 159.37***), P concentration (F ratio 48.11***) and P content (F ratio 109.12***). There was a significant benlate affect on the shoot P concentration (F ratio 36.69***). There was also a significant P – benlate interaction for shoot dry weight (F ratio 39.20***) and shoot P content. (F ratio 38.62***). All other treatments and interactions were not significant (Appendix 2, Tables 16, 17 and 18).

<u>Shoot Dry Weight</u>

Fig 2.18 presented shoot dry weight data for all plants. From Fig 2.18, for plants grown in soil not treated with benlate, there was an increase in shoot dry weight as a result of the Po and Pi treatments. For plants grown in benlate treated soil there was no increase in dry weight as a result of the 100Po treatment, but there was a large increase from the 400Po and 100Pi

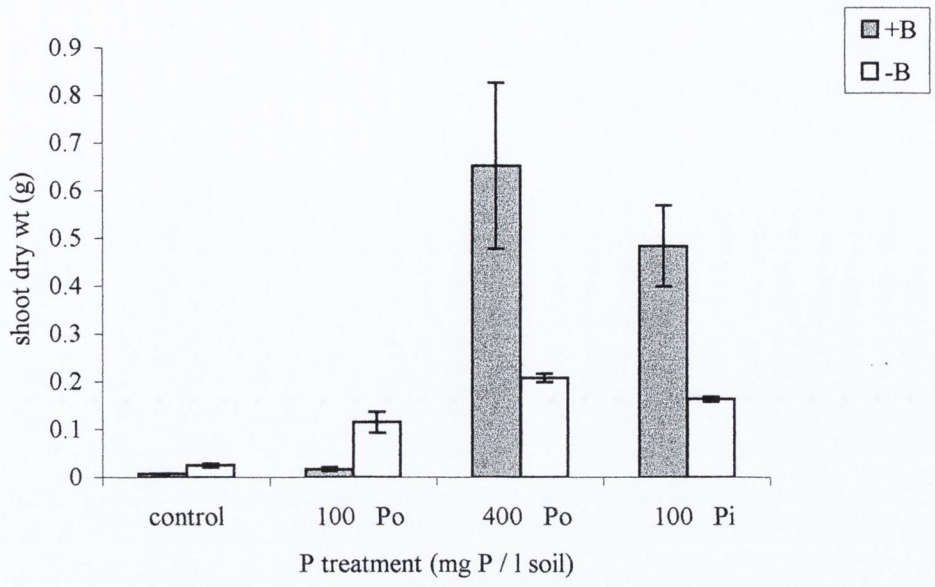


Fig 2.18: shoot dry weight for the control and P treatment plants of the *Plantago lanceolata* expeiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

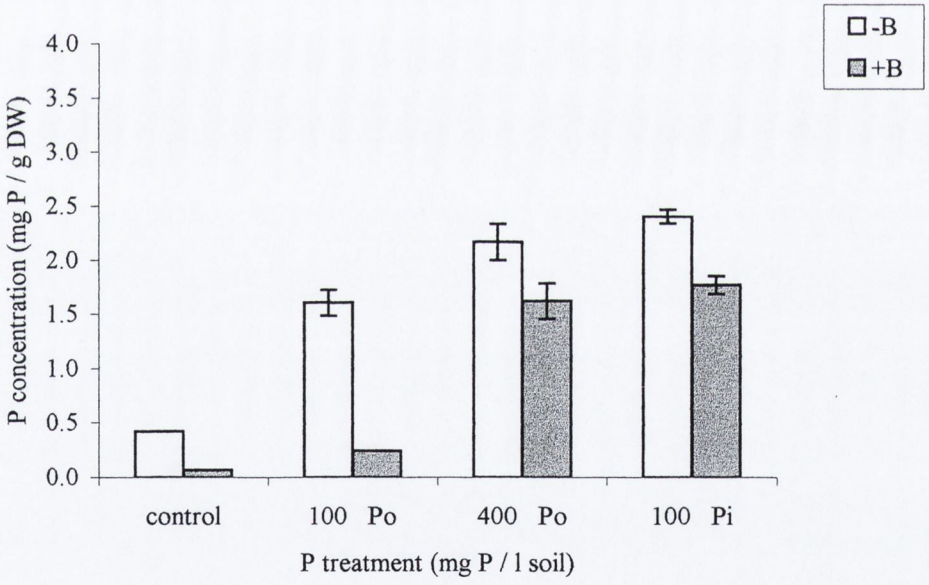


Fig 2.19: shoot P concentration for the control and P treatment plants of the *Plantago lanceolata* experiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

treatments. This significant affect of P treatment on shoot dry weight was examined using a Scheffe post hoc test (Table 2.21).

Table 2.21: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot dry weights for the *Plantago l.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	***
control < 400Po	***
control < 100Pi	***
400Po > 100Po	***
400Po - 100Pi	ns
100Pi > 100Po	***

The significant interaction between P treatment and benlate treatment was examined using a Scheffe post hoc test (Table 2.22)

Table 2.22: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected shoot dry weight for the *Plantago l.* experiment. Soil without and with benlate treatment was represented as –B and +B respectively.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-B, control - +B, control	ns
-B, 100Po - +B, 100Po	ns
-B, 400Po < +B, 400Po	**
-B, 100Pi < +B, 100Pi	**
-B, control < -B, 100Po	***
-B, control < -B, 400Po	***
-B, control < -B, 100Pi	***
+B, control < +B, 100Po	*
+B, control < +B, 400Po	***
+B, control < +B, 100Pi	***

From Table 2.22 the 100Po, 400Po and 100Pi treatments increased the shoot dry weight in soil treated and untreated with benlate. At 400Po and 100Pi, benlate treatment enhanced the phosphate effect, but at 100Po and in the control, benlate treatment had no significant effects.

Shoot P concentration

Fig 2.19 presented shoot P concentration data for all plants. From Fig 2.19, for plants grown in soil untreated and treated with benlate, the shoot P concentration increased as a result of the Po, and Pi treatments. This significant affect of P treatment on shoot P concentration was examined using a Scheffe post hoc test (Table 2.23).

Table 2.23: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P concentrations for the *Plantago l.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	*
control < 400Po	***
control < 100Pi	***
400Po > 100Po	***
400Po - 100Pi	ns
100Pi > 100Po	***

There was a statistically significant affect of benlate treatment on the shoot P concentration (Appendix 2, Table 17). Plants grown in benlate treated soil had a lower shoot P concentration than those grown in soil with no benlate treatment (Fig 2.19).

Shoot P content

Fig 2.20 presented shoot P content data for all plants. From Fig 2.20, for plants grown in soil not treated with benlate, there was an increase in shoot P content as a result of the Po and Pi treatments. For plants grown in benlate treated soil there was a small increase in P content as a result of the 100Po treatment, but there was a large increase from the 400Po and 100Pi treatments. This significant affect of P treatment on shoot P content was examined using a Scheffe post hoc test (Table 2.24).

Table 2.24: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P content for the *Plantago l.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	***
control < 400Po	***
control < 100Pi	***
400Po > 100Po	***
400Po - 100Pi	ns
100Pi > 100Po	***

The significant interaction between P treatment and benlate treatment was examined using a Scheffe post hoc test (Table 2.25).

From Table 2.25 the 100Po, 400Po and 100Pi treatments increased the shoot P content in soil treated and untreated with benlate. At 400Po and 100Pi, benlate treatment enhanced P content, but at 100Po and in the control there was no significant benlate effect.

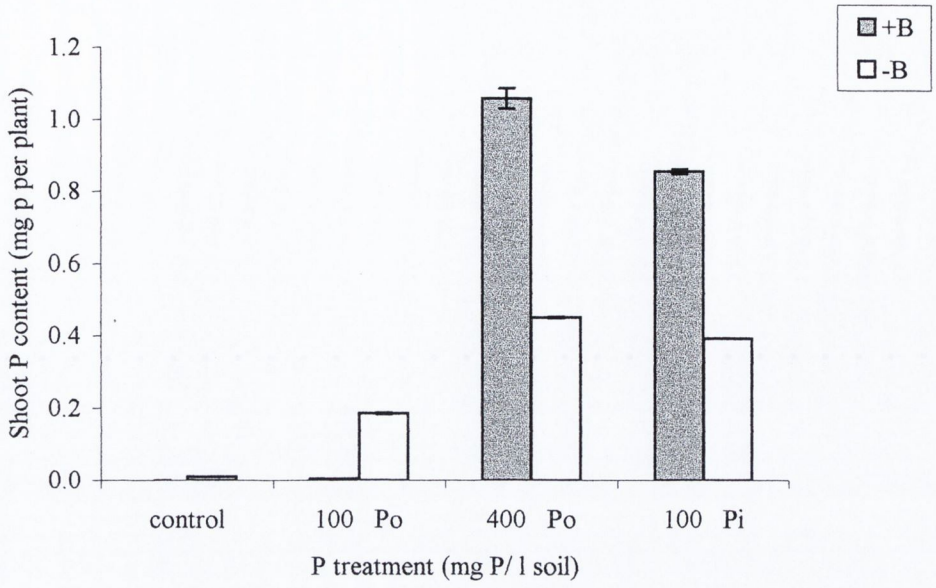


Fig 2.20: shoot P content for the control and P treatment plants of the *Plantago lanceolata* experiment, without (-B) and with (+B) benlate treatment . Each value represents the mean +/- SE of 3 replicates.

Table 2.25: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected shoot P content for the *Plantago l.* experiment. Soil without and with benlate treatment was represented as –B and +B respectively.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison			P value
-B, control	-	+B, control	ns
-B, 100Po	-	+B, 100Po	ns
-B, 400Po	<	+B, 400Po	**
-B, 100Pi	<	+B, 100Pi	**
-B, control	<	-B, 100Po	***
-B, control	<	-B, 400Po	***
-B, control	<	-B, 100Pi	***
+B, control	<	+B, 100Po	*
+B, control	<	+B, 400Po	***
+B, control	<	+B, 100Pi	***

2.3.3 *Agrostis stolonifera*

2.3.3.1 Soil phosphate

The soil total P (Tp), inorganic P (Pi) and organic P (Po) concentration data were presented in Fig 2.21 (a, b), 2.22 (a, b) and 2.23 (a, b) respectively. Three way ANOVAs were carried out to determine the affect of P treatment and benlate treatment and location of soil on soil Tp, Pi and Po concentration. These ANOVAs were presented in Appendix 2, Table 19, 20 and 21.

For Tp, Pi and Po there was a highly significant P affect (Tp F ratio 242.07***, Pi F ratio 91.02***, Po F ratio 191.28***). All other P treatments and interactions were not significant (Appendix 2, Tables 19, 20 and 21).

Total P

Fig 2.21 (a) presented soil Tp concentration data for soil untreated with benlate. Fig 2.21 (b) presented soil Tp concentration data for soil treated with benlate.

From Fig 2.21 (a, b) there was an expected increase in start soil Tp levels with increasing levels of P added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Tp concentrations. There was a significant decrease in Tp level for the 100Pi treatment inner and outer soil with benlate addition (Fig 2.21 b). There was significant decrease in Tp level for the 100Pi treatment inner soil with no benlate addition (Fig 2.21 a).

There was no significant affect of benlate treatment on the soil Tp concentration of the postharvest soils (Appendix 2 Table 19 and Fig 2.21 a, b).

From Fig 2.21 (a, b) for the post harvest soil there was an increase in total P concentration with increasing levels of P addition all soils. This significant affect of P treatment on Tp concentration was examined using a Scheffe post hoc test (Table 2.26).

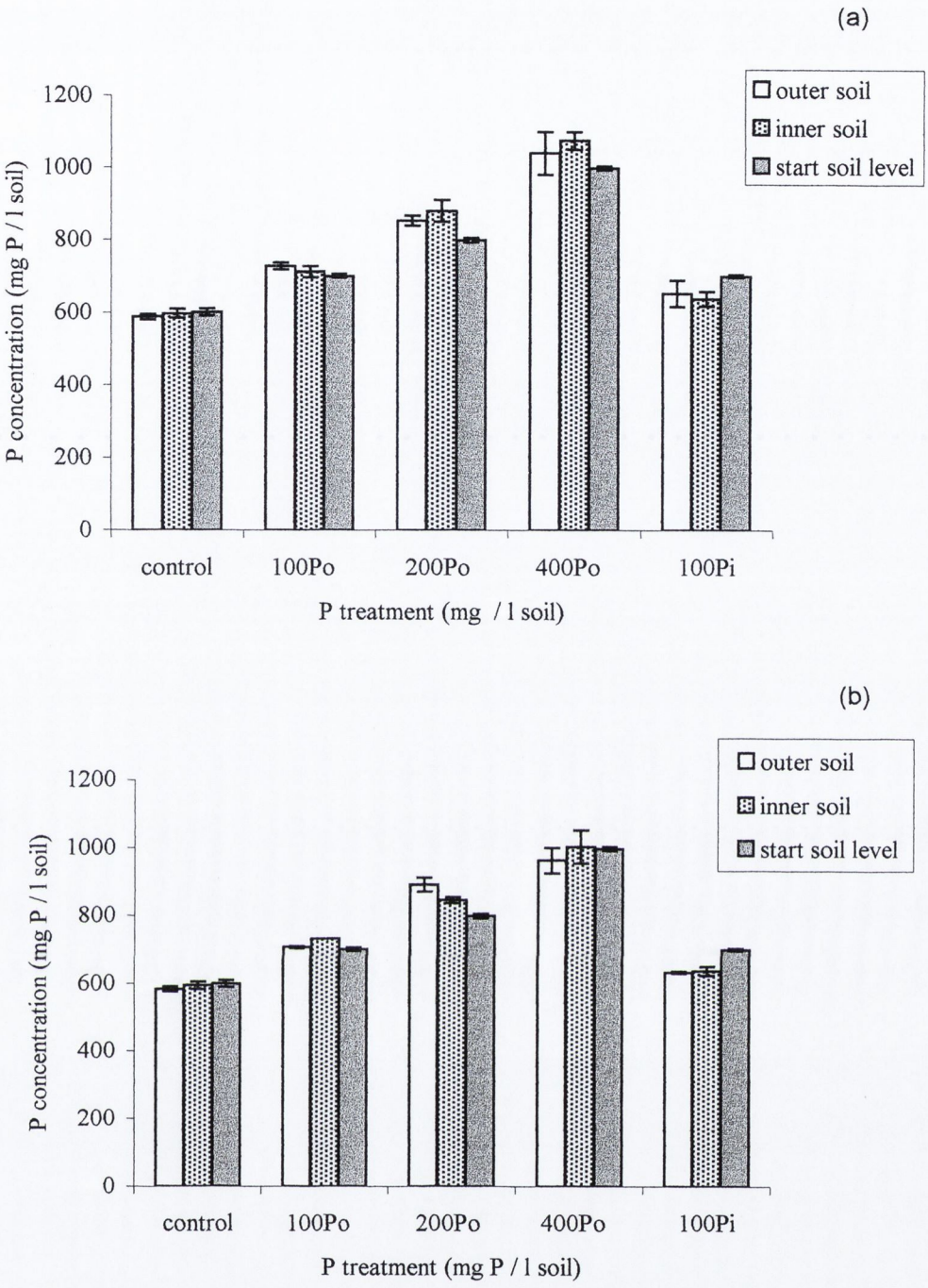


Fig 2.21: soil total phosphate concentration for the control and P treatment soils of the *Agrostis stolonifera* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

Table 2.26: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Tp concentration for the *Agrostis s.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	***
control < 200Po	***
control < 400Po	***
control < 100Pi	***
200Po > 100Po	***
400Po > 100Po	***
400Po > 200Po	***
400Po > 100Pi	***
100Pi < 100Po	***
100Pi < 200Po	***

Inorganic P

Fig 2.22 (a) presented soil Pi concentration data for soil untreated with benlate. Fig 2.22(b) presented soil Pi concentration data for soil treated with benlate.

From Fig 2.22 (a, b) the only significant change in start soil Pi concentration was in the 100Pi treatment. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Pi concentrations. There was a significant increase in Pi level for the 400Po and 100Pi treatments inner and outer soil with benlate addition (Fig 2.22 b). There were significant increases in Pi level for all Po and the Pi treatments, inner and outer soil with no benlate addition (Fig 2.22 a).

There was no significant affect of benlate treatment on the soil Pi concentration of the postharvest soils (Appendix 2, Table 20. and Fig 2.22 a, b).

From Fig 2.22 (a, b) for the post harvest soil there was an increase in Pi concentration in both soil treated and untreated with benlate. This significant affect of P treatment on Pi concentration was examined using a Scheffe post hoc test (Table 2.27).

Table 2.27: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Pi concentration for the *Agrostis s.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	***
control < 200Po	***
control < 400Po	***
control < 100Pi	***
200Po - 100Po	ns
400Po > 100Po	***
400Po > 200Po	***
400Po - 100Pi	ns
100Pi > 100Po	***
100Pi > 200Po	***

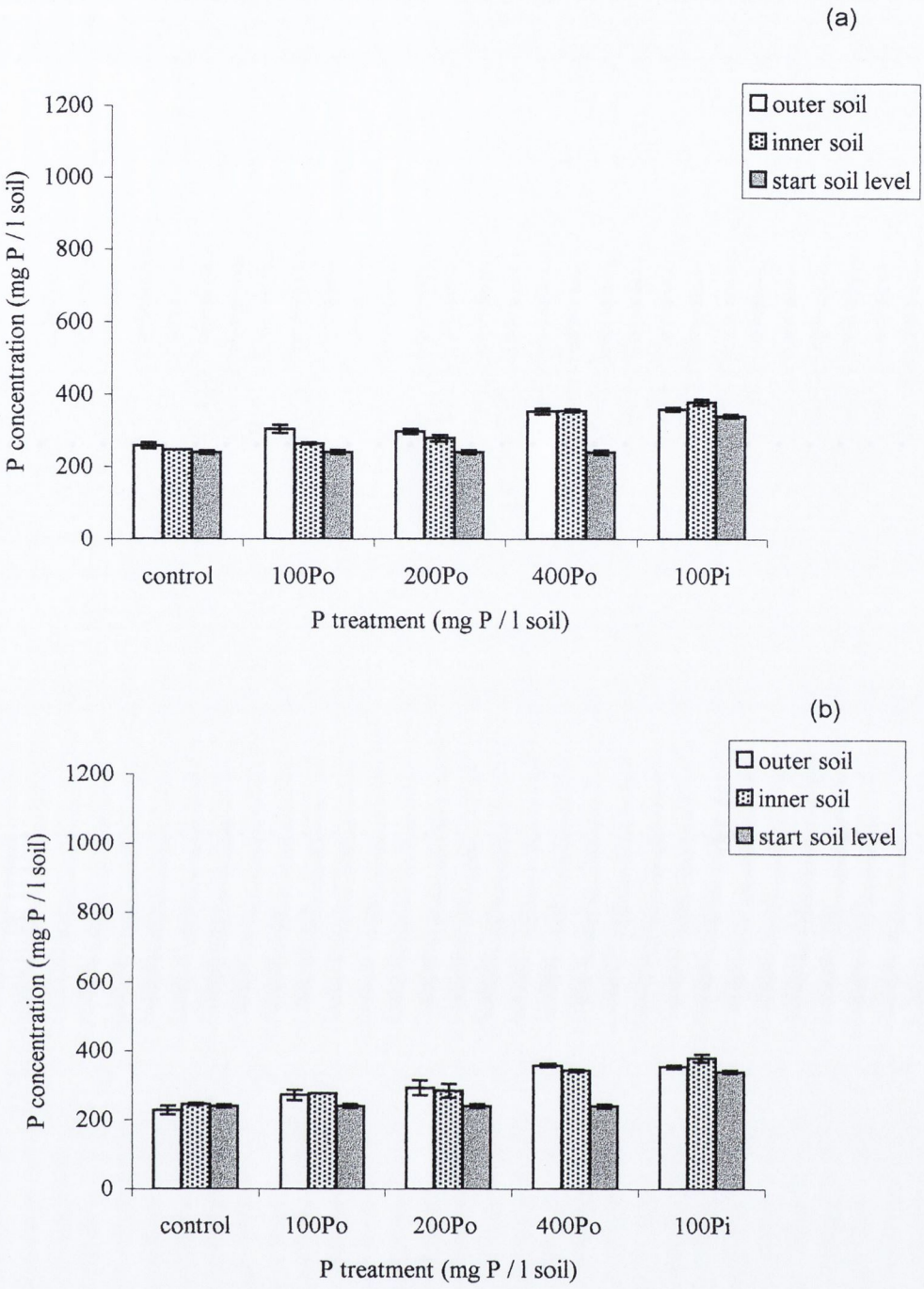


Fig 2.22: soil inorganic phosphate concentration for the control and P treatment soils of the *Agrostis stolonifera* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

Organic P

Fig 2.23 (a) presented soil Po concentration data for soil untreated with benlate. Fig 2.23 (b) presented soil Po concentration data for soil treated with benlate.

From Fig 2.23 (a, b) there was an increase in start soil Po levels with increasing levels of P added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Po concentrations. There was a significant decrease in Po level for the 400Po treatment outer soils and the 100Pi treatment outer and inner soil with benlate addition (Fig 2.23 b). There was significant decrease in Po level for the 100Po treatment outer soil and 100Pi treatment inner soils with no benlate addition (Fig 2.23 a).

There was no significant affect of benlate treatment on the soil Po concentration of the postharvest soils (Appendix 2, Table 21 and Fig 2.23 a, b).

From Fig 2.23 (a, b) for the post harvest soil there was an increase in Po concentration with increasing levels of P addition for all soils. This significant affect of P treatment on Po concentration was examined using a Scheffe post hoc test (Table 2.28).

Table 2.28: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Po concentration for the *Agrostis s.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	***
control < 200Po	***
control < 400Po	***
control < 100Pi	***
200Po > 100Po	***
400Po > 100Po	***
400Po > 200Po	**
400Po > 100Pi	***
100Pi < 100Po	***
100Pi < 200Po	***

2.3.3.2 Colonisation of Roots by VAM

The colonisation of roots by vesicular arbuscular mycorrhizal (VAM) fungi was measured by % root length colonisation (%RLC). The %RLC data was presented in Fig 2.24. A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on %RLC. This ANOVA was presented in Appendix 2, Table.22.

For %RLC there was a highly significant P affect (F ratio 7.82***) and B affect (F ratio 13.99***). There was no significant interaction (Appendix 2, Table 22).

Figure 2.24 presented %RLC data for all *Agrostis s.* plants. From Fig 2.24 the %RLC, for all P treatments, was lower in plants grown in benlate treated soil compared to plants from non-

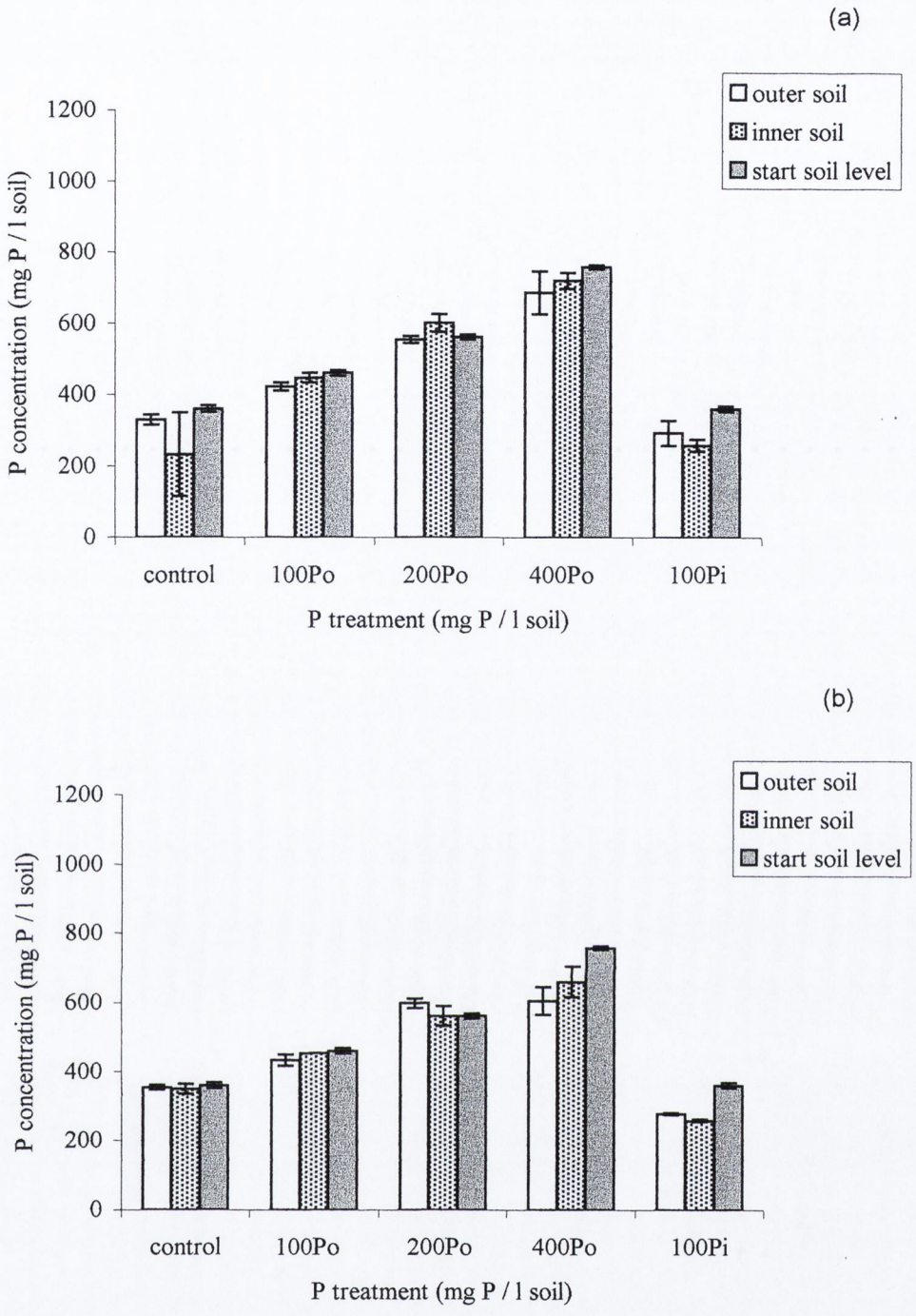


Fig 2.23: soil organic phosphate concentration for the control and P treatment soils of the *Agrostis stolonifera* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

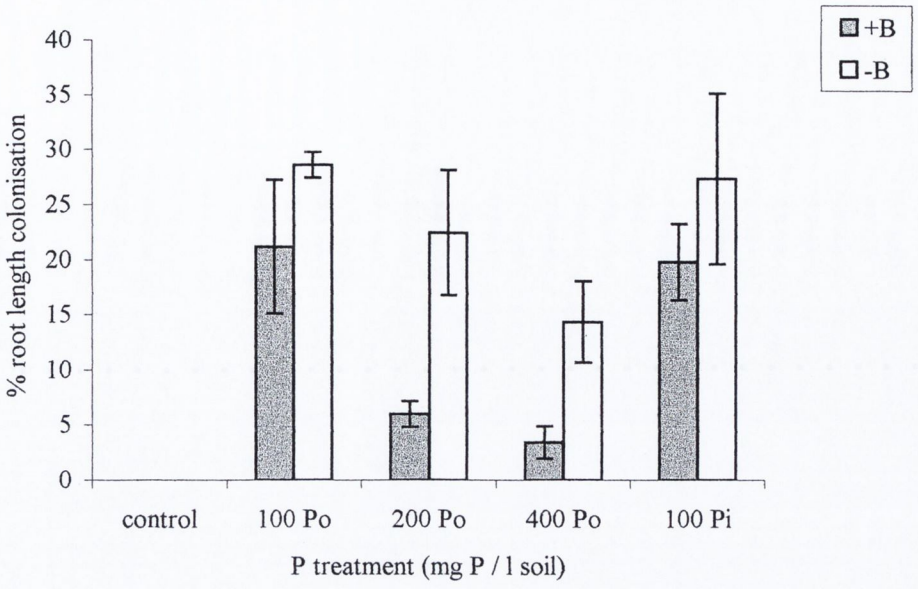


Fig 2.24: % root length colonisation for the control and P treatment plants of the *Agrostis stolonifera* experiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

benlate treated soil. This affect of benlate treatment on %RLC was significant (Appendix 2, Table 22).

Decreasing %RLC with increasing Po concentration in the soil for benlate treated and untreated plants was observed (Fig 2.24). The 100Pi treatment plants had a %RLC similar to that of the 100Po treatment plants. This significant affect of P treatment on %RLC was examined using a Scheffe post hoc test (Table 2.29).

Table 2.29: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on %RLC for the *Agrostis s.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control - 200Po	ns
control - 400Po	ns
control - 100Pi	ns
200Po - 100Po	ns
400Po < 100Po	**
400Po - 200Po	ns
400Po < 100Pi	**
100Pi - 100Po	ns
100Pi - 200Po	ns

2.3.3.3 Soil and root monoesterase activity

The phosphatase activity results are presented as follows:

soil acidic monoesterase data in Fig 2.25 (a, b) and the root acidic monoesterase data in Fig 2.26. A three way ANOVA was carried out to determine the affect of P treatment, benlate treatment and location of soil on soil acidic monoesterase activity. A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on root acidic monoesterase activity. These ANOVAs were presented in Appendix 2, Tables 23 and 24 respectively.

For soil acidic monoesterase activity there was a highly significant P affect (F ratio 7.84***) and a significant location of soil affect (F ratio 6.38*). For root acidic monoesterase activity there was a significant P affect (F ratio 7.98***). All other treatments and interactions were not significant (Appendix 2, Tables23 and 24).

<u>Soil acidic monoesterase</u>

Fig 2.25 (a) presented soil acidic monoesterase activity data for soil not treated with benlate. From Fig 2.25 (a), for outer and inner soil, there was a decrease in soil monoesterase activity with increasing concentrations of Po in the soil. The soil monoesterase activity in the 100Pi treatment soil was similar to the activity in the 100Po treatment soil. Fig 2.25 (b) presented soil acidic monoesterase activity data for soil treated with benlate. The same pattern of changing soil monoesterase activity with P treatments described for the soil untreated with benlate was again

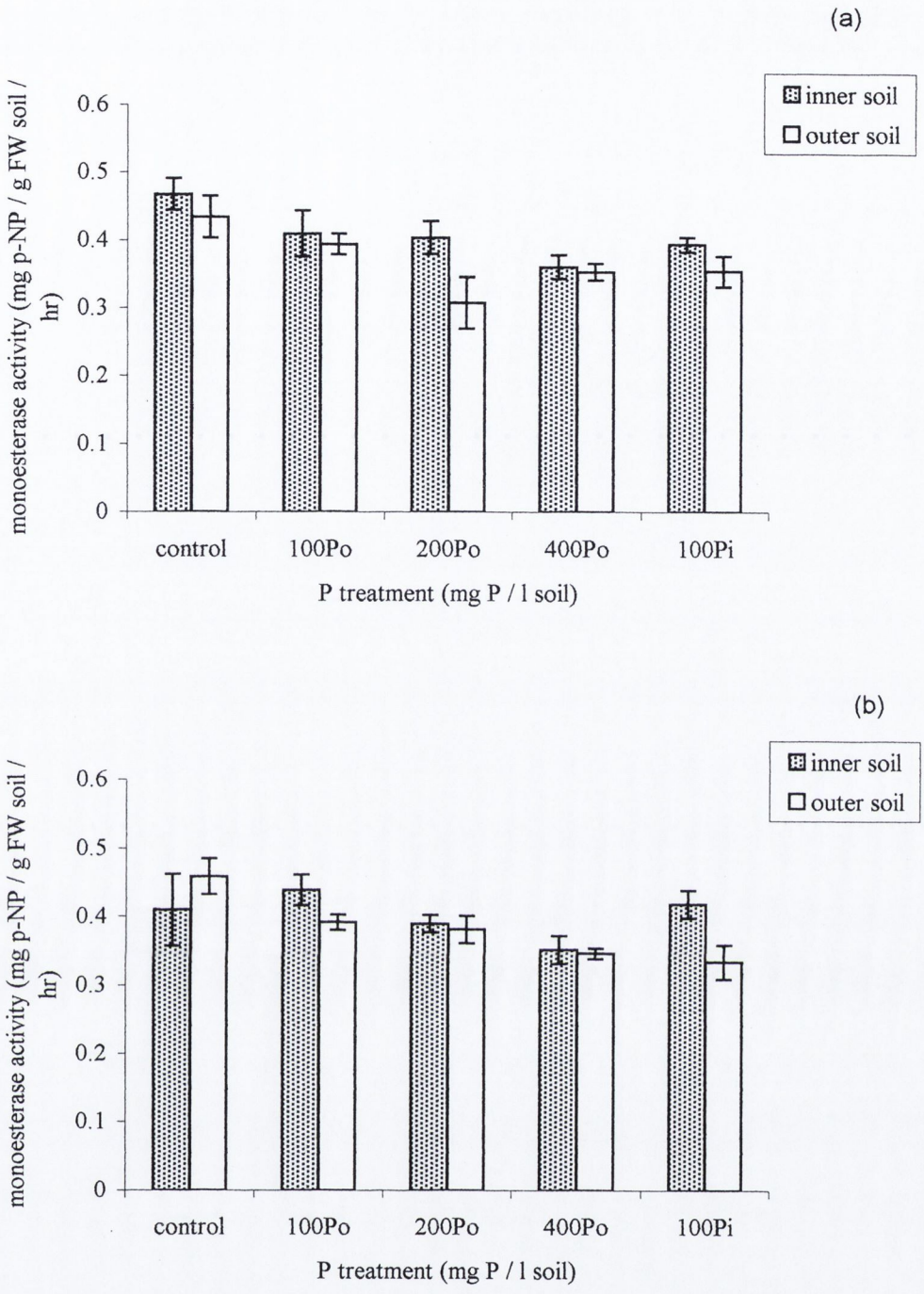


Fig 2.25: soil acidic monoesterase activity for the control and P treatment soils for the *Agrostis stolonifera* experiment for (a) soil with no benlate treatment and (b) soil with benlate treatment. Each value represents the mean +/- SE of 3 replicates.

repeated for soil treated with benlate for both inner and outer soil. There was a significant affect of location of soil on monoesterase activity levels (Appendix 2, Table 23). The monoesterase activity was higher in the inner soil than in the outer soil (Fig 2.55 a, b).

The significant affect of P treatment on soil acidic monoesterase activity was examined using a Scheffe post hoc test (Table 2.30).

Table 2.30: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on soil acidic monoesterase activity for the *Agrostis s.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control > 200Po	**
control > 400Po	***
control > 100Pi	*
200Po - 100Po	ns
400Po - 100Po	ns
400Po - 200Po	ns
400Po - 100Pi	ns
100Pi - 100Po	ns
100Pi - 200Po	ns

Root acidic monoesterase

Fig 2.26 presented root acidic monoesterase activity data for plants grown in soil treated and untreated with benlate. From Fig 2.26, there was a dramatic decrease in root acidic monoesterase activity as a result of all the Po and Pi treatments. This was the case for plants grown in benlate treated and untreated soil. This significant affect of P treatment on root acidic monoesterase activity was examined using a Scheffe post hoc test (Table 2.31).

Table 2.31: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root acidic monoesterase activity for the *Agrostis s.* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control - 200Po	ns
control - 400Po	ns
control - 100Pi	ns
200Po < 100Po	*
400Po - 100Po	ns
400Po > 200Po	**
400Po - 100Pi	ns
100Pi - 100Po	ns
100Pi > 200Po	***

2.3.3.4 Shoot and root dry weight and P concentrations

The shoot and root dry weight data was presented in Figs 2.27 and 2.28 respectively. The shoot and root P concentration data was presented in Figs 2.29 and 2.30 respectively. Two way

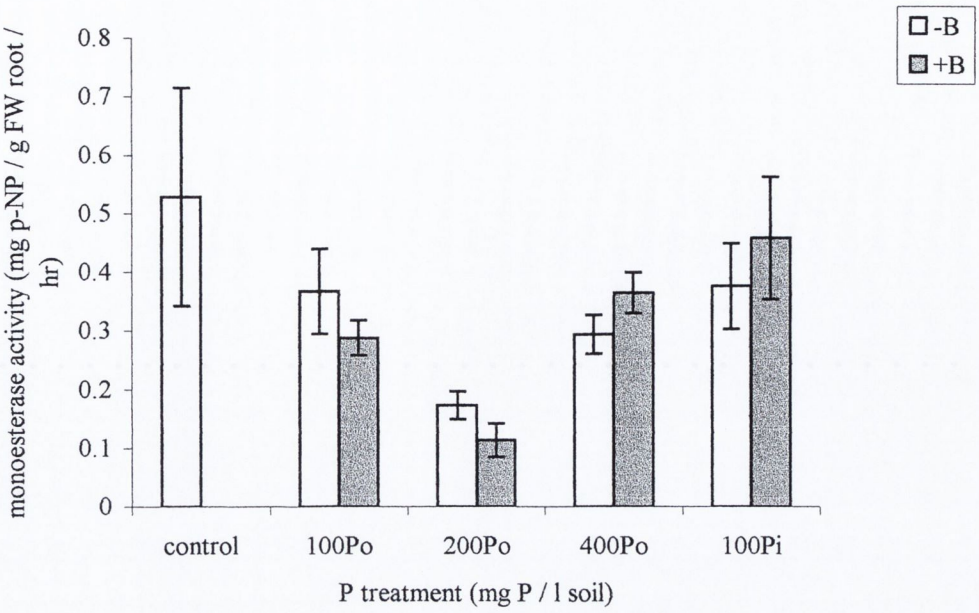


Fig 2.26: root acidic monoesterase activity for the control and P treatment plants, for the *Agrostis stolonifera* experiment, without (-B) and with (+B) benlate treatment. Each replicate value represents the mean +/- SE of 3 replicates.

ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot and root dry weights and on shoot and root P concentrations. These ANOVAs were presented in Appendix 2, Tables 25, 26, 27 and 28 respectively.

There was a highly significant benlate affect for shoot dry weight (F ratio 14.55***) and root dry weight (F ratio 15.75***). All other treatments and interactions were not significant (Appendix 2, Tables 25 and 26). There was a significant P affect for root P concentration (F ratio 25.32***). All other treatments and interactions were not significant (Appendix 2, Tables 27 and 28).

Shoot and root dry weight

Fig 2.27 presented shoot dry weight data for all plants. Fig 2.28 presented root dry weight data for all plants.

From Fig 2.27 for plants grown in soil not treated with benlate, there was an increase in shoot dry weight, with increasing levels of Po addition to the soil. The shoot dry weight of the 100Pi treatment plants was similar to that of the 100Po treatment plants. For plants grown in benlate treated soil there was an increase in dry weight with increasing levels of Po added to the soil (Fig 2.27). The 100Pi treatment plants showed the lowest shoot dry weight. There was no data available for control plants treated with benlate as there was poor growth of these plants followed by death before the harvest date (Fig 2.27). This trend of increasing shoot dry weight with increased levels of P addition to the soil was not significant. There was no statistically significant affect of P treatment on shoot dry weight (Appendix 2, Table 25).

There was a statistically significant affect of benlate on the shoot dry weights (Appendix Table 25). The shoot dry weight of the benlate treated plants was significantly greater than the plants grown in non-benlate treated soil (Fig 2.27).

From Fig 2.28, there was no difference in root dry weight of plants grown under different P treatments in benlate treated soil. This was also true for plants grown in non benlate treated soil (Fig 2.28). There was no data available for control plants treated with benlate because there was poor growth of these plants followed by death before the harvest date. The roots from plants untreated with benlate were required for other tests such as root acidic monoesterase activity as quantification of the %RLC by VAM (Fig 2.28). There was no statistically significant affect of P treatment on root dry weight (Appendix 2, Table 26).

There was a statistically significant affect of benlate on the root dry weights (Appendix 2, Table 26). The root dry weight of the benlate treated plants was significantly greater than the plants grown in non benlate treated soil (Fig 2.28).

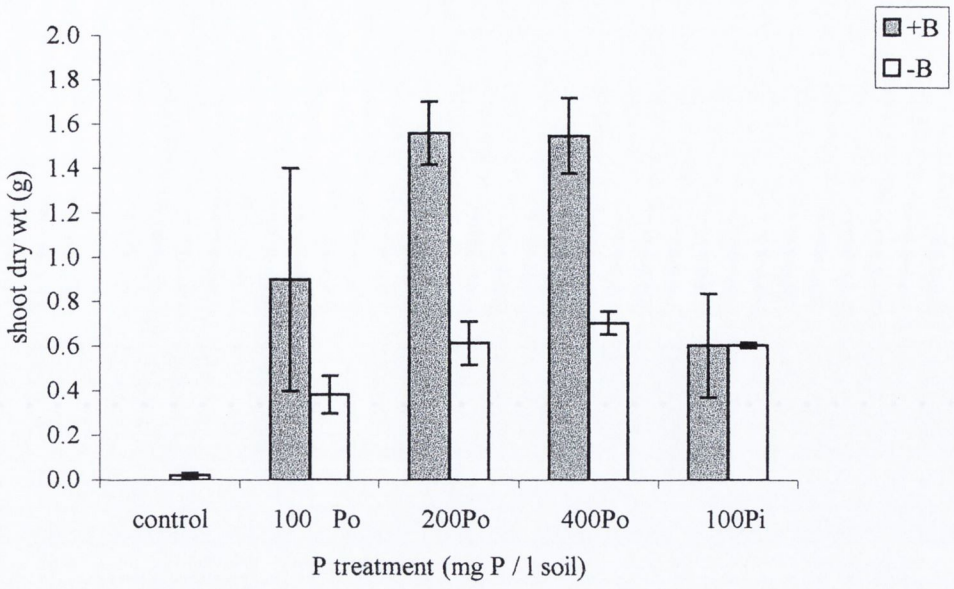


Fig 2.27: shoot dry weight for the control and P treatment plants for the *Agrostis stolonifera* experiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

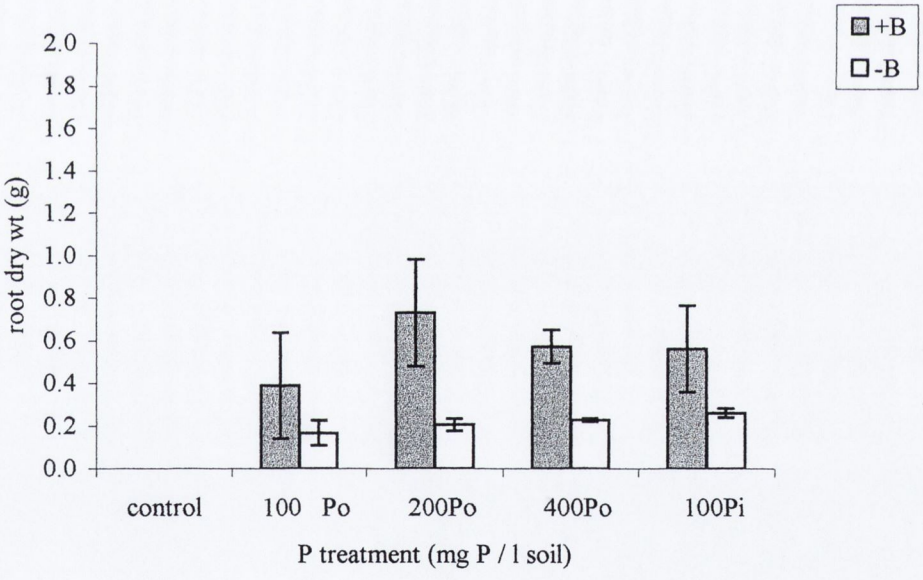


Fig 2.28: root dry weight for the control and P treatment plants fpr the *Agrostis stolonifera* experiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

Shoot and Root P Concentration

Fig 2.29 presented shoot P concentration data for all plants. Fig 2.30 presented root P concentration data for all plants.

From Fig 2.29 for plants grown in soil untreated with benlate, there was an increase in the shoot P concentration as a result of the Po and Pi treatments. The plants from these treatments had similar shoot P concentrations at the point of harvest. (Fig 2.29). There was a similar trend for plants grown in benlate treated soil (Fig 2.29). The Po and Pi treatments resulted in similar shoot P concentrations. There was no data available for control plants treated with benlate as there was poor growth of these plants followed by death before the harvest date (Fig 2.29).

There was no significant affect of benlate treatment on shoot P concentration (Appendix 2, Table 27).

From Fig 2.30, for plants grown in soil untreated with benlate, the root P concentration increased steeply from that of the 100Po treatment plants to the 200Po and 400Po treatment plants. The root P concentration of the 100Pi treatment plants was between that of the 200Po and 400Po treatment plants (Fig 2.30). This trend was repeated for the benlate treated plants (Fig 2.30). There was no data available for control plants treated with benlate as there was poor growth of these plants followed by death before the harvest date and the roots of plants not treated with benlate were required for other analyses (Fig 2.30). This significant affect of P treatment on root P concentration was examined using a Scheffe post hoc test (Table 2.32).

Table 2.32: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root P concentration for the *Agrostis s.*. experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
100Po < 200Po	*
100Po < 400Po	***
100Po < 100Pi	***
200Po < 400Po	***
100Pi - 200Po	ns
100Pi - 400Po	ns

There was no significant affect of benlate treatment on root P concentration (Appendix 2, Table 28).

Total plant P content

Fig 2.31 presented total plant P content data for plants grown in soil treated and untreated with benlate. A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on total plant P content. This ANOVA was presented in Appendix 2, Table 29.

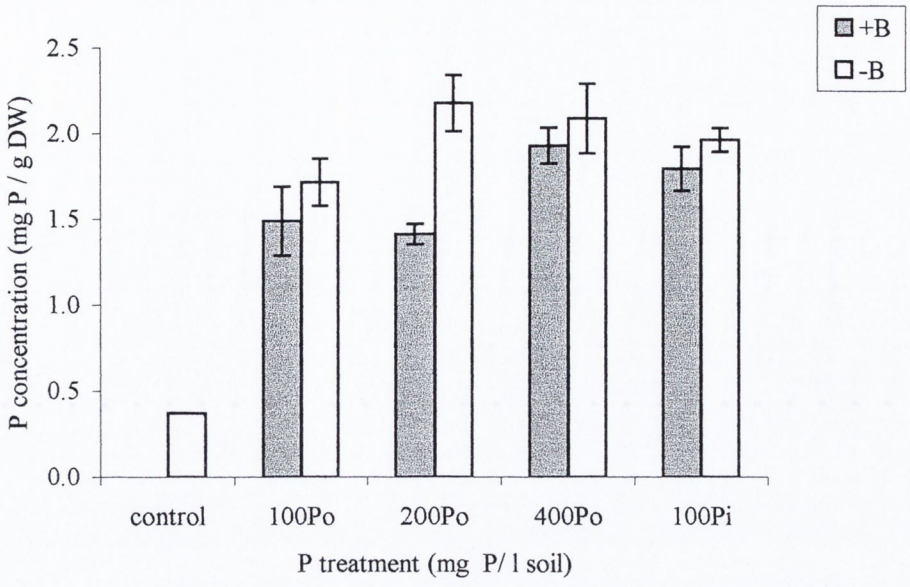


Fig 2.29: shoot P concentration for the control and P treatment plants, for the *Agrostis stolonifera* experiment, without (-B) and with (+B) benlate treatment.Each value represents the mean +/- SE of 3 replicates.

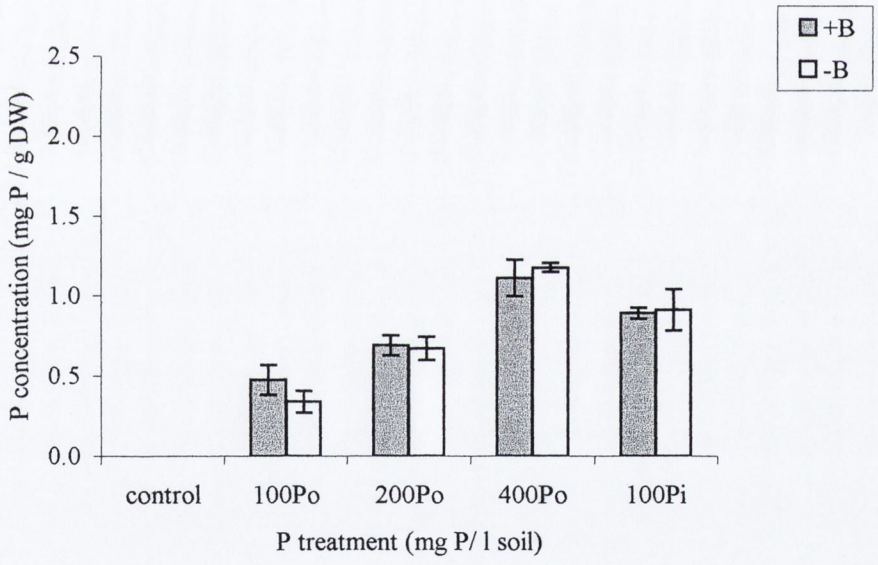


Fig 2.30: root P concentration for the control and P treatment plants, for the *Agrostis stolonifera* experiment, without (-B) and with (+B) benlate treatment.Each value represents the mean +/- SE of 3 replicates.

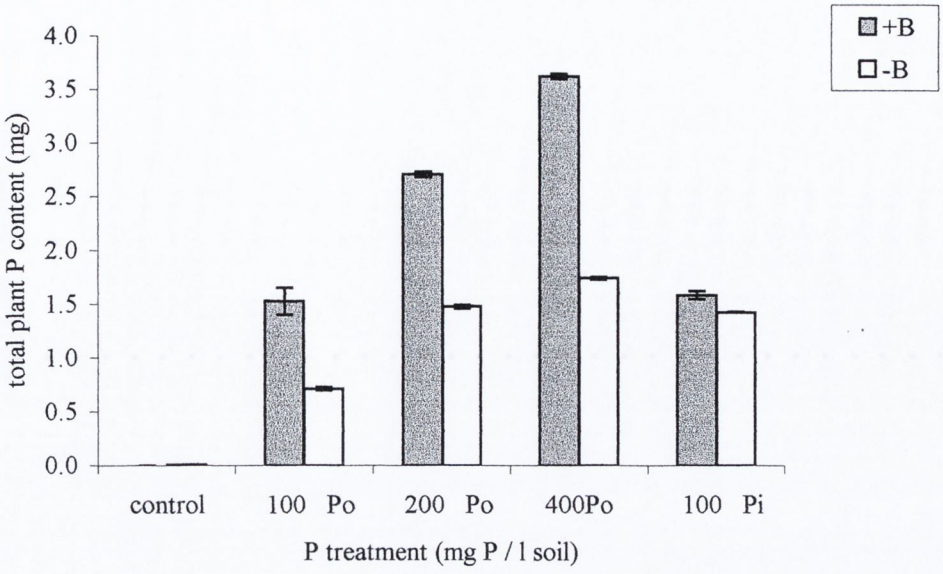


Fig 2.31: total plant P content for the control and P treatment plants, for the *Agrostis stolonifera* experiment, without (-B) and with (+B) benlate treatment. Each value represents the mean +/- SE of 3 replicates.

There was a highly significant benlate affect (F ratio 21.68***) and a significant P treatment affect (F ratio 6.94**) for total plant P content (Appendix 2, Table 29).

From Fig 2.31 there was an increase in total plant P content with increasing levels of Po in the soil. The 100Pi treatment also resulted in an increase in total plant P content but this increase was lower than that obtained with the highest Po treatment. This was true for plants grown in benlate treated and untreated soil. (Fig 2.31). This significant affect of P treatment on total plant P content was examined using a Scheffe post hoc test (Table 2.33).

Table 2.33: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on total plant P content for the *Agrostis s.*. experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control - 200Po	ns
control - 400Po	ns
control - 100Pi	ns
200Po - 100Po	ns
400Po > 100Po	**
400Po - 200Po	ns
400Po - 100Pi	ns
100Pi > 100Po	*
100Pi - 200Po	ns

There was a statistically significant affect of benlate treatment on the total plant P content (Appendix 2, Table 29). The total plant P content of plants grown in benlate treated soil was higher than plants grown in soil with no benlate treatment (Fig 2.31).

Section B: Results from experiment 2 – The hyphal exploration experiment

In the description of the results that follows, P treatment was the addition of IHP organic phosphate to the soil at the single concentration employed, 60 mg P / l soil, abbreviated to 60Po for the purpose of graphs and the results and discussion section. The 60Po treatment plants referred to plants grown in pots with the outer soil amended with 60Po, and the inner soil un-amended with P. The control treatment was PP1 soil with no phosphate addition. Where control plants were discussed this referred to plants grown in pots with inner and outer soil un-amended with P.

Benlate treatment referred to the addition of the fungicide benlate to the soil. Where soil was treated with benlate, the benlate was applied to both the inner and outer soil.

The term 'location' referred to either inner root compartment soil or the outer hyphal compartment soil, which are abbreviated to inner and outer soil respectively. Start soil referred to the soil as prepared at the start of the experiment, with the relevant P and benlate treatment,

but before contact with any plants. Post harvest soil refers to soil from which the plants have been harvested after their 12 week growth period.

2.3.4 *Plantago lanceolata*

2.3.4.1 Soil phosphate

The soil total P (Tp), inorganic P (Pi) and organic P (Po) concentration data were presented in Fig 2.32 (a, b), 2.33 (a, b) and 2.34 (a, b) respectively. Three way ANOVAs were carried out to determine the affect of P treatment and benlate treatment and location of soil on soil Tp, Pi and Po concentration. These ANOVAs were presented in Appendix 2, Tables 30, 31 and 32 respectively.

For Tp there was a highly significant P affect (F ratio 37.84***), soil location affect (F ratio 49.33***) and benlate affect (F ratio 4.82*). There was also a significant P – location of soil interaction (F ratio 22.64***) for Tp. All other treatments and interactions were insignificant. There were no significant treatments or interactions for the Pi or Po. (Appendix 2, Tables 30, 31 and 32).

Total P

Fig 2.32 presented soil Tp concentration data for control and 60Po treatment soils, treated and untreated with benlate.

From Fig 2.32 there was an expected increase in start soil Tp levels with increasing levels of P added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Tp concentrations. There was a significant decrease in Tp concentration, of the inner and outer soils of the control and 60Po treatments, with and without benlate treatment (Fig 2.32).

There was a significant affect of location of soil on the Tp concentration (Appendix 2, Table 30). However from Fig 2.32 this was not evident. There was no difference in the Tp concentration of the inner and outer postharvest soils (Fig 2.32).

There was a significant affect of benlate treatment on the soil Tp concentration of the postharvest soils (Appendix 2, Table 30). However from Fig 2.32 this was not evident. There was no difference in the Tp concentration of the benlate treated and untreated postharvest soils (Fig 2.32).

From Fig 2.32 for the post harvest soil there was an increase in total P concentration in the soil with the 60Po treatment for both the soil treated and untreated with benlate. This affect of P treatment on Tp concentration was statistically significant (Appendix 2, Table 30).

The significant interaction between P treatment and the location of soil was examined using a Scheffe post hoc test (Table 2.34).

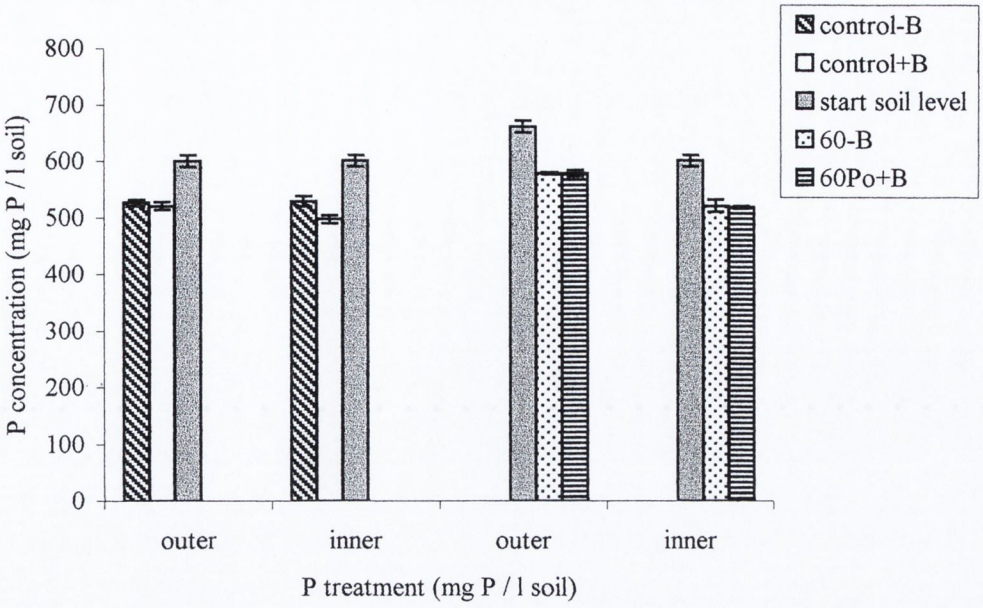


Fig 2.32: soil total phosphate concentration for the control and P treatment soil without (-B) and with (+B) benlate for the *Plantago lanceolata* hyphal exploration experiment. Each value represents the mean +/- SE of 3 replicates.

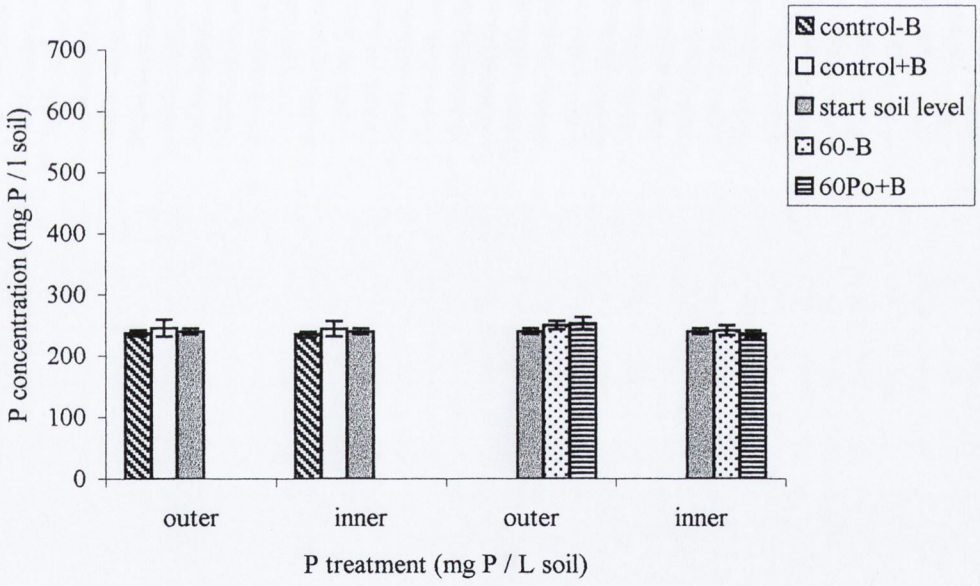


Fig 2.33: soil inorganic phosphate concentration for the control and P treatment soil without (-B) and with (+B) benlate for the *Plantago lanceolata* hyphal exploration experiment. Each value represents the mean +/- SE of 3 replicates.

Table 2.34: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors location of soil and P treatment, that affected soil Tp concentration for the *Plantago l.* hyphal exploration experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
Outer, control - Inner, control	ns
Outer, 60Po > Inner, 60Po	***
Inner, 60Po - Inner, control	ns
Outer, 60Po > Outer, control	***

Inorganic P

Fig 2.33 presented soil Pi concentration data for control and 60Po treatment soils, treated and untreated with benlate.

From Fig 2.33 there was no increase in start soil Pi levels with increasing levels of Po added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Pi concentrations. There were no significant differences in Pi concentration (Fig 2.33). There was no significant affect of location of soil on the Pi concentration (Appendix 2, Table 31).

There was no significant affect of benlate or P treatment on the soil Pi concentration of the postharvest soils (Appendix 2, Table 31). There was no interaction between any of the sources of variation that had a significant affect on the soil Pi concentration (Appendix 2, Table 31).

Organic P

Fig 2.34 presented soil Po concentration data for control and 60Po treatment soils, treated and untreated with benlate.

From Fig 2.34 there was an increase in start soil Po levels with increasing levels of Po added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Po concentrations. There were no significant differences (Fig 2.34). There was no significant affect of location of soil on the Po concentration (Appendix 2, Table 32).

There was no significant affect of benlate or P treatment on the soil Po concentration of the postharvest soils (Appendix 2, Table 31). There was no interaction between any of the sources of variation that had a significant affect on the sol Po concentration (Appendix 2, Table 32).

2.3.4.2 Colonisation of Roots by VAM

The colonisation of roots by vesicular arbuscular mycorrhizal (VAM) fungi was measured by % root length colonisation (%RLC). The %RLC data was presented in Fig 2.35. A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on %RLC. This ANOVA was presented in Appendix 2, Table 33.

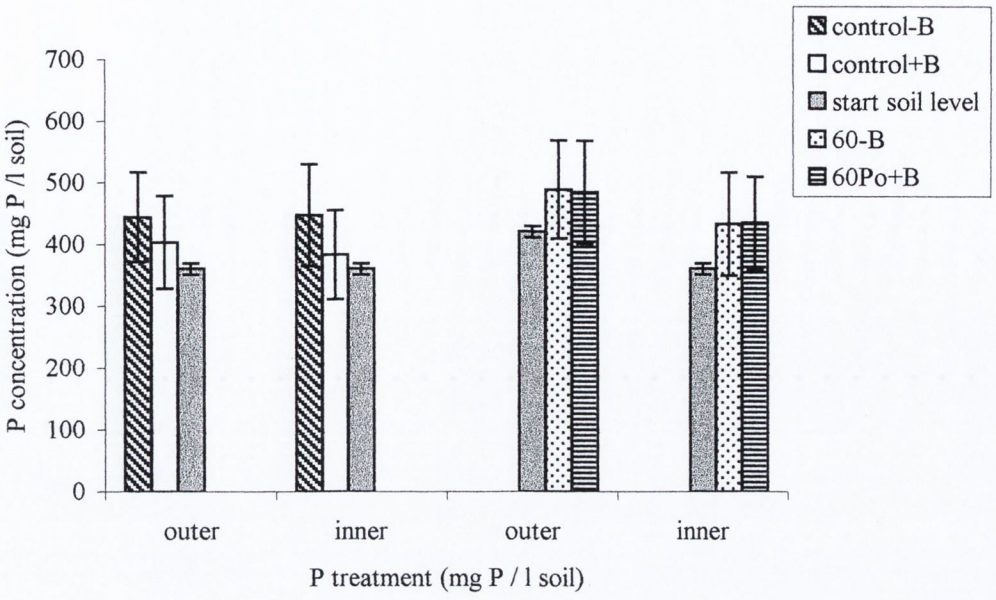


Fig 2.34: soil organic phosphate concentration for the control and P treatment soil without (-B) and with (+B) benlate for the *Plantago lanceolata* hyphal exploration experiment. Each value represents the mean +/- SE of 3 replicates.

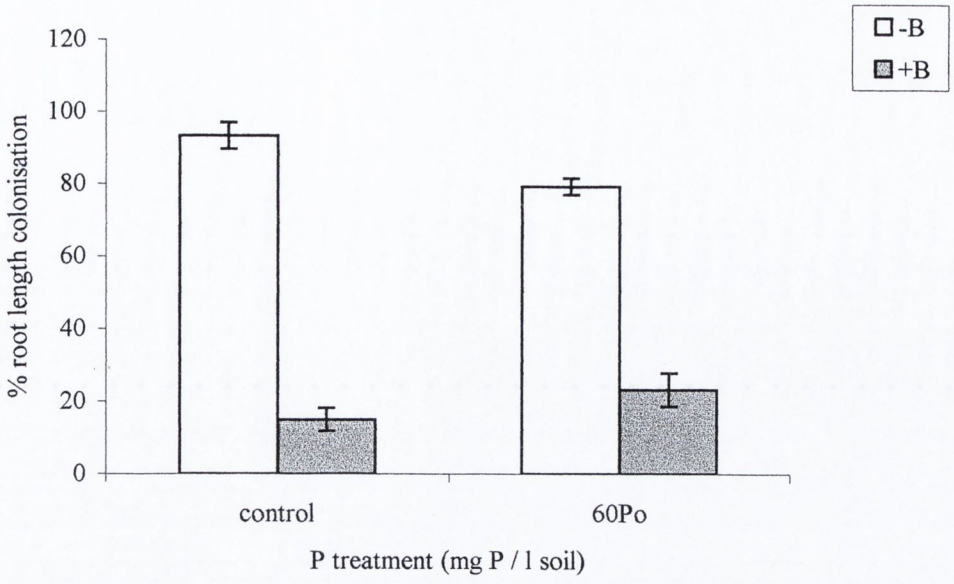


Fig 2.35: % root length colonisation for the control and P treatment plants, without (-B) and with (+B) benlate treatment, for the *Plantago lanceolata* hyphal exploration experiment. Each value represents the mean +/- SE of 3 replicates.

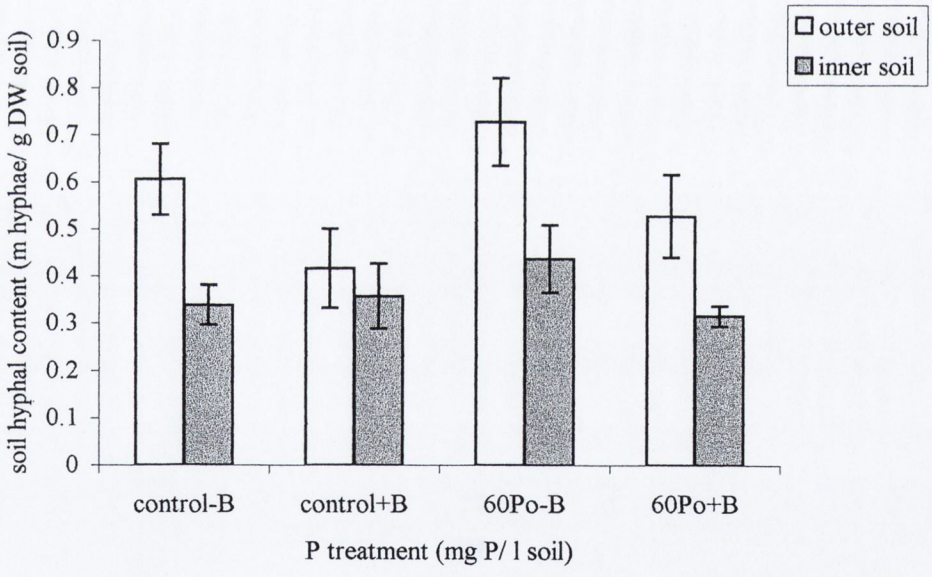


Fig 2.36: soil hyphal content for the control and P treatment soils, without (-B) and with (+B) benlate treatment, for the *Plantago lanceolata* hyphal exploration experiment. Each value represents the mean +/- SE of 3 replicates.

There was a highly significant benlate affect (F ratio 255.13***) and a significant P – benlate interaction (F ratio 8.28*) (Appendix 2, Table 33)

Figure 2.35 presented %RLC data for all *Plantago l.*. From Fig 2.35 there was a decrease in %RLC, for the control and 60Po treatment plants as a result of benlate treatment. This affect of benlate treatment on %RLC was statistically significant (Appendix 2, Table 33).

There was a decrease in %RLC as a result of the 60Po treatment for plants with no benlate application (Fig 2.35). There was no change in %RLC as a result of the 60Po treatment for benlate treated plants (Fig 2.35). This affect of P treatment on %RLC of the non-benlate plants was not statistically significant (Appendix 2, Table 33).

The significant interaction between P treatment and benlate was examined using a Scheffe post hoc test (Table 2.35).

Table 2.35: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected %RLC for the *Plantago l.* hyphal exploration experiment. Soil without and with benlate treatment was represented as –B and +B respectively.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison			P value
+B, control	<	-B, control	***
+B, 60Po	<	-B, 60Po	***
-B, 60Po	<	-B, control	***
+B, 60Po	-	+B, control	ns

There was a significant decrease in %RLC for all plants as a result of benlate treatment. The P treatment significantly reduced %RLC in soil with no benlate application. (Table 2.35).

2.3.4.3 Soil Hyphal Content

The soil hyphal content data was presented in Fig 2.36. A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on soil hyphal content. This ANOVA was presented in Appendix 2, Table 34.

There was a statistically significant affect of location of soil (F ratio 14.39***) on the soil hyphal content (Appendix 2, Table 34). The soil hyphal content in the outer soil of the control and 60Po treatment was greater than that in the inner soils (Fig 2.36).

From Fig 2.36 there was a decrease in soil hyphal content of the outer soil and no change in the inner soil on addition of benlate to the control soil. There was a decrease in outer and inner soil hyphal content on the addition of benlate to the 60Po treatment soil (Fig 2.36). This affect of benlate treatment on the soil hyphal content was significant (F ratio 43.94*) (Appendix 2, Table 34).

All other treatments and interactions were not significant (Appendix 2, Table 34).

2.3.4.4 Shoot Dry Weight

The shoot dry weight data was presented in Fig 2.37. A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on the shoot dry weight data. This ANOVA was presented in Appendix 2, Table 35.

There was a highly significant benlate affect (F ratio 29.78***) on the shoot dry weight of *Plantago l.* in the hyphal exploration experiment. The shoot dry weight of the benlate treated plants was significantly lower than that of the plants grown in non benlate treated soil (Fig 2.37).

From Fig 2.37, for plants grown in soil not treated with benlate, there was an increase in shoot dry weight as a result of the 60Po treatment. For plants grown in benlate treated soil there was no change in shoot dry as a result of the 60Po treatment (Fig 2.37). There was no significant affect of P treatment on the shoot dry weight (Appendix 2, Table 35).

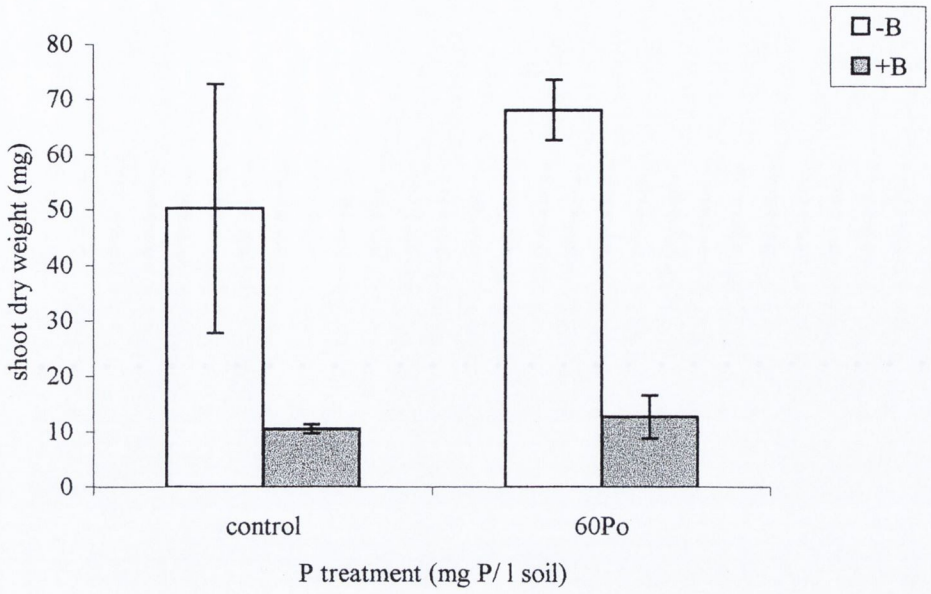


Fig 2.37: shoot dry weight for the control and P treatment plants, without (-B) and with (+B) benlate treatment, for the *Plantago lanceolata* hyphal exploration experiment. Each value represents the mean +/- SE of 3 replicates.

2.4 DISCUSSION

Section A: Discussion on Experiment 1 – the Benlate Addition Experiment

2.4.1 Published work

Hayes *et al.*, (2000) grew seedlings of three temperate grassland species, *Lolium rigidum, Danthonia richardsonii* and *Phalaris aquatica* in sterile agar or sand medium in the presence of inositol hexaphosphate. They found IHP to be a poor source of P for plant growth compared to plants supplied with an equivalent amount of inorganic P. Only by supplementing the agar containing the IHP with phytase from *Aspergillus niger* did sufficient P become available to enable plants supplied with IHP to grow as well as those supplied with Pi. These results indicate that the use of IHP as a source of P for plant growth is limited by the hydrolysis of this Po compound.

Richardson *et al.*, (2001) grew *Danthonia richardsonii* and *Phalaris aquatica* and also found a limited ability by these species to use P from IHP when grown in sterile agar culture or sand vermiculite culture, compared to their ability to use Pi. The poor ability of plants to utilise P from IHP resulted in significantly lower tissue P concentrations and reduced dry weight accumulation compared to plants that received Pi. The P nutrition of plants supplied with IHP was significantly improved by inoculating media with a cultured population of total soil microorganisms. Soil microorganisms provided a source of phytase for the breakdown of IHP for use by plants.

Tarafdar and Marschner (1995) used heat sterilised low P soil supplied with Na-phytate to grow wheat in a pot experiment. Inoculation with a VAM fungus *Glomus mossae* increased root and shoot dry weight and shoot P concentration. The results showed that Po in the form of Na-phytate was used efficiently by VAM.

Andropogon gerardii was grown in a sterilised sand medium inoculated with *Glomus etunicatum* to examine the potential of the plant to utilise phytic acid as a source of P for plant growth by Jayachandran *et al.*, (1992). Plant growth and P uptake by non VAM plants was not affected by amendment of the growth medium with phytic acid. However mycorrhizal plants did benefit from the presence of phytic acid in the soil. Mycorrhizal plants were larger and had a greater total P uptake than non VAM plants.

Unlike the published work described above, the work described in this chapter used a natural soil with its indigenous VAM population. Under these conditions IHP was found to be an available source of P to plants. As natural an environment as possible is best for such experiments because as demonstrated by the published work above the addition of soil microorganisms to a sterile culture can change the outcome of an experiment. The effectiveness of VAM fungi in providing phosphorus to the host plant depends very much on the VAM

species (Marschner, 1995). The 100Po and 400Po treatments and also the 100Pi treatments resulted in an increase in shoot dry weight, P concentration and P content for *Dactylis* and *Plantago*. The increase as a result of the 100Po treatment was equal to that of the 100Pi treatment. This indicates that IHP was an available source of P for plant growth. The growth of the plant is a monitor of Pi release. The positive affect of P treatment indicates the release of orthophosphate from the IHP, which was then taken up by the plants. At equivalent concentrations, IHP was a source of available P equal to that of Pi as indicated by biomass production. The increase in dry weight and P concentration reinforces the idea that up to a point greater P concentrations lead to a higher dry matter accumulations (Antunes and Cardoso, 1991). As there were no control plants for *Agrostis* it was not possible to determine the affect of increased P concentration in the soil on plant growth.

With regards to the benefit of VAM fungi in the use of IHP by plants, the reduction of VAM presence in the soil because of the application of benlate did not result in a significant change in the shoot dry weight, P concentration or P content for *Dactylis*. This was also true for *Plantago* with the exception of the P concentration, which was decreased in benlate treated soil. It was expected that a reduction in VAM presence would decrease the biomass and P accumulation as VAM are important in the uptake of P by plants (Marschner, 1995). The importance of VAM in the uptake of P by plants was demonstrated in Chapter 5 Section 5.4.1. Here IHP was added to the soil at 100Po, 200Po and 400Po and plants were grown in growth bags as described for this chapter. There was greater depletion of Po form the outer soil accessible to hyphae only than there was from the inner soil where the roots were located. This depletion was linked to the uptake of P by plants from the soil. In the current chapter there was no interaction between benlate and P treatment that had a significant affect on these characters. Such an interaction may have indicated that a reduction in VAM presence had a detrimental affect on plant growth at lower P concentrations but no affect at increased levels of plant available P. It is thought that VAM are most beneficial to plants at low P availability (Marschner, 1995). There was however a trend in the dry weight and P content data as a result of benlate treatment.

The addition of benlate to the soil resulted in plants of reduced VAM presence (Fig 2.9, 2.22, 2.38). Where no benlate was applied to the soil these plants are referred to as VAM plants. The presence of VAM fungi increased the dry weight and P content of *Dactylis* and *Plantago* in the control soil and the lowest Po treatment 100Po compared to plants with reduced VAM presence. This indicates a beneficial affect of the VAM fungi at these low soil P concentrations. However for the higher Po treatment 400Po and the 100Pi treatment the VAM reduced plants had a higher dry weight and P content, than the VAM plants. For these treatments the VAM fungi are having an adverse affect on plant growth.

While the formation of VA mycorrhizas under P limiting conditions results in general growth increases, root colonisation may result in growth depressions under conditions of ample nutrient supply (Jakobsen, 1995).

In order to test the hypothesis that high levels of soluble P applied in combination with VAM fungi can cause growth depressions, Antunes and Cardoso (1991) conducted a greenhouse experiment. Citrus plants were grown with four levels of soluble P, 0, 50, 100 and 200 mg P/l soil and half the plants were inoculated with *Glomus etunicatum*. At harvest shoot dry weight and root colonisation were determined. Significant increases in shoot dry weight due to VAM inoculation at the zero and 50 mg P/l soil were found. At 100 mg P/l soil the development of the VAM plants was equivalent to that of the non VAM plants and at 200 mg P/l soil growth was significantly less than that of non VAM plants. A similar pattern was visible for the leaf P content. As root colonisation was reduced at higher P levels, in this experiment it was concluded that the growth depression of the VAM plants at the higher P level was likely due to P toxicity.

In my experiment Po was used and the natural soil VAM population and growth depression was also seen at high P levels. Root colonisation was decreased by high P treatments for *Plantago* but not for *Dactylis* therefore the presence of VAM fungi may be involved in this growth depression for *Dactylis*. Abbott and Robson (1986) presented a discussion on several hypothesis that could explain the plant growth depression. These included introduction of a plant pathogen together with the mycorrhizal inoculum, competition for P, P toxicity and competition between the host plant and the VAM fungus for carbohydrates. VAM inoculum was not used in this experiment and it is unlikely that a plant pathogen from another source was the cause of the growth depression seen here because there was no disease incidence in the plants. Competition for P is also an unlikely cause because at lower levels of P VAM fungi promoted significant growth increases. It is possible that there was a drain on C resources in the case of *Dactylis*. Increased below–ground allocation of C can be responsible for growth reduction when the C drain imposed by the fungus is not compensated for by an increased rate of photosynthesis (Jakobsen, 1995). There was no reduction in VAM infection of the roots with increased P level in the soil in *Dactylis* which would reduce the C drain on the plant. Furthermore when P is not limiting for plant growth more P will be available for carbohydrate phosphorylation which is fundamental for active carbon transport from host to VAM fungus. Therefore at higher soil P levels active carbon transport can possibly proceed at higher rates resulting in greater drain from the host (Antunes and Cardoso, 1991). P toxicity is possible but unlikely for *Dactylis* because shoot P concentration was not significantly different in the 400Po and 100Pi treatment plants to that in the 100Po treatment plants. Therefore in the case of *Dactylis* it is most likely that a drain on the C reserve of the plant was responsible for the growth depression at high soil Po concentrations. For *Plantago* there was a reduction in the root colonisation at the 400Po and

100Pi treatments. In this case C drain from the host plant seems very unlikely as the reason for the growth depression (Abbott and Robson, 1986). P toxicity is most likely responsible for the depression in *Plantago*. The shoot P concentration in the 400Po and 100Pi treatment plants was significantly higher than that in the 100Po treatment plants. The plant growth depression that occurs in the grass *Dactylis* and the herb *Plantago* apparently occurs for two different reasons.

There was a significant affect of benlate treatment on the shoot and root dry weight and the total plant P content for *Agrostis*. The reduction in VAM presence as a result of the benlate treatment increased the dry weight and P content. For this species at concentrations of 100Po, 200Po and 400Po the presence of VAM had a detrimental affect on these characters. As there was no decrease in the root colonisation with P treatment it is possible that even at the low P treatment, 100Po the drain on the C reserves of the plant was too great and resulted in growth depression at all P treatments.

Tarafdar and Marschner (1994) found Po, in the form of IHP at 200 mg P/l soil increased the % of infected root length. The results demonstrate a stimulatory affect of Po in the form of IHP on VAM infection. Thingstrup *et al.*, (2000) observed a negative affect of increased soil Pi level on the colonised root length as in accordance with many previous studies. This affect has been attributed to a regulatory role of the plant P concentration. Although the underlying mechanisms have not been elucidated, the reduced mycorrhizal formation at high P levels would result from both a reduced growth rate of infection units and a reduced production of secondary external hyphae (Thingstrup *et al.*, 2000). Many factors can influence the development of mycorrhizal infection in a root system but one of the most important factors is soil and plant P. It has still not been determined whether P affects fungal development in the soil or the infection and development process in the root. Mycorrhizal infection may be regulated by soil P and there may be a threshold level below which VAM infection can be maintained at a high level. Regulation of infection by the P content of the plant is also possible but how this occurs is not clear. Plant P content can affect soluble carbohydrate content of root material and exudates and therefore affect the carbon source available for a VAM fungus. Permeability of root cell membranes has been proposed as a mechanism for the control of VAM infection. Under low P nutrition, increased root cell membrane permeability leads to increased loss of metabolites, which can sustain VAM fungal infection of roots. The VAM fungi then improve the P nutrition of the plant, root cell membrane permeability decreases, and so therefore does the loss of metabolites and the VAM infection. (Cooper, 1986).

The addition of organic or inorganic phosphate to the soil at the concentrations used in this experiment did not have a significant affect on the %RLC of the grass species *Dactylis* or *Agrostis*. %RLC was approximately 35% for the control and P treatments. It was expected that the addition of IHP to the soil would result in a decrease in the %RLC because, mycorrhizas are

thought to be most affective in phosphate nutrition at low to moderate levels of phosphorus in the growth medium. In general mycorrhizal affects decrease with increasing levels of available phosphate. Although this is mainly explained by the better phosphorus nutrition of non VAM plants increasing P can also affect development and physiology of mycorrhizal infection. (Gianinazzi-Pearson and Gianinazzi, 1989). The theory that mycorrhizas are most affective in aiding plants at low levels of P in the medium is based mainly on evidence from studies using artificial medium and selected strains of VAM fungi. IHP is an available source of P to plants as was shown by an increase in growth on the addition of IHP to the soil as will be discussed later and would therefore be expected to decrease %RLC. It would appear that the natural VAM in this soil are not inhibited by IHP concentrations in the soil up to 400 mg P/l soil. The 100Pi treatment also did not result in a significant decrease in the %RLC for these two species. Again it was expected that as a readily available source of P to plants the superphosphate would reduce the %RLC as the reliance of plants on VAM should be reduced. It is possible that in a natural soil the relationship between low P and VAM presence does not hold true. A natural soil with indigenous fungi, bacteria and microorganisms poses a more complicated environment than the simplified system with one species of inoculated VAM.

In contrast to the grass species, for the herb species *Plantago* there was a significant decrease in the %RLC from approximately 85% to 50% as a result of the higher Po treatment, 400Po and the 100Pi treatment. This was as expected for the reasons explained above. It appears that the reaction to high P levels in the soil differs between grasses and herbs.

There was a decrease in the percentage root length colonisation (%RLC) by VAM fungi for the three species, *Agrostis, Dactylis* and *Plantago* as a result of the addition of benlate to the soil. This was as expected, and was found by many other workers (Menge, 1982; Fitter and Nicholas, 1988; Habte, 1997). The addition of benlate to the soil at the rate of 0.89 mg/kg soil successfully inhibited VAM hyphal growth for the duration of the experiment, 9 weeks but did not eliminate it totally. Thingstrup *et al.*, (2000) observed successful inhibition of hyphal production by benlate at a rate of application of 20 mg/kg soil but some mycorrhizal colonisation was found in plants grown in benlate treated field soil.

Joner and Johansen (2000) extracted extraradical hyphae of *Glomus intraradices* and *G. claroideum* from sand and examined fungal phosphatase activity using the substrate p-nitrophenyl phosphate. Maximum activity was found in acid pH in the range 5.2-5.6 for both fungi. They stated this was evidence that phosphatases produced by external hyphae of AM fungi can hydrolyse extracellular phosphate ester bonds. Separation of the phosphatase activity into external soluble, wall bound and internal fractions revealed that up to 70% of the measured activity was associated with the hyphal wall and the rest with internal structures. However based on estimates of VAM fungal biomass in soil they conclude that VAM fungal contribution to

overall phosphatase activity in soil is low. Koide and Kabir (2000) demonstrated that the extraradical hyphae of *Glomus intraradices* can hydrolyse the Po compounds 5-bromo-4-chloro-3-indolyl phosphate and phenolphthalein diphosphate in a sterile gel culture. Although they did not demonstrate the hydrolysis of IHP they found significantly more P was transferred to roots when the hyphae had access to phytic acid. Joner and Johansen (2000) and Koide and Kabir (2000) examined the phosphatase activity of species *Glomus* cultures available commercially and grown in artificial media.

Joner *et al.*, (1995) tested the ability of VAM roots and hyphae to produce extracellular phosphatases. Cucumber plants were grown in a sterilised sand soil mix inoculated with *Glomus invermaium*. No differences in phosphatase activity were found between soil with or without a dense VAM fungal mycelium. VAM hyphae seem to release none or very small amounts of extracellular phosphatases.

Joner and Jakobsen (1995) investigated the influence of addition of soil organic matter to a soil on the growth of VAM hyphae and changes in phosphatase activity. Soil was sterilised and inoculated with *Glomus invermaium* and organic matter was added in the form of chopped wheat straw. In this study, the acid phosphatase activity was not altered by the presence of AM hyphae but the quantity exuded by the mycorrhizal roots was reduced. These results support the conclusions of Dodd *et al.*, (1987) and Joner *et al.*, (1995) that extracellular phosphates are not produced extraradical hyphae of the AM symbiosis. The influence of VAM hyphae on phosphatase activity in root free soil seems to be indirect through interactions with other microorganisms.

Tarafdar and Marschner (1994) examined the phosphatase activity of VAM fungi in the rhizosphere and bulk soil of mycorrhizal and non mycorrhizal wheat plants. The soil was sterilised and inoculated with *Glomus mosseae* and plants were grown in compartment pots separating the root and hyphal zones. In the root and hyphal compartments acid phosphatase was enhanced by mycorrhizal infection.

Previous studies of extracellular phosphatase in the rhizosphere of VAM plants have shown increased activity (Dodd *et al.*, 1987; Tarafdar and Marschner, 1994), no difference (Dodd *et al.*, 1987) and reduced activity (Azcon *et al.*, 1982) due to mycorrhizal colonisation. Results are conflicting overall.

In the work described in this chapter using a natural soil an its indigenous VAM fungi no affect of VAM fungi was seen on the soil acidic monoesterase activity. Soil acidic monoesterase activity in relation to benlate and P treatment was examined. While this aspect of soil acidic monoesterase activity was examined in Chapter 4, a chapter which focused in-depth on phosphatase enzymes, including soil acidic monoesterase, the work in the present chapter works

with much higher concentrations of soil Po additions. This was in order to determine if increasing concentration of Po alters the outcome. For soil planted with *Dactylis, Agrostis* and *Plantago* the addition of benlate to the soil did not have a significant affect on the soil acidic monoesterase activity. As the benlate reduced the presence of VAM fungi in the soil it indicates the VAM fungi do not make a large contribution to soil acidic monoesterase activity, even with increased levels of IHP in the soil. Results in Chapter 4 also indicated the VAM fungi did not make a large contribution to soil acidic monoesterase activity at lower concentrations of 30Po and 60Po. Acid monoesterase activity is attributed to plant roots and associated mycorrhizal and saprophytic fungi (Joner and Jakobsen, 1995). However, increasing Po concentrations up to 400Po did not stimulate a production of acidic monoesterase activity by the VAM fungi which then contributed to soil of acidic monoesterase activity.

Non mycorrhizal and VA mycorrhizal *Bouteloua gracilis* was inoculated with *Glomus fasciculatus* and grown in sterile agar in the presence of calcium phytate by Allen *et al.,* (1981). Mycorrhizal plants grown in phytate medium did not have significantly different acidic root phosphatase activity than the non mycorrhizal plants. That is mycorrhizae did not contribute to root monoesterase activity. In this chapter, the root acidic monoesterase activity of *Dactylis* and *Agrostis* was not significantly affected by a reduction in VAM presence through benlate application to the soil. This would indicate that for these species VAM hyphae are not contributing to the acidic monoesterase activity of roots. This agrees with the results of Allen *et al.,* (1981). Root activity was originating from the roots themselves. Both of these species have %RLC of approximately 30%. For *Plantago* there was a significant increase in root acidic monoesterase activity. This would suggest that a reduction in the presence of VAM hyphae induced the root to increase its own production of monoesterase and that this species was reliant on its associated VAM fungus to contribute to its root monoesterase. This is in contrast to the grasses *Dactylis* and *Agrostis* where the contribution of VAM to root phosphatase appears insignificant. The herb *Plantago* also has, at approximately 80%, the highest %RLC of the three species, also indicating a different relationship between it and its VAM fungi than for the grasses. Work in Chapter 4 section 4.4.1.4 also indicated that for *Agrostis* and *Dactylis* VAM did not contribute to root acidic monoesterase activity. There was no interaction between P treatment and benlate treatment that had a significant affect on root acidic monoesterase activity that may have indicated that VAM contributed to root monoesterase activity depending on the amount of P and therefore substrate in the soil. As VAM did not contribute to the soil acidic monoesterase activity it was not expected that they would contribute to the root acidic monoesterase activity.

2.4.2 Information Gained

It was expected that the soil acidic monoesterase activity would be higher in the inner soil than in the outer soil. This was because this activity originates from plant roots which were confined to the inner soil, and from mycorrhizal and saprophytic fungi which would be expected to be more plentiful in the inner soil which includes the rhizosphere. The rhizosphere, the zone of soil in contact with the roots, has a higher bacterial and fungal population than soil more distant from the roots because of root exudates and decaying root material that are substrates for the growth of microorganisms. Only for soil planted with *Agrostis* was the activity in the inner soil significantly higher than that in the outer soil. It is possible that *Agrostis* encouraged the growth of bacteria and fungi in its rhizosphere that had a higher capability for acid monoesterase activity which contributed to the activity detected in the inner soil, than bacteria and fungi present in the soil of the *Dactylis* and *Plantago* pots. It would be necessary to isolate and culture the microorganisms from the rhizosphere of each of the plants to determine if populations changed depending on plant species.

Soil acidic monoesterase activity for the three species was in the range from 0.4 to 0.9 mg p-NP/g FW soil/hr. That is despite the presence of different species in the soil overall the monoesterase activity remained the same. A range of these soil acidic monoesterase activities from the literature was presented in Chapter 4 section 4.4.2.

IHP is a monoester P compound and as such it is a substrate for the soil acidic monoesterase activity. An increase in IHP in the soil was be expected to increase soil acidic monoesterase activity. For *Dactylis* the 100Po and 400Po treatments did not result in a significant change in soil acidic monoesterase activity. For *Plantago* the 400Po but not the 100Po treatment resulted in a significant increase in activity. The reaction to Po appears to be species dependent. The 100Po treatment may not be sufficient to result in an increase in activity but the 400Po did result in an increase for one species. It is possible that although the added IHP was a substrate for the monoesterase that the production of this enzyme was limiting. Only the soil environment of *Plantago* was able to increase acidic monoesterase production. This would again suggest that the plant species may affect the microorganism population of a soil which in turn may affect soil acidic monoesterase activity. For *Agrostis* again no change in monoesterase activity was found with the 100Po treatment but a decrease in activity was found with the 400Po treatment and the intermediate 200Po treatment. This was unexpected as an increase was expected. It is possible that the soil environment for this species was capable of and did indeed increase soil acidic monoesterase activity which increased the Pi content of the soil. This Pi may not have been taken up rapidly enough by plants and the soil biota resulting in an increase in orthophosphate in the soil that would ultimately decrease monoesterase activity. Orthophosphate is a competitive inhibitor of monoesterase activity (Juma and Tabatabai, 1978;

Tabatabai 1994). It would be necessary to carry out assays for soil acidic monoesterase activity over a period of time in order to determine how activity changes over time in order to examine this theory further.

As orthophosphate is a competitive inhibitor of monoesterase activity it was expected that the 100Pi treatment would result in a decrease in monoesterase activity in the soil. However this was only the case for soil planted with *Agrostis*. For soil planted with *Dactylis* the 100Pi treatment resulted in an increase in activity and for soil planted with *Plantago* there was no change in activity. Again as the affect of Pi treatment changes with species it would suggest that different microorganisms are present in the soil planted with different species. This organisms have different capabilities for monoesterase production and the affect of increased Pi concentration in their surroundings on the monoesterase activities depends on the properties of the enzyme they produce. In the natural soil environment increased Pi may not always result in decreased monoesterase activity. Not enough research on enzymes has been done in natural soil systems. For all species however the activity in the 100Po and 100Pi treatments soils were equal. At this concentration Po and Pi sources do not affect soil monoesterase activity differently. A full discussion of soil acidic monoesterase activity, including background knowledge and assay methods can be found in Chapter 4, sections 4.1.4 and 4.1.6.

For all three species there was a decrease in root acidic monoesterase activity as a result of the 100Po, 400Po and 100Pi treatments. This decrease was significant for *Dactylis* and *Plantago* but not for *Agrostis* because of the large standard errors associated with this data. This decrease was as expected. Root acidic monoesterase activity is closely tied to plant demand for phosphorus (Kramer and Green, 2000). Activity is increased in plants that are P deficient (Barrett-Lennard *et al*, 1993; Frossarrd 1995; Richardson *et al*, 2000). The activity was highest in the control plants as these plants were P deficient. This was indicated by the increase in biomass as a result of the addition of P to the soil. The activity was decreased with increasing concentration of P to the soil as these plants were no longer P deficient. IHP was an available source of P as shown by increased biomass resulting from the Po treatments. The Pi added was readily available superphosphate. Phosphatase activity measured in the soil indicates a potential to use Po (Fox and Comerford, 1992). The importance of organic P as a source of P to plants depends on the amount of organic P and the presence of appropriate phosphatase to mineralise Po because Po must be hydrolysed to inorganic P before it can be utilised by plants (Fox and Comerford, 1992). As Po must first be broken down before it can be used by plants it is likely that for the first period of growth there was activity similar to that present in the control plants in order to breakdown the added IHP. But the breakdown of this IHP resulted in an increase in production of orthophosphate in excess of that which plants could absorb in a given time. Orthophosphate therefore built up and inhibited root monoesterase activity resulting in a lower

harvest activity than the control plants. The added IHP as a monoesterase substrate, may have induced an increase in root monoesterase activity in the early stages of growth to obtain P for the plant before being inhibited by increased orthophosphate levels. This theory was also discussed in Chapter 4 section 4.4.3 where even the smaller Po additions of 30Po and 60Po to the soil resulted in decrease in activity. Mineralisation of Po is required for the use of Po sources by plants Root acidic monoesterase, rather than soil acidic monoesterase, is most likely to be the major contributor to Po breakdown as it shows a consistent depletion with increased soil P levels. Chapter 4 however examines other phosphatase enzymes and their relationship to Po breakdown.

Depletion of soil phosphate is an indicator of P uptake by plants. In this experiment there was depletion of soil Tp concentration from some P treatment soils but this depletion was not consistent with any pattern of P treatment. Where depletions in Po concentration occurred this was not always matched to depletions in soil Tp. Depletions in soil Po concentration were in some cases also not indicated as a decrease in Tp. To further complicate this situation there were increases in soil Pi concentration. It was not possible therefore to isolate Po depletion by uptake by plants from mineralisation of Po to the Pi pool. There was no significant affect of location of soil on the P concentration remaining in the soil following the harvest of plants. It was expected that a difference in the P concentration in the inner and outer soil would indicate greater depletion from either of these areas. This depletion could then be related to root uptake from the inner soil or hyphal uptake from the outer soil. For these experiments however this method did not prove successful. This method has however been used successfully in Chapter 4 section 4.3.1.6, 4.3.2.6, 4.3.3.6 and Chapter 5 section 5.3.6. In both these Tp was depleted as a result of Po depletion and in the latter chapter more depletion of soil Po was indicated as being due to VAM hyphae than plant roots.

Section B: Discussion on Experiment 2 – the Hyphal Exploration Experiment

2.4.3 Information Gained

In this experiment control plants were grown in PP1 soil. This soil is P limited as was discussed in section 2.2.3 in this chapter. The addition of IHP the soil results in an increase in biomass production. In order to examine the contribution of VAM hyphae to plant growth by obtaining P outside the reach of the roots the growth bag method was used. Soil within the bag as PP1 soil with no P amendment in all control and P treatments. Soil outside the bag was PP1 soil amended with 60 mg P/l soil. This low concentration of P was chosen in order to determine if this small increase in soil Po concentration could result in a significant increase in biomass production through VAM hyphal capture only. It was also of interest to determine if the soil hyphal content was affected by this increase in soil P concentration. A control and 60Po treatment were also carried out with application of benlate to the soil to reduce mycorrhizal

presence. It was expected that any increase in plant growth as a result of hyphal transport of P to the plant from the 60Po outer soil would be decreased by a reduction in VAM presence.

As expected there was a decrease in the %RLC as a result of the application of benlate to the soil. As expected, the soil hyphal content was also significantly decreased by the application of benlate to the soil. Benlate successfully reduces VAM presence in the soil. Neither the root length colonisation nor the hyphal content of the soil was eliminated completely.

There was however no significant affect of the application of 60Po to the soil on the %RLC. For the experiments in section A, there was no significant affect of higher Po concentrations, 100Po, 200Po and 400Po on %RLC. It was not possible to discern therefore if a higher soil P concentration outside the zone of the roots would affect %RLC because this higher soil P concentration was accessible to hyphae, that would transport P to the plant and increase the plant P status. Root contact with this area of increased P may be necessary for a decrease in %RLC. A higher concentration of Po than that used to date would be necessary to achieve a decrease in %RLC under any circumstances, and examine this hypothesis. The hyphal content in the soil was also not affected by the increase in soil P concentration. A decrease would be expected as VAM hyphae are thought to be most advantageous to plant growth in low P soil, with a decrease in their presence likely as soil P increases. It is most likely that the 60Po treatment was not high enough to achieve any decrease if VAM quantity is indeed reactive to soil Po concentration. As mentioned above a concentration of 400 mg P/l soil did not affect %RLC.

There was however a significant affect of the location of the soil on the soil hyphal content. The soil outside the growth bag had a higher quantity of VAM hyphae than the soil inside the bag. There was no interaction between location of soil and P treatment that may have indicated that this proliferation of VAM hyphae in the outer soil was a response to the addition of Po to this soil. It therefore appears that either in the control soil or the 60Po treatment soil that the hyphal presence is greater in the outer soil than in the inner root zone. Hyphae are more plentiful at a distance to the root. This way they increase the volume of soil from which plants can absorb P and they are not removing P from soil that plants have access to anyway.

There was insufficient material for shoot P concentration analysis therefore shoot dry weight was measured. It has already been discussed in the experiments in section A that IHP was an available source of P for plant uptake. It was expected that the plants with access to a higher soil Po concentration, 60Po, in the outer soil through VAM hyphae would have a higher shoot dry weight than the control plants where no Po was added to the outer soil. From Fig 2.37 the 60Po treatment plants do indeed have a higher shoot dry weight. This would indicate that the plants are indeed able to access Po outside their zone of growth through VAM hyphae even when Po is

present in such a low concentration. This would also indicate VAM are important for the uptake of P by plants. But this increase in the dry weight of the 60Po treatment plants was not significantly higher than the control plants. This is because of the large standard error associated with the dry weights of the control plants. If the experiment was repeated with more replicates this may eliminate this large error. There was a significant decrease in the shoot dry weight, both for the control and 60Po treatment plants and as a result of the application of benlate to the soil. VAM hyphae were helping P uptake in the control plants where no Po had been added to the soil and in the 60Po treated plants. There was an 80% decrease in shoot dry weight for the control plants and a 79% decrease for the 60Po treatment plants as a result of the reduction of VAM presence in the soil. This substantial decrease is an indication of the importance of VAM hyphae for plant growth.

Pearson and Jakobsen (1993) investigated the relative contribution of hyphae and roots to the total uptake of P by plants. Cucumber plants were grown in sterilised soil inoculated with one of three species of VAM fungus, *Scutellospora calospora, Glomus sp.* and *Glomus caledonium.* Compartment pots were used, with 32P applied to the hyphal compartment and 33P applied to the root compartment. They found the hyphal uptake of P varied between the three species used, from 7 to 100% of the uptake of P by the roots. The contribution of roots to the total P uptake varied between mycorrhizal treatments. Indeed the roots of the plants inoculated with *G. caledonium* appeared to be inactive when compared with hyphal P uptake. Root colonised with *S. calospora* had higher rates of root P uptake that uninoculated controls, suggesting that the root P uptake systems were stimulated by the presence of the fungus.

In the experiment described in this chapter, soil phosphate concentration was examined to determine if there was a decrease in the concentration from that present at the start of the experiment to that in the post harvest soil. With regards to soil phosphate there was a decrease in soil Tp (Fig 2.32) for the control soil but this decrease was equal in the inner and outer soil, both for soil with and without benlate treatment. This decrease indicates that soil Tp was depleted from the soil most likely as a result of the uptake of P by plants. It was expected that the depletion of Tp from the inner and outer soil would be different, indicating that either root uptake from the inner soil or hyphal uptake from the outer soil depleted more soil Tp. This was not seen. It was also expected that the addition of benlate to the soil would reduce the depletion of soil Tp. This again was not seen. The same pattern was seen for the 60Po treatment soil. Again depletion was equal from the inner soil with and without benlate treatment and from the outer soil with and without benlate. More Tp remained in the postharvest outer soil from the 60Po treatment but this was as expected as Po was added to this soil at the start. There was no decrease in the soil Pi or Po concentration for the control or the P treatment soil, with or without

benlate treatment. It was not possible therefore to link depletion in soil Tp with depletion in soil Po or with uptake of P due to changing VAM presence in the soil with P and benlate treatments.

2.5 SUMMARY

- IHP was an available source of P for plant uptake. VAM hyphae were most abundant outside the rooting zone and plants were able to access Po outside their root area through these hyphae.
- The presence of VAM fungi was of benefit to plant growth at low soil Po concentrations, but at high concentrations the presence of these fungi resulted in growth depressions.
- Increasing concentrations of Po in the soil did not affect %RLC by VAM fungi in the grass species, *Agrostis s.* and *Dactylis g.*, but did significantly reduce the %RLC in the herb *Plantago l.*.
- VAM fungi were not involved in the production of soil acidic monoesterase. VAM fungi were also not involved in the production of root acidic monoesterase activity for *Agrostis s.* and *Dactylis g.* but were indicated as having an affect on the production of this enzyme in *Plantago l.*.
- The reaction of soil acidic monoesterase activity to increasing soil Po concentration varied according to the species present, whereas root acidic monoesterase activity decreased for all species.

CHAPTER 3: THE ROLE OF BACTERIA IN THE USE OF ORGANIC PHOSPHATE BY PLANTS

3.1 Introduction

3.1.1 Objectives of the Experiment

The objectives of the experiment described in this chapter were:

- to examine the role played by indigenous soil bacteria in the breakdown of soil organic phosphate for utilisation by plants
- to determine any affect increasing concentrations of inositol hexaphosphate in soil may have on the soil bacteria numbers
- to examine the extent to which soil bacteria are involved in the production of soil and root acidic monoesterase. Monoesterases are important for the mineralisation of organic phosphate (Hysek and Sarapatka, 1998), to generate inorganic phosphate that is then available for uptake by plants (Firsching and Claassen, 1996).

The unique features of this experiment are that, in order to carry out this experiment two natural soils, collected from the PP1 and PP2 sites (for site descriptions refer to Chapter 1 section 1.3), un-amended with artificial growth substrates and with their indigenous soil microflora, were used. In addition, the plant species, *Dactylis glomerata*, used in the course of this experiment was grown from seeds collected from plants growing on the PP1 site.

3.1.2 Experimental Approach

In order to examine the extent to which soil bacteria breakdown organic P for plant uptake the approach of inhibition of soil bacteria was adopted. Plants were grown in soil with no modification of the soil bacteria and in soil in which the bacterial numbers had been reduced. The antibiotic, Streptomycin, was added to the soil with the aim of reducing bacteria numbers. Streptomycin targets the soil bacteria directly without damaging the soil fungi and fauna and soil structure. The approach of previous workers examining soil bacteria in relation to soil nutrients, has been sterilisation of the soil and re-inoculation with a chosen species of bacteria. However this results in highly atypical growth conditions. Steaming and γ-irradiation, the methods most often used for soil sterilisation, result in increases in soil toxins and changes in the soil chemical and physical properties. Chemical sterilants also result in the destruction of soil biota and nutrient cycles. Total sterilisation eliminates soil bacteria but also fungi and soil fauna, all of which interact, and so complicates results (Fitter and Nicholas, 1988). Re-inoculation of soil, even with several species of bacteria, does not reproduce a natural soil environment with indigenous soil bacteria.

As there has been very little work done on the application of streptomycin directly to a soil to inhibit bacterial growth for the duration of a pot experiment, this experiment was a preliminary experiment to determine the effectiveness of streptomycin under such conditions

Inositol hexaphosphate (IHP) was chosen as the organic P compound to add to the soil as this compound is one of the principal forms of soil organic phosphate (Stevenson and Cole, 1999). Soil from the PP1 site was chosen as the control soil on which to base the treatments. This was because, of the two field soils collected the PP1 soil has a lower total P concentration (Chapter 1, Section 1.3). IHP was added to this PP1 soil at concentrations of 100 and 400 mg P/l soil. An inorganic P treatment, based on superphosphate addition, was included at 100 mg P/l soil in order to compare the performance of plants grown under equal concentrations of Po and Pi. Finally a treatment using soil from the PP2 site, with no additional P, was used. The PP1 and PP2 soils were both natural soils. A PP2 treatment was included, as the PP2 soil was naturally higher in total P and organic P than the PP1 soil. This treatment therefore provided an indication of plant growth achieved in a soil with a natural, higher organic phosphate concentration. The 400mg P/l soil Po treatment resulted in total P and organic P concentrations obtained using IHP amendments, similar to that present naturally in the PP2 soil.

It was hypothesised that if sufficient reduction in bacterial numbers was achieved by streptomycin, a decrease in Po hydrolysis would result, and therefore a reduction in growth of plants grown in streptomycin soil would be observed. The plant growth was measured through shoot dry weight, P concentration and P content data. It was also hypothesised that a reduction in bacterial numbers would result in no change in soil acidic monoesterase activity but a decrease in root acidic monoesterase activity. This was based on the observation that soil acidic phosphomonoesterase is secreted mainly by plant roots and associated mycorrhizal and several saprophytic fungi (Tabatabai 1994; Joner and Jakobsen, 1995). Bacteria are not thought to contribute to soil acidic monoesterase activity. Acidic phosphomonoesterase enzymes associated with the root surface of plants may originate from mycorrhizal or saprotrophic fungi, root exudates or bacteria (Johnson *et al.*, 1999).

This experiment would indicate if soil bacteria are contributing to the acidic monoesterase activity of roots. Although soil alkaline monoesterase activity has been attributed to soil bacteria along with microorganisms, fungi and fauna (Tabatabai 1994; Joner and Jakobsen, 1995; Kramer and Green, 2000), only soil acidic monoesterase activity was assayed in this experiment. This was because, as discussed in Chapter 4 Section 4.4.2, in the PP1 soil acidic monoesterase activity is dominant over alkaline monoesterase activity. This was as expected as the PP1 soil has an acid pH (pH 6.7) and acid monoesterase is predominant over alkaline monoesterase in acidic soils (Tabatabai, 1994).

3.1.3 Soil bacteria and phosphate breakdown

Bacteria are of importance to plant nutrition as they allow plants to utilise inorganic phosphorus from sources that are otherwise unavailable and so increase plant available P in the soil system. Microbial solubilisation of inorganic P under pure culture conditions has been shown many times. Indeed the solubilisation of a precipitated calcium phosphate in agar medium has been used as the initial criterion for isolation and enumeration of P solubilizing bacteria (Kucey *et al.*, 1989)

Inorganic phosphate solubilizing bacteria have been found in almost all soils tested although numbers vary with soil type and history (Kucey *et al.*, 1989). Many reports have examined the ability of different bacterial species to solubilise inorganic phosphate compounds of low solubility, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate (Raghu and MacRae, 1966; Saber *et al.*, 1977; Datta *et al.*, 1982; Kundu and Guar, 1984; Laheurte and Berthelin, 1988; Yahya and Al-Azawi, 1989; Gaind and Guar, 1991; Belimov *et al.*, 1995; de Freitas *et al.*, 1997; Taiwo and Oso, 1997; Pal, 1998; Kim *et al.*, 1998; Chabot *et al.*, 1998). The solubilisation of inorganic forms of P by bacteria is thought to be due to the excretion of organic acids that directly dissolve phosphatic materials and/or chelate cationic partners of the P ion (Kucey *et al.*, 1989).

To make the wide range of organic P substrates present in the soil available for plant nutrition they must be hydrolysed to inorganic P. In the 1950's in the USSR a fertiliser containing spores of the bacterium *Bacillus megatherium* var. *phosphaticum* was added to a large proportion of the agricultural soils. The fertiliser was termed phosphobacterin. The bacteria were reputed to increase the rate of organic P mineralisation in the soil resulting in the release of plant available P. Yield increases of 10-20% were obtained from over half the crops inoculated with the bacterium, including vegetable crops, grains and potatoes. However experiments conducted in the USA, using wheat, tomatoes, oats and sorghum, on the effectiveness of phosphobacterin did not show the positive results obtained in the USSR. The conclusion that phosphobacterin was of no practical use in farming in the USA was reached. (Kucey *et al.*, 1989).

The literature on the use of bacteria to increase plant-available P through Po mineralisation is somewhat contradictory. This is partly due to poorly designed and analysed experiments and it is therefore difficult to determine the validity of the conclusions based on some trials. (Kucey *et al.*, 1989). Although phosphobacterin has been reported to release inorganic P from Po sources in soil (Molla *et al.*, 1984), and cause the mineralisation of nucleic acid P, other studies have concluded that *B. megatherium* was unable to do so (Martin, 1973).

The volume of soil at the soil-root interface is termed the rhizosphere and extends only a few millimetres from the root (Steer and Harris, 2000). Microbial activity is most prominent in this

rhizosphere as plant roots are the main sources of substrates for the soil bacterial community. Plant roots will also influence the composition of the microbial community in the rhizosphere (Steer and Harris, 2000). Changes in root exudates and litter quality and quantity will influence microbes and therefore microbially mediated processes such as P mineralisation.

Barea *et al.*, (1976) isolated 50 bacteria from rhizosphere soil from several crop plants that were able to solubilise calcium phytate and sodium phytate in agar medium. These bacteria belonged to many genera including *Bacillus, Agrobacterium* and *Arthrobacter.*

Chabot *et al.*, (1996) selected two strains of rhizobium and detected the ability to hydrolyse phytic acid in plate medium. However neither of these studies considered the breakdown of organic phosphate in a natural soil environment and it is difficult to assess the contribution of bacteria to the phosphorus nutrition of the plant particularly when the information concerning microbial degradation of P compounds is from short-term *in vitro* studies.

Greaves and Webley (1965) carried out a survey to examine the presence of organic phosphate mineralising bacteria in the rhizosphere soil of pasture grasses. The total numbers of bacteria capable of decomposing sodium phytate were higher on the root surface and in the rhizosphere soil than in the non-rhizosphere soil and approximately 30-48% of culturable soil and rhizosphere microorganims utilised phytate. However the assessment of Po hydrolysis was carried out on agar plates. In a later study, Greaves and Webley (1969) incubated soil and a sand-soil mixture with sodium phytate. No plants were present. No release of inorganic orthophosphate could be detected when phytate was incubated in soil or sand–soil mixture and there was no affect on bacterial populations. In sand rapid mineralisation of the substrate occurred and the bacterial populations showed immediate increase.

Although several phosphate solubilising bacteria occur in soil it is usually considered that their numbers are not high enough to compete with other bacteria commonly established in the rhizosphere. Thus the amount of P liberated by them is generally not sufficient for a substantial increase in *in situ* plant growth. (Rodriguez and Frage, 1999).

In *E.coli* there are phosphate starvation inducible genes which act as a phosphate starvation rescue system by scavenging phosphate from organic esters and facilitating the transport of this scavenged Pi across the cell membrane. While a great deal remains to be learned about this system, it is clear that bacteria have evolved an organic phosphate-solubilising system that can act as a phosphate starvation relief mechanism. (Goldstein, 1986). It is clear that further work is required to determine the role that bacteria play in the mineralisation of soil organic phosphate.

3.1.4 The antibiotic streptomycin

Streptomycin is a highly potent antibacterial agent that acts by interfering with the binding of formylmethionyl-tRNA to ribosomes and thereby prevents the correct initiation of protein

synthesis. Streptomycin also leads to misreading of mRNA (Stryer, 1988). The antibiotic is active against a range of gram positive and gram negative bacteria (Glasby, 1992)

Streptomycin is a popular antibiotic used in the study of soil systems. Westover *et al.*, (1997) tested the tolerance of bacteria isolated from soil to streptomycin in a sterile growth medium. Da Gloria Brittode Oliveira *et al.*, (1995) examined the affect of streptomycin on a genetically modified bacterial strain. Bronstad *et al.*, (1996) assessed the antibiotic resistance of bacteria isolated from soil using a disc diffusion test. Velvis (1997) assessed the sensitivity of bacteria isolated from the soil to streptomycin using dilution plate technique. Droby and Coffey (1991) added streptomycin to soil solutions to reduce bacterial population and determine any affect this may have on the breakdown of the fungicide metalaxyl in soil. All this work was carried out on bacteria outside the soil system in sterile growth conditions.

Some work has been carried out on the addition of streptomycin directly in soils. Nakas *et al.*, (1987) added streptomycin to soil and examined the affect on soil alkaline monoesterase levels. Landi *et al.*, (1993) incubated soil with streptomycin to determine the contributions of bacteria to nitrogen mineralisation. Badalucco *et al.*, (1994) studied the degradation of streptomycin in soil by incubating soil and a solution of streptomycin. Schmidt *et al.*, (2000) studied the relationship between microbial biomass and the role of microbivore grazing on nutrient turnover after the addition of streptomycin to soil at field sites. There have been no studies, found by this author, employing streptomycin directly added to soils to examine the role of bacteria in organic phosphate mineralisation and the affect on plant growth.

3.1.5 Methods for bacterial enumeration

The dilution plate count is one of the most widely used techniques for the enumeration of microorganisms. It is however open to criticism. Plate counts yield only a small fraction of the true total bacterial population, only those bacteria able to grow under the specific cultural conditions chosen by the investigator. Plate count results should therefore be expressed as numbers of recoverable viable bacteria obtained using a given set of parameters. It is also assumed in the estimation of numbers of viable bacteria using plate count techniques that all viable cells in a sample will produce a visible colony on the agar medium used for enumeration. Despite these disadvantages, plate counting is a useful tool for making comparisons of the affects of various soil treatments on those bacteria that are recoverable under the conditions defined by the experimenter. Dilution plate counts provide an estimate of the recoverable viable bacteria in a sample under well-defined conditions imposed by the choice of culture medium and incubation conditions. (Zuberer, 1994)

Phosphate buffered saline is a popular dilutant used in the recovery and enumeration of soil bacteria and is often used in the study of bacteria and phosphate nutrition (de Freitas *et al.*,

1997). A buffered solution adjusts the tonicity of the suspending medium to that of the cell to avoid osmotic shock. (Zuberer, 1994).

3.2 Methods

3.2.1 Collection and storage of soil and seeds

As described in Chapter 2, Section 2.2.1.

3.2.2 Preparation of soil

As described in Chapter 2, Section 2.2.2.

3.2.3 Seed Germination and growth conditions

All seeds were germinated in seed trays containing soil from the PP1 site. After 5/6 days seedlings of uniform size were selected and transplanted into the experimental pots (4-inch plastic plant pots). All pots were placed in a heated greenhouse maintained at a constant 15°C in a random arrangement. An automatic watering system using capillary matting was in place.

3.2.4 Phosphate addition and experimental layout

This experiment was based on the PP1 site soil and had a control (no P addition) and 4 P treatments. The P treatments are: P added at 2 rates 100 and 400 mg P/l soil in the form of Po, P added at 1 rate 100 mg P/ l soil in the form of Pi, and a treatment based on the PP2 soil with no P addition. This was carried out for soil treated and untreated with the antibiotic streptomycin. Each control and treatment had 3 replicate pots, giving a total of 30 pots. (Table 3.1)

Table 3.1: Experimental layout for the streptomycin antibiotic experiment. Included are the total P, inorganic P and organic P concentrations for the control and P treatment soils following the relevant P additions. There were 3 replicates per treatment. This layout was duplicated for soil treated with streptomycin at a rate of 3 mg/g DW soil.

			P Concentration (mg P/l soil)		
P Treatments	**Conc P added**	**Source of P**	**Tp**	**Po**	**Pi**
Control (PP1 soil)	no P addition		584.79	360.3	224.55
100Po (PP1 soil)	100 mg P/l soil	IHP	684.79	460.13	224.55
400Po (PP1 soil)	400 mg P/l soil	IHP	985.04	760.49	224.55
100Pi (PP1 soil)	100 mg P/l soil	Pi fertiliser	684.68	360.30	324.38
PP2 soil	no P addition		1085.21	603.67	481.54

This experiment was carried out using the species *Dactylis glomerata*.

Organic phosphate was added in the form of phytic acid (myo-inositol hexakis [dihydrogen phosphate], Sigma Chemicals) which was added to the soil as a powder and was thoroughly mixed with soil before putting the soil into the plant pots. Inorganic phosphate was added in the form of commercially available Super phosphate fertiliser.

Streptomycin (Sigma Chemicals) was added to the soil at the rate of 3mg streptomycin /g DW soil, as a solution (1g Streptomycin dissolved in 100mls distilled water) and was thoroughly mixed with the soil. This is the dosage used by Badalucco *et al.*, (1994).

Each pot was filled with 400 g soil. One seedling was transplanted to each pot.

3.2.5 Harvesting

The experiment was harvested 9 weeks after transplanting seedlings into treatment pots. On harvesting each pot, soil was put into separate labelled plastic bags and stored at 4°C until analysis. All soil analyses, soil Tp, Pi, and Po, soil acidic monoesterase activity and quantification of the soil bacteria colony forming units were carried out for each replicate pot. Above ground plant material was excised from the roots. Above ground material was oven dried for dry weight measurements and total P analysis. Roots were stored in distilled water until root monoesterase analysis was carried out.

3.2.6 Assay for soil acidic monoesterase activity

As described in Chapter 4, section 4.2.6.1.

3.2.7 Assay for root acidic phosphomonoesterase activity

As described in Chapter 4, Section 4.2.6.2.

3.2.8 Soil bacteria enumeration

The enumeration of the soil bacteria was carried out using a dilution plate count where a dilution series was prepared and then the chosen dilution were transferred to growth medium using the spread plate method. This was carried out for the control and each treatment soils in duplicate. A ten-fold dilution series was used to prepare a serial dilution of the soil solution.

A 10 g sample of moist soil and a 10 g replicate for determination of the moisture content were weighed out. The 10 g sample was transferred to a square dilution bottle containing 90 ml of sterile phosphate buffered saline and hand shaken vigorously for 60 seconds. The bottle was placed on a shaker at 200 mot/min for 20 minutes. This constitutes the 10^{-1} dilution.

After shaking the bottle was removed from the shaker and allowed to stand for approximately 30 seconds. Using a 1 ml sterile pipette 1 ml was removed from the middle region of the suspension and transferred to a McCartney bottle containing 9 ml of sterile phosphate buffered saline to achieve the 10^{-2} dilution. This bottle was shaken vigorously by hand for 30 seconds.

This was repeated a further three times to obtain 10^{-3}, 10^{-4} and 10^{-5} dilutions. When this dilution series was complete the 10^{-2}, 10^{-3} and 10^{-4} dilutions were selected for spread plating. Petri plates were prepared containing Tryptic Soy Agar (Sigma Chemicals; as used by Velvis (1997), Westover *et al.*, (1997), de Freitas *et al.*, 1997 Chabot *et al.*, 1996) at 40 g Tryptic Soy Agar/l and allowed to dry.

0.1 ml aliquots of each of the selected dilutions were pipetted onto the agar surface in the centre of the plate. 2 replicate plates were used for each sample. When the inoculum had been delivered to the plates a freshly flamed glass spreader was used to spread the inoculum over the agar surface by using reverse strokes while rotating the plate for approximately 15 seconds. The

spreader was flamed between successive dilutions. Plates were allowed to stand lid uppermost until the moisture had been absorbed. The plates were then inverted and incubated at 25 °C for 7 days. After this incubation period the dilution which exhibited between 30 and 300 colonies was determined. The colonies were counted using a hand lens. The colony number per plate was converted to colony forming units (CFU).

3.2.9 Shoot dry weights

As described in Chapter 2, Section 2.2.11.

3.2.10 Shoot material digest for total P

As described in Chapter 2, Section 2.2.12.

3.2.11 Total, inorganic and organic P levels in soil

As described in Chapter 2, Section 2.2.8.

3.2.12 Root length colonisation by vesicular arbuscular mycorrhiza

As described in Chapter 2, Section 2.2.6.

3.2.13 Statistical procedures

All statistical analysis was carried out using the statistics package Data Desk, version 6.0. All data was checked for normality before statistical analysis. Where necessary data was transformed to obtain a normal distribution. All data was analysed using a two way ANOVA with P treatment and streptomycin treatment as the two factors. Prior to the statistical analysis of the bacterial count data the bacterial numbers were $\log_{10}$ transformed as recommended by Zuberer (1994).

3.3 RESULTS

In the description of the results that follows, P treatment refers to the addition of organic phosphate to the soil at the 2 concentrations employed and the addition of inorganic phosphate to the soil at the single concentration employed. The two organic phosphate concentrations used were 100 and 400 mg P/l soil abbreviated to 100Po and 400Po for the purpose of graphs and the results and discussion section. The single inorganic phosphate concentration used was 100 mg P/l soil abbreviated to 100Pi. The P treatment also includes the PP2 site soil that was included as a treatment with no P amendment. The control treatment is PP1 soil with no phosphate addition. Streptomycin treatment refers to the addition of the antibiotic streptomycin to the soil

Start soil refers to the soil as prepared at the start of the experiment, with the relevant P and streptomycin treatment, but before any seedlings were transplanted. Post harvest soil refers to soil from which the plants have been harvested after their 9-week growth period.

3.3.1. Soil phosphate

The soil total P (Tp), inorganic P (Pi) and organic P (Po) concentration data were presented in Fig 3.1, 3.2 and 3.3 respectively. Two way ANOVAs were carried out to determine the affect of P treatment and streptomycin treatment on soil Tp, Pi and Po concentration. These ANOVAS were presented in Appendix 3, Tables 1, 2 and 3.

For Tp, Pi and Po there was a highly significant P affect (Tp F ratio 28.44***, Pi F ratio 23.08***, Po F ratio 55.00***). All other treatments and interactions were not significant (Appendix 3, Tables 1, 2 and 3).

Total P

Fig 3.1 presented soil Tp concentration data for all P treatment soils with and without streptomycin treatment.

From Fig 3.1 there was an expected increase in start soil Tp levels with increasing levels of Po added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Tp concentrations. There was a statistically significant decrease in the Tp concentrations from those present in the start soils to the post harvest Tp concentration for the control soil treated with streptomycin and the 100Pi treatment soil both with and without streptomycin treatment. (Fig 3.1).

From Fig 3.1 for the post harvest soil there was an increase in total P concentration in the soil with increasing levels of P addition for all soils. This significant affect of P treatment on Tp concentration examined using a Scheffe post hoc test (Table 3.2)

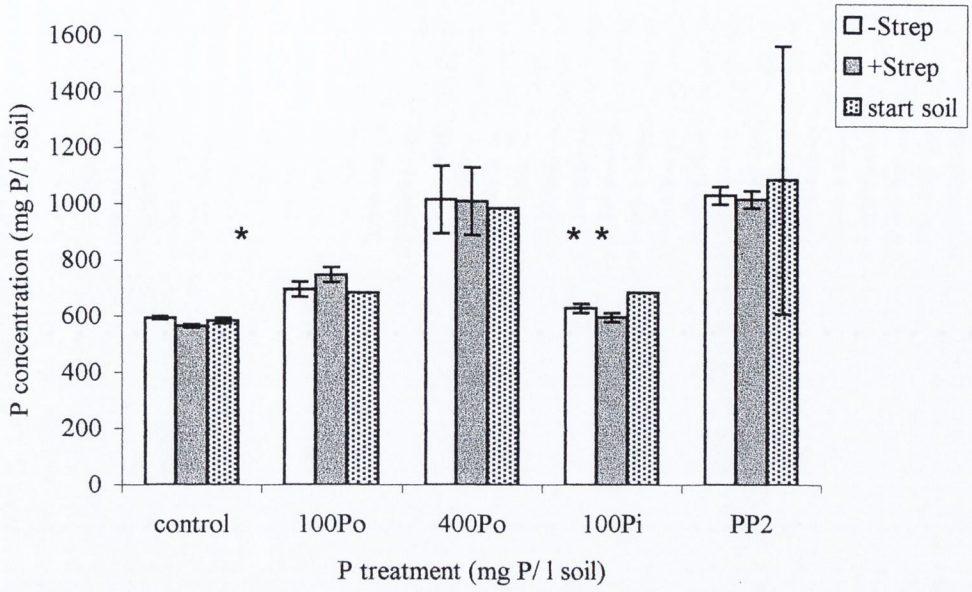


Fig 3.1: soil total phosphate concentration for the control and P treatment soils of the *Dactylis glomerata* experiment. The start soil total P concentration is represented along with the total P concentration of the postharvest soil which is divided into soil with no streptomycin application (-Strep) and soil with streptomycin application (+Strep). Each value represents the mean +/- SE of 3 replicates. * indicates a significant decrease in total P concentration from that present in the start soil.

Table 3.2: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Tp concentration for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
400Po > control	***
400Po > 100Po	**
400Po > 100Pi	***
PP2 > control	***
PP2 > 100Po	**
PP2 -> 100Pi	***
PP2 - 400Po	ns
100Pi - control	ns
100Pi - 100Po	ns
100Po - control	ns

Inorganic P

Fig 3.2 presented soil Pi concentration data for all P treatment soils with and without streptomycin treatment.

From Fig 3.2 there was no change in start soil Pi concentration with increasing levels of Po added to the soil. This was as expected as no Pi was added to any of the soils. There was a higher concentration of Pi in the start soil of the 100Pi treatment soil and the PP2 soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and postharvest soil Pi concentrations. There was no significant difference. (Fig 3.2)

From Fig 3.2 for the post harvest soil the Pi content of the 100Pi treatment soil and the PP2 soil, for soil with and without streptomycin treatment, is higher than that of the remaining treatments and control. This significant affect of P treatment on Pi concentration was examined using a Scheffe post hoc test (Table 3.3).

Table 3.3 Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Pi concentration for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant)

Comparison	P value
100Pi > control	**
100Pi > 100Po	*
400Po > control	*
PP2 > control	***
PP2 > 100Po	***
PP2 > 400Po	***
PP2 > 100Pi	**
control - 100Po	ns
400Po - 100Po	ns
400Po - 100Pi	ns

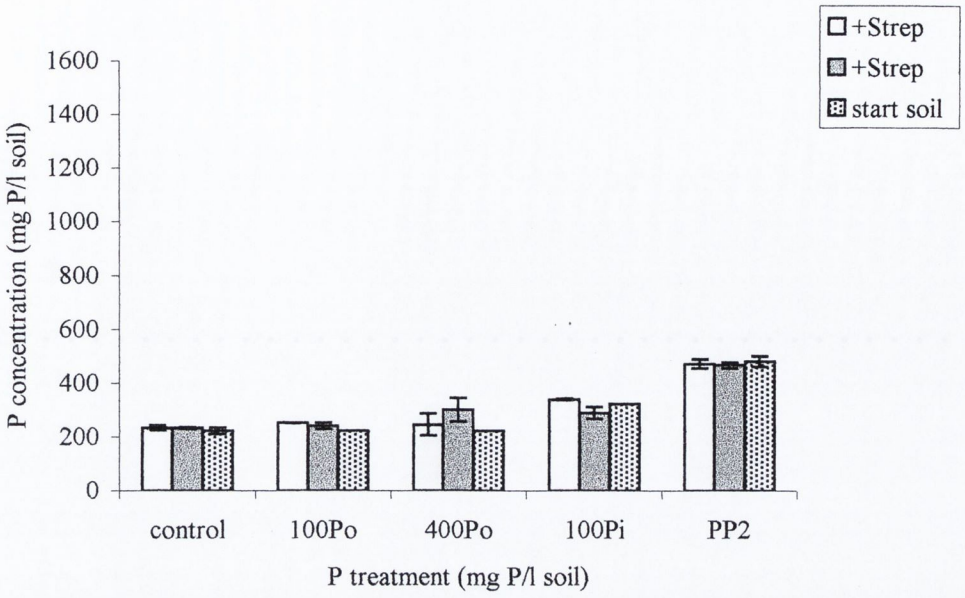


Fig 3.2: soil inorganic phosphate concentration for the control and P treatment soils of the *Dactylis glomerata* experiment. The start soil inorganic P concentration is represented along with the inorganic P concentration of the postharvest soil which is divided into soil with no streptomycin application (-Strep) and soil with streptomycin application (+Strep). Each value represents the mean +/- SE of 3 replicates.

Organic P

Fig 3.3 presented soil Po concentration data for all P treatment soils with and without streptomycin treatment.

From Fig 3.3 there was an increase in start soil Po concentration with increasing levels of Po added to the soil. The Po concentration of the PP2 soil is slightly lower than that of the 400Po treatment soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Po concentration. There was a significant decrease for the control soil with streptomycin treatment and the 100Pi treatment soil with and without streptomycin treatment. (Fig 3.3).

From Fig 3.3 for the post harvest soil there was an increase in Po concentration in the soil with increasing levels of Po addition. This significant affect of P treatment on Po concentration was examined using a Scheffe post hoc test (Table 3.4).

Table 3.4: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Po concentration for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant)

Comparison	P value
control < 100Po	**
control < 400Po	***
control > 100Pi	*
control < PP2	***
100Po > 100Pi	***
100Po < 400Po	**
100Pi < 400Po	***
100Pi < PP2	***
PP2 - 100Po	ns
PP2 - 400Po	ns

3.3.2 Phosphatase Activity

The phosphatase activity results are presented as follows:
soil acidic monoesterase data in Fig 3.4 and the root acidic monoesterase data in Fig 3.5.
Two way ANOVAs were carried out to determine the affect of P treatment and streptomycin treatment on soil and root acidic monoesterase activity. These ANOVAs were presented in Appendix 3, Table 4 and 5.

There was a highly significant P affect (F ratio 9.84***) and streptomycin affect (F ratio 14.03***) on root acidic monoesterase activity. There was also a significant P – streptomycin interaction (F ratio 4.12**) for root acidic monoesterase activity. There was no significant P or streptomycin affect or interaction for soil acidic monoesterase activity.

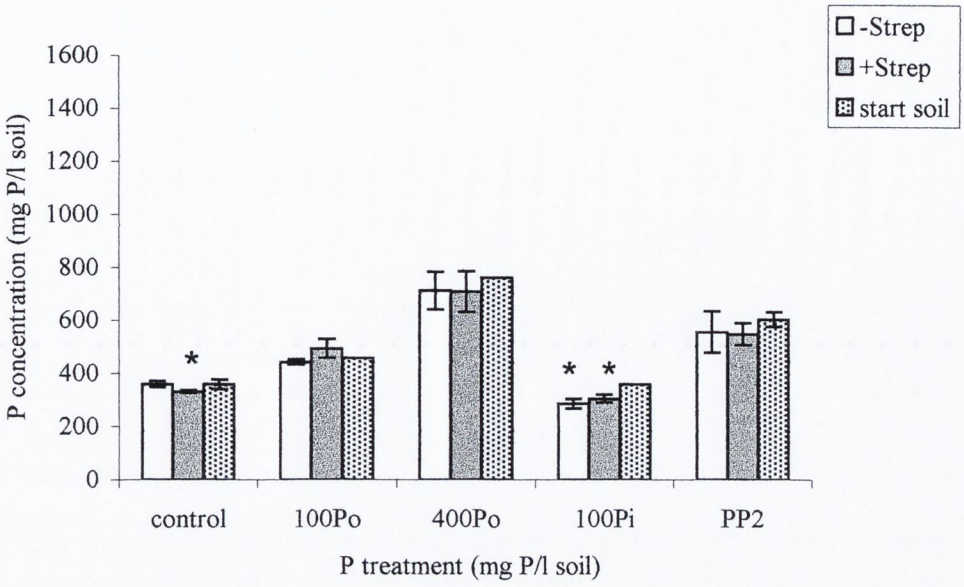


Fig 3.3: soil organic phosphate concentration for the control and P treatment soils of the *Dactylis glomerata* experiment. The start soil organic P concentration is represented along with the organic P concentration of the postharvest soil which is divided into soil with no streptomycin application (-Strep) and soil with streptomycin application (+Strep). Each value represents the mean +/- SE of 3 replicates. * indicates a significant decrease in organic P concentration from that present in the start soil.

Soil Acidic Monoesterase Activity

Figure 3.4 presented soil acidic monoesterase data for all P treatment soils, treated and untreated with streptomycin. The soil acidic monoesterase activity is in the range of 0.85 to 1.1 mg p-NP/ g FW soil/hr.

Root Acidic Monoesterase Activity

Fig 3.5 presented data for root acidic monoesterase activity. There was a decrease in root monoesterase activity for the 100Po and 400Po treatment plants. The 100Pi treatment also resulted in a decrease in root monoesterase activity. The root activity for plants grown in the PP2 soil is considerably lower than that detected in the control soil. This significant affect of P treatment on root acidic monoesterase activity was examined using a Scheffe post hoc test (Table 3.5).

Table 3.5: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root acidic monoesterase activity for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant)

Comparison	P value
control > 100Po	**
control > 400Po	**
control > 100Pi	***
control > PP2	**
100Po - 100Pi	ns
400Po - 100Pi	ns
400Po - 100Po	ns
PP2 - 100Pi	ns
PP2 - 100Po	ns
PP2 - 400Po	ns

From Fig 3.5 in the case of the 100Po treatment the root monoesterase activity of plants grown in streptomycin treated soil is higher than that of plants grown in untreated soil. This affect of streptomycin was statistically significant (Appendix 3, Table 5).

The significant interaction between P treatment and streptomycin treatment was examined using a Scheffe post hoc test (Table 3.6).

The addition of streptomycin to the soil resulted in an increase in root acidic monoesterase activity in the 100Po treatment (Fig 3.5). There was a significant decrease in root monoesterase activity as a result of the Po, Pi and PP2 treatments in soil with no streptomycin treatment. In soil, with streptomycin this decrease in root activity was no longer significant. (Table 3.6, Fig 3.5).

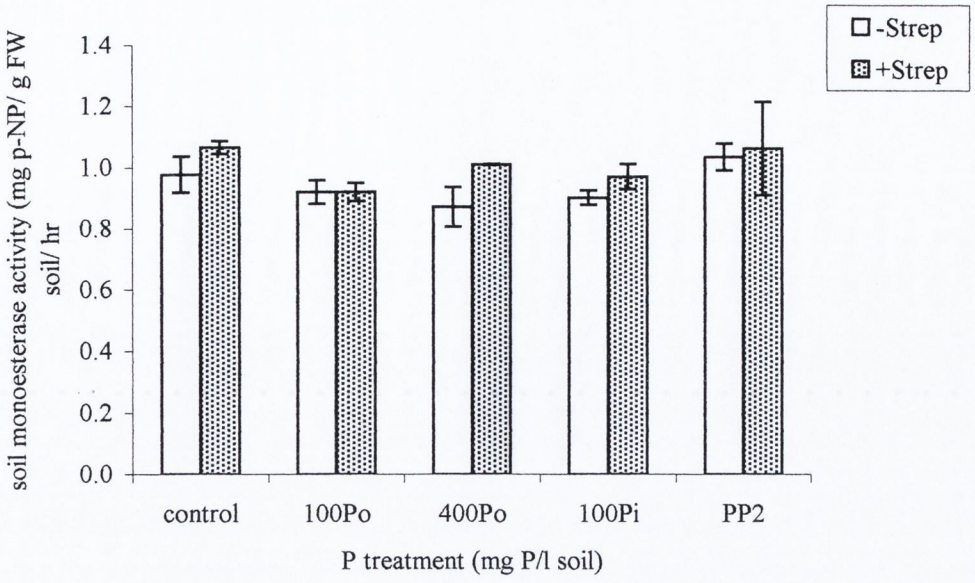


Fig 3.4: soil acidic monoesterase activity for the control and P treatment soils for the *Dactylis glomerata* experiment for soil without (-Strep) and with streptomycin (+Strep) application. Each value represents the mean +/- SE of 3 replicates.

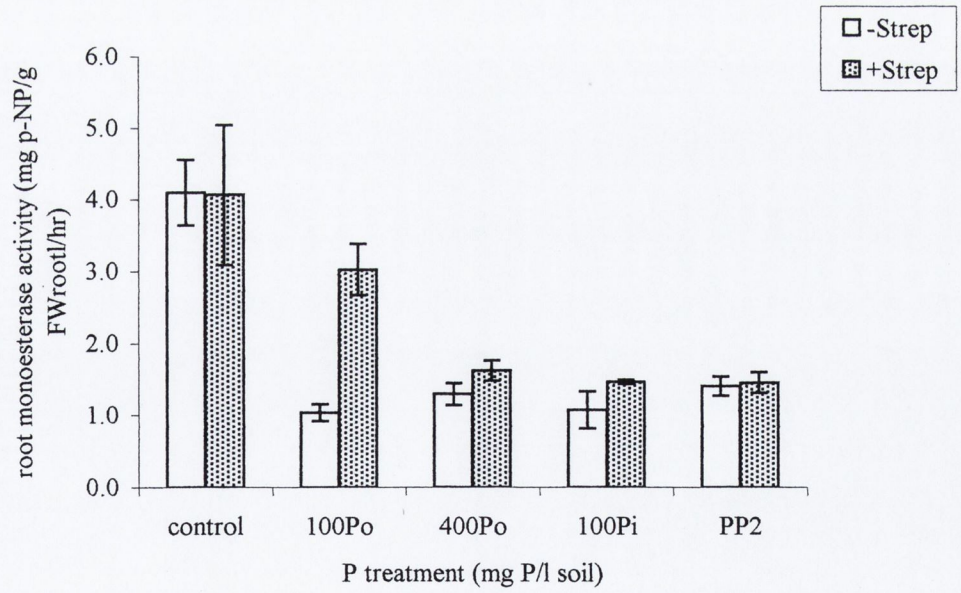


Fig 3.5: root acidic monoesterase activity for the control and P treatment soils for the *Dactylis glomerata* experiment for soil without (-Strep) and with streptomycin (+Strep) application. Each value represents the mean +/- SE of 3 replicates.

Table 3.6: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors streptomycin treatment and P treatment, that affected root acidic monoesterase activity for *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison			P value
-S, control	-	+S, control	ns
-S, 100Po	<	+S, 100Po	**
-S, 400Po	-	+S, 400Po	ns
-S, 100Pi	-	+S, 100Pi	ns
-S, PP2	-	+S, PP2	ns
-S, control	>	-S, 100Po	***
-S, control	>	-S, 400Po	*
-S, control	>	-S, 100Pi	**
-S, control	>	-S, PP2	*
+S, control	-	+S, 100Po	ns
+S, control	-	+S, 400Po	ns
+S, control	-	+S, 100Pi	ns
+S, control	-	+S, PP2	ns

3.3.3 Shoot Dry Weights

The shoot dry weight data was presented in Fig 3.6. A two way ANOVA was carried out to determine the affect of P treatment and streptomycin treatment on shoot dry weight. This ANOVA was presented in Appendix 3, Table 6.

There was a highly significant P affect (F ratio 34.07***) and streptomycin affect (F ratio 21.77***) on shoot dry weight. There was also a significant P - streptomycin interaction (F ratio 6.56***).

Fig 3.6 presented shoot dry weight data for all plants. From Fig 3.6 there was an insignificant increase in shoot dry weight as a result of the 100Po treatment. This increase was equal for plants grown in soil treated and untreated with streptomycin. There was also an increase in shoot dry weight for the 400Po and 100Pi treatments but in this case the increase for plants grown in streptomycin treated soil was substantially greater. In streptomycin treated soil the PP2 plants have a shoot dry weight similar to that of the 100Pi plants. This significant affect of P treatment on shoot dry weight was examined using a Scheffe post hoc test (Table 3.7)

There was a significant affect of streptomycin application on shoot dry weight (Appendix 3, Table 6). In the case of the 400Po, 100Pi and PP2 soil the shoot dry weight of plants grown in streptomycin treated soil was higher than those grown in non streptomycin treated soil (Fig 3.6).

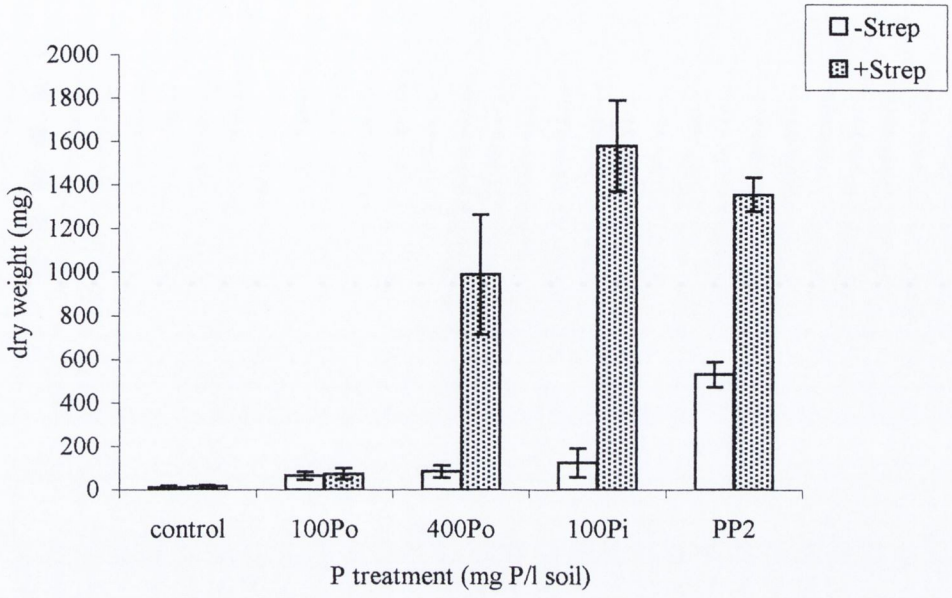


Fig 3.6: shoot dry weights for the control and P treatment plants of *Dactylis glomerata* for plants grown in soil without (-Strep) and with (+Strep) streptomycin application. Each value represents the mean +/- SE of 3 replicates.

Table 3.7: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot dry weight for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 400Po	***
control < 100Pi	***
control < PP2	***
100Po < 400Po	*
100Po < 100Pi	**
100Po < PP2	***
control - 100Po	ns
400Po - 100Pi	ns
PP2 - 100Pi	ns
PP2 - 400Po	ns

The significant interaction between P treatment and streptomycin treatment was examined using a Scheffe post hoc test (Table 3.8).

Table 3.8: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors streptomycin treatment and P treatment, that affected shoot dry weight for *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-S, control - +S, control	ns
-S, 100Po - +S, 100Po	ns
-S, 400Po < +S, 400Po	**
-S, 100Pi < +S, 100Pi	***
-S, PP2 < +S, PP2	*
-S, control - -S, 100Po	ns
-S, control < -S, 400Po	*
-S, control < -S, 100Pi	**
-S, control < -S, PP2	***
+S, control - +S, 100Po	ns
+S, control < +S, 400Po	***
+S, control < +S, 100Pi	***
+S, control < +S, PP2	***

The addition of streptomycin to the 400Po, 100Pi and PP2 treatment soils resulted in an increase in shoot dry weight (Fig 3.6). There was a significant increase in shoot dry weight as a result of the 400Po, 100Pi and PP2 treatments in soil with and without streptomycin treatment. (Table 3.8, Fig 3.6).

3.3.4 Shoot P Concentration

The shoot P concentration data was presented in Fig 3.7. A two way ANOVA was carried out to determine the affect of P treatment and streptomycin treatment on shoot P concentration. This ANOVA was presented in Appendix 3, Table 7.

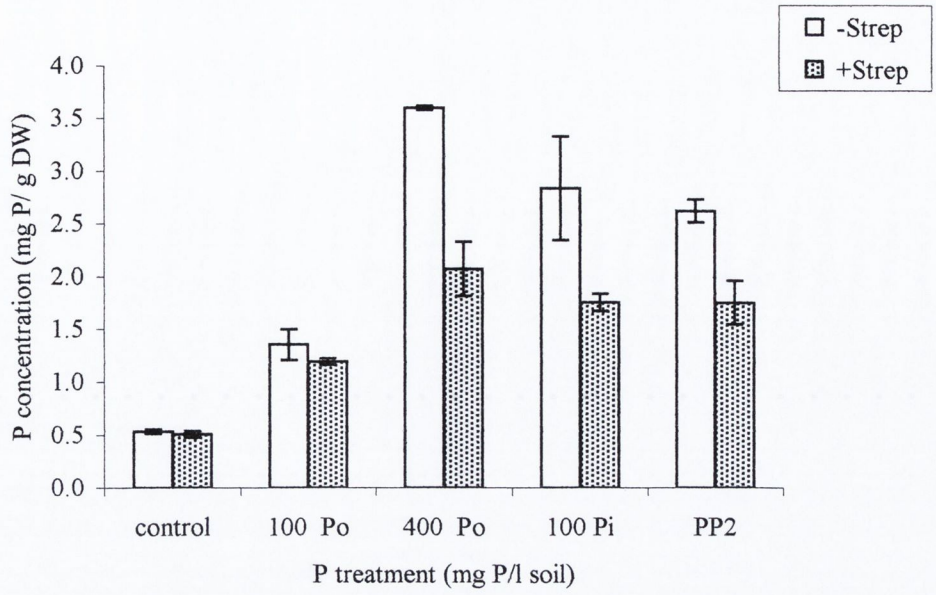


Fig 3.7: shoot P concentration for the control and P treatment plants of *Dactylis glomerata* for plants grown in soil without (-Strep) and with (+Strep) streptomycin application. Each valus represents the mean +/- SE of 3 replicates.

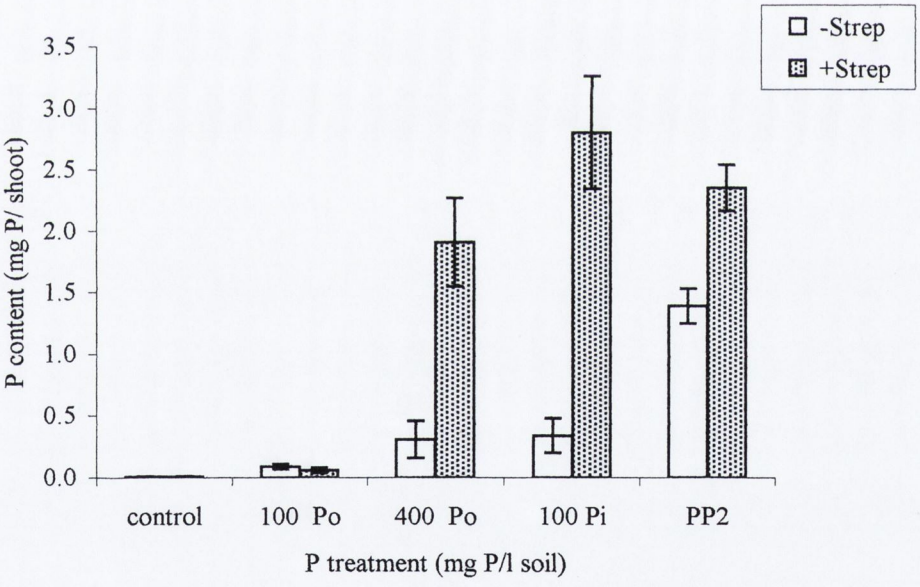


Fig 3.8:shoot P content for the control and P treatment plants of *Dactylis glomerata* for plants grown in soil without (-Strep) and with (+Strep) streptomycin application. Each value represents the mean +/- SE of 3 replicates.

There was a highly significant P affect (F ratio 65.49***) and streptomycin affect (F ratio 55.86***) on shoot P concentration. There was also a significant P - streptomycin interaction (F ratio 7.87***).

From Fig 3.7 there was an increase in shoot P with increasing levels of Po added to the soil for the 100Po and 400Po treatments. The 100Pi and PP2 treatments resulted in shoot P concentrations similar to those of the 400Po treatment. This significant affect of P treatment on shoot P concentration was examined using a Scheffe post hoc test (Table 3.9).

Table 3.9: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P concentration for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	**
control < 400Po	***
control < 100Pi	***
control < PP2	***
100Po < 400Po	***
100Po < 100Pi	***
100Po < PP2	***
400Po > PP2	**
100Pi - 400Po	ns
100Pi - PP2	ns

For the 400Po, 100Pi and PP2 treatments the shoot P concentration of the plants grown in non streptomycin treated soil was higher than that of plants grown in streptomycin treated soil. This was a statistically significant affect of streptomycin application on the shoot P concentration (Appendix3, Table 3.7).

The significant interaction between the P treatment and streptomycin treatment was examined using a Scheffe post hoc test (Table 3.10). Streptomycin had a significant effect in control and 100Po treatments, but enhanced shoot P concentration in the 400Po, PP2 and 100Pi treatments.

The application of streptomycin to the 400Po, 100Pi and the PP2 soil resulted in a significant decrease in shoot P concentration (Fig 3.7). For soil with no streptomycin application the 100Po, 400Po, 100Pi and the PP2 treatments resulted in a significant increase in shoot P concentration. In soil with streptomycin application only the 400Po, 100Pi and the PP2 soil resulted in a significant increase in shoot P concentration. (Table 3.10)

Table 3.10: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors streptomycin treatment and P treatment, that affected shoot P concentration for *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-S, control - +S, control	ns
-S, 100Po - +S, 100Po	ns
-S, 400Po > +S, 400Po	***
-S, 100Pi > +S, 100Pi	***
-S, PP2 > +S, PP2	*
-S, control < -S, 100Po	*
-S, control < -S, 400Po	***
-S, control < -S, 100Pi	***
-S, control < -S, PP2	***
+S, control - +S, 100Po	Ns
+S, control < +S, 400Po	***
+S, control < +S, 100Pi	***
+S, control < +S, PP2	***

3.3.5 Shoot P Content

The shoot P content data was presented in Fig 3.8. A two way ANOVA was carried out to determine the affect of P treatment and streptomycin treatment on shoot P content. This ANOVA was presented in Appendix 3, Table 8.

There was a highly significant P affect (F ratio 74.16***) and streptomycin affect (F ratio 12.45***) on shoot P content. There was also a significant P - streptomycin interaction (F ratio 5.41**).

From Fig 3.8 there was an increase in shoot P content with the addition of 100Po treatment and a further increase with the 400Po treatment. In the case of the 400Po treatment plants, those grown in streptomycin treated soil had a higher P content than those grown in untreated soil. The shoot P content of the 100Pi and PP2 treatment plants was similar to that of the 400Po treatment plants. This significant affect of P treatment on shoot P concentration was examined using a Scheffe post hoc test (Table 3.11).

There was a statistically significant affect of streptomycin application on shoot P content (Appendix 3, Table 8). In the case of the 400Po, 100Pi and PP2 treatment plants, those grown in streptomycin treated soils had a higher P content than those grown in untreated soil, while streptomycin treatment had no significant effect at 100Po and in the control soils (Table 3.12)

Table 3.11: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P content for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	***
control < 400Po	***
control < 100Pi	***
control < PP2	***
100Po < 400Po	***
100Po < 100Pi	***
100Po < PP2	***
400Po - 100Pi	ns
PP2 - 100Pi	ns
PP2 - 400Po	ns

The significant interaction between the P treatment and streptomycin treatment was examined using a Scheffe post hoc test (Table 3.12).

Table 3.12: probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors streptomycin treatment and P treatment, that affected shoot P content for *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-S, control - +S, control	ns
-S, 100Po - +S, 100Po	ns
-S, 400Po < +S, 400Po	*
-S, 100Pi < +S, 100Pi	**
-S, PP2 - +S, PP2	ns
-S, control < -S, 100Po	**
-S, control < -S, 400Po	***
-S, control < -S, 100Pi	***
-S, control < -S, PP2	***
+S, control < +S, 100Po	*
+S, control < +S, 400Po	***
+S, control < +S, 100Pi	***
+S, control < +S, PP2	***

The addition of streptomycin to 400Po and 100Pi treatment soils resulted in an increase in shoot P content. The 100Po, 400Po, 100Pi and PP2 treatments resulted in a significant increase in shoot P content in soil with and without streptomycin application (Table 3.12, Fig 3.8)

Phosphorus Use Efficiency

Phosphorus use efficiency (PUE) is defined as (Sanginga et al, 2000):

g shoot dry weight / mg P in shoots

PUE was calculated for the shoots of the *Dactylis* plants under different P and streptomycin treatments. The data is presented in Table 3.18

Table 3.13s: phosphorus use efficiency (g shoot dry weight per mg P in shoots) for *Dactylis* shoot material for plants grown under various combinations of P and streptomycin treatments.

	Phosphorus Use Efficiency	
P treatment	**-S**	**+S**
Control	1.17	1.95
100Po	0.72	1.21
400Po	0.27	0.52
100Pi	0.37	0.56

3.3.6 Enumeration of Soil Bacteria

The soil bacteria enumeration data was presented in Fig 3.9. A two way ANOVA was carried out to determine the affect of P treatment and streptomycin treatment on soil bacterial numbers. This ANOVA was presented in Appendix 3, Table 9.

There was a highly significant P affect (F ratio 22.53***) and streptomycin affect (F ratio 52.94***) on the soil bacterial numbers. There was also a significant P - streptomycin interaction (F ratio 13.97***).

From Fig 3.9 there was a decrease in soil bacteria numbers from that of the control soil on the addition of Po to the soil. The bacterial counts for the 100Pi and PP2 soils are similar. This significant affect of P treatment on soil bacteria numbers was examined using a Scheffe post hoc test (Table 3.19).

Table 3.14: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on soil bacterial numbers for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
100Pi < control	***
100Pi < 100Po	***
400Po > 100Pi	**
400Po < 100Po	*
PP2 > 100Pi	**
PP2 < 100Po	*
control - 100Po	ns
control - 400Po	ns
control - PP2	ns
PP2 - 400Po	ns

There was a statistically significant affect of streptomycin application on soil bacteria numbers (Appendix 3, Table 3.9). In the case of the control, 100Po, 400Po, 100Pi and PP2 treatments the bacterial numbers were lower in the soil treated with streptomycin than in the untreated soil.

The significant interaction between the P treatment and streptomycin treatment was examined using a Scheffe post hoc test (Table 3.20).

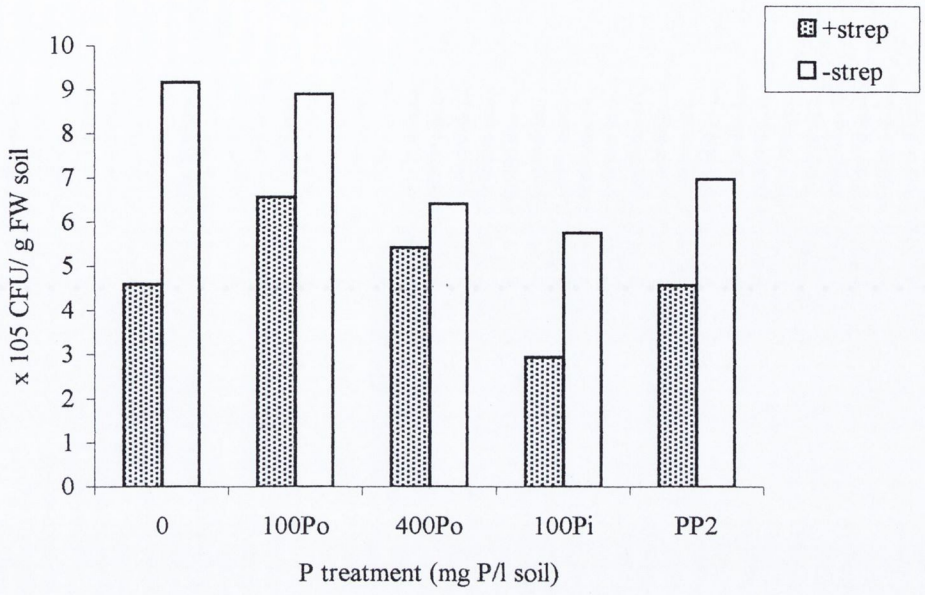


Fig 3.9: soil bacteria colony forming unit data for the control and P treatment soils without (Strep) and with (+Strep) streptomycin application for the *Dactylis glomerata* experiment.

Table 3.15: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors streptomycin treatment and P treatment, that affected soil bacterial numbers for *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison			P value
-S, control	>	+S, control	***
-S, 100Po	>	+S, 100Po	*
-S, 400Po	>	+S, 400Po	*
-S, 100Pi	>	+S, 100Pi	***
-S, PP2	>	+S, PP2	*
-S, control	<	-S, 100Po	***
-S, control	-	-S, 400Po	ns
-S, control	>	-S, 100Pi	**
-S, control	-	-S, PP2	ns
+S, control	-	+S, 100Po	ns
+S, control	>	+S, 400Po	*
+S, control	>	+S, 100Pi	**
+S, control	-	+S, PP2	ns

There was a significant decrease in soil bacterial numbers in all soils on the addition of streptomycin. In the case of soil with no streptomycin application there was a significant increase in soil bacterial numbers as a result of the 100Po treatment and decrease in soil bacterial numbers as a result of the 100Pi treatment. In soil with streptomycin application there was a significant decrease in soil bacterial numbers as a result of the 400Po and 100Pi treatments (Table 3.20, Fig 3.9)

3.3.7 % Root length colonised by vesicular arbuscular mycorrhiza

In order to confirm that streptomycin had no affect on the % root length colonisation (%RLC) by vesicular arbuscular mycorrhiza a one way ANOVA was carried out to determine the affect of streptomycin treatment on %RLC. This ANOVA was presented in Appendix 3, Table 1
There was no significant affect of the application of streptomycin to the soil on %RLC.

3.4 DISCUSSION

3.4.1 Published work

Streptomycin does not kill existing bacteria but inhibits the synthesis of new cells so that the action depends on the microbial growth rate (Schmidt *et al.*, 2000). Schmidt *et al.*, (2000) in a study of grazing on nutrient turnover in a field site were unable to detect any affect of the streptomycin addition on microbial biomass C. They suggested this was due to the absorption of the antibiotic in their organogenic soils combined with a short half-life or because the amount added was not sufficient. In a study of the affect of the addition of amino acids on soil phosphatase activity Nakas *et al.*, (1987) added streptomycin to soil and achieved a reduction in soil bacteria over a 26 day period. Badalucco *et al.*, (1994) incubated soil with streptomycin at a rate of 3 mg streptomycin/g DW soil and found a reduction in bacterial numbers over a period of a week. In this experiment the dose of 3 mg streptomycin/g DW soil successfully reduced bacterial presence over a nine week period.

3.4.2 Information gained

The application of streptomycin to the soil resulted in a significant decrease in the number of colony forming units (CFU) present in the control and P treatment soils (Table 3.19). This indicates that under the conditions of this experiment the dose of 3 mg streptomycin/g DW soil successfully reduced bacterial presence over a nine week period. It is therefore possible in the remainder of the discussion to talk about changes due to streptomycin treatment as being due to a reduction in bacterial numbers.

The dilution plate count for the culture and enumeration of soil bacteria has the disadvantage of culturing only those bacteria that will grow on the selected media and under the defined cultural conditions. However it is still a useful method of determining the affects of various soil treatments on the bacterial presence in the soil. As this was a preliminary experiment on the affect of streptomycin on the indigenous soil bacteria, in the presence of plants, in a greenhouse experiment, a general cultural medium tryptic soy agar was used. As the streptomycin was shown to be affective in reducing bacterial numbers further experiments on varying the dosage of the antibiotic need to be carried out as well as the use of selective media to examine the affect on different populations of soil bacterial, for example organic phosphate solubilising bacteria.

There was also a significant affect of P treatment on the number of CFU (Table 3.19). The addition of 100Pi to the soil resulted in a significant decrease in the number of CFU. Increasing the levels of Po in the soil both by the addition of IHP at either 100Po or 400Po to the soil or in the naturally high P of the PP2 soil did not affect the bacterial numbers in the soil. The 400Po and PP2 treatments had equal numbers of soil bacteria. At equal concentrations of Po and Pi in the soil, the 100Po and 100Pi, treatments only the Pi resulted in a decrease in bacterial presence. Only inorganic P resulted in a reduction in bacterial presence. It is possible that the addition of

readily available super P to the soil, requiring no breakdown resulted in a decreased demand for P breakdown and therefore P solubilising bacteria. An increase in bacterial numbers with increased P addition was expected as the Po and Pi should provide a source of P for bacterial growth. In addition, the amendment of the soil with P would be expected to increase the bacterial numbers in an indirect way. As will be discussed in shortly, there was an in increase in dry weight with the addition of P to the soil. This increase in plant growth would be expected to result in an increase in root deposition and exudates and root litter, all of which are important sources of substrates for soil bacteria (Steer and Harris, 2000). This increase in available resources, carbohydrates and nitrogen from the roots would have been expected to further increase bacterial numbers.

The reduction in bacterial numbers in the soil because of the application of streptomycin to the soil resulted in a significant increase in shoot dry weight for the 400Po, PP2 and 100Pi treatments (Table 3.9). The highest increase was for the 100Pi treatment with a 12-fold increase, followed by the 400Po and PP2 treatments with a 10 fold and 2-fold increase respectively. Although there was an increase in root acidic monoesterase activity with the application of streptomycin to the soil (Table 3.8) this increase was only for the 100Po treatment, and cannot account for increases in dry weight in the 400Po, PP2 or 100Pi treatments. Indeed the increase in root monoesterase activity, and so the ability to solubilize Po, for the 100Po treatment plants did not result in a significant increase in shoot dry weight.

It is possible that the streptomycin inhibited a section of the bacterial community allowing another to proliferate. This thriving population may have produced plant growth substances, such as indoleacetic acid, gibberellins and cytokinins, which would cause plant growth stimulation. This would result in a higher plant dry weight. (Kucey *et al,* 1989). A more likely explanation is that there had been bacterial competition for available soil P. A reduction in the number of soil bacteria may have increased the amount of P that was available to the plants alone and so increased dry weight. If this were the case a 12-fold increase in the Pi treatment soil would indicate that bacteria are utilising this P source most followed by the IHP at 400Po and then the Po of the PP2 soil. It is unlikely that the addition of streptomycin to the soil and so a decrease in bacterial numbers stimulated fungal activity as there was no affect of streptomycin on the % root length colonisation of the roots by vesicular arbuscular mycorrhizae. An increase in fungal activity would be expected to include an increase in %RLC.

There was also a significant affect of streptomycin treatment on shoot P concentration (Table 3.12). As with dry weight this affect occurred for the 400Po, 100Pi and PP2 treatments. The addition of streptomycin to the soil resulted in a significant decrease in shoot P concentration.

Finally streptomycin application significantly increased shoot P content of the 400Po, 100Pi and PP2 treatment plants (Table 3.15).. This increase in nutrient accumulation is most likely because of reduced bacterial competition for available nutrients as discussed for shoot dry weight.

It is necessary to consider shoot dry weight, P concentration and content together (Jarrell and Beverly, 1981). The addition of streptomycin to the soil resulted in an increase in yield and P content but a decrease in the P concentration of the shoot compared to the control plants. This implies that a reduction in soil bacteria increased the amount of available P in the soil and result in an increase in dry matter and P accumulation. However the relative rate of dry matter accumulation increased more rapidly than the rate of nutrient accumulation, resulting in a lower final concentration in treated plants.

There was a significant overall affect of P treatment on shoot dry weight (Table 3.9). The addition of 400Po, 100Pi and PP2 to the soil resulted in a significant increase in dry weight. Plant growth can be used to monitor the release of orthophosphate from P sources, the form of P most readily taken up by plants. This increase in shoot dry weight indicated a release of orthophosphate from the 400Po and PP2 treatments significantly large to result in a significant increase in dry weight. The PP1 control soil was P limiting and IHP was an available source of plant P. The addition of the super phosphate achieved a dry weight greater than that of the 100Po treatment, indicating that at equal concentrations the Pi was a better source of P for plant uptake. The dry weight of the 400Po plants was equivalent to that of the PP2 plants. IHP provided a source of P equal to that naturally present in the PP2 soil. The increases in dry weight as a result of these P treatments for plants without and without streptomycin treatment are presented in Table 3.23.

Table 3.16: Increases in shoot dry weight for *Dactylis glomerata* as a result of the P treatments expressed as a multiple of the control plants shoot dry weight. –S and +S refer to soil with no streptomycin treatment and with streptomycin treatment respectively.

	Increase in shoot dry weight (X fold increase of control dry weight)	
P treatment	**-S**	**+S**
100Po	4	4
400Po	6	59
100Pi	10	94
PP2	42	81

From Table 3.23 for soil with no streptomycin treatment the PP2 treatment resulted in the highest increase in shoot dry weight. For soil with streptomycin treatment the 100Pi treatment resulted in the highest increase in shoot dry weight. As discussed previously this is most likely as a reduction in bacteria resulted in a reduction in competition for P that in turn resulted in an increase in readily available super phosphate.

There was a significant affect of P treatment on the shoot P content (Table 3.15). The 100Po, 400Po, 100Pi and PP2 treatments soil resulted in an increase in shoot P content. The 100Pi treatment plants had a higher P content than the 100Po treatment plants. At equal concentrations, plants supplied with Pi had a higher P accumulation than those supplied with Po. The 400Po treatment resulted in an equal shoot P content to the PP2 treatment. The IHP and the natural Po of the PP2 soil were equally affective for plant P accumulation. The positive affects of the P treatments on the shoot P content indicates availability of IHP for plant use at both concentrations used and the Po in the unamended PP2 soil.

There was also a significant increase in shoot P concentration with the 100Po, 400Po, 100Pi and PP2 treatments (Table 3.12). Again the 100Pi treatment appeared a better source of plant P than IHP at an equivalent concentration as the 100Pi treatment plants had a higher shoot P concentration than the 100Po treatment plants. The 400Po treatment plants had a P concentration greater than the PP2 treatment plants.

As with the affect of streptomycin treatment the shoot dry weight, P content and concentration were considered together. As a result of the 400Po, 100Pi and PP2 treatments there was an increase in the shoot yield, the P content and the P concentration of the shoots. When the growth limiting element P was supplied the P content accumulation was more rapid than the dry matter accumulation. This therefore resulted in an increase in the overall P concentration in the shoot material.

The phosphorus use efficiency (PUE) is defined as the quantity (in grams) of shoot dry weight produced from 1 mg of P (Sanginga *et al.*, 2000). The PUE of the *Dactylis* plants was calculated based on the shoot dry weight and the P content of the shoots. The PUE decreased with increasing concentrations of Po added to the soil (Table 3.18). A 38% decrease was found on the addition of 100Po to soil without and with streptomycin treatment. On addition of 400Po to the soil a 77% and 73% decrease in PUE was found in soil without and with streptomycin treatment respectively. That is with increasing concentrations of Po added to the soil, there was a lower quantity of shoot dry weight production per mg of P in the shoot. This luxury and uneconomical use of P is probably due to the greater availability of P in the soil. There was a 67% and 70% decrease in PUE in the PP2 soil compared to the control soil without and with streptomycin treatment respectively. These decreases are similar to those observed for the 400Po treatment soil. The 400Po and PP2 soils afford the same luxury with regards to the use of P by the plants. There was a 68% and 71% decrease in PUE on the addition of 100Pi to the soil in soil without and with streptomycin treatment respectively, similar to that found with the 400Po and PP2 soils. This indicates that the 400Po, PP2 and 100Pi soils increase the availability of P to the plants and decrease the need for economical use of P resources.

There was a decrease in the total P content from that present in the soil at the start of the experiment to that found following the harvesting of the plants (Fig 3.1). But this decrease was only detected for the control soil with streptomycin treatment and the 100Pi soil with and without streptomycin treatment. There was no decrease in the Pi concentration for any of the soils (Fig 3.2). The decrease in total P concentration was matched by a decrease in the organic P concentration for the control soil with streptomycin treatment and the 100Pi soil with and without streptomycin treatment (fig 3.3).

It was expected that a decrease in total P concentration, matched by a decrease in Po concentration would have been visible for the control and 100Po, 400Po and the PP2 soils. This is because an increase in dry weight and P content was observed for these treatments. Yet depletion from the soil P pool is not evident. It was also expected that a decrease in the Tp and Pi and Po would have been visible in the 100Pi treatment soil due to the addition of readily available superphosphate. While a lower level of depletion may be expected in the soil with streptomycin treatment due to a reduction in bacterial presence and P uptake this may be masked by an increased uptake of P by plants. A reduction in competition for P as a result of reduced numbers of bacteria would increase the P available to plants.

A decrease in total P and organic P following harvesting of plants of *Agrostis, Holcus* and *Dactylis* grown in control PP1 soil and PP1 soil with much lower P amendments to the soil was found in Chapter 4 Section 4.4.1.8. Such decreases indicate a depletion of soil Tp most likely as a result of P uptake by plants and that this depletion has been from the Po pool. In this experiment only in soil without increased concentrations of Po, the control and 100Pi treatment soil, was depletion in Tp and Po seen. Even in the superphosphate amended soil, the 100Pi soil, no depletion of Pi was seen. In this experiment the quantification of soil Tp, Pi and Po does not indicate from which pool the P was removed by plant uptake.

Phosphatase enzymes act to breakdown organic phosphate to forms readily available for uptake by plants. The soil acidic monoesterase activity is associated with plant roots and associated mycorrhizal fungi and is secreted into the soil (Joner and Jakobsen, 1995). The inositol hexaphosphate (IHP) added to the soil is a form of monoester P and as such should provide a substrate for acid monoesterase. It was expected that the addition of IHP, and therefore monoesterase substrate, to the soil would stimulate acidic monoesterase production and result in an increase in the acidic monoesterase activity. However there was no affect of the addition of IHP to the soil at either 100Po or 400Po on soil acidic monoesterase activity (Table 3.6). The PP2 soil naturally higher in Po concentration than the PP1 did not have a significantly different soil acidic monoesterase activity. It is possible that there was sufficient levels of soil acidic monoesterase activity already present in the soil to breakdown the IHP added to the soil.

No increase in the activity of this soil enzyme with the addition of Po to the soil may also indicate that this enzyme is limiting in soil. One plant per pot with its associated mycorrhizal fungi was possibly insufficient to produce additional enzyme. This would also account for the reason that the control, Po treated soils and the PP2 soil do not have significantly different levels of acidic monoesterase activity in the soil. Only one plant is present per pot and the plant is *Dactylis* in all cases. In order to determine if limitation on enzyme production was the reason for a lack of affect of P treatment on soil acidic monoesterase it would be necessary to carry out an experiment increasing the numbers of plant per pot at increased concentrations of Po. This would determine if the number of plants in an area significantly affect the soil acidic monoesterase production. Soil collected form the field may be analysed directly for soil acidic monoesterase activity the number of species contributing to this activity would be difficult to quantity.

There was also no affect of the addition of Pi to the soil on soil acidic monoesterase activity (Table 3.6). Pi was added to the soil at 100 mg P/l soil which corresponds to 10 μmole PO_4^{3-}/g soil. Orthophosphate is a competitive inhibitor of acid and alkaline phosphatase in soil (Joner and Jakobsen, 1995). Juma and Tabatabai (1978) found that at 10 μmole PO_4^{3-}/g soil inhibition of soil acidic monoesterase activity ranged from 21 to 42 percent. In this experiment. no decrease in soil acidic monoesterase activity was found as a result of increasing the orthophosphate concentration. The Pi added in the 100Pi treatment was possibly taken up by the plants for growth and was reduced rapidly to a level that did not inhibit the activity at the time of harvest.

There was no affect of the reduction of bacterial numbers by streptomycin on soil phosphatase activity (Table 3.6). This was as expected as soil acidic monoesterase is secreted mainly by plant roots and associated mycorrhizal and several saprophytic fungi (Tabatabai 1994; Joner and Jakobsen, 1995). Bacteria are not thought to be responsible for soil acidic monoesterase.

A significant increase in root phosphatase activity was detected with streptomycin application to the 100Po treatment soil (Table 3.8). The reduction in bacteria numbers with streptomycin application, as indicated by the plate dilution method, induces an increase in root phosphatase activity for this Po treatment. It was expected that a reduction in bacterial numbers would result in a decrease in root acidic monoesterase activity. This is based on the fact that acidic phosphomonoesterase enzymes associated with the root surface of plants may originate from mycorrhizal or saprotrophic fungi, root exudates or bacteria (Johnson *et al*, 1999). This reduction was not detected and indeed an increase was found for the 100Po treatment. The increase in the activity of the 100Po treatment plants with a reduction in bacterial numbers could be explained as, the antibiotic reduced the number of phosphatase producing bacteria and the roots produced more phosphatase to compensate for this loss. However a consistent increase

was nor evident for the other Po treatments that also had a significant reduction in soil bacterial numbers.

As no reduction in root acidic monoesterase activity was detected with a reduced number of soil bacteria this would indicate that bacteria do not make a substantial contribution to this root acidic monoesterase activity of *Dactylis*. Or it is possible that the reduction in the number of soil bacteria was insufficient to result in a detectable decrease in root activity.

Root acidic monoesterase activity is closely tied to plant demand for phosphorus (Kramer and Green, 2000). Activity is increased in plants that are P deficient (Barrett-Lennard *et al*, 1993; Frossarrd 1995; Richardson *et al*, 2000). There was a significant affect of P treatment on root phosphatase activity (Table 3.6). The 100Po, 400Po and PP2 treatments resulted in significant and equal decreases in root acidic monoesterase activity. This is as expected, and indicates that as a result of the addition of Po to the soil the plants were no longer as P deficient as the control plants. The IHP added to the soil was an available source of P for plant growth. This is enforced by the increase in dry weight and P content with Po addition to the soil. As the 100Po and 400Po treatments resulted in decreases in root activity of equal magnitude, the 100Po treatment was sufficient to alleviate the P deficiency as indicated by the root acidic monoesterase assay. 100Po did significantly increase the shoot P content but 400Po was required for a significant increase in shoot dry weight.

IHP must be must be hydrolysed to inorganic P before it can be utilised by plants (Fox and Comerford, 1992). It is possible that increasing the levels of IHP in the soil did cause an increase in root monoesterase activity early in the growing period in order to breakdown this additional substrate. But the activity subsequently decreased due to the build up in the soil of orthophosphate, from the Po hydrolysis, in excess of that absorbed by the plant.

The 100Pi treatment has a root activity significantly lower than the control. This decrease on the addition of Pi was as expected as orthophosphate inhibits phosphatase enzyme activity (Spiers and McGill, 1979).

3.5 Summary:

- Under the conditions of this experiment the addition of streptomycin, at the rate of 3 mg/g DW soil, to the soil did result in a significant decrease in bacterial colony forming units up to 9 weeks.
- Streptomycin sensitive bacteria were in competition with the *Dactylis* plants for the available P in the soil. Plant shoot biomass and P accumulation possibly increased as a result of a decrease in the numbers of streptomycin sensitive bacteria. This would indicate that the streptomycin sensitive bacteria were not responsible for the breakdown of soil Po for utilisation by plants
- Monoesterase is the enzyme responsible for the breakdown of IHP to orthophosphate, which is available to plants. Streptomycin sensitive bacteria were not involved in the production of soil acidic monoesterase activity. Streptomycin sensitive bacteria were not indicated as making an important contribution to root acidic monoesterase activity.
- Phytic acid was a source of plant available P as indicated by increases in dry weight and P content on the addition of IHP to the soil.
- There was no affect of increased concentrations of soil Po on the numbers of soil bacteria as quantified by the dilution plate method under the terms described in this experiment

CHAPTER 4: SOIL ORGANIC PHOSPHATE AND SOIL PHOSPHATASE ACTIVITY

4.1 Introduction

4.1.1 Objectives of Experiment

The objectives of the experiments described in this chapter were:

- to determine phosphatase enzyme activity at different levels of soil organic phosphate
- to examine phosphatase enzyme activity detected at different levels of soil organic phosphate in relation to plant growth and plant P uptake
- to examine phosphatase enzyme activity detected at different levels of soil organic phosphate in relation to levels of soil total, inorganic and organic phosphate
- to examine the role of mycorrhizas in the production of phosphatase enzymes

A natural soil, collected from the PP1 site (for site description refer to Chapter 1 section 1.3), un-amended with artificial growth substrates and with its indigenous soil microflora, was used. The plant species used in the course of this experiment were grown from seeds collected from plants growing on the PP1 site.

In order to take a rounded approach to soil organic phosphate and soil phosphatase, the activity of four phosphatase enzymes was quantified: soil acid and alkaline phosphomonoesterase activity, soil phosphodiesterase activity and root acidic phosphomonoesterase activity. The experiment was repeated for three species, *Agrostis stolonifera, Dactylis glomerata* and *Holcus lanatus* in order to compare the responses of different species under the same growth conditions and establish if species affected the phosphatases of their surrounding soil differently.

4.1.2 Experimental Approach

Inositol hexaphosphate (IHP) was chosen as the organic P compound to add to the soil as this compound is one of the principal forms of soil organic phosphate (Stevenson and Cole, 1999). It was added at concentrations of 30 and 60 mg P/l soil. These concentrations were the lowest concentrations of IHP added to soil in any of the experiments in this thesis. These concentrations were chosen as phosphatase analysis was carried out in Chapters 2 and 3 where higher concentrations of IHP had been added to soil. In the experiments in this chapter the affect of lower concentrations of IHP on phosphatase activity was examined.

In order to examine the role of vesicular arbuscular mycorrhizas in the production of root and soil phosphatase enzymes, the fungicide, Benlate, was added to the soil in half of the pots. This fungicide reduces the development of vesicular arbuscular mycorrhizas (Menge, 1982). (further details on Benlate: Chapter 2, Section 2.1.2.2) This results in plants and soils with a low vesicular arbuscular mycorrhizal infection that can be compared to soils and plants untreated with Benlate, with naturally occurring infection levels.

With the aim of separating the contribution of vesicular arbuscular mycorrhizal hyphae and plant roots to the production of soil phosphatase enzymes the 'growth bag' method was employed in these experiments (further details on the bag method: Chapter 2, section 2.1.2.1).

4.1.3 Definition of Phosphatase

Phosphatase is the general term that has been used to describe a broad group of enzymes that catalyse the hydrolysis of both esters and anhydrides of phosphoric acid (Tabatabai, 1994). These enzymes have been classified into five major groups by the commission on enzymes of the International Union of Biochemistry (Tabatabai, 1994). Two of these groups are of interest in the work discussed in this chapter – the phosphoric monoester hydrolases (EC 3.1.3) and the phosphoric diester hydrolases (EC 3.1.4).

A considerable part of soil phosphorus is organically bound (Helal, 1990; Barrett *et al.*, 1998). Harrison (1987) estimates that from 30 to 65% of total soil P is present in organic forms. Phosphatases are thus important for the mineralisation of this organic phosphate (Hysek and Sarapatka, 1998) and the hydrolysis of Po compounds, to generate inorganic phosphate that is then available for uptake by plants (Firsching and Claassen, 1996). The relationship between phosphatase activity and total organic phosphorus content is complicated, as negative and positive correlations have been found. Such relationships are difficult to detect due to factors such as varying site geology and vegetation type. (Harrison, 1987) Phosphatases also contribute to the extracellular degradation of complex polymeric molecules to more readily assimilated monomers. (Grierson and Comerford, 2000).

The addition of magnesium salts to soil has been found to stimulate phosphatase activity and in a range of acid soils a positive correlation has been found between phosphatase activity and extractable magnesium content. Though manganese can replace magnesium in phosphatase activation it can also inhibit the enzyme. Conversely a reduction in phosphatase activity has been found on the addition of calcium ions to soils. Activity in poor soils may be particularly sensitive to pollution by heavy metals. Heavy metals and other trace elements such as Zn, Mo, F, Cu, V, As, Al, Sn, Ni, Pb, Cr and Fe occurring as soil pollutants are known to inhibit phosphatase activity, though molybdenum may also be a stimulant of phosphatase. (Harrison, 1987)

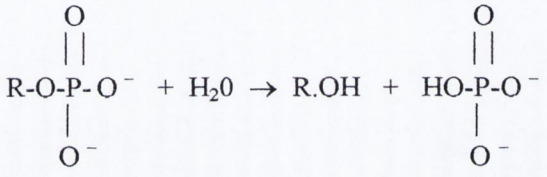
According to Burns (1982) at any one time the total activity of an enzyme in soil is comprised of activities associated with different soil constituents, for example: enzyme secreted by living cells during normal cell growth, enzyme associated with non-proliferating cells, cell debris, leaked from intact cells or lysed cells, absorbed to clay minerals or associated with humic colloids. Some of these activities probably do not provide a significant contribution to overall activity. Enzyme in dead cells or cell debris and free enzyme is probably short lived while

enzyme in spores and cysts are unlikely to reveal any activity in routine assays (Nannipieri *et al.*, 1996). Soil enzymes rarely occur free in soil solution but are more frequently associated with cells or cell debris or as immobilised molecules bound to clay minerals or coupled with humic colloid polyphenols (De Cesare, 2000). Enzymes immobilised on soil clays and humates constitute a persistent extracellular catalytic activity (Rojo *et al.*, 1990)

4.1.4 Phosphoric Monoester Hydrolases

The phosphoric monoester hydrolases, commonly referred to as phosphomonoesterases, catalyse the hydrolysis of monoesterases of orthophosphoric acid (Feder, 1973). These phosphomonoesterases contain the enzymes orthophosphoric monoester phosphohydrolyse EC 3.1.3.2 and orthophosphoric monoester phosphohydrolase EC 3.1.3.1 commonly referred to as acid phosphomonoesterase and alkaline phosphomonoesterase respectively (Tabatabai, 1994).

The phosphomonoesterases have been shown to catalyse the P-O bond cleavage (Feder, 1973) and the general equation of the reaction catalysed by acid and alkaline phosphatases is



These enzymes are known to hydrolyse a variety of phosphomonoesterases. The hydrolysis in soils of B-glycerophosphophosphate, phenylphosphate, b-naphthyl phosphate and p-nitrophenyl phosphate has been reported (Tabatabai, 1994).

The acid phosphomonoesterase activity shows optimum activity in the acid range and is predominant in acid soils. Alkaline phosphomonoesterase activity shows optimum activity in the alkaline range and is predominant in alkaline soils (Rojo *et al.*, 1990; Tabatabai, 1994). The optimum pH range for phosphatase activity is generally quite narrow (Rojo, 1990). The inverse relationship between acid and alkaline phosphomonoesterase activity and soil pH implies that the stability of these enzymes in soil, or the rate of synthesis and release of the enzymes by soil microorganisms, is related to soil pH (Juma and Tabatabai, 1978). The natural pH of a soil is most often not the pH for optimum enzyme activity (Harrison, 1987).

Juma and Tabatabai (1978) investigated the activity of acid and alkaline phosphomonoesterase in a variety of soils with pH ranging from 5.8 to 8.0. In acidic soils they detected a large amount of acid phosphomonoesterase activity, but also a small amount of alkaline activity. In alkaline soils they detected mainly alkaline phosphomonoesterase activity but also a small amount of acidic activity. Although this information is specific to the soils and conditions used in this experiment, this information indicates that there is no cut off point, in terms of soil pH, where both acid and alkaline phosphomonoesterase cannot be detected.

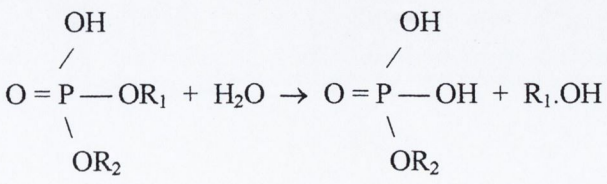
Acidic phosphomonoesterase is secreted mainly by plant roots and associated mycorrhizal and several saprophytic fungi (Tabatabai, 1994; Joner and Jakobsen, 1995). Acidic phosphomonoesterase enzymes associated with the root surface of plants may originate from mycorrhizal or saprotrophic fungi, bacteria or root exudates (Johnson *et al.*, 1999). The root acidic phosphomonoesterase is located mainly in the epidermis, cortex and root cap of roots (Juma and Tabatabai, 1988) and is an extracellular enzyme secreted, by the roots particularly in apical zones (Marschner, 1995). McLachlan found that the optimum pH for root surface acidic phosphomonoesterase activity lay in the range pH 5 to 6 when he tested a number of plant species, but found no evidence of alkaline phosphatase activity (Dodd *et al.*, 1987). Furthermore alkaline activity is absent from the rhizosphere of plants grown axenically (Joner and Jakobsen, 1995). Higher plants are considered to have no alkaline phosphatase activity and this activity has been attributed to soil bacteria, microorganisms, fungi and fauna (Tabatabai 1994; Joner and Jakobsen, 1995; Kramer and Green, 2000). Evidence has accumulated that suggests that microbial phosphomonoesterase activity plays a significant role in soil phosphorus transformations (Helal, 1990).

The role of phosphatases in the soil depends on the concentrations and chemical state of soil P (Dodd *et al.*, 1987). Phosphomonoesterase activity is influenced by the availability of Po to hydrolytic cleavage. For example, extracellular phosphomonoesterase activity of roots may be stimulated in the presence of easily hydrolysed substrates but repressed by non-hydrolyzable forms of Po (Joner and Jakobsen, 1995).

Phosphorus is taken up by plants mainly as orthophosphate ions from the soil solution (Stevenson and Cole, 1999). Orthophosphate is the product of phosphate mineralisation and is also added to the soil in the form of fertiliser. Kinetic studies on the affect of orthophosphate on acid and alkaline phosphomonoesterases showed that it inhibited the activity of these enzymes in soil. Orthophosphate is a competitive inhibitor of acid and alkaline phosphomonoesterases (Juma and Tabatabai, 1978; Tabatabai, 1994). Phosphatase enzymes are however inducible under conditions of low Pi. (Grierson and Comerford, 2000)

4.1.5 Phosphoric Diester Hydrolases

Orthophosphoric diester phosphohydrolase EC 3.1.4.1, is commonly referred to as phosphodiesterase, in soils. Phosphodiesterase catalyses the overall reaction of the type



where R1 and R2 represent either alcohol or phenol groups or nucleosides (Tabatabai, 1994).

Phosphodiesterase activity has been found in various plants, microorganisms and animals (Tabatabai, 1994). Among bacteria phosphodiesterse activity has been shown to be present in *Escherichia coli* and *Micrococcus lysodeikticus* (Browman and Tabatabai, 1978). Phosphodiesterase is best known for its ability to degrade nucleic acid. Studies have shown that at 5 mM orthophosphate is a competitive inhibitor of this enzyme (Tabatabai 1994). Studies of enzyme activity on soil profiles have shown that the activity usually decreases with increase in sample depth (Browman and Tabatabai, 1978). However phosphodiesterase is the least studied of the phosphatases in the soil and information on this enzyme is scarce.

4.1.6 Phosphatase Assays

Several methods have been proposed for the estimation of phosphomonoesterase activities in soils (Tabatabai, 1994). Soil phosphomonoesterase activity has been estimated by determining the phenol released when soil was incubated with phenyl phosphate (Tabatabai, 1994). This substrate has been used by several workers (Kramer and Yerdei, 1959; Halstead, 1964). Skujins *et al* (1962) incubated soil with β-glycerophosphate, and the amount of this substrate hydrolysed, estimated by analysis for extractable total and inorganic P, provided a measure of due to phosphomonoesterase. However this method is time consuming and has low precision. Raminez-Martinez and McLaren (1966) proposed a method involving fluorimetric assay of the β-naphthol released by incubation of the soil with β-naphthyl phosphate. However β-naphthol is sorbed by soil constituents and this complicates the method and makes it time consuming, as the capacity of each soil to sorb β-naphthol must be determined and allowed for in the calculation of results. The main difference between these methods is the substrate used and the technique used for measuring the product of hydrolysis of the substrate by phosphatase enzymes.

The most accurate and precise method for the estimation of phosphomonoesterase activity involves colorimetric estimation of *p*-nitrophenol, released due phosphomonoesterase activity when soil is incubated with buffered sodium *p*-nitrophenyl phosphate solution (Tabatabai, 1994). The basic method was developed by Tabatabai and Bremner (1969). Workers have used various modifications of this basic method. In this work the modified method of Dodd *et al* (1987) and Joner *et al* (1995) were used to determine the acidic and alkaline phosphomonoesterase activity respectively. An acidic buffer, pH 5.2, is used for determination of acid phosphatase activity (Dodd *et al*, 1997) and an alkaline buffer, pH 8.5, is used to determine alkaline phosphatase activity (Joner *et al*, 1995). The colorimetric procedure used to quantify the *p*-nitrophenol released by phosphomonoesterase activity is based on the fact that alkaline solutions of this phenol have a yellow colour. Acid and alkaline solutions of sodium *p*-nitrophenyl phosphate and acid solutions of *p*-nitrophenol are colourless. (Tabatabai, 1994)

The principle of the assay used to quantify phosphodiesterase activity is similar to that of the assay used for phosphomonoesterase activity. The main difference is the substrate used.

Phosphodiesterase activity is detected in soils using the substrate *bis-p*-nitrophenyl phosphate (BPNP). The *p*-nitrophenol released from this substrate due to phosphodiesterase activity is extracted and determined colourimetrically (Tabatabai, 1994). The soil phosphodiesterase activity measured with BPNP helps in the hydrolyses of the oligonucleotides and cyclic nucleotides in soil (Hayano, 1987).

Because of the location of enzymes on and within soil particles and aggregates, the substrate concentrations generally used in soil enzymes assays to ensure saturation are orders of magnitude higher than those used in soil free systems (De Cesare, 2000).

Air drying of fresh field moist soil probably destroys extracellular enzymes, which includes enzymes responsible for dissolution of a nutrient such as phosphatase and may accelerate their absorption to soil colloids (Burns, 1982). Air drying also causes the lysis of microorganisms and short term increases in enzyme activity due to release of previously intracellular enzymes (Burns, 1982). Marked changes in the composition of soil microflora will result from the air drying of soil, with the presence of non-proliferating cells such as fungal spores, protozoan cysts and microorganisms which are surrounded by polysaccharide-based gums able to survive under dry conditions, and in the absence of fresh organic substrates (Nannipieri *et al.*, 1996). Generally the contribution of the enzyme activity associated with non-proliferating cells to overall enzyme activity of soil is believed to be negligible. However this may not be the case when air-dry soils have been stored for long periods before being analysed (Nannipieri *et al.*, 1996). Air drying of field moist soil results in increases in acid phosphatase and a decrease in alkaline phosphatase activity (Tabatabai, 1994).

4.1.7 Background to Study

Organic phosphate accounts for a large percentage of soil phosphate. The infiltration of Po compounds further into the soil profile than inorganic fertilisers and the subsequent action of phosphatases of soil, microbes or plant roots may lead to release of orthophosphate in deeper parts of soil profile where it may become available to roots (Juma and Tabatabai, 1988). However, the nutritional significance of soil Po is generally underestimated. Most mathematical models simulating P uptake by growing plants have been constructed on an entirely inorganic basis (Helal, 1990).

Most research relating to soil phosphomonoesterases has therefore focused on the relationship between inorganic phosphate and enzyme production. For example, Fox and Comerford (1992) examined the affect of inorganic phosphate addition on soil acidic phosphomonoesterase activity in a forest soil. P fertilisation decreased phosphomonoesterase activity in the rhizosphere soil. They defined the rhizosphere as, the soil adhering to the roots following shaking to remove the bulk soil. Because they detected phosphatase activity in the soil they

concluded that potential to utilise organic P existed in their soils. Their only examination of this theory on soil Po was to add commercially available phosphatase (Sigma chemicals), derived from whet germ, to the soil. They determined the amount of soluble organic P in a soil extract that could be hydrolysed by this acidic phosphomonoesterase added to the soil. They found only 20-30% of the soluble Po in the extract was hydrolysed, indicating that simple phosphomonoesterases were a minor component of soluble organic P in these soils. This added phosphatase was possibly not representative of the naturally occurring soil phosphatase. They did not investigate the relationship between soil phosphatase present in the soil and soil Po.

Tando *et al.*, (1993) examined the activity of acidic phosphomonoesterase secreted by the roots of nine crop species when grown in nutrient solutions containing inorganic P as their phosphate source. Speirs and McGill (1979) examined how acidic phosphomonoesterase activity and production in some American soils was related to soil properties and past inorganic P fertiliser history. P fertilisation was found to increase and decrease phosphatase activity depending on the soil type.

Work has also been carried out to relate phosphomonoesterase activity to environmental factors. Kramer and Green (2000) have examined the seasonal dynamics of acidic and alkaline phosphomonoesterase activity, along with the affect of soil water potential and temperature on the enzymes. Speir and Cowling (1991) studied the phosphomonoesterase activity of soil and roots in high and low fertility pasture sites to determine the affect of seasonal changes and soil moisture.

The significance of phosphatases for the utilisation of Po by plants is not well understood (Firsching and Claassen, 1996: Antibus *et al.*, 1997). Where phosphomonoesterases and Po have been examined, workers used artificial growth substrates and conditions. Results have indicated that roots could hydrolyse a variety of Po compounds (Juma and Tabatabai, 1988). However this work by Juma and Tabatabai (1988) was performed with sterile roots of corn and soybean, which had been germinated on agar, suspended in solutions of various Po compounds including glucose phosphate and sodium glycerophosphate and incubated for 2 days – very un-natural conditions. The Po compounds used did not include IHP. The amount of orthophosphate released into the solutions was taken as an indicator of root acidic phosphomonoesterase activity.

Barrett *et al.*, (1998) examined the phosphomonoesterase activity of wheat seedling roots grown in solution culture and sterile agar medium containing IHP or Pi at elevated atmospheric CO_2 concentrations. They found plants grown in the presence of IHP to have a higher root phosphatase activity than those grown with Pi at ambient and elevated CO_2 concentrations. They were no control plants grown with no P present.

Helal (1990) studied the affect of IHP or Pi on root acidic phosphomonoesterase activity of varieties of bean plants. Plants were grown in sand culture with the phosphate supplied as a nutrient solution.

Barrett-Lennard *et al.*, (1993) examined the root acidic phosphatase activity of root segments of clover plants, which had been grown in agar culture, when IHP and other Po compounds such as cAMP, were present as the substrate in the assay solution. They detected little activity when IHP was present and concluded that it was poorly hydrolysed and a poor source of P for growth, compared to cAMP for which they detected a higher activity. The nucleic acid, cAMP, less common in soil than IHP, appeared to be a better source of P for growth resulting in higher plant P concentrations and contents. Barrett- Barrett-Lennard *et al* concluded that root phosphomonoesterases are important in the use of Po by plants for nutrition but it depends on the particular Po compound.

Richardson *et al* (2000) examined the root acidic phosphomonoesterase activity of wheat seedlings grown in agar with IHP as the P source. No difference in root activity was detected when plants were grown in the presence if IHP than when they were grown with no P at all and plants showed limited ability to obtain P from IHP. A soil bacterium with phosphatase activity was introduced to the growth environment and was found to enhance the utilisation of P from IHP. In soil environments plants may similarly be reliant on rhizosphere microoganisms to increase nutrient availability (Richardson *et al.*, 2000). This cannot be explored using such an artificial growth system that does not take naturally occurring soil microflora into account.

Phosphodiesterase is the least studied of the phosphatases in the soil. References to phosphodiesterase activities in the literature are scarce and generally unsystematic Trasar-Cepeda *et al.*, (2000). Root surface phosphodiesterase activity of tree roots of beech, grey birch and red maple was examined by Antibus *et al.*, (1997). All these root types had phosphodiesterase activity indicating their potential for Po mineralisation. Naseby and Lynch (1997) tested an assay for determining soil phosphodiesterase activity using only small soil samples. The enzyme was first extracted from the soil and then the extract was analysed for phosphodiesterase activity. It was found the enzyme activity decreased with depth. The addition of a mix of urea, colloidal chitin and glycerophosphate did not affect phosphodiesterase activity. Trasar-Cepeda *et al.*, (2000) examined the phosphodiesterase activity, along with phosphomonoesterase activity of soils from an oakwood forest in NW Spain, with a view to establishing a database of biochemical parameters of soils in the area. They did not correlate this activity to soil phosphate content or plant growth. In a literature search no work studying the affect of soil Po on diesterase activity was found by this author.

As demonstrated, many of the studies to date have focused on soil and root phosphatase activity in relation to soil inorganic P. Studies on soil and root phosphatase activity and their relationship to soil organic P and plant P uptake, have used nutrient solution culture, sterile agar medium and sand culture. None of these environments represent the real growth conditions to which plants are normally exposed, leaving the importance of phosphatases in providing plants with P from Po sources poorly understood. There is therefore a need for detailed investigation of precise role and functions of phosphatase enzymes in soil Po dynamics (Frossard *et al.*, 2000). The work in this chapter has a different approach to that adopted by workers to date. Field-collected soil is used, un-amended with any artificial growth medium or any artificial phosphatases. A natural soil with its indigenous soil microbial population is a more natural situation allowing microorganisms to play their part in plant P nutrition. Furthermore the majority of work on phosphatases and soil Po has focused on phosphomonoesterases, and in particular to acid phosphatase (Tabatabai, 1994). There is little analysis related to alkaline phosphomonoesterases activity and phosphodiesterase activity. The work in this chapter relates to acidic and alkaline phosphomonoesterases and phosphodiesterase activity in order to take a balanced approach.

4.2 METHODS

4.2.1 Collection and storage of soil and seeds

As described in Chapter 2, Section 2.2.1

4.2.2 Preparation of soil

As described in Chapter 2, Section 2.2.2

4.2.3 Seed Germination and growth conditions

All seeds were germinated in seed trays containing soil from the PP1 site. After 5/6 days seedlings of uniform size were selected and transplanted into the experimental pots (4-inch plastic plant pots). The 'growth bag' method was used in this experiment in order to distinguish between contributions of roots and of mycorrhizal hyphae to soil phosphatase activity (further details on the bag method: chapter 2, Sections 2.1.2.1 and 2.2.3). All pots were placed in a heated greenhouse maintained at a constant 15°C in a random arrangement. An automatic watering system using capillary matting was in place.

4.2.4 Phosphate addition and experimental layout

This experiment was based on the PP1 site soil and had a control (no P addition) and 2 treatments, where phosphorus was added at two rates, 30 and 60 mg P/l soil, in the form of Po. This was carried out for soil treated and untreated with Benlate. Benlate is a fungicide known to reduce mycorrhizal development (Menge, 1982). (further details on Benlate: Chapter 2, Section 2.1.2.2). Each control and treatment had 3 replicate pots, giving a total of 18 pots. (Table 4.1)

Table 4.1: Experimental layout for the soil phosphatase experiment. There were 3 replicates per treatment. This layout was replicated for soil treated with benlate at a rate of 0.89g benlate/kg soil

P Treatments	Conc P added	Source of P
Control (PP1 soil)	no P addition	
30Po (PP1 soil)	30 mg P/l soil	IHP
60Po (PP1 soil)	60 mg P/l soil	IHP

This experiment was carried out concurrently for the species *Agrostis stolonifera*, *Dactylis glomerata* and *Holcus lanatus*.

Organic phosphate was added in the form of phytic acid (myo-inositol hexakis [dihydrogen phosphate], Sigma Chemicals) which was added to the soil as a powder and was thoroughly mixed with soil before the soil was put into the 'growth bags' and plant pots.

Benlate (Du Pont Chemicals) was added to the soil at the rate of 0.89 g benlate/kg soil, as a solution (0.89 g benlate in 28 ml distilled water) and was thoroughly mixed with the soil before the soil was put into the 'growth bags' and plant pots. Where soil was not treated with benlate 28 ml of water per kg soil was added to the soil to ensure all treatment received the same levels of moisture.

Each pot was filled with 400 g soil (200 g in 'growth bag', 200 g in surrounding area) of the appropriate treatment soil. One seedling was placed in each pot.

4.2.5 Harvesting

Experiments were harvested 12 weeks after transplanting seedlings into treatment pots. On harvesting each pot, soil from inner root compartment and outer hyphal compartment was put into separate labelled plastic bags and stored at 4°C until analysis. All soil analyses: soil phosphatase activity and soil total, inorganic and organic P, were carried out for inner and outer soil for each replicate pot.

Above ground plant material was excised from the roots and was dried for dry weight measurements and total P analysis. Roots were stored in distilled water until root phosphatase analysis was carried out. The remaining root material was dried for dry weight measurements and total P analysis.

4.2.6 Soil and root enzyme assays

Phosphomonoesterase and phosphodiesterase activity were quantified by measuring the amount of *p*-nitrophenol (*p*-NP) released from a buffered substrate by the phosphatase activity during an incubation period of 1 hour. The amount of *p*-NP released was quantified using absolute quantities of *p*-NP to create a standard curve.

For phosphatase activity of soil and roots the results were expressed as mg *p*-NP/g FW soil or root/hr, as expressing such data on a fresh weight (FW) basis has been found to be more reproducible than using dry weights (Johnson *et al.*, 1999). The control analysis allowed for the presence of trace amounts of *p*-nitrophenol in some commercial samples of *p*-nitrophenyl phosphate and *bis-p*-nitrophenyl phosphate and for colour not derived from *p*-NP released by phosphatase activity. (Tabatabai, 1994)

Air drying of field moist soil results in increases in acid phosphatase and a decrease in alkaline phosphatase activity (Tabatabai, 1994). For these reasons all phosphatase analyses carried out in the duration of this work used fresh soil which was stored for a maximum of two weeks at 4 °C. Phosphatase activity remains stable over 28 days in moist soils stored at 4 °C (Kramer and Green, 2000).

The assays used to quantify the soil and root acidic phosphomonoesterase activity, soil alkaline phosphomonoesterase activity and soil phosphodiesterase activity are similar. They differ on the following points:

- The phosphomonoesterase assays used *p*-nitrophenyl disodium phosphate as the enzyme substrate. The phosphodiesterase assay used *bis-p*-nitrophenyl phosphate as the substrate.
- The acidic phosphomonoesterase assays used 0.1 M sodium acetate buffer, pH 5.2 (Dodd *et al*, 1987). The alkaline phosphomonoesterase assay used 0.1 M $NaHCO_3$ buffer, pH 8.5

(Joner *et al*, 1995). The diesterase assay used 0.05 M THAM buffer, pH 8.0 (Browman and Tabatabai, 1978).

- In the phosphomonoesterase assays the NaOH added after incubation, stopped the phosphatase activity and developed the yellow colour used to estimate the quantity of *p*-nitrophenol released. In the phosphodiesterase assay THAM buffer, pH 12, was used in the place of NaOH because the diesterase substrate *bis-p*-nitrophenyl phosphate is not stable in NaOH solutions. (Tabatabai, 1994).

4.2.6.1 Assay for soil acidic phosphomonoesterase activity

Soil acid monoesterase activity was measured using the method of Dodd *et al.*, 1987. Fresh soil, 0.5 g, was weighed into a centrifuge tube. 4 ml of 0.1 M sodium acetate buffer, pH 5.2 and 1 ml of 25 mM *p*-nitrophenyl disodium phosphate (*p*-NPP) (Sigma Chemicals) was added. Tubes were incubated at 25 °C for 1 hour in a water bath. To terminate the reaction 5 ml of 0.5 M NaOH (Sigma Chemicals) was added. Tubes were centrifuged at 2500 g (Sanyo Harrier centrifuge 15/80) for 15 minutes. A 1 in 10 dilution of the resulting supernatant was prepared using sodium acetate buffer pH 5.2 and the absorbance was read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

A standard curve was obtained using standards prepared using *p*-nitrophenol ($NO_2C_6H_4OH$) (Sigma Chemicals) in 0.1 M sodium acetate buffer, pH 5.2 in the range 0 2, 0 4, 0 6, 0 8, and 10 mg *p*-NP/l. For each standard 5 ml was pipetted into a centrifuge tube and 5 ml of 0.5 M NaOH was added. The solution was mixed and the absorbance read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

A control was prepared by adding 0.5 g of soil to 4 ml 0.1 M sodium acetate buffer in a centrifuge tube and incubating at 25 °C for 1 hour in a water bath. 5 ml of 0.5 M NaOH followed by 1 ml of 25 mM *p*-nitrophenyl disodium phosphate were then immediately added. Tubes were centrifuged at 2500 g (Sanyo Harrier centrifuge 15/80) for 15 minutes. A 1 in 10 dilution of the resulting supernatant was prepared using sodium acetate buffer pH 5.2 and the absorbance was read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

4.2.6.2 Assay for root acidic phosphomonoesterase activity

The root acidic phosphomonoesterase assay was carried out immediately after harvesting the plants. Root acidic phosphomonoesterase activity was measured using the method of Dodd *et al.*, (1987). Fresh root material, 0.1 g, was weighed into a centrifuge tube. 4 ml of 0.1 M sodium acetate buffer, pH 5.2 and 1 ml of 25 mM *p*-nitrophenyl disodium phosphate (*p*-NPP) (Sigma Chemicals) was added. Tubes were incubated at 25 °C for 1 hour in a water bath. To terminate the reaction 5 ml of 0.5 M NaOH (Sigma Chemicals) was added. Tubes were centrifuged at

2500 g (Sanyo Harrier centrifuge 15/80) for 15 minutes. A 1 in 20 dilution of the resulting supernatant was prepared using sodium acetate buffer pH 5.2 and the absorbance was read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

A standard curve was prepared as detailed in section 4.2.6.1.

A control was prepared by adding 0.1 g of root to 4 ml 0.1 M sodium acetate buffer in a centrifuge tube and incubating at 25 °C for 1 hour in a water bath. 5 ml of 0.5 M NaOH followed by 1 ml of 25 mM *p*-nitrophenyl disodium phosphate were then immediately added. Tubes were centrifuged at 2500 g (Sanyo Harrier centrifuge 15/80) for 15 minutes. A 1 in 20 dilution of the resulting supernatant was prepared using sodium acetate buffer pH 5.2 and the absorbance was read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

4.2.6.3 Assay for soil alkaline phosphomonoesterase activity

Soil alkaline monoesterase activity was measured using the method of Joner and Jakobsen (1995). Fresh soil, 0.5 g, was weighed into a centrifuge tube. 4 ml of 0.1 M $NaHCO_3$ buffer, pH 8.5 and 1 ml of 25 mM *p*-nitrophenyl disodium phosphate (*p*-NPP) (Sigma Chemicals) was added. Tubes were incubated at 25 °C for 1 hour in a water bath. To terminate the reaction 5 ml of 0.5 M NaOH (Sigma Chemicals) was added. Tubes were centrifuged at 2500 g (Sanyo Harrier centrifuge 15/80) for 15 minutes. A 1 in 10 dilution of the resulting supernatant was prepared using $NaHCO_3$ buffer pH 8.5 and the absorbance was read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

A standard curve was obtained using standards prepared using *p*-nitrophenol ($NO_2C_6H_4OH$) (Sigma Chemicals) in 0.1M $NaHCO_3$ buffer, pH 8.5 in the range 0 2, 0 4, 0.6, 0 8, and 10 mg *p*-NP/l. For each standard 5 ml was pipetted into a centrifuge tube and 5 ml of 0.5 M NaOH was added. The solution was mixed and the absorbance read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

A control was prepared by adding 0.5 g of soil to 4 ml 0.1M $NaHCO_3$ buffer, pH 8.5 in a centrifuge tube and incubating at 25 °C for 1 hour in a water bath. 5 ml of 0.5 M NaOH followed by 1 ml of 25 mM *p*-nitrophenyl disodium phosphate (Sigma Chemicals) were then immediately added. Tubes were centrifuged at 2500 g (Sanyo Harrier centrifuge 15/80) for 15 minutes. A 1 in 10 dilution of the resulting supernatant was prepared using sodium acetate buffer pH 5.2 and the absorbance was read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

4.2.6.4 Assay for soil phosphodiesterase activity

Soil phosphodiesterase activity was measured using the method of Browman and Tabatabai, (1978). Fresh soil, 0.5 g, was weighed into a 12 ml centrifuge tube. 2 ml of 0.05 M THAM

(tris(hydroxymethyl)aminomethane) buffer, pH 8.0 was added. 0.5 ml 0.05 M *bis-p*-nitrophenyl phosphate (BPNP) (Sigma Chemicals) solution was added. Tubes were inverted to mix and incubated in a water bath at 25 °C for 1 hour. 0.5 ml 0.5 M $CaCl_2$ and 2 ml THAM-NaOH (0.01 M THAM, pH 12) extractant solution was added. Tubes were inverted to mix and centrifuged at 2500g (Sanyo Harrier centrifuge 15/80) for 15 minutes. A 1 in 25 dilution of the resulting supernatant was prepared using THAM buffer pH 10 and the absorbance was read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

A standard curve was obtained using standards were prepared using *p*-nitrophenol ($NO_2C_6H_4OH$) (Sigma Chemicals) in distilled water containing 0 2, 0 4, 0 6, 0 8, and 10 mg *p*-NP/l solution. For each standard 5 ml was pipetted into a centrifuge tube and 0.5 ml of 0.5 M $CaCL_2$ and 2 ml THAM-NaOH extractant solution were added. The solution was mixed and the absorbance read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

A control was prepared by adding 0.5 g of soil to 2 ml of 0.05 M THAM buffer, pH 8.0 in a 12 ml centrifuge tube and incubating at 25 °C for 1 hour. 0.5 ml 0.5 M $CaCl_2$ and 2 ml THAM-NaOH extractant solution was added. 0.5 ml 0.05 M BPNP solution was added.

Tubes were centrifuged at 2500 g (Sanyo Harrier centrifuge 15/80) for 15 minutes and the absorbance of a 1 in 25 dilution of the resulting supernatant read at 400 nm on a spectrophotometer (Pye Unicam SP6-550 UV/VIS spectrophotometer).

4.2.7 Plant dry weights

As described in Chapter 2, Section 2.2.11.

4.2.8 Plant material digest for total P

As described in Chapter 2, Section 2.2.12

4.2.9 Total, inorganic and organic P levels in soil

As described in Chapter 2, Section 2.2.8.

4.2.10 Statistical procedures

All statistical analysis was carried out using the statistics package Data Desk, version 6.0. All data were checked for normality before statistical analysis. Where necessary data were transformed to obtain a normal distribution. Soil phosphatase and phosphate data was analysed using a three way ANOVA with P treatment, benlate treatment and location of the soil (ie. inner or outer soil) as the three factors. Plant dry weight and P content data were analysed using a two way ANOVA with P treatment and benlate treatment as the two factors. Correlations were carried out using a Pearson Product Moment Correlation.

4.3 Results

In the description of the results that follows, P treatment refers to the addition of organic phosphate to soil at the 2 concentrations employed. These two concentrations are 30 and 60 mg P/l soil abbreviated to 30Po and 60Po respectively, for the purpose of graphs and results description and discussion. The control treatment is soil with no phosphate addition. Benlate treatment refers to the addition of the fungicide benlate to the soil. The term 'location' refers to either inner root compartment soil or the outer hyphal compartment soil, which are abbreviated to inner and outer soil respectively. Start soil refers to the soil as prepared at the start of the experiment, with the relevant P and benlate treatment, but before contact with any plants. Post harvest soil refers to soil from which the plants have been harvested after their 12 week growth period.

4.3.1 *Agrostis stolonifera*

4.3.1.1 Soil phosphatase activity

The soil phosphatase activity results were presented as follows:
soil acidic monoesterase data in Fig 4.1 (a,b), soil alkaline monoesterase data in Fig 4.2 (a,b), soil diesterase data in Fig 4.3 (a,b) .Three way ANOVAs were carried out to determine the affect of P treatment, benlate treatment and location of soil on these soil phosphatase activities. These ANOVAs were presented in Appendix 4, Tables 1, 2 and 3.

There was a significant P affect (F ratio 3.88*) on soil diesterase activity There was also a significant P - benlate interaction (F ratio 5.74**) for soil diesterase activity. All other treatments and interactions for the three soil phosphatase activities were not significant.

Soil acidic monoesterase activity

Figure 4.1 (a) presented soil acidic monoesterase activity data for soil not treated with benlate. From Fig 4.1 (a), the phosphatase activity of the inner soil was higher than that detected in the outer soil. Figure 4.1 (b) presented soil acidic monoesterase activity data for soil treated with benlate. From Fig 4.1 (b) the activity of the inner soil was again higher than that of the outer soil, except for the 60Po treatment soil, where the activity of the inner and outer soil overlapped. However there was no significant affect of the location of the soil on soil acidic monoesterase activity (Appendix 4, Table 1).

Soil Alkaline Monoesterase Activity

Fig 4.2 (a) presented soil alkaline monoesterase activity for soil not treated with benlate. In the case of the control soil the activity of the outer compartment soil was higher than that of the inner compartment soil. For the 30 Po treatment the situation was reversed and the inner soil activity was greater than that of the outer soil. The inner and outer compartment activities were the same for the 60Po treatment soil. Fig 4.2 (b) presented soil alkaline monoesterase activity

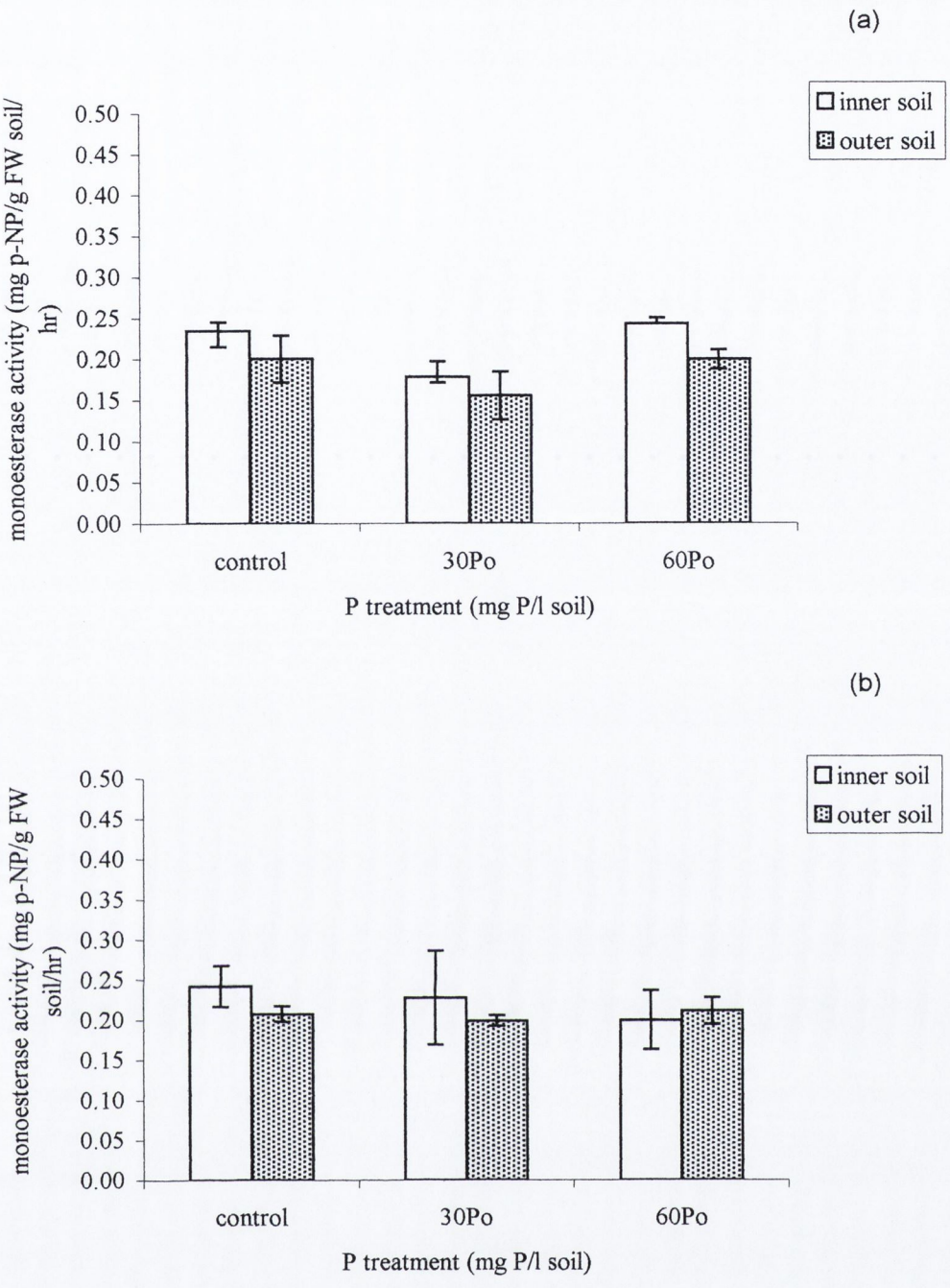
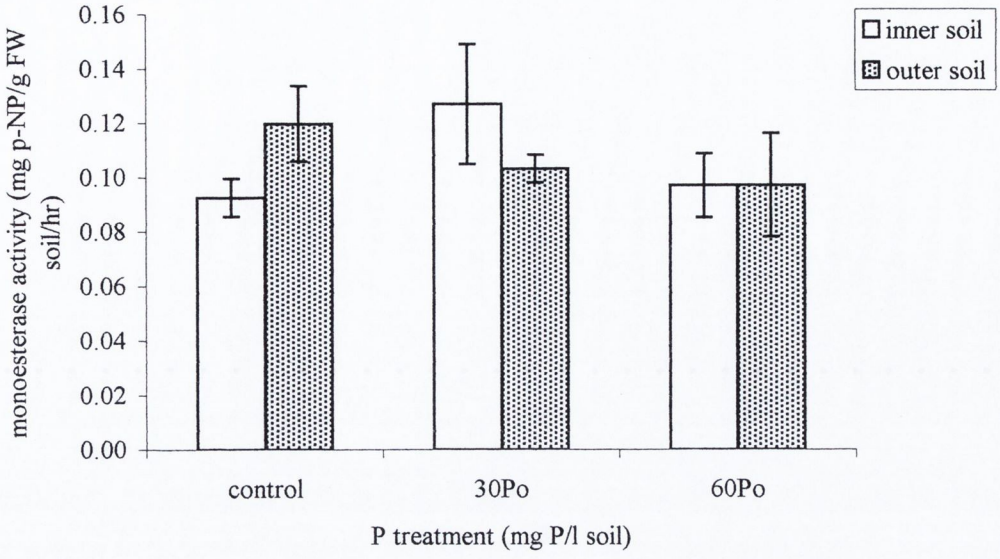


Fig 4.1: soil acidic monoesterase activity for the control and P treatment soils for the *Agrostis stolonifera* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.



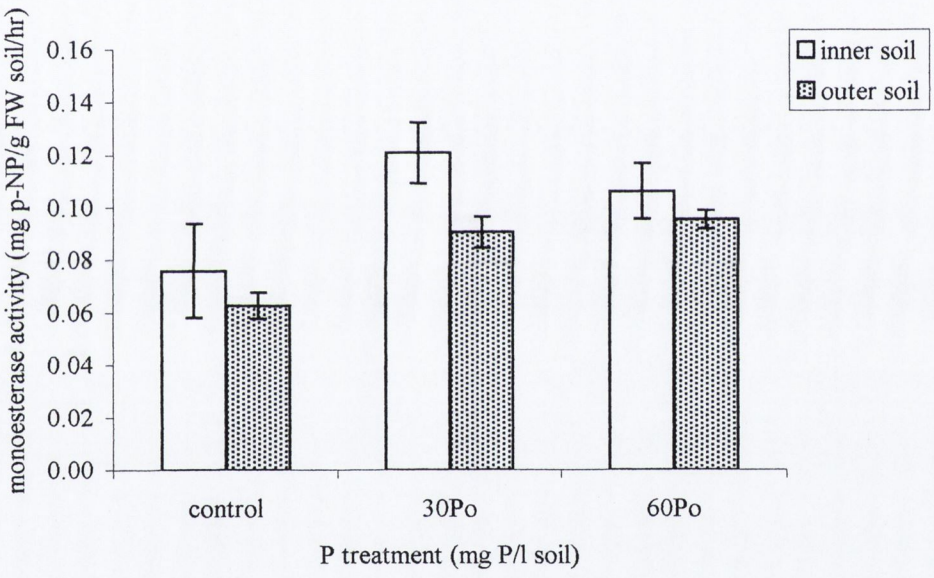


Fig 4.2: soil alkaline monoesterase activity for the control and P treatment soils for the *Agrostis stolonifera* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

for soil treated with benlate. From Fig 4.2 (b) the activity of the inner soil was greater than that of the outer soil for both control and treatment soils. However there was no significant difference in the soil alkaline monoesterase activity in the inner and outer soil (Appendix 4, Table 2).

Soil Diesterase Activity

Fig 4.3 (a) presented soil diesterase data from soil not treated with benlate. Fig 4.3 (b) presented soil diesterase activity levels from soil treated with benlate. With increasing levels of organic P added there was an increase in diesterase activity (Fig 4.3, a, b). This increase in enzyme activity with increasing levels of Po added to the soil was significant (Appendix 4, Table 3).

The significant interaction between P treatment and benlate treatment was examined using a Scheffe post hoc test (Table 4.2).

Table 4.2: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected soil diesterase activity for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-B, control - +B, control	ns
-B, 30Po - +B, 30Po	ns
-B, 60Po > +B, 60Po	*
-B, control - -B, 30Po	ns
-B, control < -B, 60Po	**
+B, control - +B, 30Po	ns
+B, control - +B, 60Po	ns

The addition of benlate to the 60Po treatment soil resulted in a significant reduction in the soil diesterase activity (Fig 4.3, a, b: Table 4.2). There was a significant increase in soil diesterase activity with the 60Po treatment in soil untreated with benlate (Fig 4.3, a: Table 4.2).

4.3.1.2 Root acidic monoesterase activity

The root acidic monoesterase activity results were presented in Fig 4.4 (a, b). A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on root acidic phosphomonoesterase activity. This ANOVA was presented in Appendix 4, Table 4.

There was no significant P affect, benlate affect or interaction between these variables for root acidic monoesterase activity.

4.3.1.3 Shoot and Root Dry Weights

The shoot and root dry weight data were presented in Fig 4.5 (a,b). Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot and root dry weights. These ANOVAs were presented in Appendix 4, Tables 5 and 6.

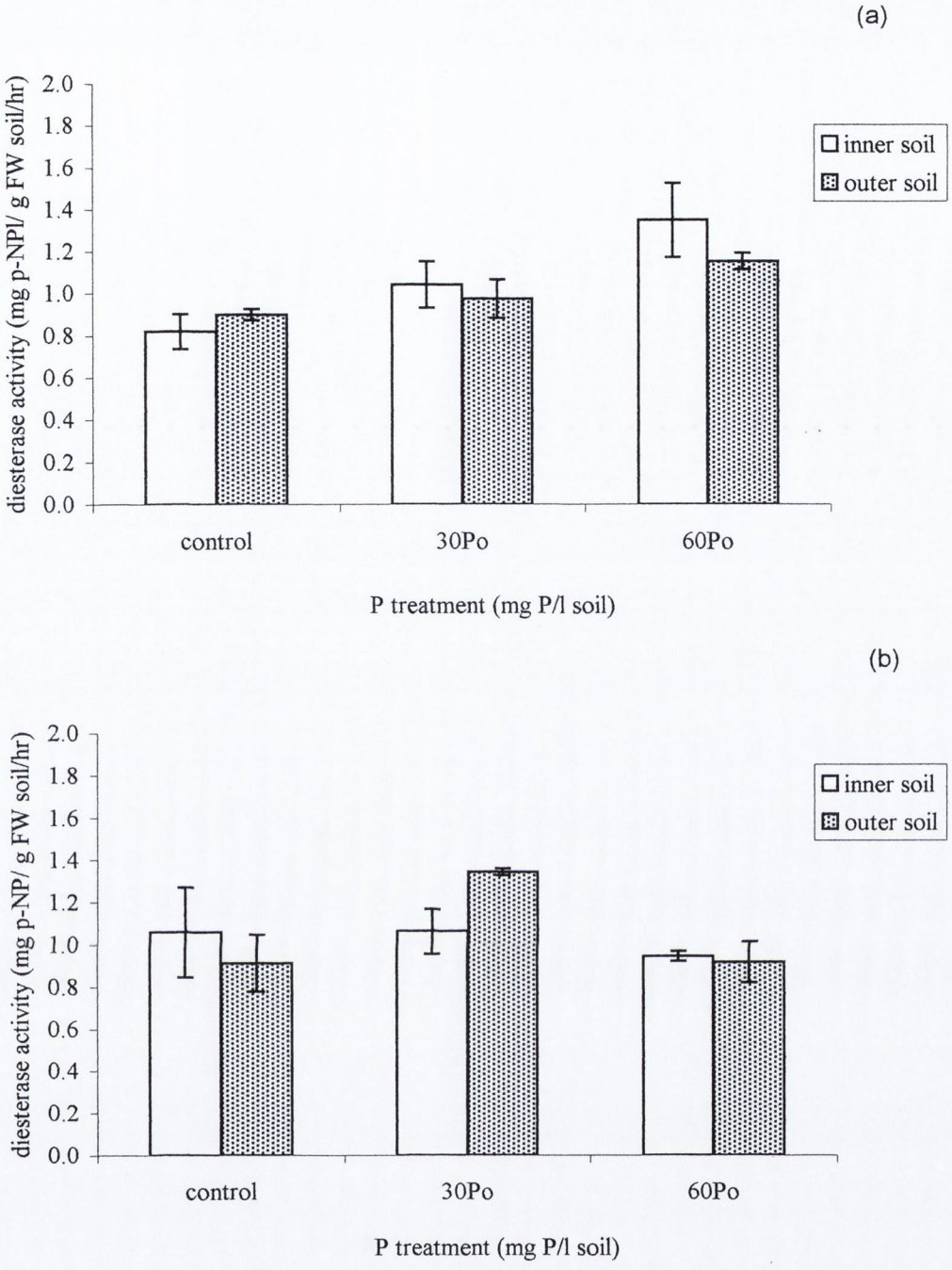
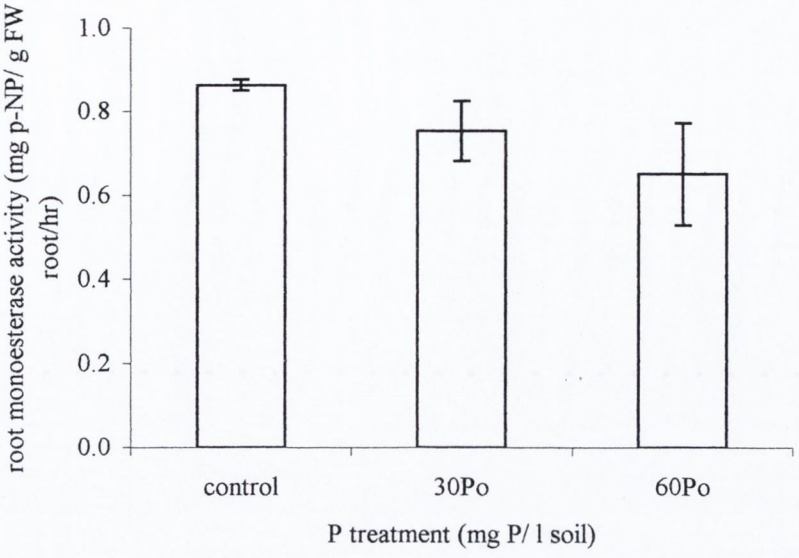


Fig 4.3: Soil diesterase activity for the control and P treatment soils for the *Agrostis stolonifera* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

(a)



(b)

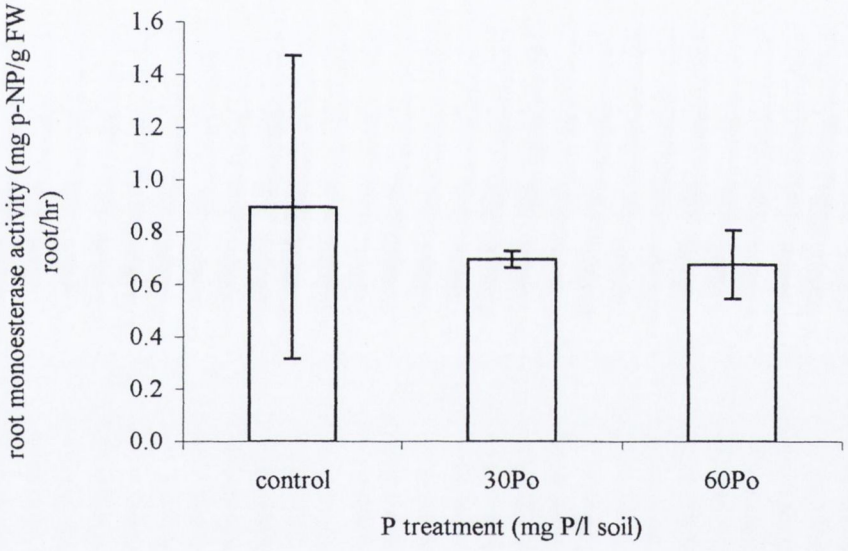


Fig 4.4: Root acidic monoesterase activity for the control and P treatment plants for the *Agrostis stolonifera* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

There was a highly significant P affect for shoot (F ratio 18.79***) and root (F ratio 514.11***) dry weight. There was a significant P - benlate interaction on root dry weight (F ratio 286.74***). All other treatments and interactions were not significant.

The shoot : root dry weight ratio for *Agrostis stolonifera* at each combination of P treatment and benlate treatment were presented in Table 4.3.

Table 4.3: shoot : root dry weight ratios for *Agrostis stolonifera* at each combination of P treatment and benlate treatment

	Shoot : Root ratio	
P treatment	**-Benlate**	**+Benlate**
control	3.7	24.7
30Po	4.3	3.3
60Po	2.7	2.5

From Table 4.3 the shoot : root ratio (r:s ratio) showed no consistent pattern of change with P treatment, in soil untreated with benlate. The 30Po treatment resulted in an increase in r:s ratio, but the 60Po treatment resulted in a decrease in r:s ratio. In soil treated with benlate, the addition of increasing concentrations of Po to the soil resulted in a decrease in r:s ratio (Table 4.3).

Shoot dry weight

Fig 4.5 (a) presented shoot dry weight data for plants grown in soil untreated with benlate. Fig 4.5 (b) presented shoot dry weight data for plants grown in soil treated with benlate. From Fig 4.5 (a, b) there was an increase in shoot dry weight from that of the control plants to that of the 30Po and 60Po treatment plants. This significant affect of P treatment on shoot dry weight was examined using a Scheffe post hoc test (Table 4.4).

Table 4.4: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot dry weight for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	***
control < 60Po	**
30Po - 60Po	ns

Root dry weight

Fig 4.5 (a) presented root dry weight data for plants grown in soil untreated with benlate. Fig 4.5 (b) presented root dry weight data for plants grown in soil treated with benlate. From Fig 4.5 (a, b) there was an increase in root dry weight from that of the control plants to that of the 30Po and 60Po treatment plants. This significant affect of P treatment on root dry weight was examined using a Scheffe post hoc test (Table 4.5).

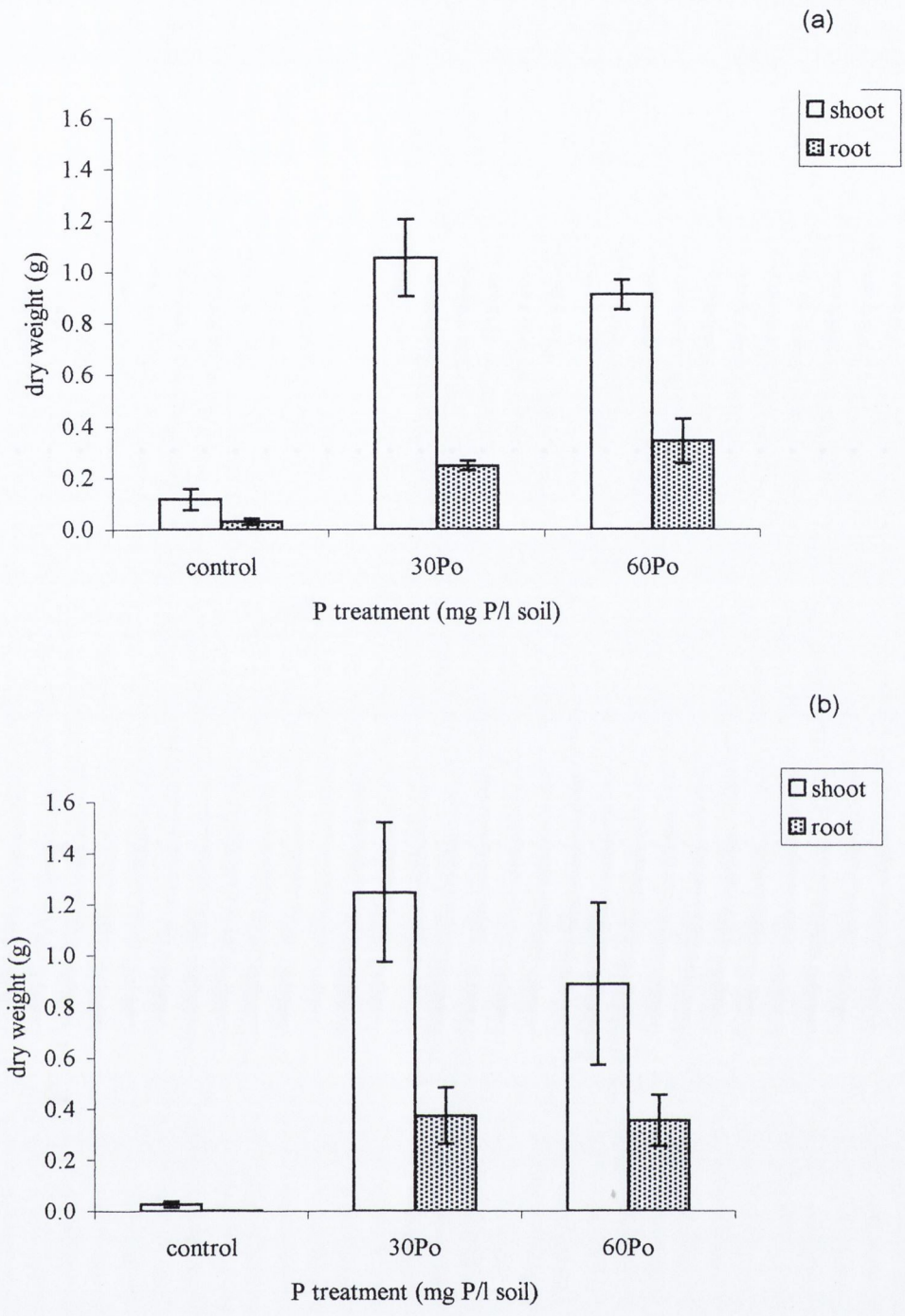


Fig 4.5: shoot and root dry weights for the control and P treatment plants of *Agrostis stolonifera* for plants grown in (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

Table 4.5: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root dry weight for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	***
control < 60Po	***
30Po - 60Po	ns

The significant interaction between P treatment and benlate treatment was examined using a Scheffe post hoc test, (Table 4.6).

Table 4.6: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected root dry weight for the *Agrostis stolonifera* experiment.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-B, control - +B, control	ns
-B, 30Po - +B, 30Po	ns
-B, 60Po - +B, 60Po	ns
-B, control < -B, 30Po	***
-B, control < -B, 60Po	***
+B, control < +B, 30Po	***
+B, control < +B, 60Po	***

.There was a significant increase in root dry weight of the *Agrostis* plants as a result of the 30Po and 60Po treatments, for plants grown in benlate treated and untreated soil (Table 4.6; Fig 4.5 a, b).

4.3.1.4 Shoot and Root P Concentration

The shoot and root P concentration data were presented in Fig 4.6 (a, b). Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot and root P concentrations. These ANOVAs were presented in Appendix 4, Tables 7 and 8.

There was a significant P affect (F ratio 3.66*) on shoot P concentration. All other treatments and interactions were not significant.

Shoot P concentration

Fig 4.6 (a) presented shoot P concentration data for plants grown in soil untreated with benlate. Fig 4.6 (b) presented shoot P concentration data for plants grown in soil treated with benlate. From Fig 4.6 (a, b) there was no difference in the shoot P concentration between the control plants and the 30Po or the 60Po treatment plants. However the 60Po treatment resulted in a higher shoot P concentration than the 30Po treatment (Fig 4.6 a, b). The significant affect of P treatment on shoot P concentration was examined using a Scheffe post hoc test (Table 4.7).

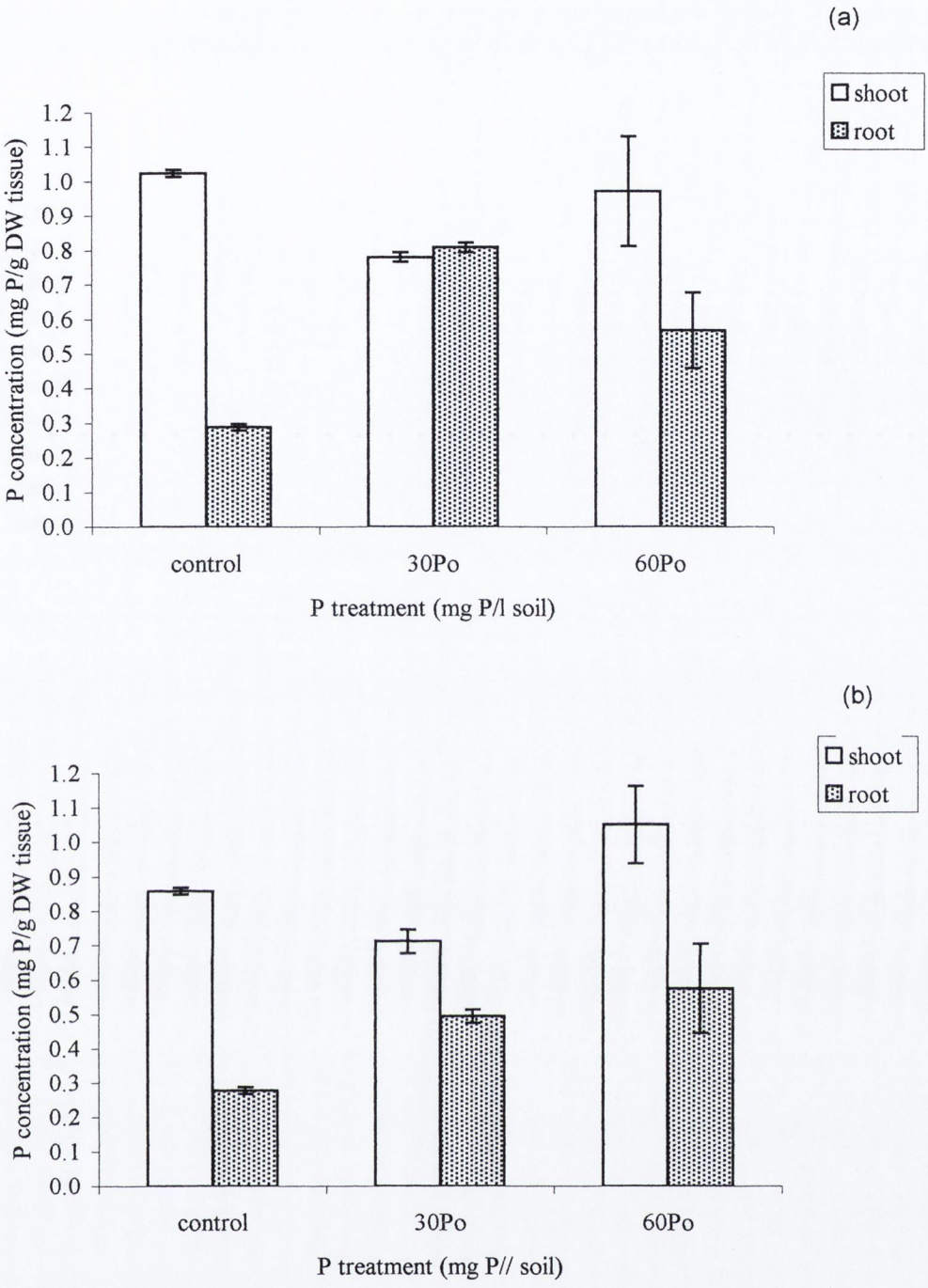


Fig 4.6: shoot and root P concentration for the control and P treatment plants of *Agrostis stolonifera* for plants grown in (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

Table 4.7: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P concentration for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 30Po	ns
control - 60Po	ns
30Po < 60Po	*

Root P concentration

Fig 4.6 (a) presented root P concentration data for plants grown in soil untreated with benlate. Fig 4.6 (b) presented root P concentration data for plants grown in soil treated with benlate. From Fig 4.6 (a, b) there was an increase in root P concentration from that of the control plants with the addition of Po for the 30Po and 60Po treatment plants. This apparent affect of P treatment was not statistically significant (Table 4.10).

4.3.1.5 Shoot and Root P Content

The shoot and root P content data were presented in Fig 4.7 (a, b). Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot and root P content. These ANOVAs were presented in Appendix 4, Tables 9 and 10.

There was a significant P affect on shoot P content (F ratio 11.03***) All other treatments and interactions were not significant.

Shoot P content

Fig 4.7 (a) presented shoot P content data for plants grown in soil untreated with benlate. Fig 4.7 (b) presented shoot P content data for plants grown in soil treated with benlate. From Fig 4.7 (a, b) there was an increase in shoot P content a result of the 30Po and 60Po treatments. This significant affect of P treatment on shoot P content was examined using a Scheffe post hoc test (Table 4.8).

Table 4.8: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P content for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	**
control < 60Po	**
30Po - 60Po	ns

Root P content

Fig 4.7 (a) presented root P content data for plants grown in soil untreated with benlate. Fig 4.7 (b) presented root P content data for plants grown in soil treated with benlate. From Fig 4.7 (a, b) there was a slight increase in root P content as a result of the 30Po and 60Po treatments. This

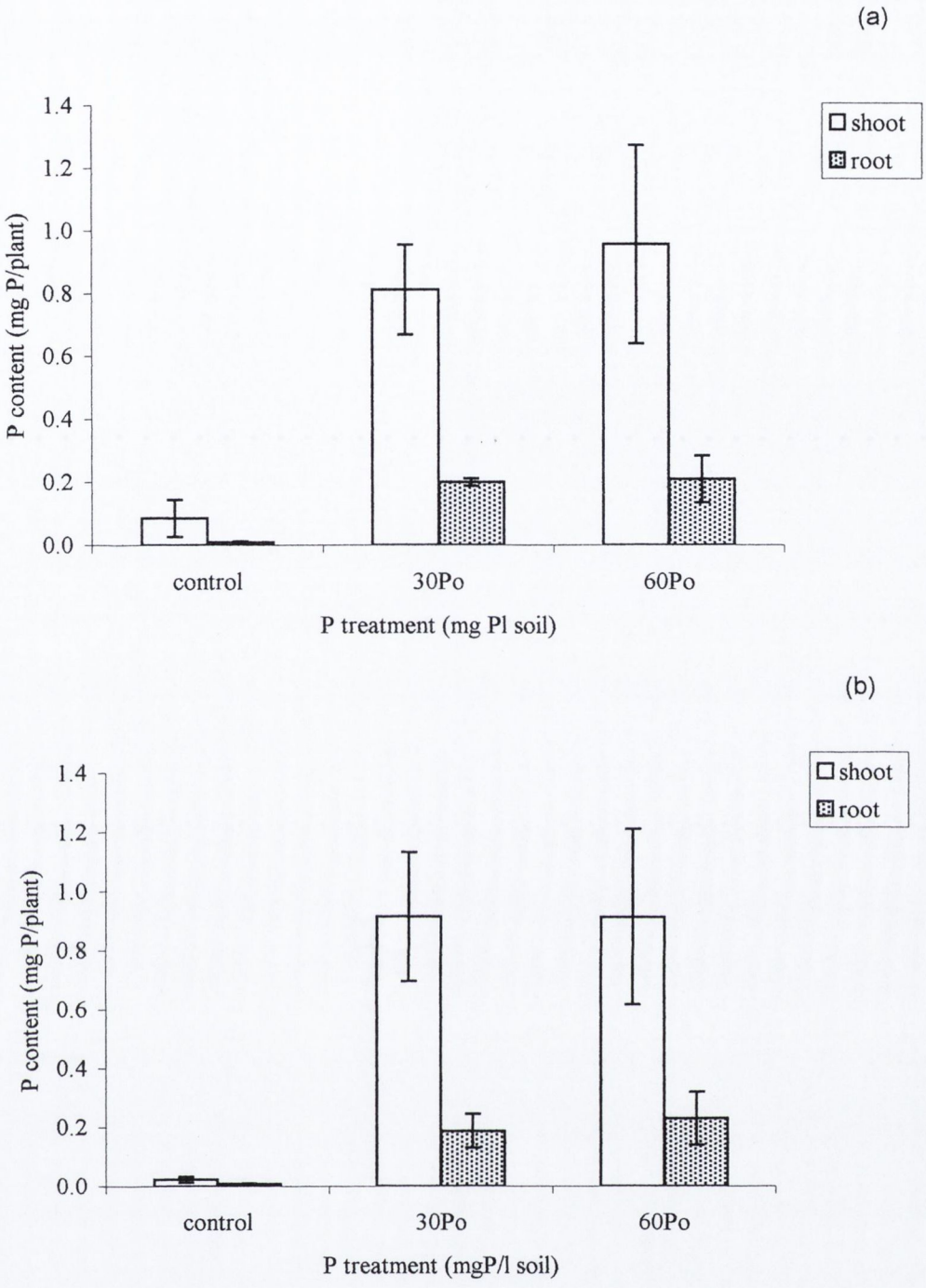


Fig 4.7: shoot and root P content for the control and P treatment plants of *Agrostis stolonifera* for plants grown in (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

increase was the same for both the 30Po and 60Po treatment. However this affect of P treatment on shoot P content was not significant (Table 4.12).

4.3.1.6 Soil phosphate

The soil total P (Tp), inorganic P (Pi) and organic P (Po) concentration data were presented in Fig 4.8 (a, b), 4.9 (a, b) and 4.10 (a, b) respectively. Three way ANOVAs were carried out to determine the affect of P treatment, benlate treatment and location of soil on soil Tp, Pi and Po concentration. These ANOVAs were presented in Appendix 4, Tables 11, 12 and 13

There was a significant P affect on soil total phosphate (F ratio 3.36*). All other treatments and interactions were not significant.

Soil Total P

Fig 4.8 (a) presented soil Tp concentration data for soil untreated with benlate. Fig 4.8 (b) presented soil Tp concentration data for soil treated with benlate.

From Fig 4.8 (a, b) there was an expected increase in start soil Tp levels with increasing levels of Po added to the soil. A pooled t test (at $p = 0.05$) was carried out to determine if there was a significant difference between the start soil and post harvest soil Tp concentrations. There was a statistically significant decrease in the Tp concentrations from those present in the start soils to the post harvest Tp concentration for the control and two P treatment soils (Fig 4.8 a, b). This decrease was in the inner and outer soils.

From Fig 4.8 (a, b) for the post harvest soil, there was an increase in total P concentration in the soil with increasing levels of P addition for both the soil treated and untreated with benlate. This significant affect of P treatment on Tp concentration was examined using a Scheffe post hoc test (Table 4.9).

Table 4.9: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Tp concentration for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 60Po	*
control - 30Po	ns
30Po - 60Po	ns

Soil Inorganic P

Fig 4.9 (a) presented soil Pi concentration data for soil untreated with benlate. Fig 4.9 (b) presented soil Pi concentration data for soil treated with benlate.

From Fig 4.9 (a, b) there was no change in start soil Pi concentration with increasing levels of Po added to the soil. This was as expected as no Pi was added to any of the soils. A pooled t test

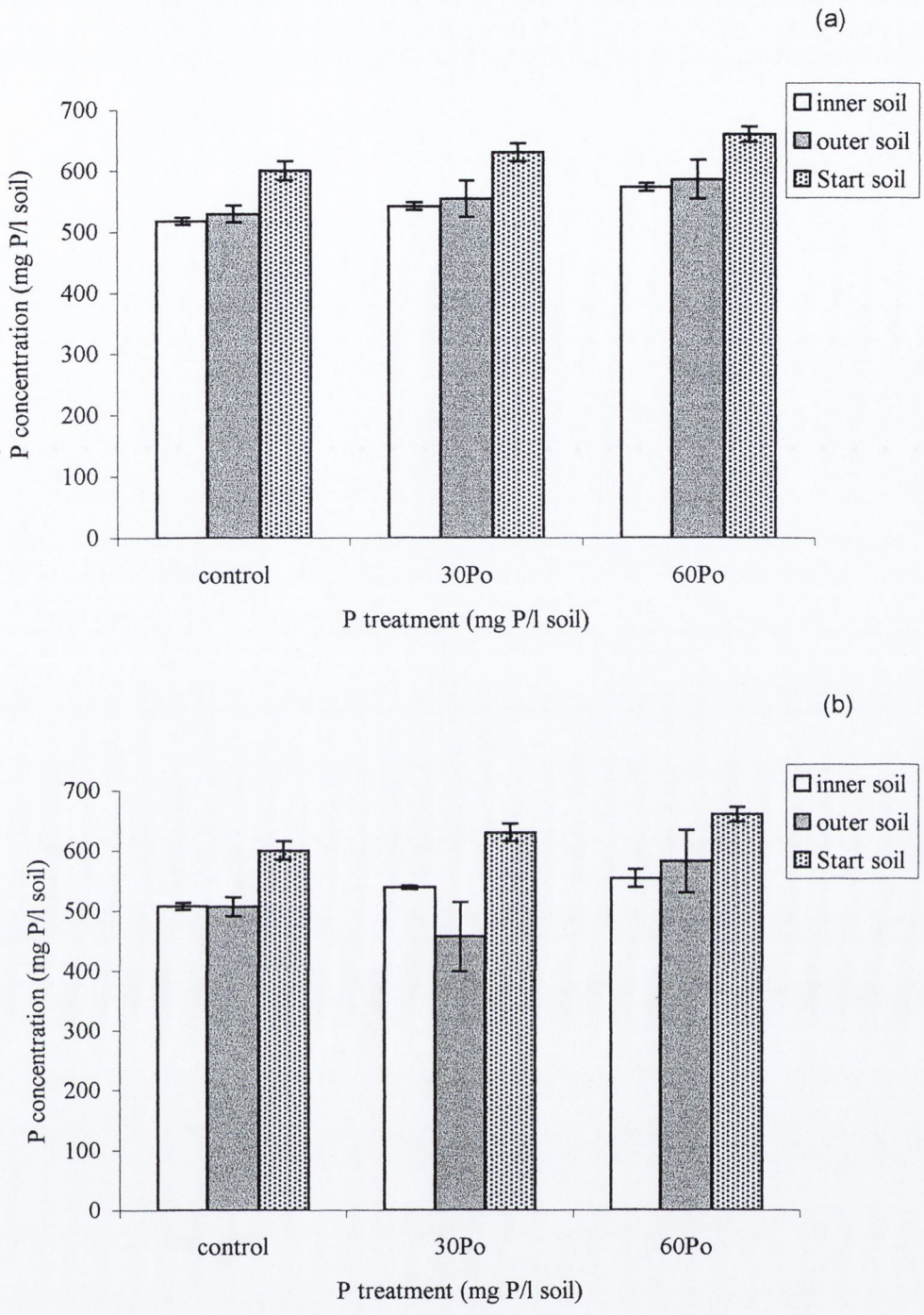


Fig 4.8: soil total phosphate concentration for the control and P treatment soils of the *Agrostis stolonifera* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

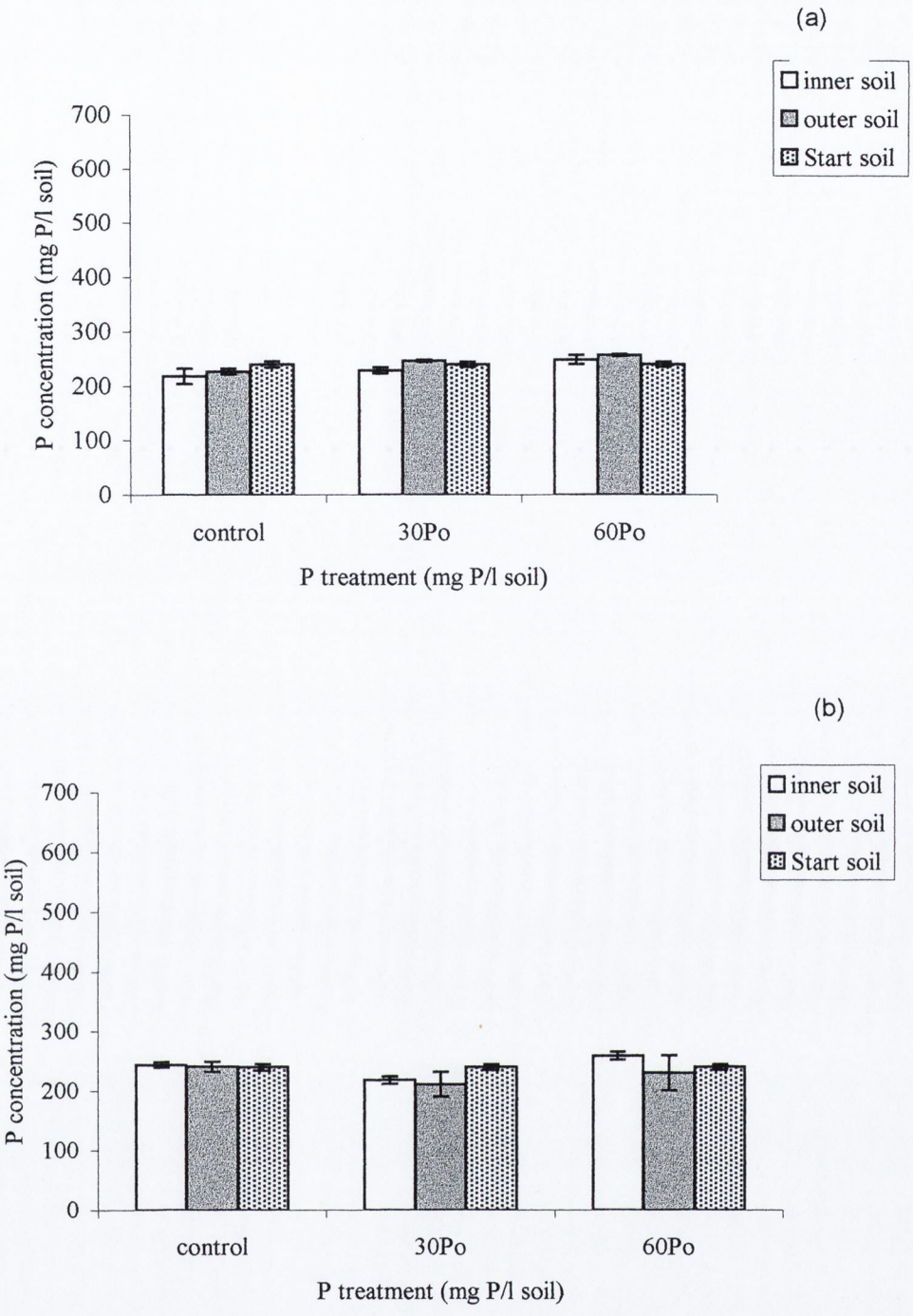


Fig 4.9: soil inorganic phosphate concentration for the control and P treatment soils of the *Agrostis stolonifera* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

(at p = 0.05) was carried out to determine if there is a significant difference between the start soil and post harvest soil Pi concentrations. There was no decrease in the Pi concentration from those present at the start of the experiment to the post harvest Pi concentration for the control and treatment soils, for the inner or outer soils (Fig 4.9 a, b).

From Fig 4.9 (a, b) for the post harvest soil there was no change in the Pi concentration in the soil with increasing levels of P addition. There was no statistically significant affect of P treatment on soil Pi concentration in the postharvest soil (Table 4.14).

Soil Organic P

Fig 4.10 (a) presented soil Po concentration data for soil untreated with benlate. Fig 4.10 (b) presented soil Po concentration data for soil treated with benlate.

From Fig 4.10 (a, b) there was an increase in start soil Po concentration with increasing levels of Po added to the soil. A pooled t test (at p = 0.05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Po concentration. There was a decrease in the Po concentrations from those present at the start of the experiment to the post harvest Po concentrations for the control and treatment soils (Fig 4.10 a, b). This decrease was in the inner and outer soils.

From Fig 4.10 (a, b) for the post harvest soil there was an increase in Po concentration in the soil with increasing levels of P addition. This increase was most noticeable in the in the soil treated with benlate. This affect of P treatment on Po concentration was not statistically significant (Table 4.14).

4.3.2 *Holcus lanatus*

4.3.2.1 Soil phosphatase activity

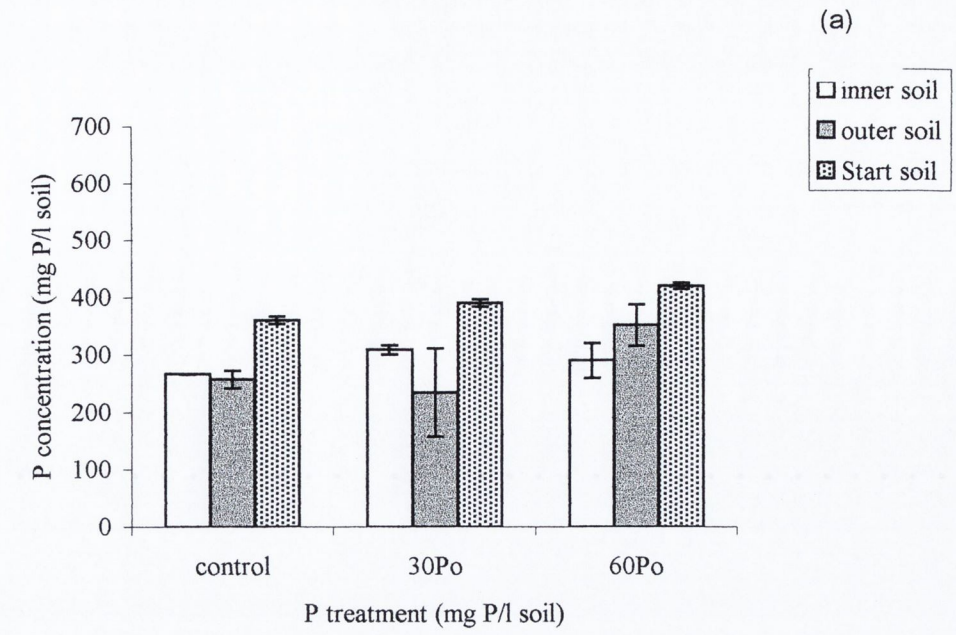
The soil phosphatase activity results were presented as follows:

soil acidic monoesterase data in Fig 4.11 (a,b), soil alkaline monoesterase data in Fig 4.12 (a,b), soil diesterase data in Fig 4.13 (a,b). Three way ANOVAs were carried out to determine the affect of P treatment, benlate treatment and location of soil on these soil phosphatase activities. These ANOVAs were presented in Appendix 4, Tables 14, 15 and 16.

There was a significant location of soil affect on the soil acidic monoesterase activity (F ratio 9.85**) and the soil alkaline monoesterase activity (F ratio 6.75**). There was also a significant P -benlate interaction for soil acidic monoesterase activity (F ratio 4.06*). All other treatments and interactions were not significant.

Soil acidic monoesterase activity

Figure 4.11 (a) presented soil acidic monoesterase activity data for soil not treated with benlate. From Fig 4.11 (a), the monoesterase activity of the inner soil was higher than that detected in



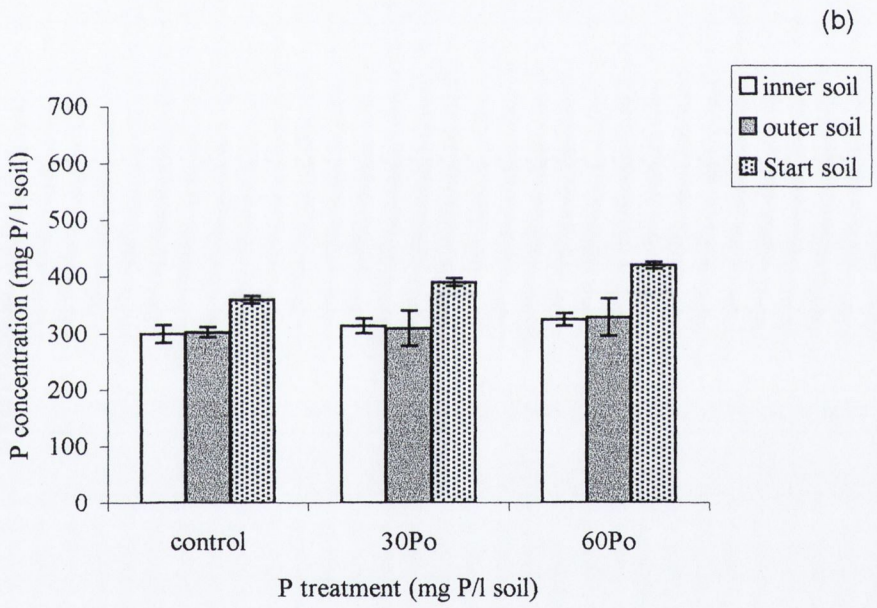


Fig 4.10: soil organic phosphate concentration for the control and P treatment soils of the *Agrostis stolonifera* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

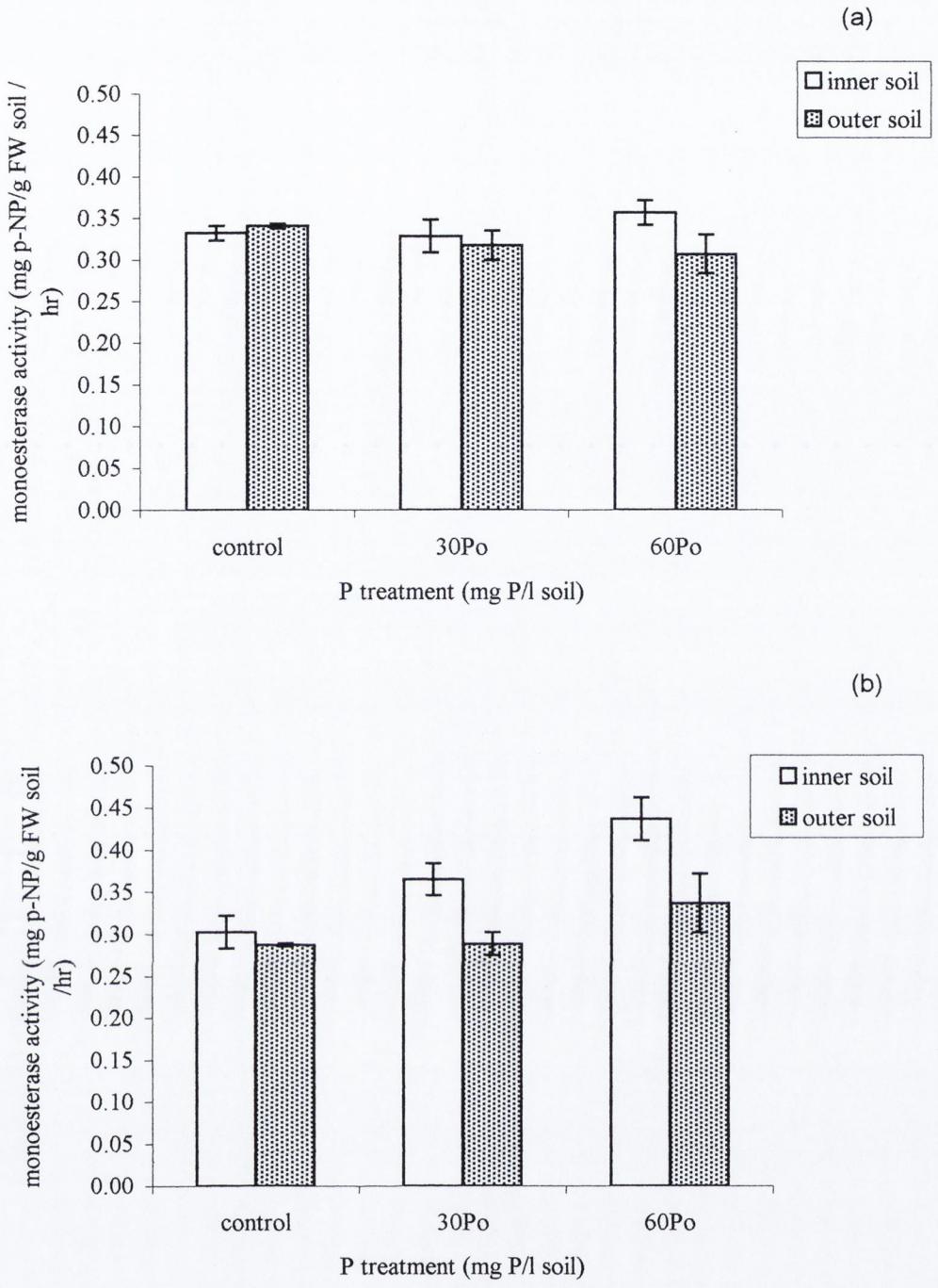


Fig 4.11: soil acidic monoesterase activity for the control and P treatment soils for the *Holcus lanatus* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

the outer soil for the 30Po and 60Po treatment soils. Figure 4.11 (b) presented soil acidic monoesterase activity data for soil treated with benlate. From Fig 4.11 (b) the monoesterase activity of the inner soil was again higher than that of the outer soil for the 30Po and 60Po treatment soils. This difference in the monoesterase activity of the inner and outer soil was significant (Appendix 4, Table 4.14).

The significant interaction P treatment and benlate treatment was examined using a Scheffe post hoc test (Table 4.10).

Table 4.10: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected soil acidic monoesterase activity for *Holcus lanatus* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison			P value
-B, control	-	+B, control	ns
-B, 30Po	-	+B, 30Po	ns
-B, 60Po	-	+B, 60Po	ns
-B, control	-	-B, 30Po	ns
-B, control	-	-B, 60Po	ns
+B, control	-	+B, 30Po	ns
+B, control	<	+B, 60Po	**

There was no statistically significant affect of P treatment on the monoesterase activity in the soils untreated with benlate. There was however a statistically significant affect of the 60Po treatment on the monoesterase activity in the soil treated with benlate. (Tale 4.10).

Soil Alkaline Monoesterase Activity

Fig 4.12 (a) presented soil alkaline monoesterase activity for soil not treated with benlate. In the case of the control and 60Po treatment soils the alkaline monoesterase activity of the inner compartment soil was higher than that of the outer compartment soil. Fig 4.12 (b) presented soil alkaline monoesterase activity data for soil treated with benlate. From Fig 4.12 (b) the monoesterase activity of the inner soil was greater than that of the outer soil for both control and treatment soils. This difference in the monoesterase activity of the inner and outer soil was statistically significant (Appendix 4, Table 4.15).

Soil Diesterase Activity

Fig 4.13 (a) presented soil diesterase activity data from soil not treated with benlate. The activity of the inner soil was slightly greater than that of the outer soil for the 30Po and 60Po treatment soil. Fig 4.13 (b) presented soil diesterase activity from soil treated with benlate. The activity of the inner soil, was equal to that of the outer soil for the control and 60Po treatment, and was greater than that of the outer soil for the 30Po treatment. There was no statistically significant difference in the diesterase activity of the inner and outer soil (Appendix 4, Table 4.16).

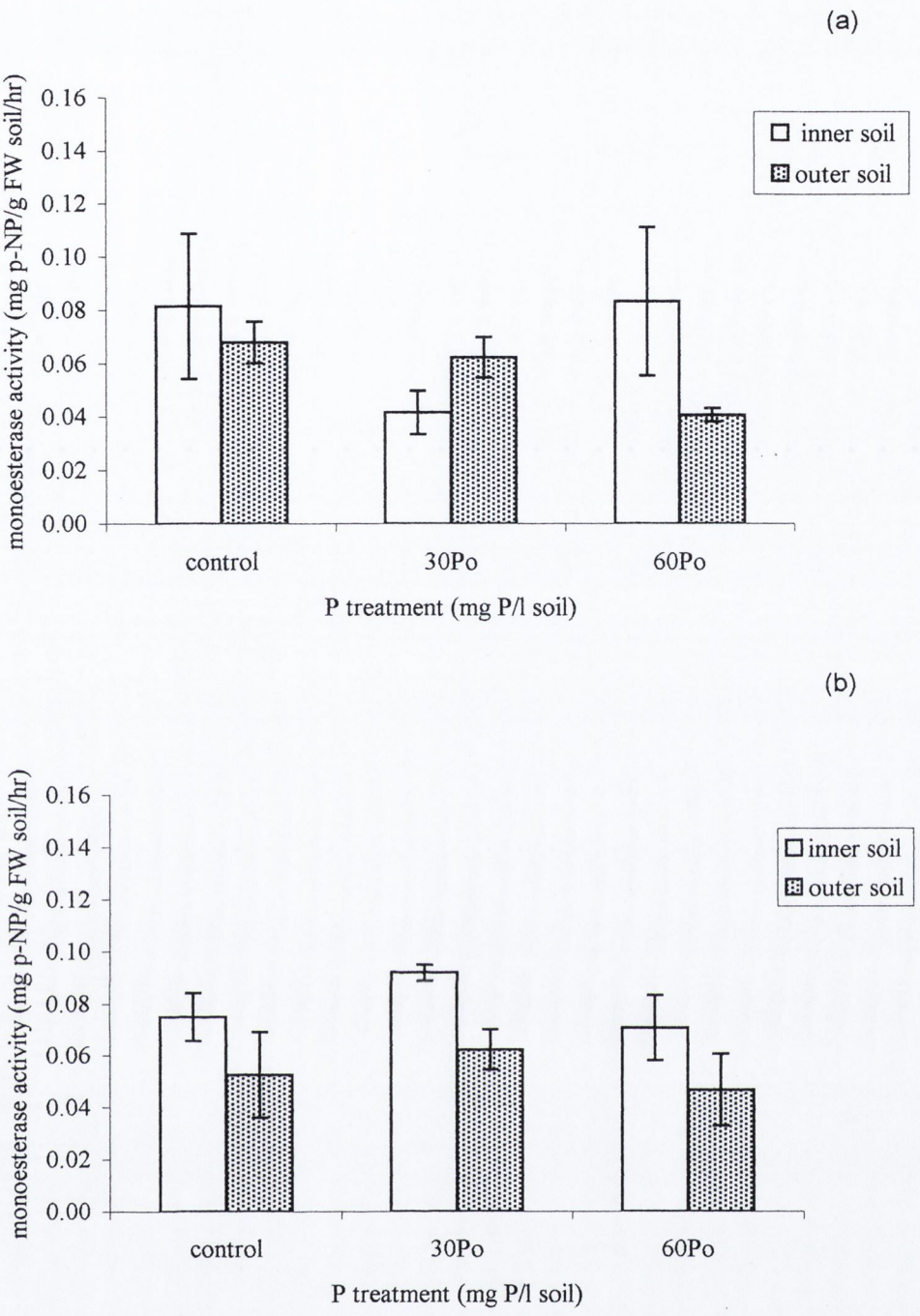


Fig 4.12: soil alkaline monoesterase activity for the control and P treatment soils for the *Holcus lanatus* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

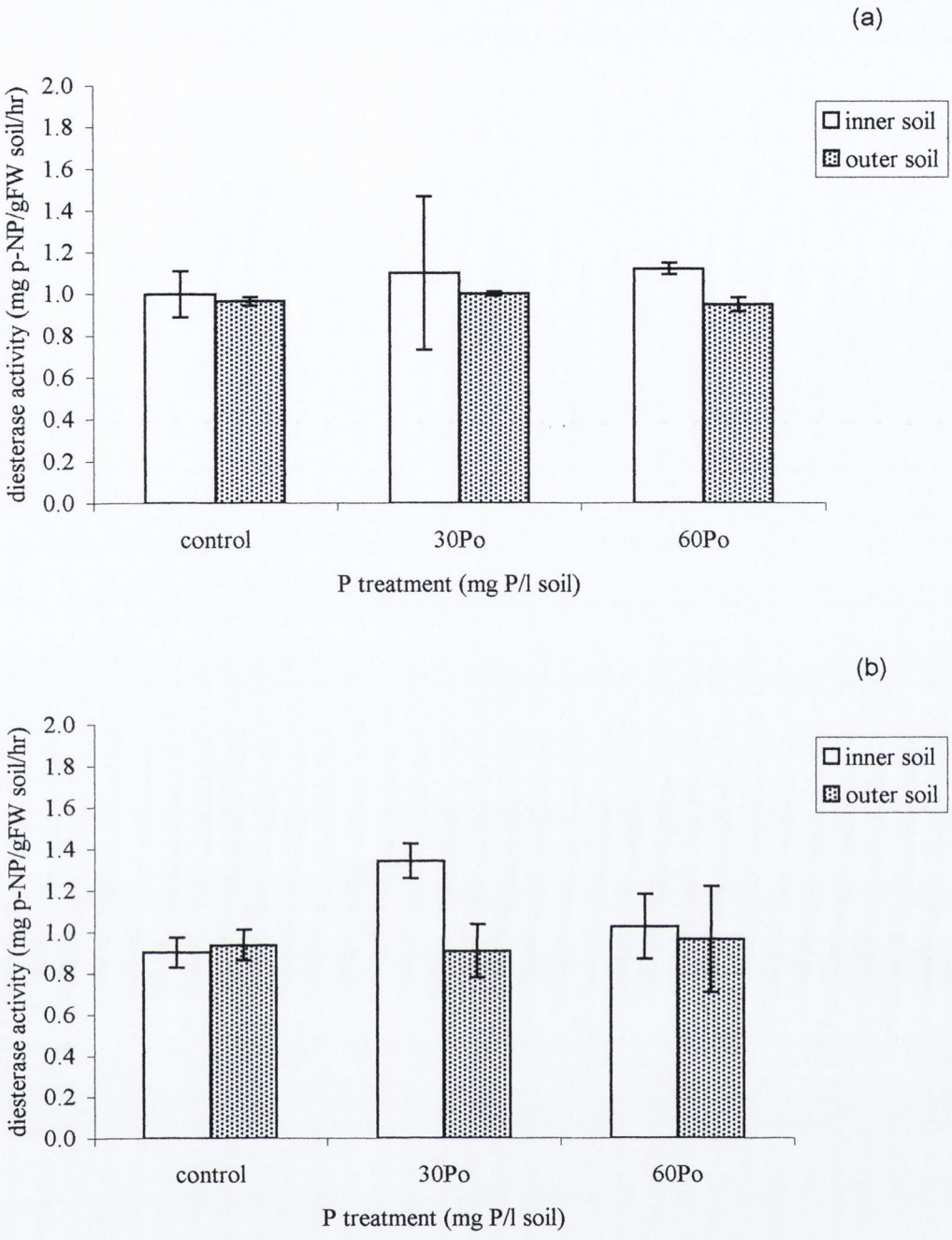


Fig 4.13: soil diesterase activity for the control and P treatment soils for the *Holcus lanatus* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

4.3.2.2 Root acidic monoesterase activity

The root acidic monoesterase activity results were presented in Fig 4.14 (a, b). A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on root acidic phosphomonoesterase activity. This ANOVA was presented in Appendix 4, Table 17.

There was a significant P affect on root acidic monoesterase activity (F ratio 6.33**). All other treatments and interactions were not significant.

Fig 4.14 (a) presented root acidic monoesterase activity from soil not treated with benlate. There was a decrease in the root monoesterase activity with increasing levels of organic P added to the soil (Fig 4.14, a). Fig 4.14 (b) presented root acidic monoesterase activity from soil treated with benlate. Again there was a slight decrease in the monoesterase activity levels with increasing levels of organic P added to the soil (Fig 4.14, b). This significant affect of P treatment on root acidic monoesterase activity was examined using a Scheffe post hoc test (Table 4.11).

Table 4.11: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root acidic monoesterase activity for the *Holcus lanatus* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control > 60Po	**
control - 30Po	ns
30Po - 60Po	ns

4.3.2.3 Shoot and Root Dry Weights

The shoot and root dry weight data were presented in Fig 4.15 (a,b). Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot and root dry weights. These ANOVAs were presented in Appendix 4, Tables 18 and 19.

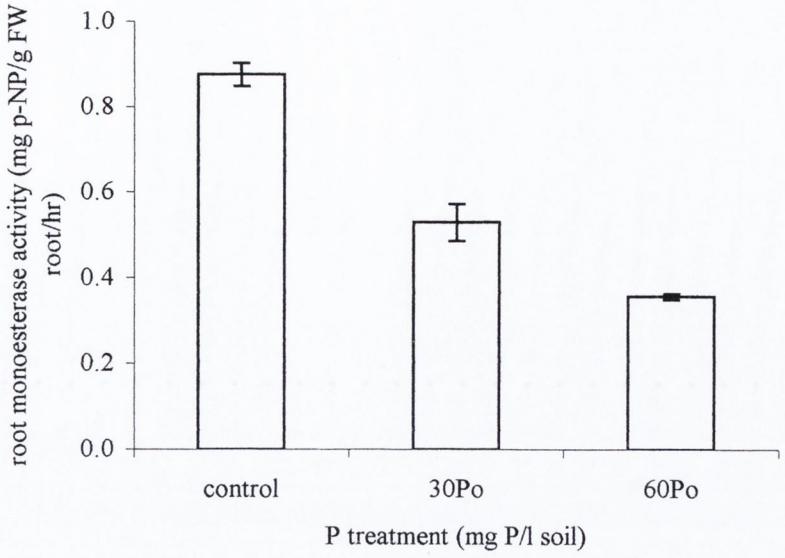
There was a significant P affect on shoot (F ratio 62.07***) and root (F ratio 20.17***) dry weight. There was also a significant P - benlate interaction that had an affect on shoot dry weight (F ratio 4.58*).

The shoot : root dry weight ratio for *Holcus lanatus* at each combination of P treatment and benlate treatment were presented in Table 4.12.

Table 4.12: shoot : root dry weight ratios for *Holcus lanatus* at each combination of P treatment and benlate treatment

	Shoot : Root ratio	
P treatment	**-Benlate**	**+Benlate**
control	3.2	7.6
30Po	1.0	1.0
60Po	0.7	1.0

(a)



(b)

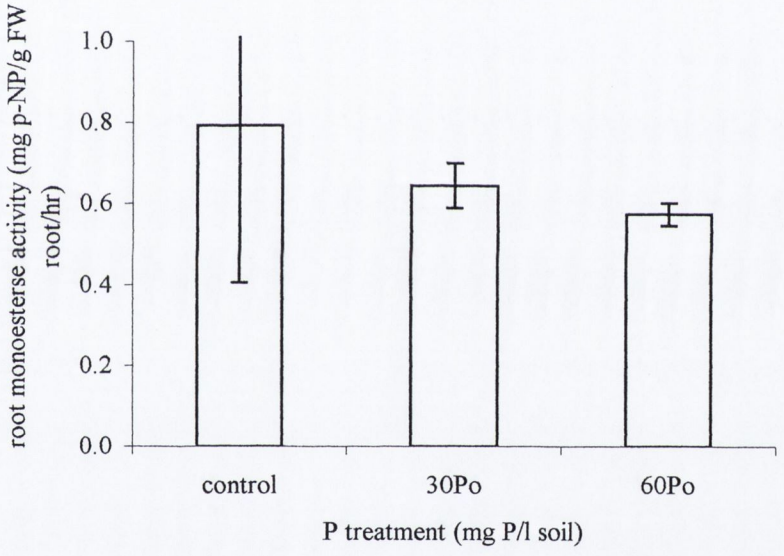


Fig 4.14: Root acidic monoesterase activity for the control and P treatment plants for the *Holcus lanatus* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

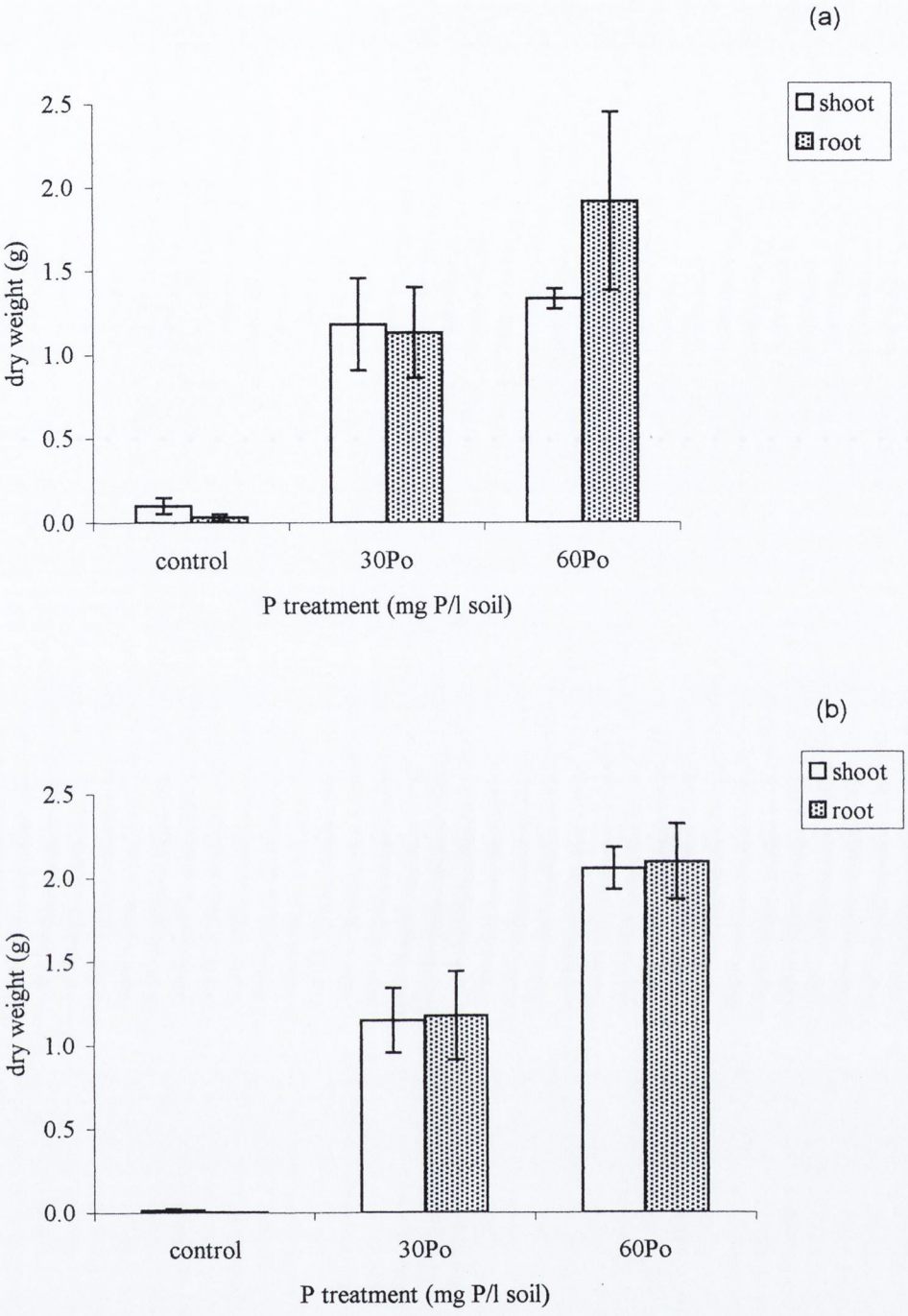


Fig 4.15: shoot and root dry weights for the control and P treatment plants of *Holcus lanatus* for plants grown in (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

From Table 4.12 the shoot : root ratio (r:s ratio) showed a decrease with increasing concentrations of Po in the soil, in soil untreated with benlate. In soil treated with benlate, the addition of Po to the soil resulted in a decrease in r:s ratio (Table 4.12).

Shoot dry weight

Fig 4.15 (a) presented shoot dry weight data for plants grown in soil untreated with benlate. Fig 4.15 (b) presented shoot dry weight data for plants grown in soil treated with benlate. From Fig 4.15 (a, b) there was an increase in shoot dry weight from that of the control plants to that of the 30Po treatment plants with a further slight increase to that of the 60Po treatment plants. This significant affect of P treatment on shoot dry weight was examined using a Scheffe post hoc test (Table 4.13).

Table 4.13: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot dry weight for the *Holcus lanatus* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	*
control < 60Po	***
30Po < 60Po	*

The significant interaction between P treatment and benlate treatment was examined using a Scheffe post hoc test (Table 4.14).

Table 4.14: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected shoot dry weight for *Holcus lanatus* experiment.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant)

Comparison	P value
-B, control - +B, control	ns
-B, 30Po - +B, 30Po	ns
-B, 60Po < +B, 60Po	*
-B, control < -B, 30Po	***
-B, control < -B, 60Po	***
+B, control < +B, 30Po	***
+B, control < +B, 60Po	***

In soil untreated with benlate the P treatments – 30Po and 60Po, resulted in significant increases in shoot dry weight. This was also true for soil treated with benlate (Table 4.14; Fig 4.15 a, b).

Root dry weight

Fig 4.15 (a) presented root dry weight data for plants grown in soil untreated with benlate. Fig 4.15 (b) presented root dry weight data for plants grown in soil treated with benlate. From Fig 4.15 (a, b) there was an increase in root dry weight with the increasing addition of Po to the soil.

This significant affect of P treatment on root dry weight was examined using a Scheffe post hoc test (Table 4.15).

Table 4.15: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root dry weight for the *Holcus lanatus* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	*
control < 60Po	***
30Po < 60Po	*

4.3.2.4 Shoot and Root P Concentration

The shoot and root P concentration data were presented in Fig 4.16 (a, b). Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot and root P concentrations. These ANOVAs were presented in Appendix 4, Tables 20 and 21.

There was a significant P affect on shoot (F ratio 11.42*)and root (F ratio 7.41*) P concentration. All other treatments and interactions were not significant.

Shoot P concentration

Fig 4.16 (a) presented shoot P concentration data for plants grown in soil untreated with benlate. Fig 4.16 (b) presented shoot P concentration data for plants grown in soil treated with benlate. From Fig 4.16 (a, b) there was an increase in shoot P concentration with increasing concentrations of Po added to the soil. This significant affect of P treatment on shoot P concentration was examined using a Scheffe post hoc test (Table 4.16).

Table 4.16: probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P concentration for the *Holcus lanatus* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	**
control < 60Po	***
30Po - 60Po	ns

Root P concentration

Fig 4.16 (a) presented root P concentration data for plants grown in soil untreated with benlate. Fig 4.16 (b) presented root P concentration data for plants grown in soil treated with benlate. From Fig 4.16 (a, b) there was an increase in root P concentration for the 30Po and 60Po treatments. This significant affect of P treatment on root P concentration was examined using a Scheffe post hoc test (Table 4.17).

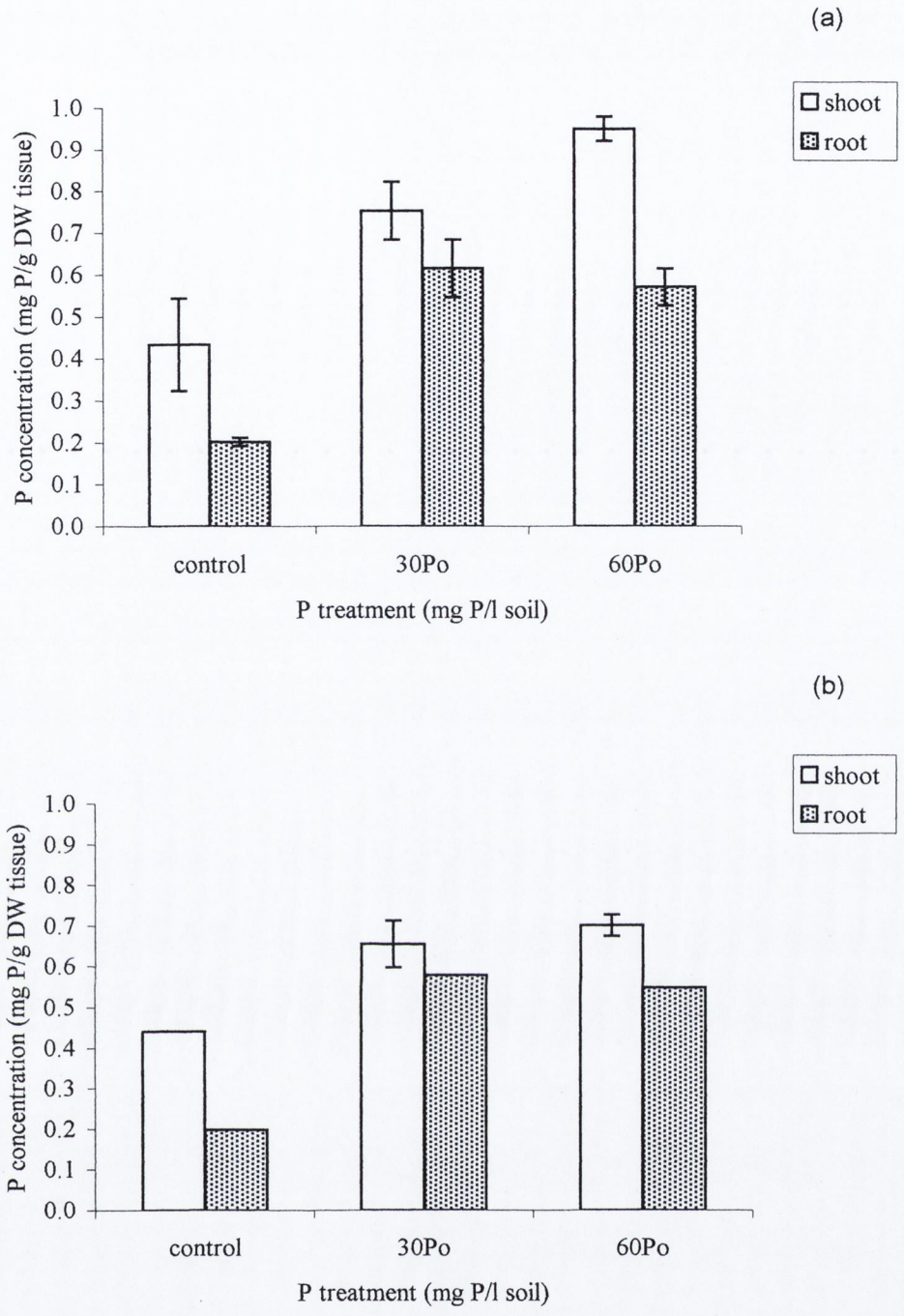


Fig 4.16: shoot and root P concentration for the control and P treatment plants of *Holcus lanatus* for plants grown in (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

Table 4.17: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root P concentration for the *Holcus lanatus* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	*
control < 60Po	***
30Po - 60Po	ns

4.3.2.5 Shoot and Root P Content

The shoot and root P content data were presented in Fig 4.17 (a, b). Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot and root P content. These ANOVAs were presented in Appendix 4, Tables 22 and 23.

There was a highly significant P affect on shoot (F ratio 217.15***) and root (F ratio 13.66***) P content. All other treatments and interactions were not significant.

Shoot P content

Fig 4.17 (a) presented shoot P content data for plants grown in soil untreated with benlate. Fig 41.7 (b) presented shoot P content data for plants grown in soil treated with benlate. From Fig 4.17 (a, b) there was an increase in shoot P content a result of the 30Po and 60Po treatments. This significant affect of P treatment on shoot P content was examined using a Scheffe post hoc test (Table 4.18).

Table 4.18: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P content for the *Holcus lanatus* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	***
control < 60Po	***
30Po < 60Po	***

Root P content

Fig 4.17 (a) presented root P content data for plants grown in soil untreated with benlate. Fig 41.7 (b) presented root P content data for plants grown in soil treated with benlate. From Fig 4.17 (a, b) there was an increase in root P content with increasing concentrations of Po added to the soil. This significant affect of P treatment on root P content was examined using a Scheffe post hoc test (Table 4.19).

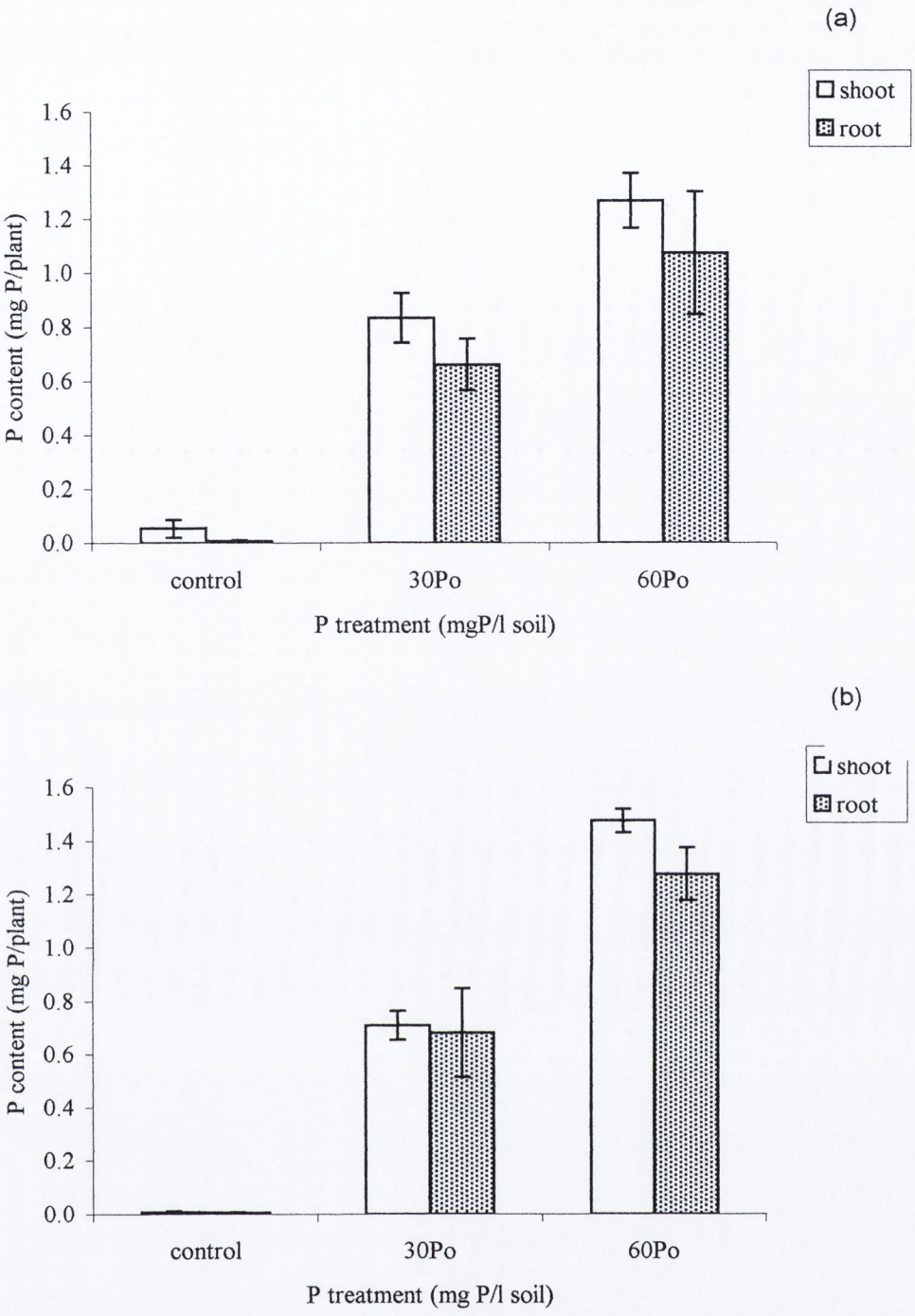


Fig 4.17: shoot and root P content for the control and P treatment plants of *Holcus lanatus* for plants grown in (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

Table 4.19: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root P content for the *Holcus lanatus* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	*
control < 60Po	**
30Po < 60Po	*

4.3.2.6 Soil phosphate

The soil total P (Tp), inorganic P (Pi) and organic P (Po) concentration data were presented in Fig 4.18 (a, b), 4.19 (a, b) and 4.20 (a, b) respectively. Three way ANOVAs were carried out to determine the affect of P treatment, benlate treatment and location of soil on soil Tp, Pi and Po concentration. These ANOVAs were presented in Appendix 4, Tables 24, 25 and 26.

There was a significant P affect on soil organic P concentration (F ratio 3.22*). All other treatments and interactions were not significant.

Soil Total P

Fig 4.18 (a) presented soil Tp concentration data for soil untreated with benlate. Fig 4.18 (b) presented soil Tp concentration data for soil treated with benlate.

From Fig 4.18 (a, b) there was an expected increase in start soil Tp levels with increasing levels of Po added to the soil. A pooled t test (at $p = 0.05$) was carried out to determine if there was a significant difference between the start soil and post harvest soil Tp concentrations. There was a significant decrease in the Tp concentrations from those present in the start soils to the post harvest Tp concentration for the control and two P treatment soils (Fig 4.8 a, b). This decrease was in the inner and outer soils

From Fig 4.18 (a, b) for the post harvest soil there was an increase in total P concentration in the soil with increasing levels of P addition for both the soil treated and untreated with benlate. This affect of P treatment on Tp concentration was not significant (Table 4.31).

Soil Inorganic P

Fig 4.19 (a) presented soil Pi concentration data for soil untreated with benlate. Fig 4.19 (b) presented soil Pi concentration data for soil treated with benlate.

From Fig 4.19 (a, b) there was no change in start soil Pi concentration with increasing levels of Po added to the soil. This was as expected as no Pi was added to any of the soils. A pooled t test (at $p = 0.05$) was carried out to determine if there is a significant difference between the start soil and post harvest soil Pi concentrations. There was no difference in the Pi concentration for the control and treatment soils, for the inner or outer soils (Fig 4.19 a, b).

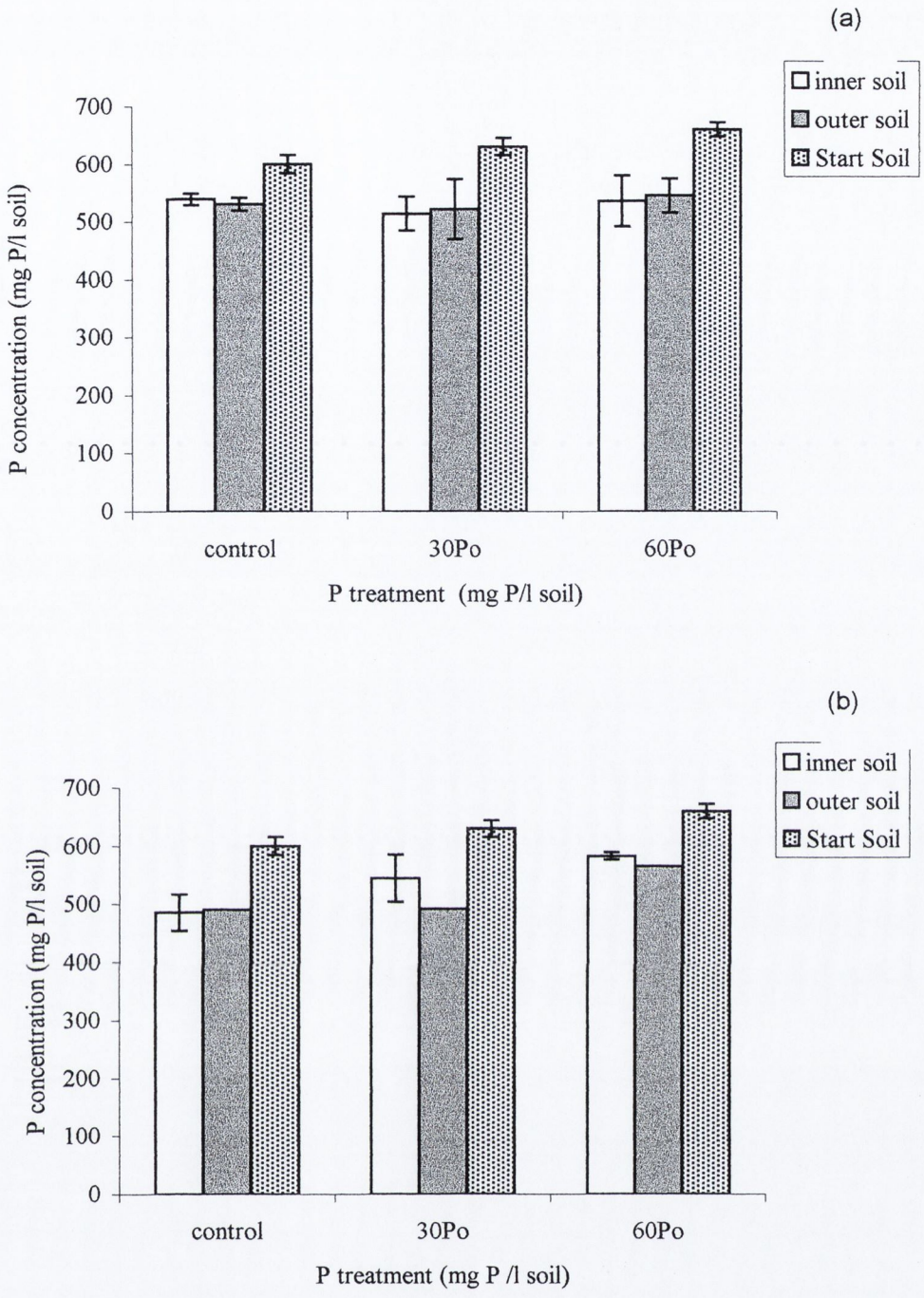


Fig 4.18: soil total phosphate concentration for the control and P treatment soils of the *Holcus lanatus* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

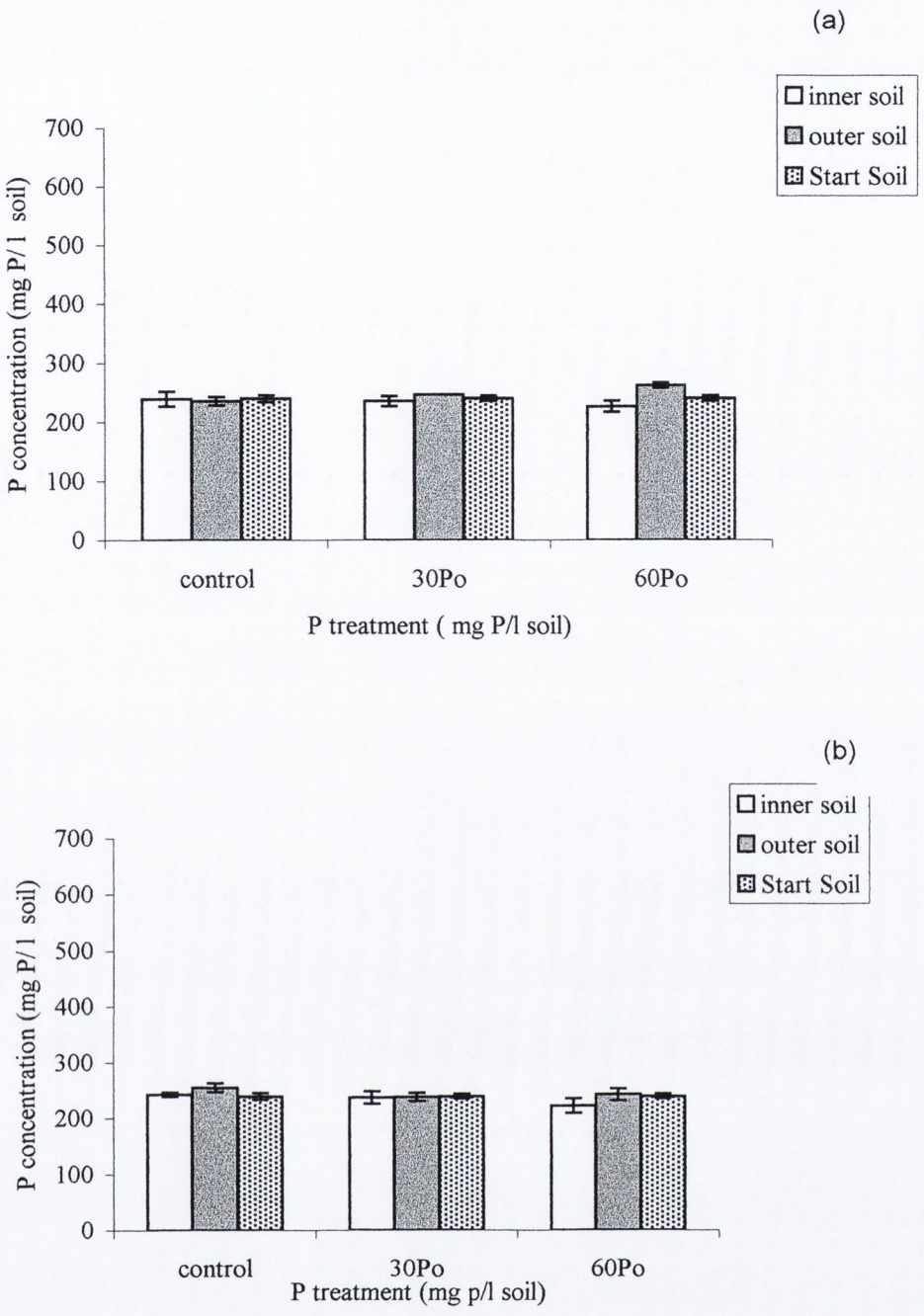


Fig 4.19: soil inorganic phosphate concentration for the control and P treatment soils of the *Holcus lanatus* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

From Fig 4.19 (a, b) for the post harvest soil there was no change in the Pi concentration in the soil with increasing levels of P addition. There was no significant affect of P treatment on soil Pi concentration in the postharvest soil (Table 4.31).

Soil Organic P

Fig 4.20 (a) presented soil Po concentration data for soil untreated with benlate. Fig 4.20 (b) presented soil Po concentration data for soil treated with benlate.

From Fig 4.20 (a, b) there was an increase in start soil Po concentration with increasing levels of Po added to the soil. A pooled t test (at $p = 0.05$) was carried out to determine if there was a significant difference between the start soil and post harvest soil Po concentration. There was a decrease in the Po concentrations from those present at the start of the experiment to the post harvest Po concentrations for the control and treatment soils (Fig 4.20 a, b). This decrease was in the inner and outer soils.

From Fig 4.20 (a, b) for the post harvest soil there was an increase in Po concentration in the soil with increasing levels of P addition. This significant affect of P treatment on Po concentration was examined using a Scheffe post hoc test (Table 4.20).

Table 4.20: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Po concentration for the *Holcus lanatus* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 60Po	*
control - 30Po	ns
30Po - 60Po	ns

4.3.3 *Dactylis glomerata*

4.3.3.1 Soil phosphatase activity

The soil phosphatase activity results were presented as follows:
soil acidic monoesterase data in Fig 4.21 (a,b), soil alkaline monoesterase data in Fig 4.22 (a,b), soil diesterase data in Fig 4.23 (a,b). Three way ANOVA were carried out to determine the affect of P treatment, benlate treatment and location of soil on these soil phosphatase activities. These ANOVAs were presented in Appendix 4, Tables 27, 28 and 29.

There was a significant location of the soil affect on the soil acidic (F ratio 4.79*) and alkaline (F ratio 21.82***) monoesterase activity and the soil diesterase (F ratio 4.30*) activity. There was a significant P - benlate interaction for soil acidic (F ratio 9.73***) monoesterase activity and soil diesterase (F ratio 3.97*) activity. There was also a significant P - location of the soil interaction for soil alkaline (F ratio 4.18*) monoesterase activity.

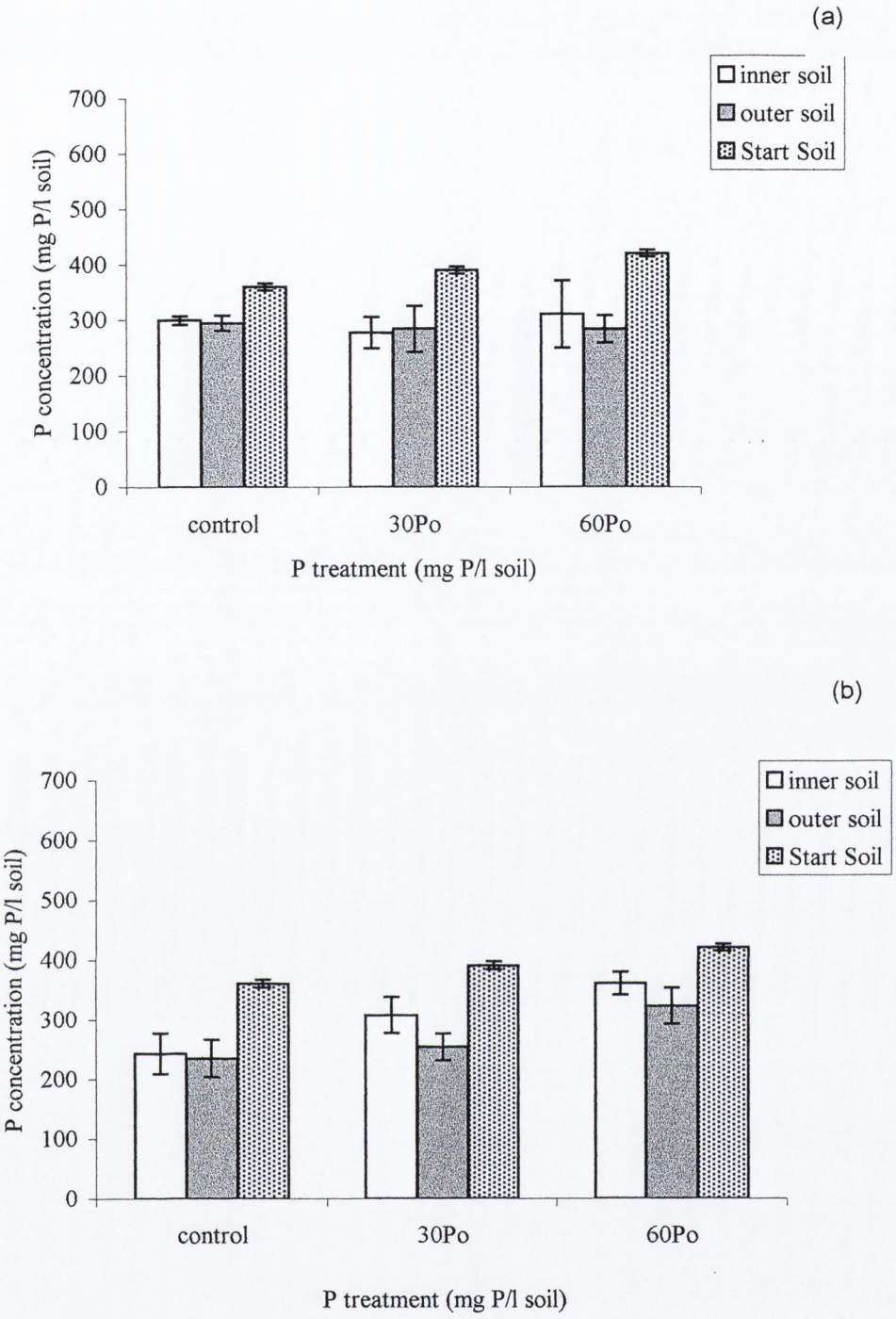


Fig 4.20: soil organic phosphate concentration for the control and P treatment soils of the *Holcus lanatus* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

Soil acidic monoesterase activity

Figure 4.21 (a) presented soil acidic monoesterase activity data for soil not treated with benlate. From Fig 4.21 (a), the monoesterase activity of the inner soil was higher than that detected in the outer soil for the control and the two P treatment soils. Figure 4.21 (b) presented soil acidic monoesterase activity data for soil treated with benlate. From Fig 4.21 (b) the monoesterase activity of the inner soil was again higher than that of the outer soil for the control and 30Po treatment soil. This difference in the monoesterase activity of the inner and outer soil was significant (Appendix 4, Table 27).

The significant interaction between P treatment and benlate treatment was examined using a Scheffe post hoc test (Table 4.21).

Table 4.21: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected soil acidic monoesterase activity for *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison			P value
-B, control	-	+B, control	ns
-B, 30Po	-	+B, 30Po	ns
-B, 60Po	<	+B, 60Po	***
-B, control	-	-B, 30Po	ns
-B, control	>	-B, 60Po	**
+B, control	-	+B, 30Po	ns
+B, control	-	+B, 60Po	ns

There was a significant increase in soil acidic monoesterase activity as a result of benlate application to the 60Po treatment soil. There was a statistically significant decrease in monoesterase activity as a result of the 60Po treatment in the soil untreated with benlate.

Soil Alkaline Monoesterase Activity

Fig 4.22 (a) presented soil alkaline monoesterase activity for soil not treated with benlate. In the case of the 30Po and 60Po treatment soils the alkaline monoesterase activity of the inner compartment soil was higher than that of the outer compartment soil. Fig 4.22 (b) presented soil alkaline monoesterase activity data for soil treated with benlate. From Fig 4.22 (b) the monoesterase activity of the inner soil was again greater than that of the outer soil for both control and treatment soils. This difference in the monoesterase activity of the inner and outer soil significant (Appendix 4, Table 4.28).

The significant interaction P treatment and location of the soil was examined using a Scheffe post hoc test, (Table 4.22).

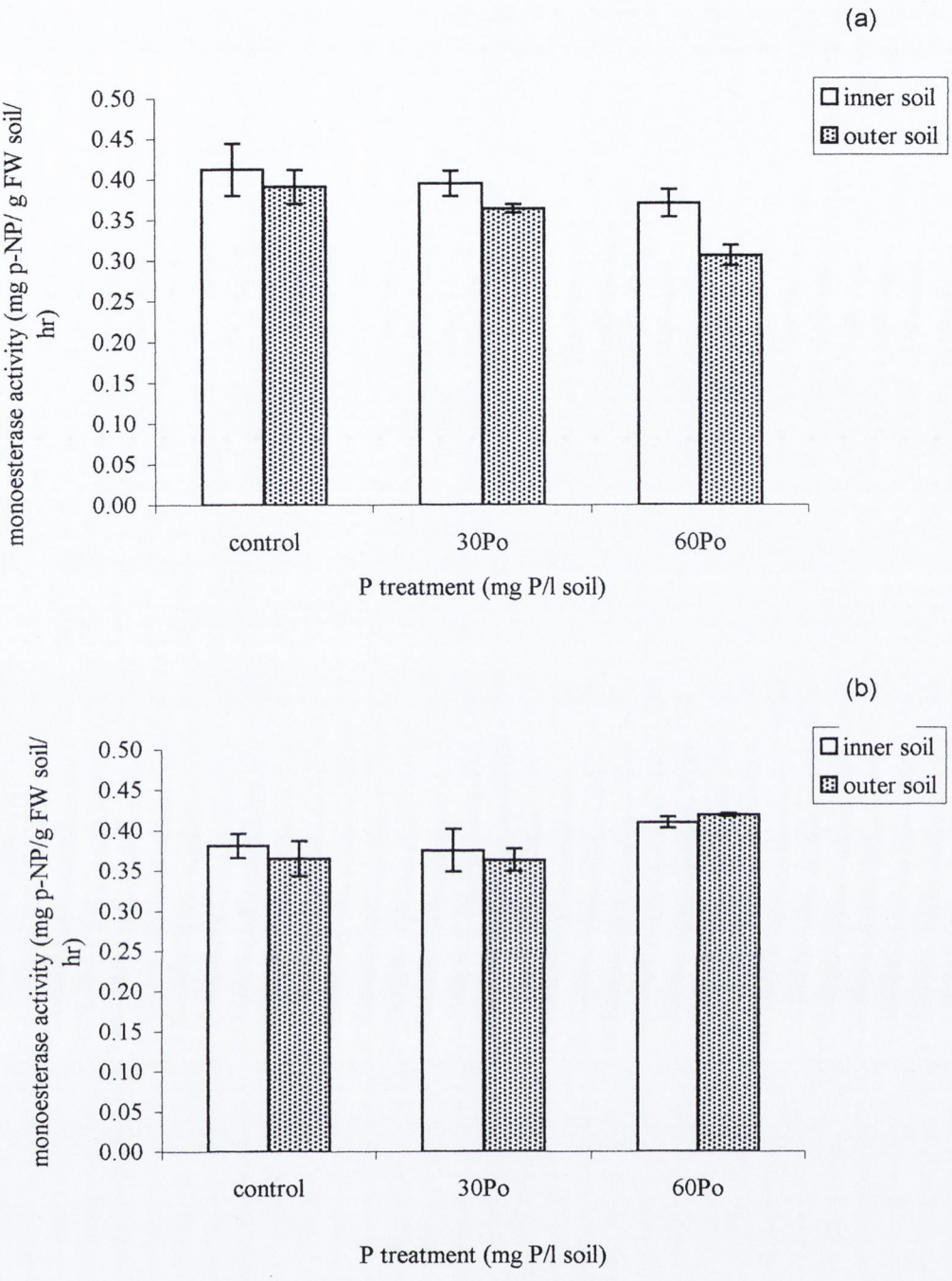


Fig 4.21: soil acidic monoesterase activity for the control and P treatment soils for the *Dactylis glomerata* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

Table 4.22: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors location of soil and P treatment, that affected soil alkaline monoesterase activity for *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison			P value
outer, control	-	outer, 30Po	ns
outer, control	-	outer, 60Po	ns
outer, 30Po	-	outer, 60Po	ns
inner, control	-	inner, 30Po	ns
inner, control	-	inner, 60Po	ns
inner, 60Po	-	inner, 30Po	ns
outer, control	-	inner, control	ns
outer, 30Po	<	inner, 30Po	**
outer, 60Po	<	inner, 60Po	***

However the soil alkaline monoesterase activity of the inner soil is higher than that of the outer soil for both the 30Po and 60Po treatments (Fig 4.22, a, b; Table 4.22).

Soil Diesterase Activity

Fig 4.23 (a) presented soil diesterase activity data from soil not treated with benlate. The activity of the inner soil was slightly greater than that of the outer soil for the 30Po and 60Po treatment soil. Fig 4.23 (b) presented soil diesterase activity from soil treated with benlate. The activity of the inner soil, was equal to that of the outer soil for the control and 60Po treatment, and was greater than that of the outer soil for the 30Po treatment. This difference in the diesterase activity of the inner and outer soil was statistically significant (Appendix 4, Table 4.29).

The significant interaction between P treatment and benlate treatment was examined using a Scheffe post hoc test (Table 4.23).

Table 4.23: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected soil diesterase activity for *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison			P value
-B, control	-	+B, control	ns
-B, 30Po	-	+B, 30Po	ns
-B, 60Po	-	+B, 60Po	ns
-B, control	-	-B, 30Po	ns
-B, control	-	-B, 60Po	ns
+B, control	-	+B, 30Po	ns
+B, control	-	+B, 60Po	ns

The post hoc test shows none of the P – benlate comparisons to be significant (Table 4.23).

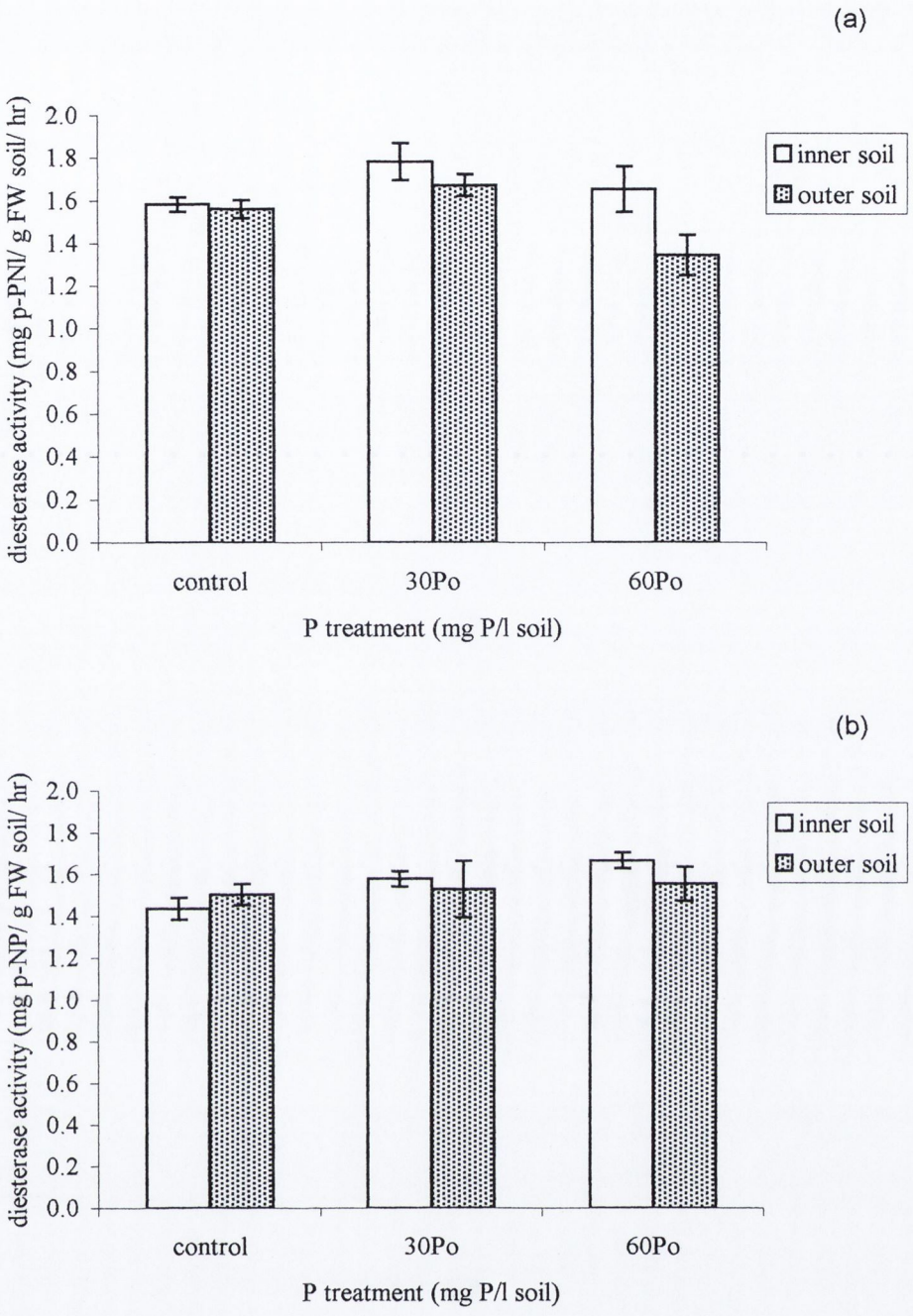


Fig 4.23: soil diesterase activity for the control and P treatment soils for the *Dactylis glomerata* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

4.3.3.2 Root acidic monoesterase activity

The root acidic monoesterase activity results were presented in Fig 4.24 (a, b). A two way ANOVA was carried out to determine the affect of P treatment and benlate treatment on root acidic phosphomonoesterase activity. This ANOVA was presented in Appendix 4, Table 30.

There was a significant P affect (F ratio 109.92***) on root acidic monoesterase activity. All other treatments and interactions were not significant.

Fig 4.24 (a) presented root acidic monoesterase activity from soil not treated with benlate. There was a decrease in the root monoesterase activity with increasing levels of organic P added to the soil (Fig 4.24, a). Fig 4.24 (b) presented root acidic monoesterase activity from soil treated with benlate. Again there was a decrease in the monoesterase activity levels with increasing levels of organic P added to the soil (Fig 4.24, b). The addition of organic phosphate to the soil significantly decreased the root monoesterase activity (Appendix 4, Table 4.30). This significant affect of P treatment on root activity was examined using a Scheffe post hoc test (Table 4.24).

Table 4.24: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root acidic monoesterase activity for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control > 60Po	***
control > 30Po	***
30Po > 60Po	***

4.3.3.3 Shoot and Root Dry Weights

The shoot and root dry weight data were presented in Fig 4.25 (a,b). Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot and root dry weights. These ANOVAs were presented in Appendix 4, Tables 31 and 32.

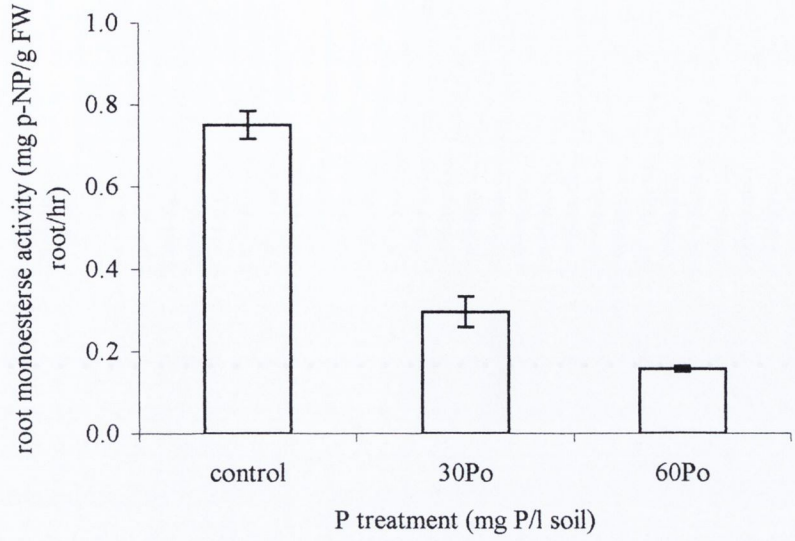
There was a highly significant P affect on shoot (F ratio 22.04***) and root (F ratio 28.68***) dry weight. All other treatments and interactions were not significant.

The shoot : root dry weight ratio for *Dactylis glomerata* at each combination of P treatment and benlate treatment were presented in Table 4.25.

Table 4.25: shoot : root dry weight ratios for *Dactylis glomerata* at each combination of P treatment and benlate treatment.

	Shoot : Root ratio	
P treatment	**-Benlate**	**+Benlate**
control	3.8	4.7
30Po	1.7	2.7
60Po	1.4	1.2

(a)



(b)

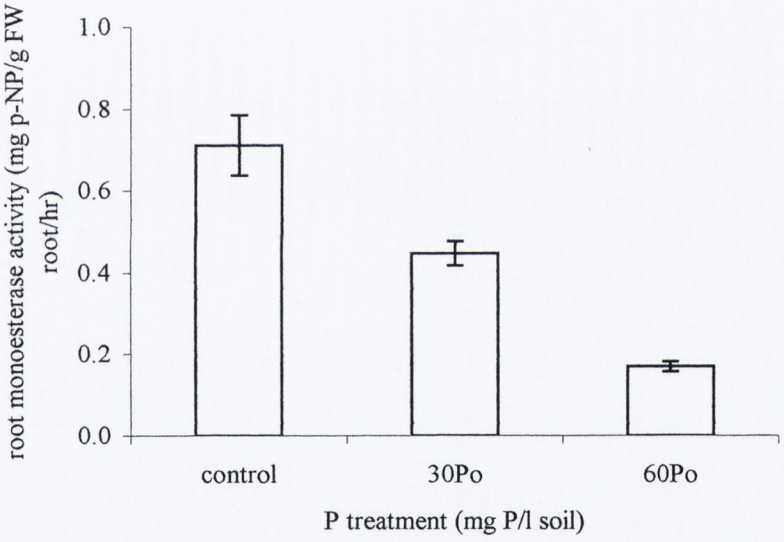


Fig 4.24: Root acidic monoesterase activity for the control and P treatment plants for the *Dactylis glomerata* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

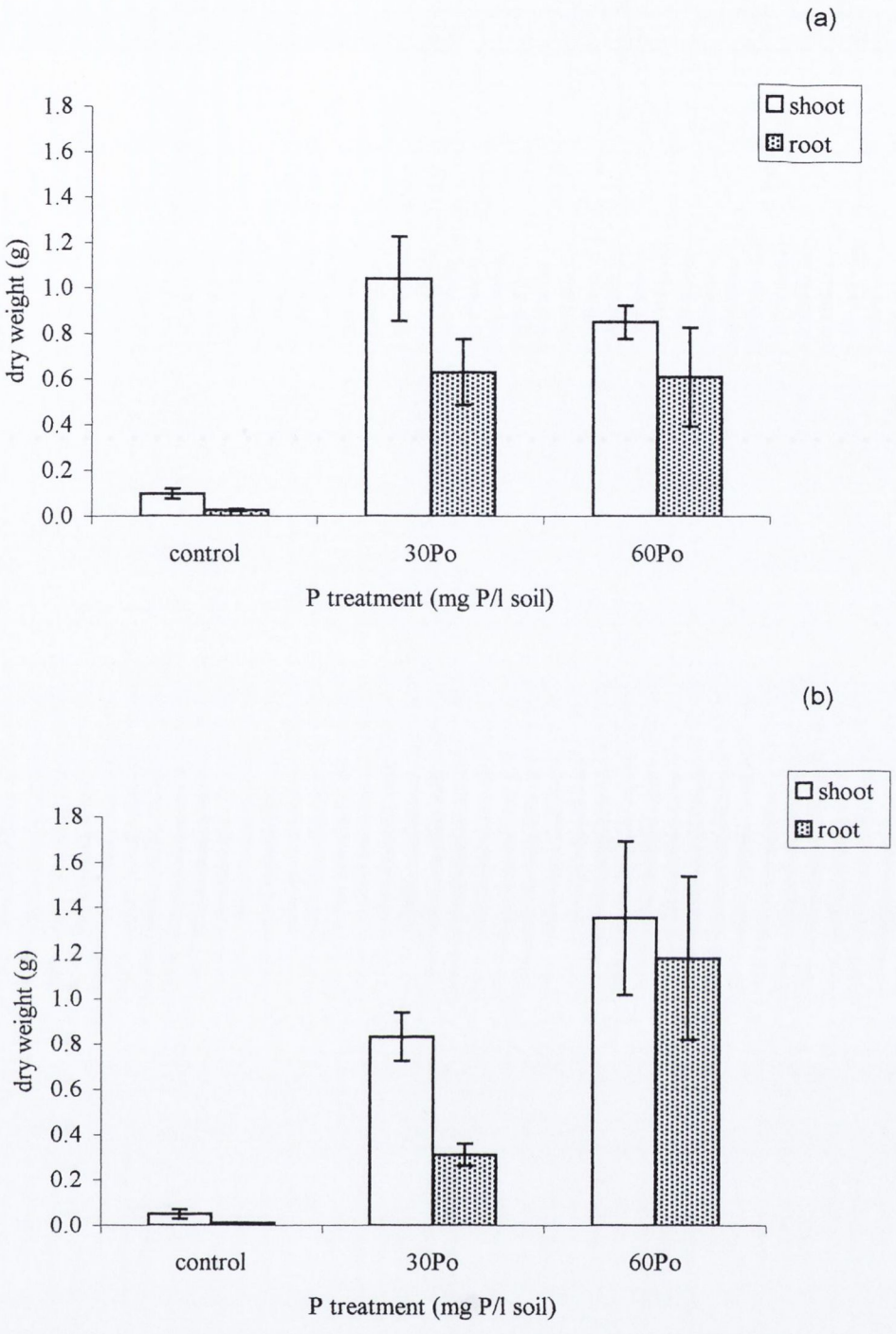


Fig 4.25: shoot and root dry weights for the control and P treatment plants of *Dactylis glomerata* for plants grown in (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

From Table 4.25 the shoot : root ratio (r:s ratio) showed a decrease with increasing concentrations of Po in the soil, in soil untreated with benlate. In soil treated with benlate, the addition of increasing levels of Po to the soil also resulted in a decrease in r:s ratio (Table 4.25).

Shoot dry weight

Fig 4.25 (a) presented shoot dry weight data for plants grown in soil untreated with benlate. Fig 4.25 (b) presented shoot dry weight data for plants grown in soil treated with benlate. From Fig 4.25 (a, b) there was an increase in shoot dry weight with the addition of 30Po with no further increase to that of the 60Po treatment plants. This significant affect of P treatment on shoot dry weight was examined using a Scheffe post hoc test (Table 4.24).

Table 4.26: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot dry weight for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	***
control < 60Po	***
30Po - 60Po	ns

Root dry weight

Fig 4.25 (a) presented root dry weight data for plants grown in soil untreated with benlate. Fig 4.25 (b) presented root dry weight data for plants grown in soil treated with benlate. From Fig 4.25 (a, b) there was an increase in root dry weight with the addition of 30Po. There was no further increase for the 60Po treatment plants. This significant affect of P treatment on root dry weight was examined using a Scheffe post hoc test (Table 4.27).

Table 4.27: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root dry weight for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	***
control < 60Po	***
30Po - 60Po	ns

4.3.3.4 Shoot and Root P Concentration

The shoot and root P concentration data were presented in Fig 4.26 (a, b). Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot and root P concentrations. These ANOVAs were presented in Appendix 4, Tables 33 and 34.

There was a significant P affect (F ratio 25.92***) and benlate affect (F ratio 12.62**) on shoot P concentration. There was also a significant P – benlate interaction (F ratio 20.19***) for shoot P concentration. For root P concentration there was a significant P affect (F ratio 8.18*). All other treatments and interactions were not significant.

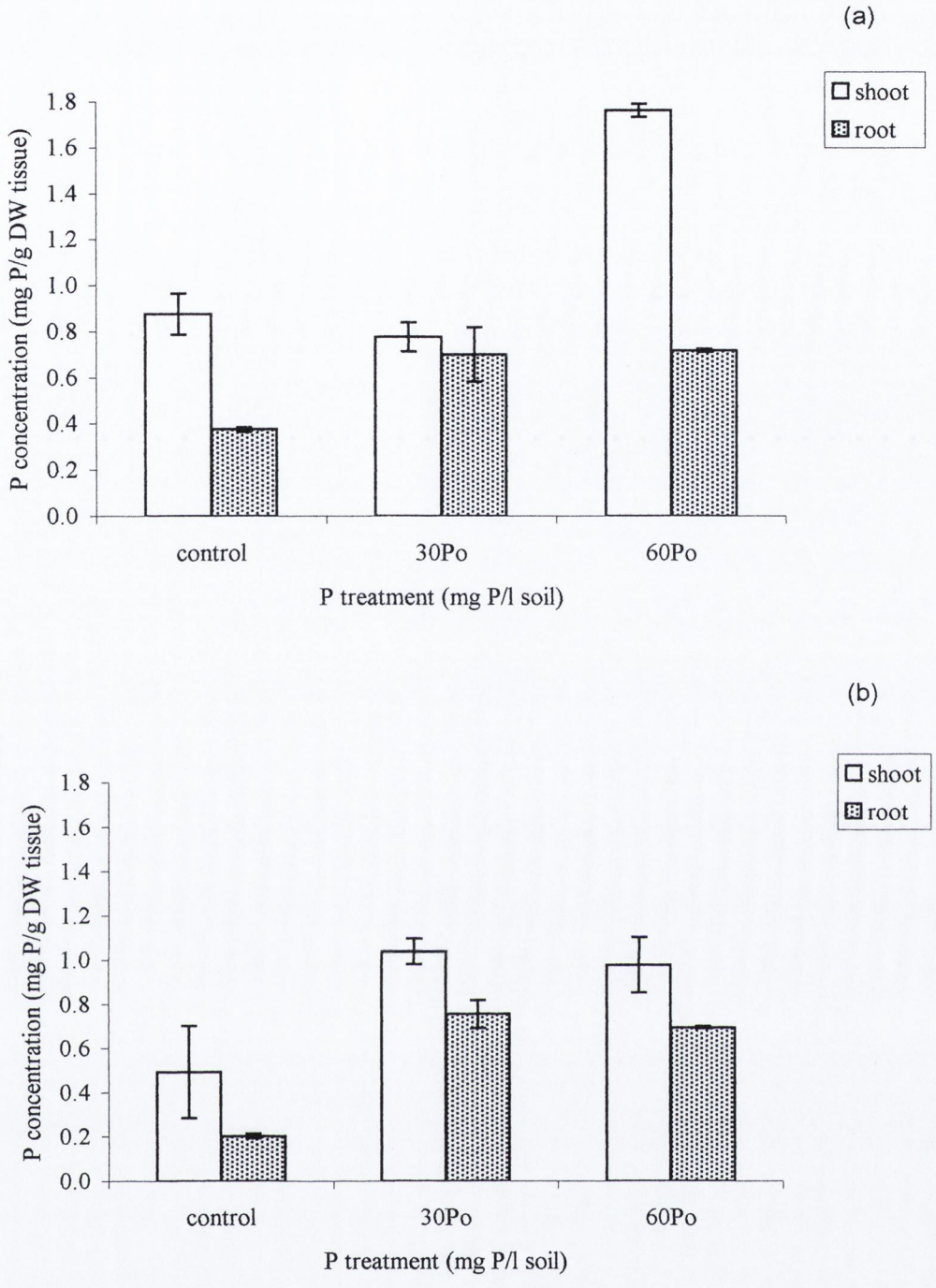


Fig 4.26: shoot and root P concentration for the control and P treatment plants of *Dactylis glomerata* for plants grown in (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

Shoot P concentration

Fig 4.26 (a) presented shoot P concentration data for plants grown in soil untreated with benlate. Fig 4.26 (b) presented shoot P concentration data for plants grown in soil treated with benlate. From Fig 4.26 (a, b) the 30Po treatment resulted in a similar shoot P concentration to the control plants. There was an increase in shoot P concentration with the 60Po treatment. This significant affect of P treatment on shoot P concentration was examined using a Scheffe post hoc test (Table 4.28).

Table 4.28: probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P concentration for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 30Po	ns
control < 60Po	***
30Po < 60Po	***

For the control and 60Po treatment plants there appeared to be a lower P concentration in the shoots of plants grown in benlate treated soil. For the 30Po treatment plants the benlate treated plants had a higher P concentration. This affect of benlate treatment on shoot P concentration was significant (Appendix 4, Table 4.33).

The significant interaction between the P treatment and benlate treatment was examined using a Scheffe post hoc test (Table 4.29).

Table 4.29: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors benlate treatment and P treatment, that affected shoot P concentration for *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-B, control - +B, control	ns
-B, 30Po - +B, 30Po	ns
-B, 60Po > +B, 60Po	***
-B, control - -B, 30Po	ns
-B, control < -B, 60Po	***
+B, control < +B, 30Po	**
+B, control < +B, 60Po	*

There was a significant decrease in the shoot P concentration on the application of benlate to the 60Po treatment soil (Fig 4.26 a, b; Table 4.29). There was no significant affect of the 30Po treatment, but there was a significant increase in shoot P concentration as a result of the 60Po treatment, for soil with no benlate application (Fig 4.26 a, b; Table 4.29). For soil that received benlate application there was a significant increase in shoot P concentration as a result of the 30Po and the 60Po treatments.

Root P concentration

Fig 4.26 (a) presented root P concentration data for plants grown in soil untreated with benlate. Fig 4.26 (b) presented root P concentration data for plants grown in soil treated with benlate. From Fig 4.26 (a, b) there was an increase in root P concentration for the 30Po treatments. There was no further increase for the 60Po treatment. This significant affect of P treatment on root P concentration was examined using a Scheffe post hoc test (Table 4.30).

Table 4.30: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root P concentration for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	*
control < 60Po	*
30Po - 60Po	ns

4.3.3.5 Shoot and Root P Content

The shoot and root P content data were presented in Fig 4.27 (a, b). Two way ANOVAs were carried out to determine the affect of P treatment and benlate treatment on shoot and root P content. These ANOVAs were presented in Appendix 4, Tables 35 and 36.

There was a highly significant P affect on shoot (F ratio 87.28***) and root (12.88***) P content. All other treatments and interactions were not significant.

Shoot P content

Fig 4.27 (a) presented shoot P content data for plants grown in soil untreated with benlate. Fig 4.27 (b) presented shoot P content data for plants grown in soil treated with benlate. From Fig 4.27 (a, b) there was an increase in shoot P content with increasing P concentrations in the soil. This significant affect of P treatment on shoot P content was examined using a Scheffe post hoc test (Table 4.31).

Table 4.31: probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P content for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	***
control < 60Po	***
30Po < 60Po	***

Root P content

Fig 4.27 (a) presented root P content data for plants grown in soil untreated with benlate. Fig 4.27 (b) presented root P content data for plants grown in soil treated with benlate. From Fig 4.27 (a, b) there was an increase in root P content by the 30Po treatment. There was no further

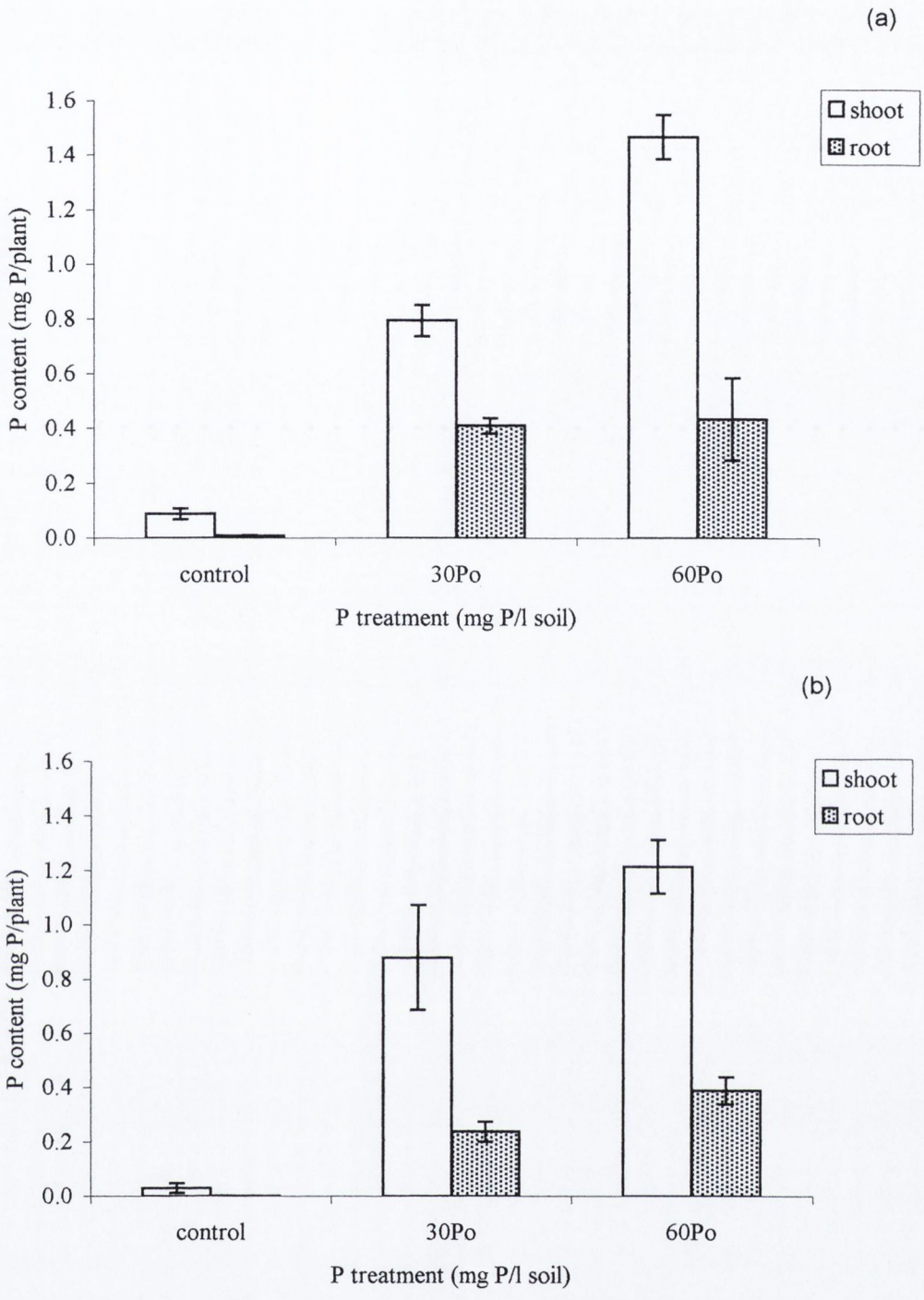


Fig 4.27: shoot and root P content for the control and P treatment plants of *Dactylis glomerata* for plants grown in (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

increase by the 60Po treatment. This significant affect of P treatment on root P content was examined using a Scheffe post hoc test (Table 4.32).

Table 4.32: probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on root P content for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 30Po	**
control < 60Po	**
30Po - 60Po	ns

4.3.3.6 Soil phosphate

The soil total P (Tp), inorganic P (Pi) and organic P (Po) concentration data were presented in Fig 4.28 (a, b), 4.29 (a, b) and 4.30 (a, b) respectively. Three way ANOVAs were carried out to determine the affect of P treatment, benlate treatment and location of soil on soil Tp, Pi and Po concentration. These ANOVAs were presented in Appendix 4, Tables 37, 38 and 39.

There was a significant P affect on total P (F ratio 7.48*) and organic P (F ratio 3.34*) concentration. All other treatments and interactions were not significant.

Soil Total P

Fig 4.28 (a) presented soil Tp concentration data for soil untreated with benlate. Fig 4.28 (b) presented soil Tp concentration data for soil treated with benlate.

From Fig 4.28 (a, b) there was an expected increase in start soil Tp levels with increasing levels of Po added to the soil. A pooled t test (at $p = 0.05$) was carried out to determine if there was a significant difference between the start soil and post harvest soil Tp concentrations. There was a statistically significant decrease in the Tp concentrations from those present in the start soils to the post harvest Tp concentration for the control and two P treatment soils (Fig 4.28 a, b). This decrease was in the inner and outer soils.

From Fig 4.28 (a, b) for the post harvest soil there was an increase in total P concentration in the soil with increasing levels of P addition for both the soil treated and untreated with benlate. This significant affect of P treatment on Tp concentration examined using a Scheffe post hoc test Table 4.33.

Table 4.33: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Tp concentration for the *Holcus lanatus* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 30Po	ns
control < 60Po	**
30Po < 60Po	*

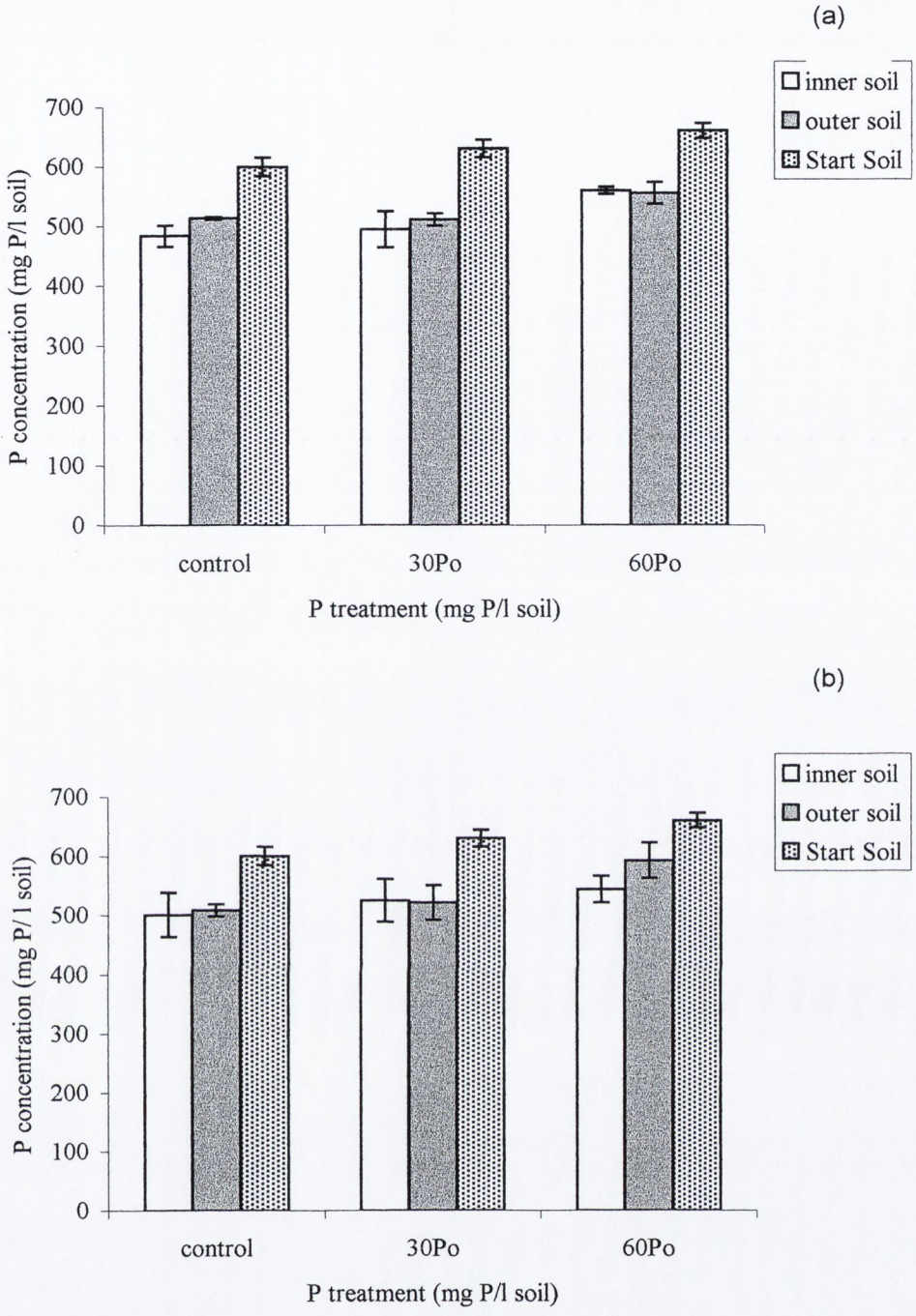


Fig 4.28: soil total phosphate concentration for the control and P treatment soils of the *Dactylis glomerata* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

Soil Inorganic P

Fig 4.29 (a) presented soil Pi concentration data for soil untreated with benlate. Fig 4.29 (b) presented soil Pi concentration data for soil treated with benlate.

From Fig 4.29 (a, b) there was no change in start soil Pi concentration with increasing levels of Po added to the soil. This was as expected as no Pi was added to any of the soils. A pooled t test (at $p = 0.05$) was carried out to determine if there is a significant difference between the start soil and post harvest soil Pi concentrations. There was no significant difference.(Fig 4.29 a, b).

Soil Organic P

Fig 4.30 (a) presented soil Po concentration data for soil untreated with benlate. Fig 4.30 (b) presented soil Po concentration data for soil treated with benlate.

From Fig 4.30 (a, b) there was an increase in start soil Po concentration with increasing levels of Po added to the soil. A pooled t test (at $p = 0.05$) was carried out to determine if there was a significant difference between the start soil and post harvest soil Po concentration. There was a significant decrease in the Po concentrations from those present at the start of the experiment to the post harvest Po concentrations for the control and treatment soils (Fig 4.30 a, b). This decrease was in the inner and outer soils.

From Fig 4.30 (a, b) for the post harvest soil there was an increase in Po concentration in the soil with increasing levels of P addition. This significant affect of P treatment on Po concentration was examined using a Scheffe post hoc test (Table 4.34).

Table 4.34: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Po concentration for the *Dactylis glomerata* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 60Po	ns
control - 30Po	ns
30Po < 60Po	*

4.3.4 Mass Balance

For each of the species the mg of Tp and Po depleted from the pot was matched with the total plant P content in order to determine how much soil P depletion was accounted for by plant uptake. This data was presented in Table 4.35.

From Table 4.35 only a very small proportion of the Tp and Po depleted from the soil was accounted for by plant uptake using the methods of soil phosphate and plant P content determination in this experiment.

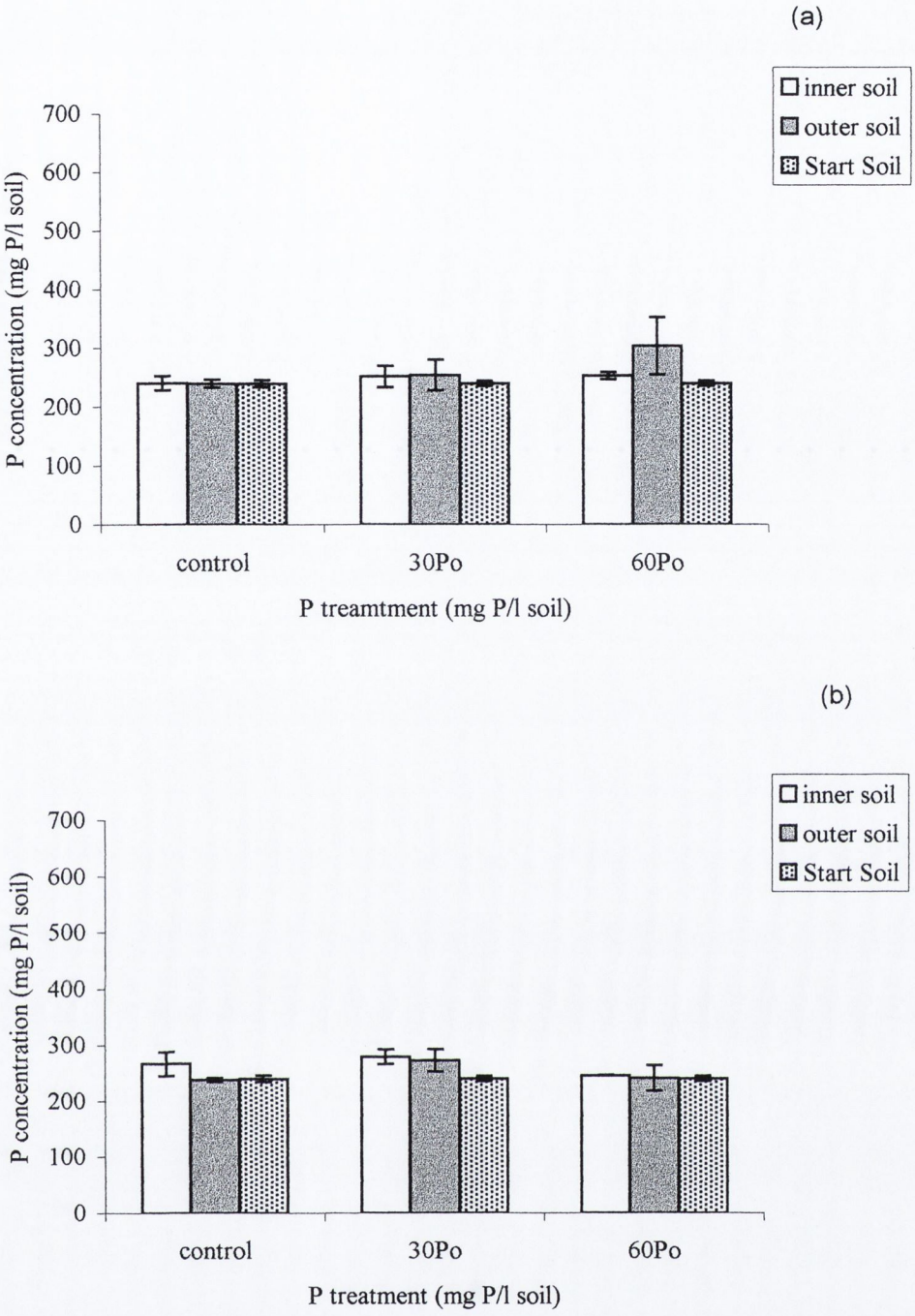


Fig 4.29: soil inorganicl phosphate concentration for the control and P treatment soils of the *Dactylis glomerata* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

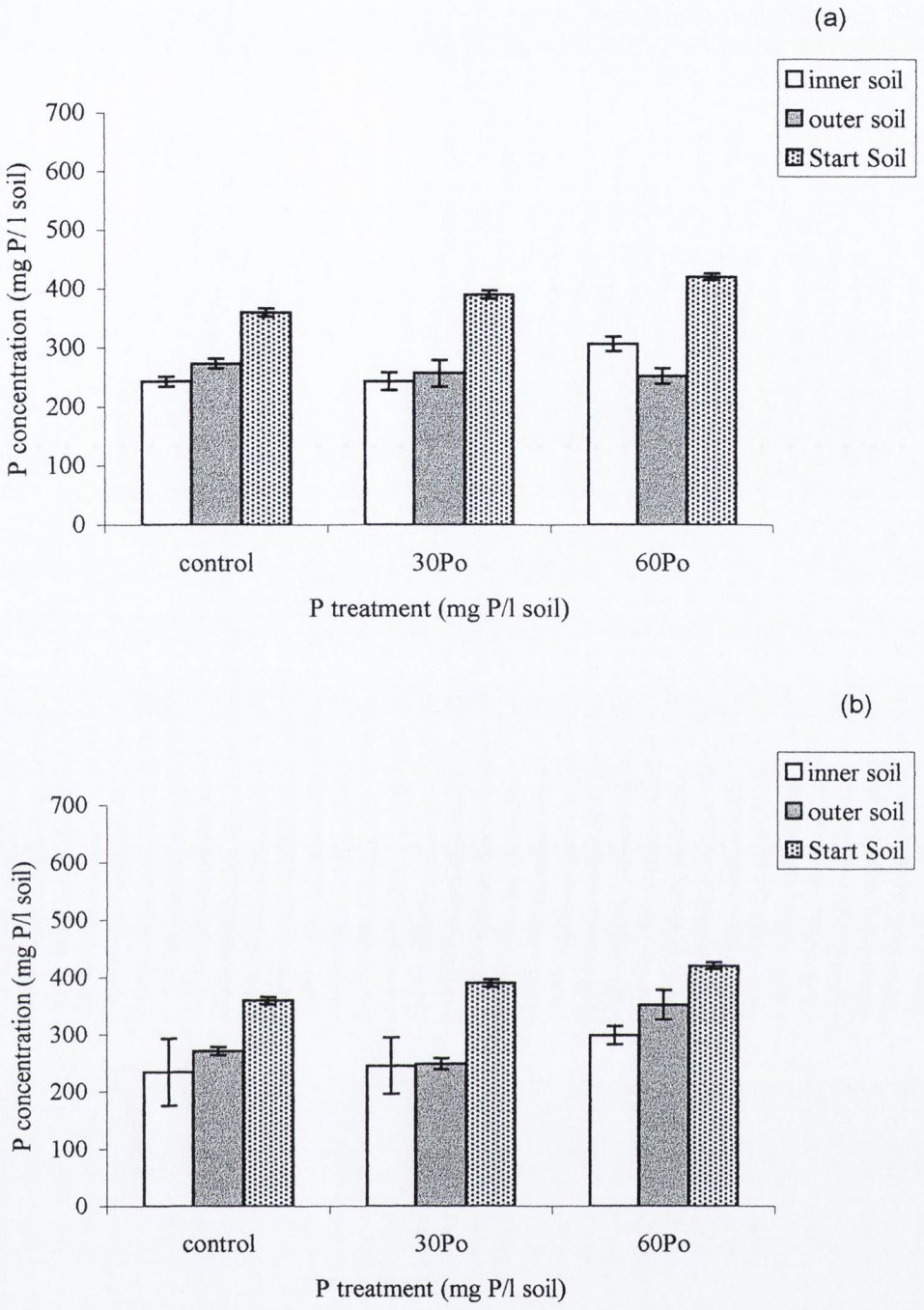


Fig 4.30: soil organicl phosphate concentration for the control and P treatment soils of the *Dactylis glomerata* experiment for (a) soil with no benlate application and (b) soil with benlate application. Each value represents the mean +/- SE of 3 replicates.

Table 4.35: Tp and Po depleted from the pot (mg P/pot) and the corresponding total plant P content (mg P/plant) for each of the species, *Agrostis s.*, *Holcus l.* and *Dactylis g.* in the control and P treatment soils.

Species	P treatment	Benlate treatment	Tp depleted (mg P/pot)	Po depleted (mg P/pot)	Plant P Content (mg P/plant)
Agrostis	Control	-B	48.76	36.12	0.09
	Control	+B	54.80	55.52	0.02
	30Po	-B	44.99	48.11	1.01
	30Po	+B	102.71	92.38	1.10
	60Po	-B	44.14	54.27	1.17
	60Po	+B	62.82	77.23	1.16
Holcus	Control	-B	41.19	38.67	0.06
	Control	+B	65.08	69.41	0.01
	30Po	-B	68.61	66.82	1.49
	30Po	+B	81.77	80.71	1.39
	60Po	-B	73.47	64.71	2.34
	60Po	+B	56.05	57.84	2.33
Dactylis	Control	-B	68.81	69.45	0.10
	Control	+B	58.52	74.35	0.03
	30Po	-B	80.23	87.19	1.21
	30Po	+B	64.15	83.55	1.12
	60Po	-B	61.84	99.42	1.90
	60Po	+B	67.18	71.96	1.22

4.3.5 Correlations

Pearson Product Moment Correlations (r) were carried out between the soil acidic monoesterase activity, the soil alkaline monoesterase activity, the soil diesterse activity and the root acidic monoesterase activity and, between these phosphatase measurements and the plant growth variables, shoot and root dry weight, P concentration and P content.

This was done for each of species, *Agrostis stolonifera, Holcus lanatus* and *Dactylis glomerata.* No statistically significant correlations were found.

4.4 DISCUSSION

In order to obtain a comprehensive picture of the phosphatase activity in the soil the presence of three soil enzymes, acid and alkaline monoesterase activity and diesterase activity, and root acidic monoesterase activity was analysed for each species, *Agrostis stolonifera, Holcus lanatus and Dactylis glomerata.* Soil phosphate concentration was also examined and plant growth parameters dry weight, P concentration and P content were measured. The aim of the experiment was to determine if increasing the soil Po content with IHP amendments had any affect on this group of enzymes and to determine how the individual enzymes reacted and monitor changes in plant growth.

4.4.1 Published Work

The soil acidic monoesterase activities measured in this experiment for soil planted with each of the species *Agrostis s., Holcus l.* and *Dactylis g.* were presented in Table 4.36.

Table 4.36: Soil acidic monoesterase activities for soil planted with *Agrostis stolonifera, Holcus lanatus,* and *Dactylis glomerata.* These values are for the control soil with no benlate treatment and are separated into activities detected in the inner and outer soil.

	Soil Acidic Monoesterase Activity (mg p-NP/g FW soil/hr)	
Species	**Inner soil**	**Outer soil**
Agrostis stolonifera	0.23	0.20
Holcus lanatus	0.33	0.34
Dactylis glomerata	0.41	0.39

The soil alkaline monoesterase activities measured in this experiment for soil planted with each of the species *Agrostis s., Holcus l.* and *Dactylis g.* were presented in Table 4.37.

Table 4.37: soil alkaline monoesterase activities for soil planted *with Agrostis stolonifera, Holcus lanatus,* and *Dactylis glomerata.* These values are for the control soil with no benlate treatment and are separated into activities detected in the inner and outer soil.

	Soil Alkaline Monoesterase Activity (mg p-NP/g FW soil/hr)	
Species	**Inner soil**	**Outer soil**
Agrostis stolonifera	0.09	0.12
Holcus lanatus	0.08	0.06
Dactylis glomerata	0.09	0.08

In order to compare the acid and alkaline monoesterase activities detected in this experiment with levels detected in soils by other workers, the control inner and outer soil values with no P or benlate treatment are presented in Table 4.36 and Table 4.37.

Comparison of enzyme activities from these experiments with published data from other investigations is confounded by the use of a number of different units and assay procedures. Furthermore the majority of phosphatase studies related to soil acidic monoesterase activity and

few studies have considered alkaline monoesterase activity (Joner and Jakobsen, 1995). Kremer (1994) observed an alkaline monoesterase activity of 0.05 –0.25 mg *p*-NP/g soil/hr in a forest soil. Trasar-Cepeda *et al.*, (2000) detected an acidic monoesterase activity of 0.31 – 6.59 mg *p*-NP/g soil/hr in an oakwood acidic soil from NW Spain. They did not measure alkaline monoesterase activity. Fox and Comerford (1992) measured an acidic monoesterase activity of 0.5- 1.5 mg *p*-NP/g soil/hr in an acidic (pH 4.2) pine plantation soil. They also did not measure alkaline monoesterase activity. The soils analysed by these workers were from natural sites with the exception of the pine plantation soil of the Fox and Comerford (1992) study that was being used for pine crop production. This pine plantation soil however showed a similar acidic monoesterase activity to the other two natural soils. The soil acidic and alkaline monoesterase activities from the work in this chapter presented in Table 4.36 and Table 4.37 were for a semi-natural grassland soil and are in the range of activities observed by these workers. The soil acidic monoesterase activity was similar for each of the three species as the soil alkaline monoesterase activity.

Kramer and Green (2000) detected an acidic monoesterase activity in an alkaline soil (pH 7.9) under a *Juniperus monosperma* canopy in northern Arizona of 0.02 mg p-NP/g soil /hr. They also measured an alkaline activity level of 0.13 mg p-NP/g soil /hr. This result of a higher alkaline than acidic monoesterase activity in an alkaline soil illustrates the point made by Tabatabai (1994) that alkaline phosphatase is predominant in alkaline soils. Tabatabai (1994) also states that acid phosphatase is predominant in acid soils. The work presented in this chapter using the PP1 site soil, which with a pH of 6.7 is acidic, confirms this theory. Overall a higher acidic than alkaline soil monoesterase activity was found.

Fox and Comerford (1992) found that the soil acidic monoesterase activity was higher in the rhizosphere soil than in the bulk soil in the presence of *Pinus elliottii*. In this case the rhizosphere soil referred to the soil adhering to the roots when the plant was shaken to remove excess soil on harvesting. Helal and Sauerbeck (1984) also detected a higher soil acidic monoesterase activity in the rhizosphere soil than in the surrounding soil in the presence of maize plants. Tarafdar and Jungk (1987) found that the rhizosphere of *Brassica oleracea, Allium cepa, Triticum aestivum* and *Trifolium alexandrinum* had higher levels of both acidic and alkaline monoesterase activity than the bulk soil. The maximum distance from the root surface at which the activity was higher than the bulk soil was 2.0 to 3.1mm for acid monoesterase and 1.2 to 1.6 for alkaline monoesterase activity.

The volume of soil at the soil-root interface is termed the rhizosphere and extends only a few millimetres from the root (Steer and Harris, 2000). In this chapter the inner soil of the growth bag extended beyond the zone of the rhizosphere as given by this definition. The inner soil was the 200 g of soil inside the growth bag that was in contact with the roots. This inner area

encompassed more soil than that adhering to the roots, as taken for the rhizosphere by Fox and Comerford (1992) and Helal and Sauerbeck (1984), and was in excess of the 3 mm distance zone from the root as measured by Tarafdar and Jungk (1987). Despite this, a higher acidic and alkaline monoesterase activity was detected in this inner soil than in the outer soil. This was true when the data was examined for all the P and benlate treatments together. In the experiment described in this chapter there was a significantly higher soil acidic monoesterase activity detected in soil planted with *Dactylis g.* and *Holcus l.* than in that planted with *Agrostis s.*. There was no significant difference in the acidic monoesterase activity of the inner and outer soil for *Agrostis s.*. For alkaline monoesterase activity as with the acidic activity there was a significantly higher activity in the inner soil than in the outer soil again for soil planted with *Dactylis g. and Holcus l.*. There was no significant difference in the inner and outer soil alkaline monoesterase activity for *Agrostis s.*.

The soil acidic monoesterase activity is associated with plant roots and associated mycorrhizal fungi and is secreted into the soil (Joner and Jakobsen, 1995) and for this reason would be expected to be higher in the rhizosphere, near the roots. The alkaline monoesterase activity is released by soil bacteria and microorganisms (Joner and Jakobsen, 1995) which are often more numerous in the root zone because they thrive on root exudates and decaying root material (Steer and Harris, 2000). Therefore the alkaline monoesterase activity would be expected to be higher in the rhizosphere zone. In the case of *Agrostis s.* there was no significant difference in acidic and alkaline monoesterase activity of the inner and outer soil. The three species had similar root acidic monoesterase actives (as presented in Table 4.38 in a following section) so it is unlikely that the *Agrostis s.* simply secreted less acidic monoesterase. The properties of monoesterase enzymes associated with roots can vary with species, for example their resistance to decomposition by the action of microorganisms in soil solution (Tadano *et al.*, 1993). The root monoesterase of the *Agrostis s.* plants once secreted into the soil, may have been decomposed by the microbial population in the inner soil and therefore soil acidic monoesterase activity in the inner soil would be similar to that in the outer soil. Isolation and purification of the soil acidic monoesterase enzymes and identification of their source and properties would be required to prove this theory. With respect to the soil alkaline monoesterase activity, it is possible that the soil bacteria and microorganisms were fewer in number, or of a different species composition, than in the soil planted with *Dactylis g.* or *Holcus l.* and so resulted in an alkaline activity in the inner soil similar to that in the outer soil. To assist in confirming either of these theories it would be necessary to enumerate the bacteria from soil planted with each of the plant species in use, and to identify the species present.

The soil diesterase activities measured in this experiment for soil planted with each of the species *Agrostis s.*, *Holcus l.* and *Dactylis g.* were presented in Table 4.38.

Table 4.38: soil diesterase activities for soil planted with *Agrostis stolonifera, Holcus lanatus,* and *Dactylis glomerata.* These values are for the control soil with no benlate treatment and are separated into activities detected in the inner and outer soil.

	Soil Diesterase Activity (mg p-NP/g FW soil/hr)	
Species	**Inner soil**	**Outer soil**
Agrostis stolonifera	0.82	0.90
Holcus lanatus	0.99	0.96
Dactylis glomerata	1.58	1.56

In order to compare the soil diesterase activities detected in this experiment with levels detected in soils by other workers, the control inner and outer soil values with no P or benlate treatment are presented in Table 4.38.

Naseby and Lynch (1997) detected an average soil diesterase activity of 4.0 mg p-NP/g FW soil/hr for an agricultural soil planted with wheat. Much lower soil diesterase activities were detected by Trasar-Cepeda *et al.,* (2000) who measured the diesterase activity of an oakwood soil from NW Spain and detected activities ranging from 0.12 to 0.50 mg p-NP/g soil/hr. The considerably higher diesterase activity of the agricultural soil (Naseby and Lynch, 1997) may be related to a high return of dead shoot and root matter from the wheat crop which contains diester P such as DNA, which is readily mineralised. The build up of this plant material over several years of crop production may have induced a higher diesterase activity. Differences in soil phosphatase activity in general have been shown to be related to soil type and vegetation present, and have been found to be correlated with organic carbon and loss on ignition (Harrison, 1987). This may also be true for diesterase. The soil diesterase activities detected in this experiment for a semi-natural soil are closer in range to those obtained Trasar-Cepeda *et al.,* (2000) for their natural wood soils.

The diesterase activities detected in this work (Table 4.38) were higher than either the soil acidic or alkaline monoesterase activities. Diesterase acts on soil diester P which includes soil phospholipids and fragments of RNA (Addiscott *et al*, 2000). Diesters are a relatively available pool of Po that is decreased or disappears with disturbance or cultivation (Preston and Trofymow, 1998; Robinson *et al*, 1988). The high levels of diesterase activity in the experimental soil compared to the monoesterase activity indicated the potential of the soil to utilise diester P and it would be expected that this high diesterase activity was due to a high diester P content of the soil. However nuclear magnetic resonance work carried out on the PP1 site soil and discussed in Chapter 7, Sections 7.3.1 and 7.3.2, identifies monoester P as being an approximately 10 times more dominant P fraction in the control PP1 soil than diester P. Phosphodiesterase activity has been found to be present in various plants and microorganisms and orthophosphate has been found to be a competitive inhibitor of this enzyme (Tabatabai, 1994). The high diesterase activity in this soil may be due to the fact that once secreted into the

soil diesterase is very resistant to breakdown by the microbial population in this soil and has a long active life in the soil. Mineralisation of Po to form orthophosphate did not result in sufficiently high levels of orthophosphate to inhibit the diesterase activity and reduce the activity to a level similar to that of monoesterase activity. Insufficient work has been carried out on soil diesterase to conclude with any certainty what controls soil diesterase activity and why activity varies between soils.

No studies relating to differences in diesterase activity between rhizosphere and bulk soil were found by this author. In this work the diesterase activity of the inner soil was higher than that of the outer soil where soil was planted with *Dactylis g.*. For soil planted with *Holcus l.* and *Agrostis* s. there was no significant difference in the diesterase activity present in the inner and outer soil. It is possible that different soil microorganisms were present in the soil, especially in the inner root soil, as a result of planting with *Dactylis g.* and they had a better ability for diesterase production and secretion. Further work on soil diesterase isolation and purification is required to identify sources and properties.

The root acidic monoesterase activities measured in this experiment for each of the species Agrostis s., Holcus l. and Dactylis g. were presented in Table 4.39.

Table 4.39: root acidic monoesterase activities for *Agrostis stolonifera, Holcus lanatus,* and *Dactylis glomerata.* These values are for plants grown in the control soil with no benlate treatment.

Species	**Root Acidic Monoesterase Activity (mg p-NP/g FW root/hr)**
Agrostis stolonifera	0.86
Holcus lanatus	0.87
Dactylis glomerata	0.75

Tando *et al.*, (1993) found root acidic monoesterases ranging from 0.27 to 1.39 mg p-NP/g root/hr for a range of crop plants with rice and radish being at the lowest and highest end of the scale respectively. Dodd *et al.*, (1987) detected root phosphatase activities of 0.27 – 1.25 mg p-NP/ g FW root tissue/hr. The root acidic monoesterase values for the species in this experiment are in the same order of magnitude. Ability to secrete acid phosphatase often differs among species (Tadano *et al.*, 1993; Frossard, 1995) however for these three species under control conditions with no P addition similar root acidic monoesterase activities were found.

4.4.2 Information Gained

Phosphatase activity measured in the soil indicates a potential to use Po (Fox and Comerford, 1992). The importance of organic P as a source of P to plants depends on the amount of organic P and the presence of appropriate phosphatase to mineralise Po because Po must be hydrolysed to inorganic P before it can be utilised by plants (Fox and Comerford, 1992).

The acidic and alkaline monoesterase enzymes act on monoester P such as inositol phosphate, to break it down to inorganic phosphate which can then be taken up by plants. Acid monoesterase activity is attributed to plant roots and associated mycorrhizal and saprophytic fungi. Alkaline monoesterase is released by soil bacteria, microorganisms, fungi and fauna (Joner and Jakobsen. 1995). In this experiment there was a higher acidic than alkaline monoesterase activity. This was as expected for this acidic PP1 site soil. This implies that the majority of monoester Po breakdown in this soil is due to acidic monoesterase secreted by roots and fungi. The potential for Po use by plants exists in this soil.

The inositol hexaphosphate (IHP) added to the soil is a form of monoester P and as such should provide a substrate for acid and alkaline monoesterase. It was expected that the addition of IHP, and therefore monoesterase substrate, to the soil would stimulate acidic monoesterase production and result in an increase in the acidic monoesterase activity. As the alkaline monoesterase activity was lower overall, little reaction to IHP addition was expected. There was however no affect of the P treatment on the soil acidic or alkaline monoesterase activity for soil planted with any of the species, *Agrostis s.* (Table 4.2), *Holcus l.* (Table 4.16) or *Dactylis g.* (Table 4.32). The addition of 30Po to the soil, or doubling this concentration to 60Po, had no significant affect on the acidic or alkaline monoesterase activity. As the addition of substrate to the soil did not affect the monoesterase enzyme it is possible that monoesterase production itself was limiting and no increase in activity of soil acidic monoesterase by the plant roots and fungi was possible. One plant and associated fungi may not have been sufficient to produce an increase in acidic monoesterase that would diffuse into the soil and be detected. It is also possible that there was no change in soil acidic monoesterase activity because sufficient levels of soil acidic monoesterase activity were already present in the soil to breakdown the IHP added, which was used for plant growth as indicated by increase biomass of the plants with Po amendment of the soil. The resulting Pi from this breakdown was taken up by plants and microbes and did not build up to a level in the soil that would cause a decrease in soil monoesterase activity.

The addition of benlate to the soil at the concentrations employed in this experiment has been shown to reduce the presence of mycorrhizal hyphae (Chapter 2, Section 2.3.1.2). The application of benlate to the soil, and so a reduction in VAM presence, had no affect on the level of the acidic or alkaline monoesterase activity. This would indicate that mycorrhizal hyphae are not making a detectable contribution to the activity levels of these enzymes, either in the control soil or with increased soil Po concentrations of the 30Po and 60Po treatment. Published results relating to the production of phosphatase by VAM hyphae for mobilisation of Po are conflicting. Smith and Read (1997) are of the opinion that there is no good evidence that mycorrhizal fungi are involved in the mineralisation of Po through phosphatase enzymes. Not

enough work has been carried out to examine the utilisation of Po by VAM plants, through increased phosphatase release from the fungal hyphae (Smith and Read, 1997). Some workers have concluded that extracellular phosphatases are not produced by extraradiacal hyphae of VAM symbiosis (Dodd *et al.*, 1987; Joner *et al.*,1995; Joner and Jakobsen, 1995). Others claim results demonstrate the production of phosphatase by mycorrhizal hyphae and their contribution towards plant P uptake (Tarafdar and Marschner, 1994). Koide and Kabir (2000) presented evidence of the hydrolysis of 2 forms of Po, the toludine salt of 5-bromo-4-chloro-3-indolyl phosphate and the tetrasodium salt of phenol-phathalein diphosphate, by VAM hyphae in sterile culture. The results presented in this chapter on soil monoesterase supported the view of Dodd *et al.*, (1987), Joner *et al.*, (1995) and Joner and Jakobsen, (1995) that VAM hyphae do not contribute to the production of phosphatase.

For the two species *Dactylis g.* and *Holcus l.* there was a significant interaction between P treatment and benlate treatment that affected the soil acidic monoesterase activity but the combinations of these two factors which significantly affect the enzyme activity level are different for each species. Both however related to the 60Po treatment. In the case of *Dactylis g.* the 60Po treatment resulted in a significant decrease in soil acidic monoesterase activity for soil with no benlate application. The addition of benlate to the soil however resulted in no such affect of the 60Po treatment. This may be that in soil with no benlate application that the VAM hyphae did help to breakdown the added IHP at the 60Po treatment, and the resulting Pi increased in concentration and inhibited the acidic monoesterase activity. On addition of benlate to the soil however, VAM presence was reduced and so was the potential for Po breakdown. The 60Po was no longer broken down to the same extent to result in Pi levels that would inhibit the acidic monoesterase. With respect to *Holcus l.* the addition of benlate and the 60Po treatment to the soil resulted in an increase in soil acidic monoesterase activity. With no benlate addition and the 60Po treatment there was sufficient levels of acidic monoesterase to breakdown the soil Po, but the levels of Po released were not high enough to inhibit acidic monoesterase activity. Reducing the VAM presence through benlate addition reduced their potential to contribute to acidic monoesterase activity. Roots and other fungi may have increased production of this enzyme to compensate for this. The role of VAM hyphae in the production of acidic monoesterase for Po breakdown may therefore depend on the soil Po concentration and indeed Po type. Only IHP was used here and the role of fungi other than VAM was not assessed.

Phosphodiesterase (EC 3.1.4.1) has been found to be present in various microorganisms and animals. References to phosphodiesterase activities in the literature are scarce and unsystematic (Trasar-Cepeda *et al.*, 2000). The factors that significantly affected soil diesterase varied across the three species. In the case of *Agrostis s.* there was a significant affect of P treatment on the soil diesterase activity. The addition of Po to the soil resulted in an increase in diesterase

activity. There was no significant affect of P treatment on the diesterase activity of soil planted with *Holcus l. or Dactylis g.*. It was not expected that the diesterase activity of the soil would be affected by the addition of IHP to the soil. IHP is a monoester P compound and diesterase acts on diester P compounds such as RNA. The increase in diesterase activity in the *Agrostis s.* soil was possibly because of a high turnover of plant material returning diester P compounds to the soil, which stimulated diesterase production. There is a high abundance of diester P such as nucleic acid compounds in plant material. (Stevenson and Cole, 1999)

There was no affect of benlate on diesterase activity of soil planted with *Agrostis s.*, *Holcus l.* or *Dactylis g.*. This was as expected as benlate at the concentration used in this experiment reduces the presence of vesicular arbuscular mycorrhiza (VAM) (Chapter 2, Section 2.3.1.2). As vesicular arbuscular mycorrhiza are not associated with the production of soil diesterase reducing the presence of VAM would not therefore affect diesterase activity. This also indicates that benlate does not have an adverse affect on any microoganisms or animals that do produce diesterase activity.

Phosphatase enzymes associated with the root surface of plants may originate from mycorrhizal or saprophytic fungi, bacteria or root exudates (Johnson *et al.*, 1999). Root acidic monoesterase activity is closely tied to plant demand for phosphorus (Kramer and Green, 2000). Activity is increased in plants that are P deficient (Barrett-Lennard *et al.*, 1993; Frossarrd 1995; Richardson *et al.*, 2000). Tadano *et al.*, (1993) found acid monoesterase activity of the roots increased under P deficient conditions in crop species including wheat rice tomato and sugar bean. It is indicated that the control plants are P deficient as the P treatment does result in a significant increase in shoot and root dry weight for *Agrostis s.* (Table 4.5), *Holcus l.* (Table 4.19) and *Dactylis g.* (Table 4.37). The addition of Po to the soil resulted in a significant decrease in root monoesterase activity for *Dactylis g.* and *Holcus l.*. This was as expected, and indicated that as a result of the addition of Po to the soil the plants were no longer as P deficient as the control plants. The IHP added to the soil was an available source of P for plant growth.

IHP must be hydrolysed to inorganic P before it can be utilised by plants (Fox and Comerford, 1992). It is possible that increasing the levels of IHP in the soil did cause an increase in soil monoesterase activity early in the growing period in order to breakdown this additional substrate. However the activity subsequently decreased due to the production of orthophosphate from the Po hydrolysis in excess of that absorbed by the plant and so built up in the soil. Orthophosphate is a competitive inhibitor of acid and alkaline phosphatase in soil. It is also possible that there was no change in root monoesterase activity in the early days of the growing period with the addition of IHP to the soil. There was sufficient monoesterase activity already present on the roots to break-up this additional Po for use by plants and result in an increase in

dry weight. The Pi resulting from this break-up may have built up and inhibited the root monoesterase activity.

For *Dactylis g.* both 30Po and 60Po resulted in a significant decrease in root activity indicating that both Po treatments alleviated the P deficiency of this species. The hydrolysis of the 30Po and 60Po most likely resulted in excess production of Pi than could be absorbed by the plant at that time and so resulted in the inhibition of the root activity. In the case of *Holcus l.* only the 60Po treatment resulted in a significant decrease in root activity. That is the higher Po treatment was required to alleviate the P deficiency in this species to a level detectable by the root monoesterase assay. In the case of *Agrostis s.* neither the 30Po nor 60Po treatment resulted in a significant decrease in root activity. The 30Po and 60Po treatments did result in a significant increase in shoot and root dry weight indicating that the IHP was used by this species, but from the root monoesterase activity assay neither treatment resulted in the release of excess Pi that could not be taken up by the plant. It would be of interest to carry out root monoesterase activity assays at various time intervals throughout the growth period. This would help to determine if the affects of P treatment on root activity changed with time and the age of the plant.

There was an increase in biomass as a result of Po addition to the soil. That is the IHP contributed to the P taken up by the plants. It is most likely that this IHP was enzymatically broken down before use. Soil organic phosphate contributes to the phosphorus nutrition of plants primarily after being mineralised into organic phosphorus (Addiscott *et al.*, 2000). Of the enzymes assayed root acidic monoesterase is most likely to have significantly helped P uptake from IHP as there was little response to Po addition by the other enzymes, soil acidic and alkaline monoesterase. Root monoesterase activity acted to breakdown the IHP for plant P uptake and the Pi subsequently released inhibited the activity. It is proposed that if the phosphatases are bound to the root only those organic compounds that reach the root surface can be hydrolysed, i.e. only those which are soluble and can be transported through the soil liquid phase towards the root surface (Firsching and Claassen, 1996). The results from this experiment disagree with this proposal. Root monoesterase appears to be very important in the use of IHP by plants in this experiment and as will be mentioned later in this discussion, IHP addition to the soil resulted in significant increases in dry weight and Tp and Po was depleted not only from the soil near the root but from the soil in the entire pot. Although the phosphatases are associated with the root it would appear that they are solubilising IHP throughout the pot. IHP is considered to be very stable and unlikely to be transported through the soil liquid phase (Stevenson and Cole, 1999).

It is also possible that the IHP was available directly for plant uptake and the root phosphatase levels dropped due to sufficient levels of available P in the soil through this IHP. Most but not all authors believe that the dephosphorylation of P from organic molecules is an indispensable

step during its utilisation by plant roots (Findenegg and Nelemans, 1993). Results obtained by Findenegg and Nelemans (1993) do not rule out the possibility of an uptake of unhydrolysed organic P. Johnson *et al.*, (1999) proposed that if root surface phosphatases have an important role in plant P supply this will most likely occur when P demand is high and is limiting plant growth. In this experiment however root monoesterase appeared vital for use of IHP for plant growth even at increased concentrations of 30Po and 60Po that are indicated as alleviating plant P deficiency. These results support view that plants can utilise Po fractions in the soil by means of phosphatase activity enriched at the soil root interface (Tarafdar and Jungk, 1987). It is proposed that if the phosphatases are bound to the root only those organic compounds that reach the root surface can be hydrolysed, i.e. only those which are soluble and can be transported through the soil liquid phase towards the root surface (Firsching and Claassen, 1996).

The addition of benlate to the soil however had no affect on the root acidic monoesterase activity for any of the three species. Benlate reduces VAM presence. The reduction in VAM therefore did not affect the root acidic monoesterase activity. This would indicate that the VAM hyphae do not contribute to the root acidic monoesterase activity. As discussed in relation to the root acidic monoesterase the role of VAM fungi in the production of monoesterases for the breakdown of Po is uncertain (Smith and Read, 1997). This work indicates that root acidic monoesterase activity is originating from the roots themselves with no significant contribution form VAM hyphae.

For all three species there was a significant affect of P treatment on shoot and root dry weight. For *Dactylis g.* and *Agrostis s.* the 30Po and 60Po treatment resulted in an equal increase in shoot and root dry weight. For *Holcus l.* the 60Po treatment resulted in a higher shoot and root dry weight than the 30Po treatment. This would indicate that IHP was available for plant P uptake and was taken up by the plants and increased biomass production. The increase in dry weight on the addition of Po to the soil would also indicate that the PP1 soil was deficient in plant available P.

Table 4.40 expressed the increase in shoot and root dry weight for each of the species as a result of the 30Po and 60Po treatment for soil treated and untreated with benlate. The increase was expressed as the number of times the dry weight had been increased as a result of the P treatments compared to the control plants dry weight.

From Table 4.40 the relative increase in dry weight when the P treatment plants are compared to the control plants, is greater for the root dry weight than for the shoot dry weight. For example, for *Dactylis g.* the 30Po and 60Po treatment resulted in a 10-fold and 8-fold increase respectively in shoot dry weight but a 23-fold increase in root dry weight.

Table 4.40: Increase in shoot and root dry weight for each species as a result of the 30Po and 60Po treatment expressed as a multiple of the control plants shoot and root dry weight. –B and +B refer to soil with no benlate treatment and with benlate treatment respectively.

	Increase in dry weight (X fold increase of control dry weight)			
	Shoot Dry Weight		Root Dry Weight	
Species/Po treatment	**-B**	**+B**	**-B**	**+B**
Dactylis g. / 30Po	10	16	23	28
Dactylis g. / 60Po	8	26	23	109
Holcus l. / 30Po	10	75	33.6	588
Holcus l. / 60Po	11	133	58	1048
Agrostis s. / 30Po	10	45	6	337
Agrostis s. / 60Po	6	31	10	321

From Table 4.40 overall there was a greater increase in the shoot and root dry weight for all species as a result of the 30Po and 60Po treatment when grown in soil with benlate application than when grown in soil with no benlate. For example, for *Dactylis g.* there was a 16 and 26-fold increase in shoot dry weight as a result of the 30Po and 60Po treatments respectively, when plants were grown in soil with benlate application. This is compared to a 10 and 8-fold increase in shoot dry weight as a result of the 30Po and 60Po treatments respectively when plants were grown in soil with no benlate application. However this affect of benlate on shoot and root dry weight was not significant.

The addition of benlate to the soil did not significantly affect the shoot or root dry weight for any of the species. The reduction in VAM presence as a result of benlate application did not affect the biomass accumulation of the species. The dry weight of the species increased as a result of P addition to the soil irrespective of the level of VAM present. This is surprising as VAM aid in the uptake of P by plants. Root infection with VAM fungi can increase efficiency of nutrient absorption and enhance the growth of mycorrhizal plants, particularly at low availability of P in the soil (Kothari *et al.*, 1990). Phosphate mineralised from P sources appears to be more readily available to mycorrhizal than non-mycorrhizal plants. In this experiment however with the species *Agrostis s.*, *Holcus l.* and *Dactylis g.* and IHP supplied at 30Po and 60Po there was no apparent affect of the naturally occurring VAM on P uptake by plants. VAM are thought to aid the P uptake of plants especially at low soil P concentrations, but for the control soil and with the amendment of this soil with Po at 30Po and 60Po the presence or absence of VAM was not significant.

It has been proposed by Barrett-Lennard *et al.*, (1993) that IHP, although a predominant form of Po in soil, is a poor source of P for growth. They found IHP was poorly hydrolysed by root segments and was a poor source of P for growth for clover when grown in sterile agar culture. Richardson *et al.*, (2000) found plants showed limited ability to obtain P from IHP as indicated by low dry matter accumulation and reduced P content of tissues. Growth and P uptake by plants supplied with IHP was comparable to plants grown in the absence of added P. They

performed this study by growing plants in sterile agar culture. Hayes *et al.*, (2000) examined the affect of the IHP addition to the growth media of three grass species, *Phalaris aquatica, Lolium rigidum* and *Danthonia richardsonii*. The growth media however consisted of sterile agar or a sand – vermiculite mix. They found IHP to be a poor source of P for plant growth. They concluded that the acquisition of IHP P was limited by availability of the compound and the capacity of roots to hydrolyse the IHP. Only by supplementing the agar containing the IHP with phytase from *Aspergillus niger* was any improvement in plant growth observed. This serves to highlight the fact that such limited artificial growth media are unsuitable for studies relating to soil Po nutrition of plants and do not represent the actual growth environment of plants in the field. In the experiment described in this chapter, a semi natural soil was used and IHP provided a source of phosphate available for plant growth and significantly increased dry matter accumulation. The work in this chapter contradicts the conclusions of Barrett-Lennard *et al.*, (1993) and Richardson *et al.*, (2000). IHP was found to be a good source of available P as was indicated by an increase in dry matter with increasing concentrations of Po in the soil.

All species showed a decrease in shoot:root ratio with increasing concentrations of Po in the soil. Plants with a lower root:shoot ratio have a smaller shoot proportion, a lower photosynthetic potential and a larger root capacity. That is although there was an increase in total plant biomass there was a larger proportion of root tissue than shoot tissue with increasing soil Po concentrations. The Po taken up by the plants was put into the production of roots that would aid in exploring larger areas of soil and acquiring more P.

The addition of Po to the soil had a significant affect on the shoot P concentration for the three species but the pattern of this affect was different for each species. In the case of *Agrostis s.* there was no significant affect of Po addition to the soil on shoot P concentration. For *Dactylis g.* there was an increase in shoot P concentration as a result of the 60Po treatment. For the *Holcus l.* the shoot P concentration was increased to the same extent by both the 30Po and 60Po treatment.

The root P concentration of *Agrostis s.* was not affected by the P treatment. The root P concentration of *Dactylis g.* did however increase with the addition of 30Po and 60Po to the soil. For *Holcus l.*, as with the shoot P concentration, the root P concentration increased to an equal extent with 30Po and 60Po treatment. The higher concentration of Po in the soil and the increased root growth resulting in a higher proportion of root material to shoot material probably helped in the uptake of P by the plants leading to a higher concentration of P in the tissues.

There was no affect of benlate addition on P concentration for any of the species. As with root and shoot dry weight this again would indicate that VAM do not play an important role in P uptake at these low concentrations of Po.

The shoot content was affected by the P treatment for all the species. For *Agrostis s.* the application of 30Po and 60Po resulted in a similar increase from that of the control plant values. For the *Dactylis g.* and *Holcus l.* there was an increase in content with increasing levels of Po added to the soil.

For *Agrostis s.* there was no significant affect of P treatment on the root P content. The root content of both Dactylis *g.* and *Holcus l.* was significantly increased by the addition of Po to the soil. For *Dactylis g.* the increase due to the addition of 30Po and 60Po was not significantly different. In the case of *Holcus l.* there was an increase with the addition of 30Po and a further increase with the 60Po treatment.

There was no significant affect of benlate on root or shoot content for any of the species. This was as expected as there was no significant affect of benlate application on the shoot and root dry weight or P concentration as discussed above. The P content depends on the dry weight and P concentration.

It was necessary to consider shoot and root dry weight, P concentration and P content together in order to obtain a clear picture of the affect of P treatment on plant growth. This was the approach proposed by Jarrell and Beverly (1981). Changes in shoot and root dry weight, P concentration and plant P content are presented together in Table 4.41.

Table 4.41: changes in the plant variables, shoot and root dry weight, P concentration and P content as a result of P treatment for *Agrostis stolonifera, Holcus lanatus* and *Dactylis glomerata.* The symbol ↑ indicates an increase in the variable value as a result of P treatment and 0 indicates no change in the variable as a result of P treatment.

Plant species	**Change in P content of shoot/root**	**Change in dry weight of shoot/root**	**Change in P concentration of shoot/root**
Agrostis s. – shoot	↑	↑	0
Agrostis s. – root	↑	↑	0
Holcus l. – shoot	↑	↑	↑
Holcus l. – root	↑	↑	↑
Dactylis g. – shoot	↑	↑	↑
Dactylis g. – root	↑	↑	↑

When P was applied to *Agrostis s.* there was an increase in shoot/root dry weight but there was also an equal increase in the P acquisition as indicate by the shoot/root P content. Therefore overall there was no change in the P concentration of the shoot/root. When P was applied to *Holcus l.* or *Dactylis g.* there was an increase in shoot/root dry weight but there was a larger

increase in the shoot/root P content. This therefore resulted in an increase in shoot/root P concentration. At the 30Po and 60Po treatments used in this experiment *Holcus l* and *Dactylis g.* were capable of a larger P acquisition than biomass accumulation.

The importance of Po as a source of P to plants depends on the amount and forms of Po and the presence of appropriate phosphatases to mineralise Po (Fox and Comerford, 1992). These dry weight and P content results indicate that IHP has been a source of P available and used by the plants. An increase in the soil IHP content has directly led to an increase in dry weight and P content of the plant tissue. The increases with the addition of P to the soil would indicate that P was a limiting nutrient and this has been corrected by organic P addition to the soil.

As expected there was a decrease in the Tp levels from those present at the start of the experiment to those found in the post harvest soil for all species. This would indicate that there was uptake of phosphate by the plants. There was no change in the soil inorganic P concentration from that present in the start soil to that of the postharvest soil. No depletion due to plant uptake or increase due to mineralisation of Po to Pi was detected in soil Pi concentration. There was however a decrease in the soil organic P concentration. The decrease in total P concentration was matched by a decrease in soil organic P concentration. This indicated that it was the Po pool that had contributed to the P taken up by the plants. The contribution of Po to plant P uptake was proportional to the rate at which Po is mineralised in the soil (Harrison 1987) It is likely that this Po was mineralised by the phosphatase enzymes before uptake. Any Pi resulting from this mineralisation was taken up by the plants and did not enter the Pi pool. The Po pool which is often though to play an insignificant role in plant P nutrition has been shown here to provide P for plant growth. The depletion in soil total and organic P occurred to equal extents in the inner and outer soil. The inner soil was accessible to roots and VAM hyphae, but the outer soil was accessible to VAM hyphae only. Both the roots and VAM hyphae and the VAM hyphae alone are depleting the soil Tp and Po reserves to equal extents.

Tarafdar and Jungk (1987) observed a depletion of soil Tp and Po at the root surface but only up to a distance of 1.5 mm, of a number of crop species including clover and wheat. This depletion zone so near to the root was explained as the area in which the root phosphatases acted. They proposed that the depletion of Tp might be explained by the utilisation of P by plants and microorganisms. In the work in this chapter the depletion in soil Tp and Po was from the entire pot.

There was no significant affect of benlate on the soil Tp or Po concentrations of the post harvest soil. A reduction in mycorrhizal presence in the soil and roots due to benlate application had no affect on the Tp or Po depleted from the soil. This was not as expected as VAM play an

important role in P uptake that is then translocated to the plants (Kothari *et al.*, 1990). It was thought that a reduction in VAM as a result of benlate application would result in less depletion from the soil Tp and Po pools. This re-enforces the idea that VAM do not play an important part in plant P nutrition at such low Po concentrations, 30Po and 60Po as used in this experiment. There was no interaction between benlate treatment and location of the soil that had a significant affect on the Tp or Po depleted from the soil. A reduction in VAM presence as a result of benlate application would be expected to reduce the depletion observed from the outer soil as no roots can access this outer soil only VAM hyphae. This however was not observed.

Correlations were carried out between the soil acidic and alkaline monoesterase, soil diesterase and root acidic monoesterase and between these measurements and the plant growth variables shoot and root dry weight, P concentration and P content. However no significant correlations were found.

Generally plant phosphatases are not good indices of plant yield or P status (Speir and Cowling, 1991). Speir and Cowling (1991) found the number of significant relationships between yield parameters and enzyme activities was small and decided that there was little prospect of using plant phosphatase activities to predict pasture productivity in field situations. Some studies have shown strong relationships between root surface phosphatase and P uptake whereas others have found inconsistent relationships (Johnson *et al.*, 1999). In the work presented in this chapter no relationship was found between root surface phosphatase activity and shoot and root P concentrations. Like Johnson *et al.*, (1999) no significant correlation between root surface phosphatase activity and the P status of the soils.

4.5 SUMMARY

- Soil acidic monoesterase activity was higher than the alkaline monoesterase activity as expected for this acidic soil.
- Soil diesterase activity showed a higher activity than the soil acidic or alkaline monoesterase.
- Soil acidic or alkaline monoesterase activity and soil diesterase activity was not affected by the addition of benlate or IHP to the soil.
- Root acidic monoesterase was indicated as being the most important enzymes of those analysed with respect to the use of the Po compound, IHP, for growth by the species *Agrostis stolonifera, Holcus lanatus* and *Dactylis glomerata.*
- The addition of IHP to the soil resulted in a decrease in the root acidic monoesterase activity. There was no affect of benlate addition to the soil on root acidic monoesterase activity.
- IHP was shown to be an available source of P for plant uptake for the above species as indicated by the increase in plant biomass and plant P content when added to the soil.
- A decrease in the total P concentration of the soil was matched by a decrease in the Po concentration of the soil indicating that P removed by plants for growth was taken from the Po pool.

CHAPTER 5: THE AFFECT OF NITROGEN AND ORGANIC PHOSPHATE ON PLANT GROWTH

5.1 Introduction

5.1.1 Objectives of Experiment

The objectives of the experiment described in this chapter were:

- to determine the affect of increasing concentrations of soil organic phosphate on plant growth when soil nitrogen concentration was also increased.
- to investigate if the plant availability of soil organic phosphate was affected by an increased nitrogen concentration.
- to determine if an increased concentration of nitrogen in the soil, either alone or in combination with soil organic phosphate, affected root or soil acidic monoesterases.
- to determine if an increased concentration of nitrogen in the soil influenced the infection of roots by vesicular arbuscular mycorrhiza (VAM) and to examine affects on plant growth resulting from any change in infection levels.

From the experiment described in Chapter 2 it is evident that increasing the levels of Po in the soil results in an increase in the growth of plants, but it is not known what affect, if any, increased levels of soil nitrogen would have on this result.

The unique features of this experiment are that, natural soil collected from the PP1 site (for site descriptions refer to Chapter 1 Section 1.3), un-amended with artificial growth substrates and with its indigenous soil microflora, was used. In addition, the plant species, *Agrostis stolonifera*, used in the course of this experiment was grown from seeds collected from plants growing on the PP1 site. Nitrogen was added to the soil in combination with P in an organic form, inositol hexaphosphate.

5.1.2 Experimental Approach

Inositol hexaphosphate (IHP) was chosen as the organic P compound to add to the soil as this compound is one of the principal forms of soil organic phosphate (Stevenson and Cole, 1999). Soil from the PP1 site was chosen as the control soil on which to base the treatments. IHP was added to the soil at increasing concentrations, 100, 200 and 400 mg P/l soil. An inorganic P treatment, based on superphosphate addition, was included at 100mg P/l soil in order to compare the performance of plants grown under equal concentrations of Po and Pi.

The rates of application of nitrogen to agricultural grasslands are not determined by N availability or mineralisation rates, but by the land use and the management of the grassland (MacKenzie and Taureau, 1997). Teagasc recommend application rates varying from 0 kg N/hectare for a good clover sward with extensive grazing to 400 kg N/hectare for grasslands under extensive silage production (Byrne, 1996). They recommend 125 kg N/hectare for

grasslands with two or more silage cuts a year (MacKenzie and Taureau, 1997). A literature review by Byrne (1996) indicated that the average N application was 100 kg/ hectare. This rate was chosen by Byrne (1996) who carried out work on the PP1 site in a study investigating the interactive affects of nutrient addition and cutting intensity on floristic composition and biomass production, with the aim of producing management prescriptions from a nature conservation point of view. Bagayoko *et al.*, (2000) examined changes in nutrient availability in an African soil as affected by fertilisation with calcium ammonium nitrate at rates of 30, 60 and 90 kg/hectare. In a study of the affect of nitrogen application to soil on egg plant growth Lopez-Cantareo *et al.*, (1998) applied N at 150, 225 and 300 kg/hectare. These rates were very high rates in comparison to rates used by other workers. In the experiment described in this chapter N was added in the form of calcium nitrate at a rate of 68 kg/hectare. This rate was chosen as the PP1 site is a semi-natural grassland site and it was of interest to examine how a natural site would respond to N application but at a lower rate than normally applied to soils for agricultural purposes. This rate is lower than that recommended for silage production by Teagasc and is lower than that used by most workers in investigations of N nutrition.

With the aim of separating the contribution of VAM hyphae and plant roots to changes in soil phosphate and monoesterase activity and the uptake of P by plants, the 'growth bag' method was employed in these experiments. (further details on the growth bag method: Chapter 2, Section 2.1.2.1).

It was hypothesised that increasing levels of Po added to the soil, with no additional nitrogen, would result in increased growth of the *Agrostis* plants. The dry matter production of plants is increased by increasing levels of soil nitrogen in a typical response curve (Marschner, 1995). Therefore, the addition of N to the soil was expected to result in a further increase above that found with the addition of P alone. An increase in plant growth as a result of N addition to the soil was expected to result in a greater depletion of soil phosphate, as larger plants will remove more P from the soil. It was expected that the addition of nitrate to the soil would decrease the percentage root length colonisation by VAM because in general additions of nitrate to the soil decreases the presence of these fungi (Haynes, 1986).

5.1.3 Forms of nitrogen

Nitrogen is central to plant growth because of its role in substances such as proteins, nucleic acids, chlorophyll and nucleotides. In these and other organic materials N exists in a chemically reduced state and commonly constitutes 1.5 to 5% of the dry weight of plants (Haynes, 1986). There is no mechanism for long term storage of plant available N in soils, and approximately 97 to 99% of soil N is present in organic forms that are unavailable to plants. Some of this N slowly becomes available through microbial decomposition of soil organic matter with the release of mineral forms of N (Goh and Haynes, 1986).

Nitrogen deficiency symptoms are most closely associated with restricted chlorophyll synthesis. N deficiency is characterised by a reduction in plant growth and a general loss of green colour progressing to yellow coloration particularly in the older leaves. It first occurs in the older leaves as N is translocated to developing areas of the plant under N stress. As the severity of the deficiency increases the entire plant turns yellow and the older leaves turn brown and die and growth ceases. When supply of soil N is adequate plant growth is normally vigorous and plants are a dark green colour. (Goh and Haynes, 1986)

When N is the major growth limiting factor, the simplest response of plants to applied N is an increase in dry matter with increasing rates of N, up to a maximum. The biomass then either stays constant or declines with further increases of N. The magnitude of the positive response to applied N is likely to be primarily dependant on the size of the available and potentially available pool of N in the soil and the demand for N by the crop as determined by its potential dry matter production (Goh and Haynes, 1986).

However responses to N do not necessarily follow a pattern similar to this. In highly fertile soils with an abundant N supply, applications of N can have no affect or even decrease crop yields. If some factor other than N is limiting growth then even if the supply of soil N is low applications of fertiliser N are not likely to have a significant affect on plant growth or yields (Goh and Haynes, 1986).

Nitrogen, which is taken up at the greatest rates of all minerals, is available as either the anion nitrate or the cation ammonium for uptake by the roots of higher plants (Peuke *et al.*, 1998). Bailey (1998) varied the ratio of ammonium to nitrate supplied to perennial rye grass in order to examine the affects on nitrogen absorption and assimilation and plant growth found both forms absorbed at equal rates. Most of the ammonium has to be incorporated into organic compounds in the roots, whereas nitrate is readily mobile in the xylem and can also be stored in the vacuoles of roots, shoots and storage organs with no need for assimilation. Nitrate is then reduced to ammonia in order to be incorporated into organic structures and fulfil its function as a plant nutrient. In most plant species both roots and shoots are capable of nitrate reduction (Marschner, 1995)

Most agronomic research has shown that NH_4^+ and NO_3^- as forms of fertiliser are virtually interchangeable because in most well drained soil conditions NH_4^+ is transformed to NO_3^- within a few days (Goh and Haynes, 1986). The process whereby NH_4^+ is oxidised to NO_3^- is called nitrification.

Generally rhizosphere pH decreases when NH_4^+ forms of N fertiliser are used and increases when NO_3^- forms are used because of the release of H^+ and OH^- and $HCO3^-$ respectively (Zoysa *et al.*, 1998). The forms of N supplied therefore exert a strong influence on the plant availability

of P in the rhizosphere soil through their influence on pH, which determines the rate of dissolution of rock phosphate and P fixation by soil colloids (Zoysa *et al.*, 1998). Acidification of the rhizosphere soil associated with a predominant NH_4^+-N uptake compared to NO_3^- -N resulted in increased availability and uptake of sparingly soluble nutrients such as P from rock phosphate (Ortas *et al.*, 1996; Ruan *et al.*, 2000). P deficiency can cause a restriction in N transport and metabolism (Ameziane *et al.*, 1997). No work was found by this author addressing the relationship between N and Po and any affect N may have on the availability of Po.

Mycorrhizal associations can be important factors influencing N nutrition of plants particularly when the relatively immobile NH_4^+ rather than NO_3^- is the major source of plant available N (Haynes, 1986). VAM fungus might be expected to be most important with respect to the utilisation of NO_3^- as plants associated with them are common in soils with high rates of nitrification. However the high mobility of NO_3^- in soils probably means that mycorrhizal associations have little influence on NO_3^- uptake. In general additions of NO_3^- -N to the soil decreases the VAM infection by reducing the infectivity of mycorrhizal propagules such as spores or infected root fragments)(Haynes, 1986).

5.1.4 Current phosphorus and nitrogen research

Nitrogen and phosphate are two of the most important plant nutrients in agriculture and are frequently applied to soils in the form of fertilisers. Therefore many studies have been carried out to determine the affects of varying the rates of P and N, when applied in combination, on nutrient availability and the growth of a variety of plant species. However the studies to date have concentrated on the application of nitrogen with inorganic P because of the use of Pi as a fertiliser.

Ameziane *et al.*, (1997) studied the affects of nitrate and phosphate nutrition, using combinations at various rates, on chicory tap root development and the development of the chicon that is consumed as a salad. The plants were grown in sand culture and P was applied as inorganic P. Both P and N were supplied as nutrient solutions. P content, sucrose enzymes and amino acid content of roots were used to measure affects of the treatments. With N limitation, P accumulation was decreased by 40-60% over the experimental period. A high N and P fertility was associated with a poor chicon yield, however the presence of low P during vegetative growth moderated the adverse affects of high nitrate and improved the chicon yield. Ryser *et al.*, (1997) examined the growth response of three grass species to varying levels of P and nitrate, but in solution culture, and with inorganic P.

Lopez-Cantareo *et al.*, (1998) studied the affect that application of nitrogen (as nitrate) and phosphorus (as Pi), at various rates, exerted on some parameters of phosphorus metabolism in eggplant. The plants were grown in soil with a total N content of 3.5 g/kg soil but no indication

of total P or organic P content of the soil was given. For a Pi treatment of 240 kg P/hectare, increasing the N treatment from 150 kg/hectare to 300 kg/hectare resulted in an increase in the total P content of the leaf material. The higher P treatment of 360 kg P/hectare resulted in higher leaf total P content for all N treatments. Zoysa *et al.*, (1998) determined the affect of ammonium and nitrate additions on the mobilisation and plant uptake of P from rock phosphate in the rhizosphere of tea. The soil was collected in Sri Lanka but no information was provided on the total N or P content or the organic P content of the soil. Both forms of N significantly increased shoot dry matter yield over the control treatment with no N additions.

Ruan *et al.*, (2000) examined the affect of nitrogen form, nitrate or ammonium, and phosphorus source on the growth, nutrient uptake and rhizosphere soil property of tea (*Camellia sinensis*). The phosphate was applied as soluble $Ca(H_2PO_4)_2$ or the less soluble rock phosphate. The plants were grown in a sand soil mix. The soil had a total N concentration of 0.76 g/kg soil but no total P or organic P concentrations were provided. The leaf dry matter production of tea was significantly greater in the treatments with NH_4^+ than NO_3^-. Addition of phosphorus as either source did not influence dry matter production. With soluble P addition shoot P concentration was greater in the NH_4^+ than in the NO_3^- treatment. With rock phosphate addition the shoot P concentration was hardly affected by the nitrogen form. Bagayoko *et al.*, (2000) measured changes in soil pH and nutrient availability of a West African soil, as affected by distance from the root surface and by fertilisation with inorganic superphosphate and calcium ammonium nitrate at various rates. Regardless of the level of mineral fertiliser applied the soil pH increased by two units from the bulk to the rhizosphere soil. Changes in pH lead to changes in nutrient availability close to the roots. P availability was expressed as water extractable P and Bray P.

Gordon *et al.*, (2001) investigated the affects of nitrogen and phosphorus on high Arctic heath vegetation using combinations of nitrogen and phosphorus as nitrate solution and inorganic P solution. No background soil nutrients for this field site were provided. Nitrogen decreased lichen cover but increased bryophyte presence. The combination of nitrogen and phosphorus altered species composition. No work was found by this author examining the affect of nitrogen application in combination with organic P.

Hodge *et al.*, (2000) examined the ability of an arbuscular mycorrhizal fungal inoculum added to soil already containing an indigenous AM fungal community to enhance N capture by *Plantago lanceolata* from a complex organic patch. Plants were grown in sand soil mixture. Although the added arbuscular mycorrhizal fungal inoculum benefited *Plantago* seedlings in terms of P content of tissues it did not increase total N capture or affect the form in which N was captured by P roots.

Liu *et al.*, (2000) conducted a study to evaluate the affect of N and P supply levels on mycorrhizal formation and nutrient uptake in corn plants. A sand soil mix was used as the growth substrate. N was applied as ammonium nitrate (3 rates 0, 70, 140 mg N/kg soil) and P as potassium phosphate (0, 40, 80 mg P/kg soil). Phosphorus application decreased the percentage of arbuscular mycorrhizal fungi colonisation. Mycorrhizal plants had a significantly higher shoot dry weight and P concentration than non-mycorrhizal plants. Nitrogen application significantly increased the shoot dry weight of mycorrhizal and non-mycorrhizal plants. Under conditions where N was sufficient in soil P utilisation in biomass production was increased. This resulted in an increase in growth and dilution of P in the tissues.

Ortas *et al.*, (1996) examined the affect of ammonium and nitrate N and various rates of Pi application on P uptake by sorghum plants with and without mycorrhizal inoculation. Soils from two sites were used. Monocalcium phosphate was supplied at 0, 20, 40, and 60 mg P/kg soil. N was added as ammonium sulphate or calcium nitrate at 120 mg N/kg soil. In all P treatments ammonium as the N source resulted in higher dry matter than nitrate as the N source. Inoculation with VAM enhanced differences between N sources. Sorghum plants infected with mycorrhiza had nearly 3 times higher shoot dry matter yields than non-inoculated controls. The same was true for total P content. Mycorrhizal infection increased with increasing P application up to 40 mg P/kg soil then slightly decreased. Ammonium resulted in a higher percentage of VAM infection than nitrate in soil from the first site. For the second site the opposite was true and nitrate resulted in a higher percentage of VAM infection than ammonium.

5.2 Methods

5.2.1 Collection and storage of soil and seeds

As described in Chapter 2, Section 2.2.1

5.2.2 Preparation of soil

As described in Chapter 2, Section 2.2.2

5.2.3 Seed Germination and growth conditions

All seeds were germinated in seed trays containing soil from the PP1 site. After 5/6 days seedlings of uniform size were selected and transplanted into the experimental pots (4-inch plastic plant pots). The 'growth bag' method was used in this experiment in order to distinguish between contributions of roots and of mycorrhizal hyphae to the uptake of P by plants. (further details on the growth bag method: Chapter 2, sections 2.1.2.1 and 2.2.3). All pots were placed in a heated greenhouse maintained at a constant 15°C in a random arrangement. An automatic watering system using capillary matting was in place.

5.2.4 Phosphate addition and experimental layout

This experiment was based on the PP1 site soil and had a control (no P addition) and 4 P treatments. The P treatments are: P added at 3 rates 100, 200 and 400 mg P/l soil in the form of Po and P added at 1 rate 100 mg P/ l soil in the form of Pi. The Pi treatment was included in order to compare the affect on plant growth of Po and Pi at the same concentration. This was carried out for soil treated and untreated with N in the form of nitrate as $Ca(NO_3)_2.4H_2O$ at a rate of 68 mg N /l soil (equivalent to 68 kg N / hectare). Each control and treatment had 3 replicate pots, giving a total of 30 pots (Table 5.1). The total N content of the PP1 soil before any addition of N was 5271 mg N/ l soil (equivalent to 5271 kg N / hectare).

Table 5.1: Experimental layout for the phosphate and nitrogen experiment. There were 3 replicates per treatment. This layout was replicated for soil treated with $Ca(NO_3)_2.4H_2O$ at a rate of 68 mg N/l soil.

P Treatments	Conc P added	Source of P
Control (PP1 soil)	no P addition	
100Po (PP1 soil)	100 mg P/l soil	IHP
200Po (PP1 soil)	200 mg P/l soil	IHP
400Po (PP1 soil)	400 mg P/l soil	IHP
100Pi (PP1 soil)	100 mg P/l soil	Pi fertiliser

This experiment was carried out using the species *Agrostis stolonifera*.

Organic phosphate was added in the form of phytic acid (myo-inositol hexakis [dihydrogen phosphate], Sigma Chemicals) which was added to the soil as a powder and was thoroughly mixed with soil before the soil was put into the 'growth bags' and plant pots. Inorganic phosphate was added in the form of commercially available Super phosphate fertiliser in the form of a powder. Nitrate was added to the soil in the form of $Ca(NO_3)_2.4H_2O$ at the rate of

68mg N /l soil, as a solution and was thoroughly mixed with the soil. Each pot was filled with 400 g soil (200 g in 'growth bag', 200 g in surrounding area) of the appropriate treatment soil. One seedling was placed in each pot.

5.2.5 Harvesting

Experiments were harvested 9 weeks after transplanting seedlings into treatment pots. On harvesting each pot, soil from inner root compartment and outer hyphal compartment was put into separate labelled plastic bags and stored at 4 °C until analysis. All soil analyses: soil phosphatase activity and soil total, inorganic and organic P, were carried out for inner and outer soil for each replicate pot.

Above ground plant material was excised from the roots and dried for dry weight measurements and total P analysis. Roots were stored in distilled water until root phosphatase analysis was carried out.

5.2.6 Assay for soil acidic phosphomonoesterase activity

as described in Chapter 4, Section 4.2.6.1

5.2.7 Assay for root acidic phosphomonoesterase activity

as described in Chapter 4, Section 4.2.6.2

5.2.8 Shoot dry weights

As described in Chapter 2, Section 2.2.11

5.2.9 Shoot material digest for total P

As described in Chapter 2, Section 2.2.12

5.2.10 Total, inorganic and organic P levels in soil

As described in Chapter 2, Section 2.2.8

5.2.11 Statistical procedures

All statistical analysis was carried out using the statistics package Data Desk, version 6.0. All data was checked for normality before statistical analysis. Where necessary data was transformed to obtain a normal distribution. Soil phosphatase and phosphate data was analysed using a three way ANOVA with P treatment, N treatment and location of the soil (ie. inner or outer soil) as the three factors. Plant dry weight and P concentration and content data were analysed using a two way ANOVA with P treatment and N treatment as the two factors. Correlations were carried out using a Pearson Product Moment Correlation.

5.3 RESULTS

In the description of the results that follows, P treatment refers to the addition of organic phosphate to the soil at the 3 concentrations employed and the addition of inorganic phosphate to the soil at the single concentration employed. The 3 organic phosphate concentrations used were 100, 200 and 400 mg P/l soil abbreviated to 100Po, 200Po and 400Po for the purpose of graphs and the results and discussion section. The single inorganic phosphate concentration used was 100 mg P/l soil abbreviated to 100Pi. The control treatment was PP1 soil with no phosphate addition.

N treatment refers to the addition of nitrate to the soil at a rate of 68 mg N/l soil. Start soil refers to the soil as prepared at the start of the experiment, with the relevant P and N treatment, but before contact with any plants. Post harvest soil refers to soil from which the plants have been harvested after their 9-week growth period. The term 'location' refers to either inner root compartment soil or the outer hyphal compartment soil, which are abbreviated to inner and outer soil respectively.

5.3.1 Shoot Dry Weight

The shoot dry weight data was presented in Fig 5.1. A two way ANOVA was carried out to determine the affect of P treatment and N treatment on shoot dry weight. This ANOVA was presented in Appendix 5, Table 1.

There was a highly significant P affect (F ratio 12.08***) on the shoot dry weight. All other treatments and interactions were not significant.

Fig 5.1 presented shoot dry weight data for all plants. From Fig 5.1 there was an increase in shoot dry weight with increasing concentration of P in the soil for both N treated and untreated plants. The shoot dry weight of the plants treated with 100Pi was similar to that of the 200Po treatment plants. This significant affect of P treatment was examined using a Scheffe post hoc test (Table 5.2).

Table 5.2: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot dry weight for the *Agrostis stolonifera* experiment.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control < 200Po	**
control < 400Po	***
control < 100Pi	***
100Po - 200Po	ns
100Po < 400Po	*
100Po - 100Pi	ns
200Po - 100Pi	ns
200Po - 400Po	ns
400Po - 100Pi	ns

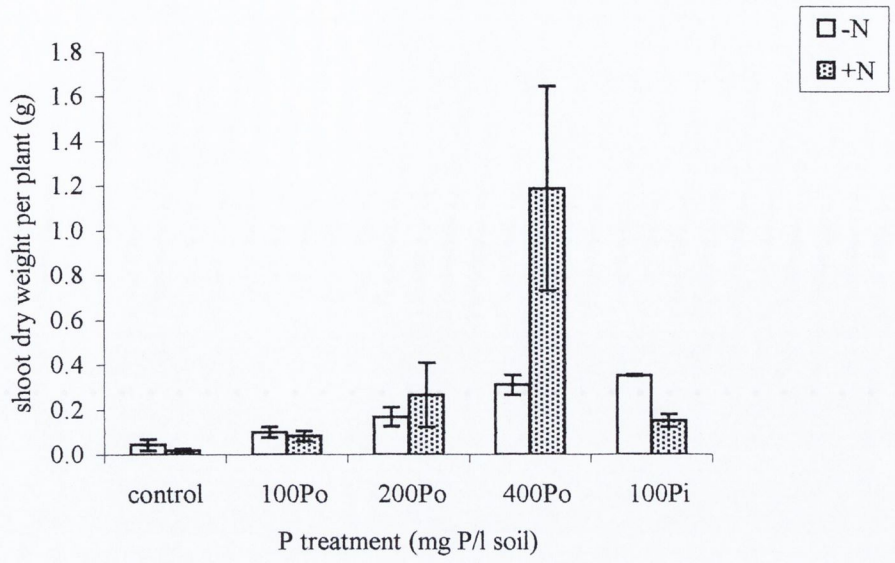


Fig 5.1 : Shoot dry weight for the control and P treatment plants of *Agrostis stolonifera* for plants grown in soil without (-N) and with (+N) N treatment. Each value represents the mean +/- SE for 3 separate determinations.

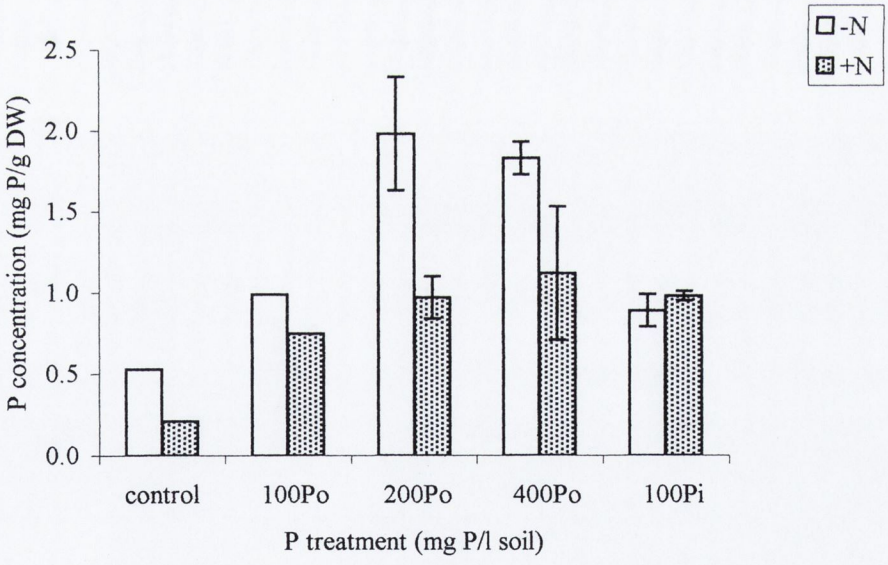


Fig 5.2 : Shoot P concentration for the control and P treatment plants of *Agrostis stolonifera* for plants grown in soil without (-N) and with (+N) N treatment.Each value represents the mean +/- SE for 3 separate determinations.

5.3.2 Shoot P Concentration

The shoot P concentration data was presented in Fig 5.2. A two way ANOVA was carried out to determine the affect of P treatment and N treatment on shoot P concentration. This ANOVA was presented in Appendix 5, Table 2.

There was a significant P affect (F ratio 4.69*) on shoot P concentration. All other treatments and interactions were not significant.

From Fig 5.2 there was an increase in shoot P concentration with the 100Po, 200Po and 400Po treatments. The concentration of the 100Pi treatment shoots is similar to that of the 100Po treatment plants. The N treated and untreated plants follow this pattern. This significant affect of P treatment on shoot P concentration was examined using a Scheffe post hoc test (Table 5.3).

Table 5.3: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P concentration for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	ns
control < 200Po	*
control < 400Po	*
control - 100Pi	ns
100Po - 200Po	ns
100Po - 400Po	ns
100Po - 100Pi	ns
200Po - 400Pi	ns
200Po - 100Pi	ns
400Po - 100Pi	ns

5.3.3 Shoot P Content

The shoot P content data was presented in Fig 5.3. A two way ANOVA was carried out to determine the affect of P treatment and N treatment on shoot P content. This ANOVA was presented in Appendix 5, Table 3.

There was a significant P affect (F ratio 25.14***) on the shoot P content. All other treatments and interactions were not significant.

From Fig 5.3 there was an increase in shoot P content with increasing level of Po in the soil, from 100Po to 200Po with a further increase for the 400Po treatment. The shoot P content of the 100Pi plants was similar to that of the 100Po plants. The N treated and untreated plants followed this pattern. This significant affect of P treatment on shoot P content was examined using a Scheffe post hoc test (Table 5.4).

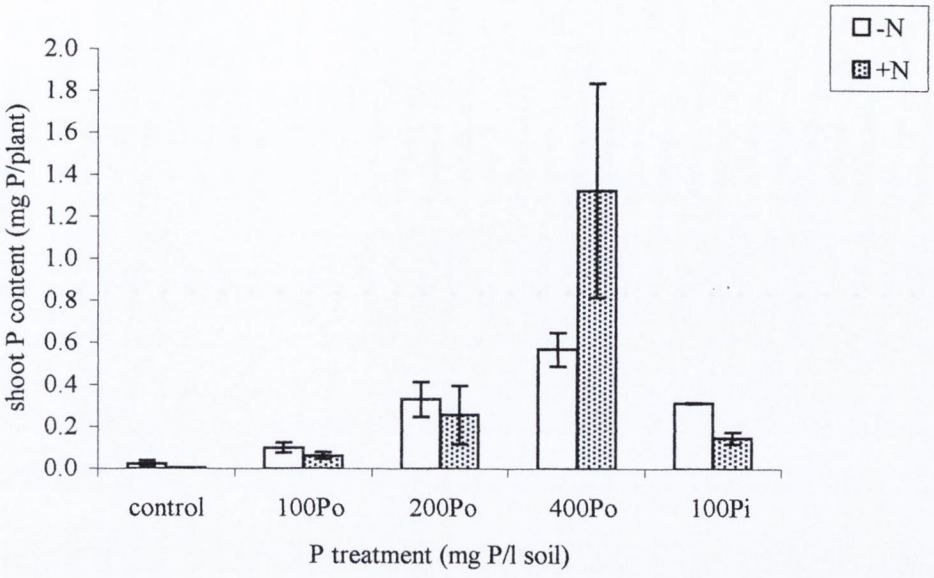


Fig 5.3: Shoot P content for the control and P treatment plants of *Agrostis stolonifera* for plants grown in soil without (-N) and with (+N) N treatment. Each value represents the mean +/- SE for 3 separate determinations

Table 5.4: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on shoot P content for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control < 100Po	**
control < 200Po	***
control < 400Po	***
control < 100Pi	***
100Po - 200Po	ns
100Po < 400Po	**
100Po - 100Pi	ns
200Po - 400Po	ns
200Po - 100Pi	ns
400Po - 100Pi	ns

Phosphorus Use Efficiency

Phosphorus use efficiency (PUE) is defined as (Sanginga *et al.*, 2000):

g shoot dry weight / mg P in shoots

PUE was calculated for the shoots of the *Agrostis* plants under different P and N treatments. The data is presented in Table 5.5.

Table 5.5: Phosphorus use efficiency (g shoot dry weight per mg P in shoots) for *Agrostis* shoot material for plants grown under various combinations of P and N treatments.

	P use efficiency	
P treatment (mg P/l soil)	**-N**	**+N (68 mg N/ l soil)**
Control	1.89	4.76
100Po	1.01	1.33
200Po	0.51	1.03
400Po	0.55	0.90
100Pi	1.12	1.02

There was a decrease in PUE from that for the control plants with increasing levels of Po added to the soil. The PUE of the Pi treatment plants is below that of the control plants. Within P treatments the addition of N results in an increase in PUE. A two factorial ANOVA was carried out to determine the affect of P treatment and N treatment on PUE. The affects on PUE by the P and N treatment described were statistically significant (Appendix 5, Table 4).

5.3.4 Soil Phosphatase Activity

The soil acidic monoesterase data was presented in Fig 5.4 (a, b). A three way ANOVA was carried out to determine the affect of P treatment and N treatment and location of soil on soil acidic monoesterase activity. This ANOVA was presented in Appendix 5, Table 5.

There was a significant P affect (F ratio 2.67*) on the soil acidic monoesterase activity. All other treatments and interactions were not significant.

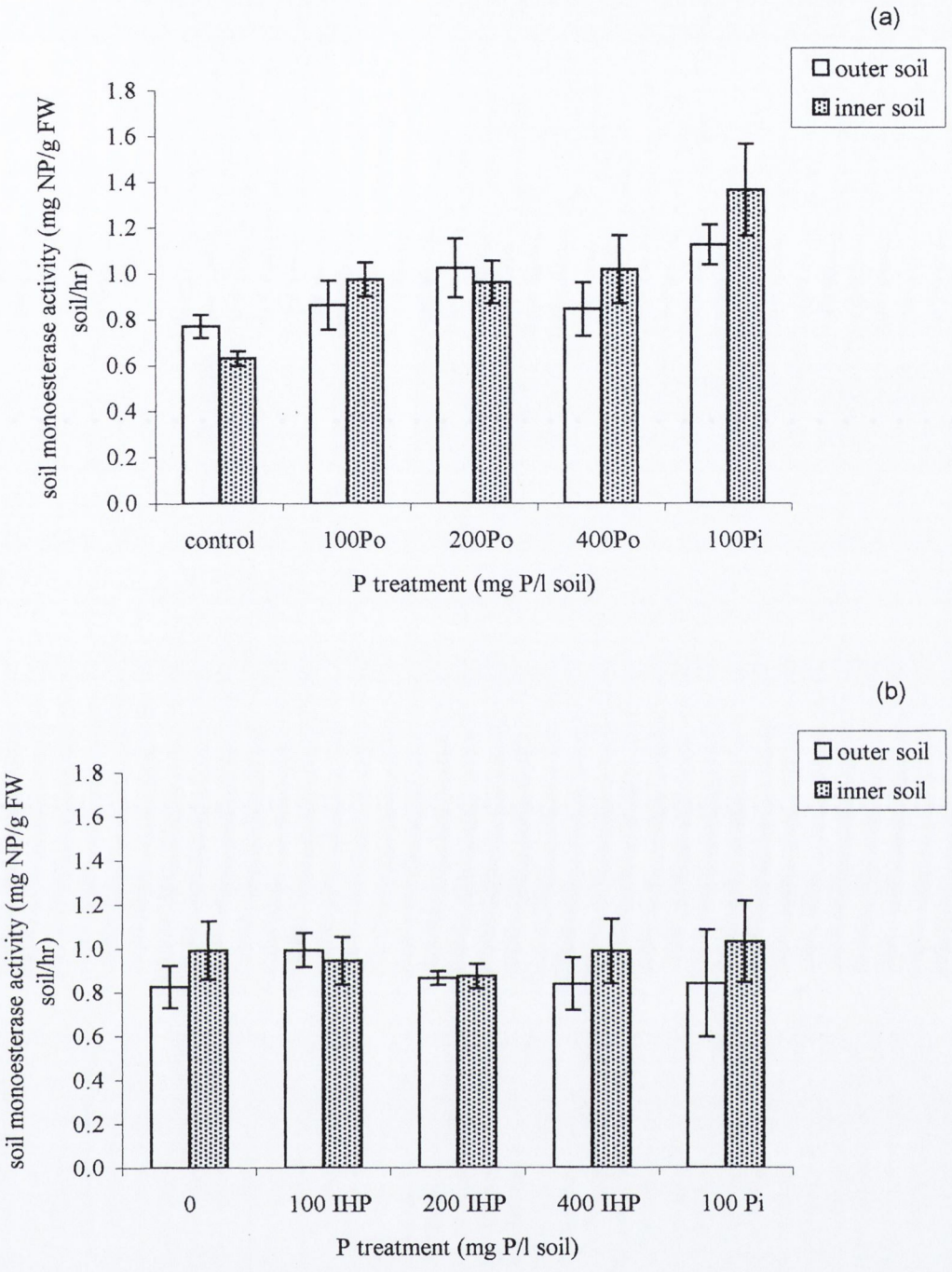


Fig 5.4: Soil acidic monoesterase activity for the inner and outer soils for the control and P treatments for the *Agrostis stolonifera* experiment for (a) soil with no N treatment and (b) soil with N treatment at 68 mg N / l soil. Each value represents the mean +/- SE of 3 separate determinations.

Figure 5.4 (a) presented soil acidic monoesterase activity data for soil not treated with N. There was a slight increase from the phosphatase activity level in the control soil to that in the Po treated soils. All the Po treatments resulted in an increase of similar magnitude. The highest increase in phosphatase activity was for the 100Pi treatment. Figure 5.4 (b) presented soil acidic monoesterase activity data for soil treated with N. There was no increase in activity level from that present in the control soil to that in the soils treated with Po at any concentration. The 100Pi treatment soil had a slightly higher activity level compared to the control soil.

The significant affect of P treatment of soil monoesterase activity was examined using a Scheffe post hoc test (Table 5.6).

Table 5.6: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on soil acidic monoesterase activity for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant)

Comparison	P value
control - 100Po	ns
control - 200Po	ns
control - 400Po	ns
control < 100Pi	*
100Po - 200Po	ns
100Po - 400Po	ns
100Po - 100Pi	ns
200Po - 400Po	ns
200Po - 100Pi	ns
400Po - 00Pi	ns

5.3.5 Root Phosphatase Activity

The root acidic monoesterase data was presented in Fig 5.5. A two way ANOVA was carried out to determine the affect of P treatment and N treatment on root acidic monoesterase activity. This ANOVA was presented in Appendix 5, Table 6.

There was a significant N affect (F ratio 12.08**) on root acidic monoesterase activity. There was an increase in root monoesterase activity on the addition of N to the soil for all the Po treatments and the Pi treatment (Fig 5.5). All other treatments and interactions were not significant.

5.3.6 Soil phosphate

The soil total P (Tp), inorganic P (Pi) and organic P (Po) concentration data were presented in Fig 5.6 (a, b), 5.7 (a, b) and 5.8 (a, b) respectively. Three way ANOVAs were carried out to determine the affect of P treatment, N treatment and location of soil on soil Tp, Pi and Po concentration. These ANOVAs were presented in Appendix 5, Tables 7, 8 and 9.

There was a significant P affect on soil Tp (F ratio 6.07***) and soil Po (F ratio 4.71**). There was also a significant location of soil affect on soil Tp (F ratio 10.94**) and soil Po (F ratio

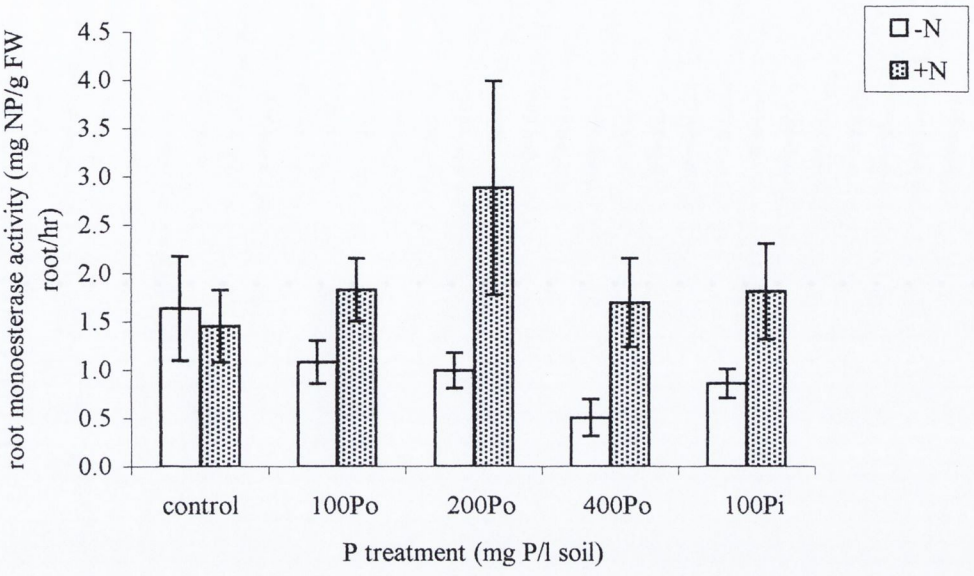


Fig 5.5: Root acidic monoesterase activity for the control and P treatment plants for the *Agrostis stolonifera* experiment for soil without (-N) and with (+N) N treatment. Each value represents the mean +/- SE of 3 separate determinations.

13.19***) There was a significant P - N interaction for Tp (F ratio 5.03**) and Po (F ratio 4.28**). All other treatments and interactions were not significant.

It was of interest to determine any affect of location of soil may have had on soil phosphate concentrations. This will show if there was a significant difference in phosphate concentrations between the inner and outer postharvest soils. As significance difference will indicate that more P was depleted from either the inner root compartment or the outer mycorrhizal compartment. The affects of P treatment and N treatment on postharvest soil P concentrations were also included in the ANOVA as it was necessary to determine if there is any interaction between the location of the soil and either of these variables. An interaction would indicate that the affect of the location of the soil on soil P concentration would change depending on the P or N treatment.

Total P

Fig 5.6 (a) presented soil Tp concentration data for soil untreated with N. Fig 5.6 (b) presented soil Tp concentration data for soil treated with N.

From Fig 5.6 (a, b) there was an expected increase in start soil Tp levels with increasing levels of Po added to the soil. A pooled t test (at $p = 0.05$) was carried out to determine if there was a significant difference between the start soil and post harvest soil Tp concentrations. There was a significant decrease in the Tp concentrations from those present in the start soils to the post harvest Tp concentration for the three Po treatment soils and the one Pi treatment soil (Fig 5.6 a, b). This depletion was greater in the outer soils (Fig 5.6 a, b). This affect of location of soil on the Tp concentration was significant (Appendix 5, Table 7). That is, the Tp concentrations in the outer mycorrhizal zone have been depleted significantly more than in the inner root zone.

From Fig 5.6 (a, b) there was a slight increase in the Tp concentration of the postharvest soils with increasing levels of Po added to the soil at the start of the experiment. This significant affect of P treatment was examined using a Scheffe post hoc test (Table 5.7).

Table 5.7: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Tp concentration for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control - 200Po	ns
control < 400Po	**
control - 100Pi	ns
100Po - 200Po	ns
100Po - 400Po	ns
100Po - 100Pi	ns
200Po - 400Po	ns
200Po - 100Pi	ns
400Po > 100Pi	**

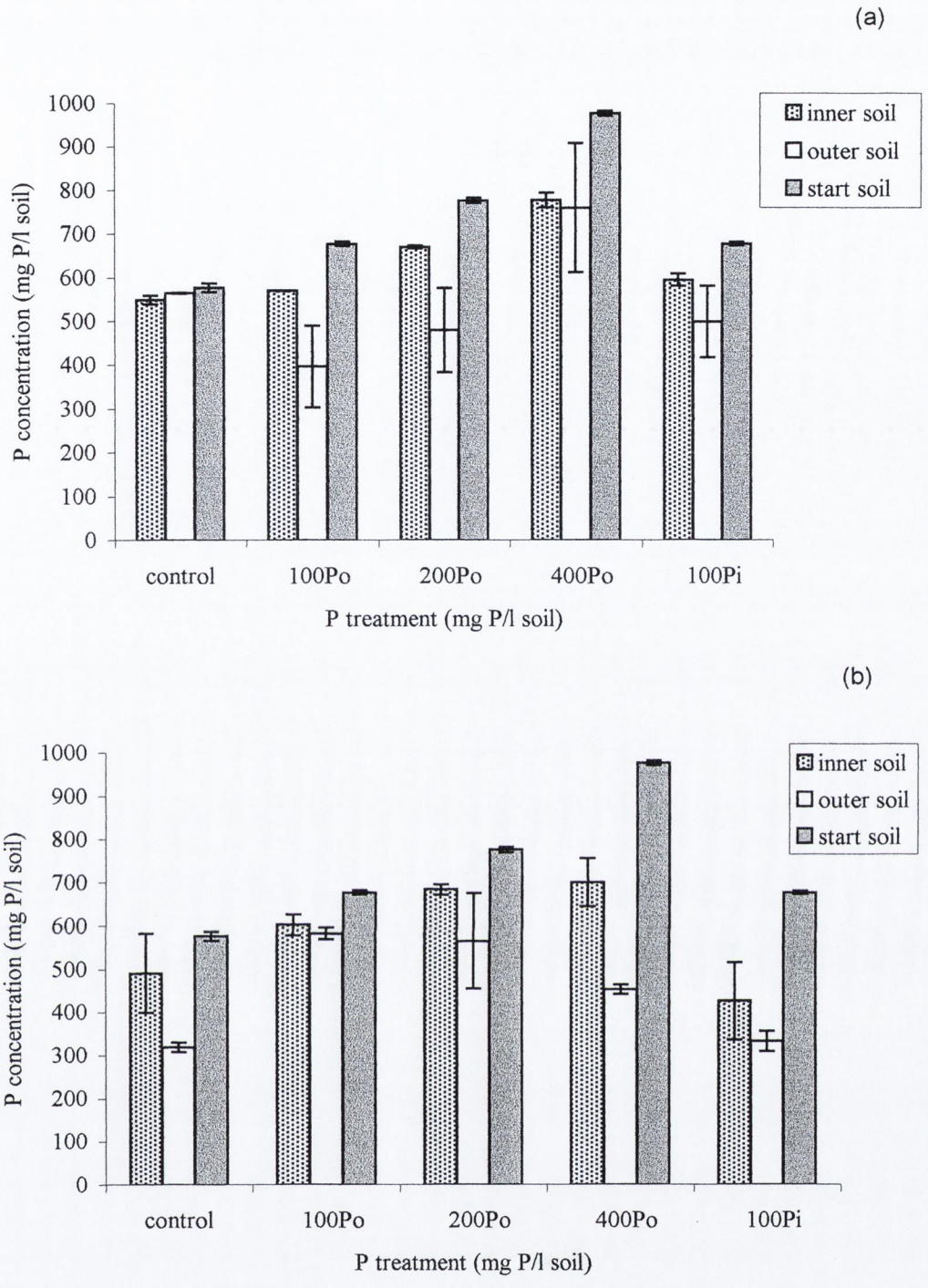


Fig 5.6: soil total phosphate concentration for the control and P treatment soils of the *Agrostis stolonifera* experiment for (a) soil with no N treatment and (b) soil with N treatment. Each value represents the mean +/- SE of 3 separate determinations.

The significant interaction between P treatment and the N treatment was examined using a. Scheffe post hoc test (Table 5.8).

Table 5.8: probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors N treatment and P treatment, that affected soil Tp concentration for *Agrostis stolonifera* experiment. Soil without and with N application are indicated as –N and +N respectively.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-N, control - +N, control	ns
-N, 100Po - +N, 100Po	ns
-N, 200Po - +N, 200Po	ns
-N, 400Po - +N, 400Po	ns
-N, 100Pi - +N, 100Pi	ns
-N, control - -N, 100Po	ns
-N, control - -N, 200Po	ns
-N, control - -N, 400Po	ns
-N, control - -N, 100Pi	ns
+N, control - +N, 100Po	ns
+N, control - +N, 200Po	ns
+N, control - +N, 400Po	ns
+N, control - +N, 100Pi	ns

Table 5.8 indicates that there are no significant differences between soil Tp at all combinations of P and N treatment.

Inorganic P

Fig 5.7 (a) presented soil Pi concentration data for soil untreated with N. Fig 5.7 (b) presented soil Pi concentration data for soil treated with N.

From Fig 5.7 (a, b) there was no difference in start soil Pi levels with increasing levels of Po added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Pi concentrations. There were no significant differences (Fig 5.7 a, b).

Organic P

Fig 5.8 (a) presented soil Po concentration data for soil untreated with N. Fig 5.8 (b) presented soil Po concentration data for soil treated with N.

From Fig 5.8 (a, b) there was an increase in start soil Po levels with increasing levels of Po added to the soil. A pooled t test (at p .05) was carried out to determine if there was a significant difference between the start soil and post harvest soil Po concentrations. There was a statistically significant decrease in the Po concentrations from those present in the start soils to the post harvest Po concentration for the three Po treatment soils (Fig 5.8 a, b). Where depletion of Po occurs, this was greater in the outer soils (Fig 5.8 a, b). This affect of location of soil on

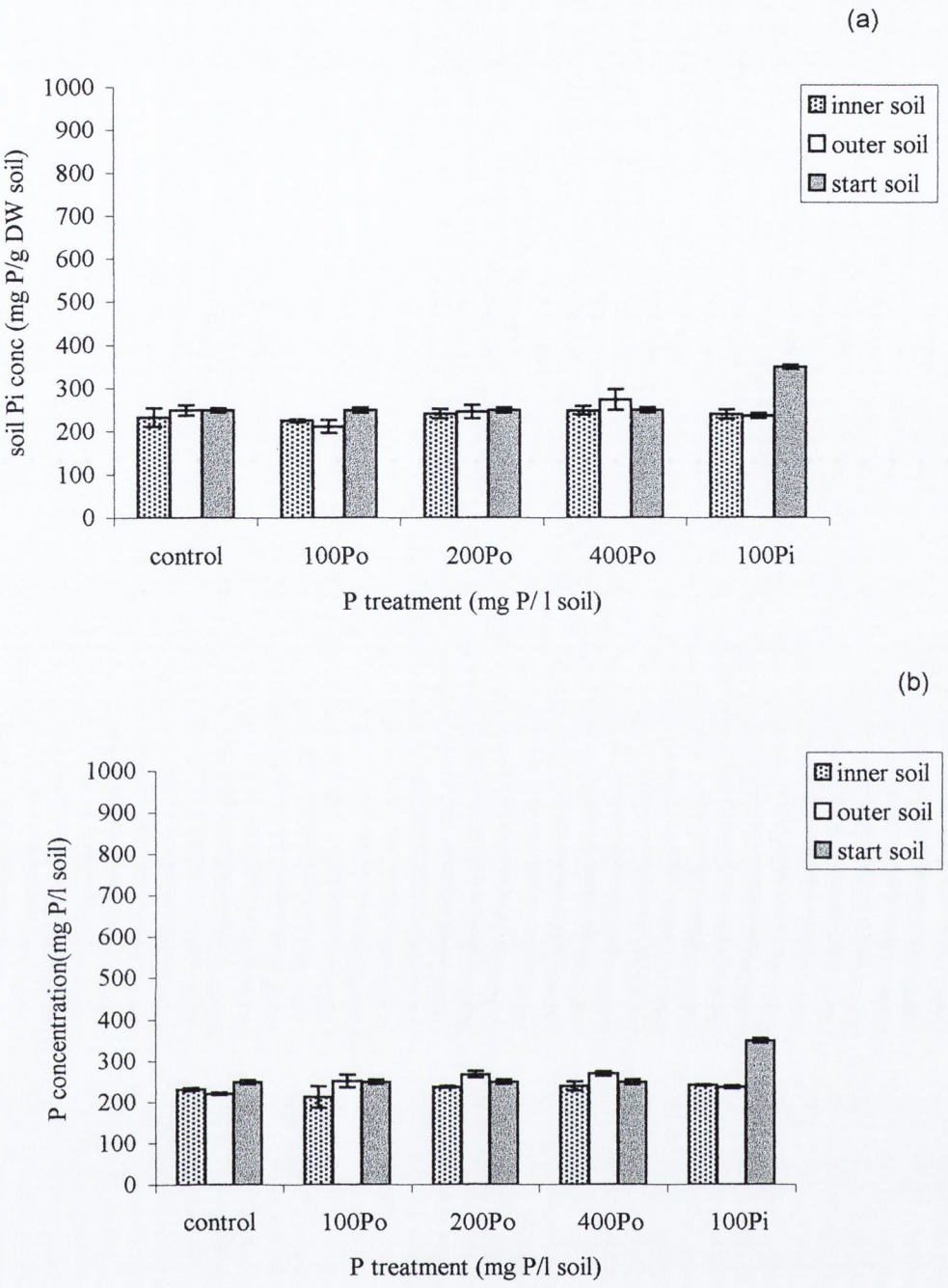


Fig 5.7: soil inorganic phosphate concentration for the control and P treatment soils of the *Agrostis stolonifera* experiment for (a) soil with no N treatment and (b) soil with N treatment. Each value represents the mean +/- SE of 3 separate determinations.

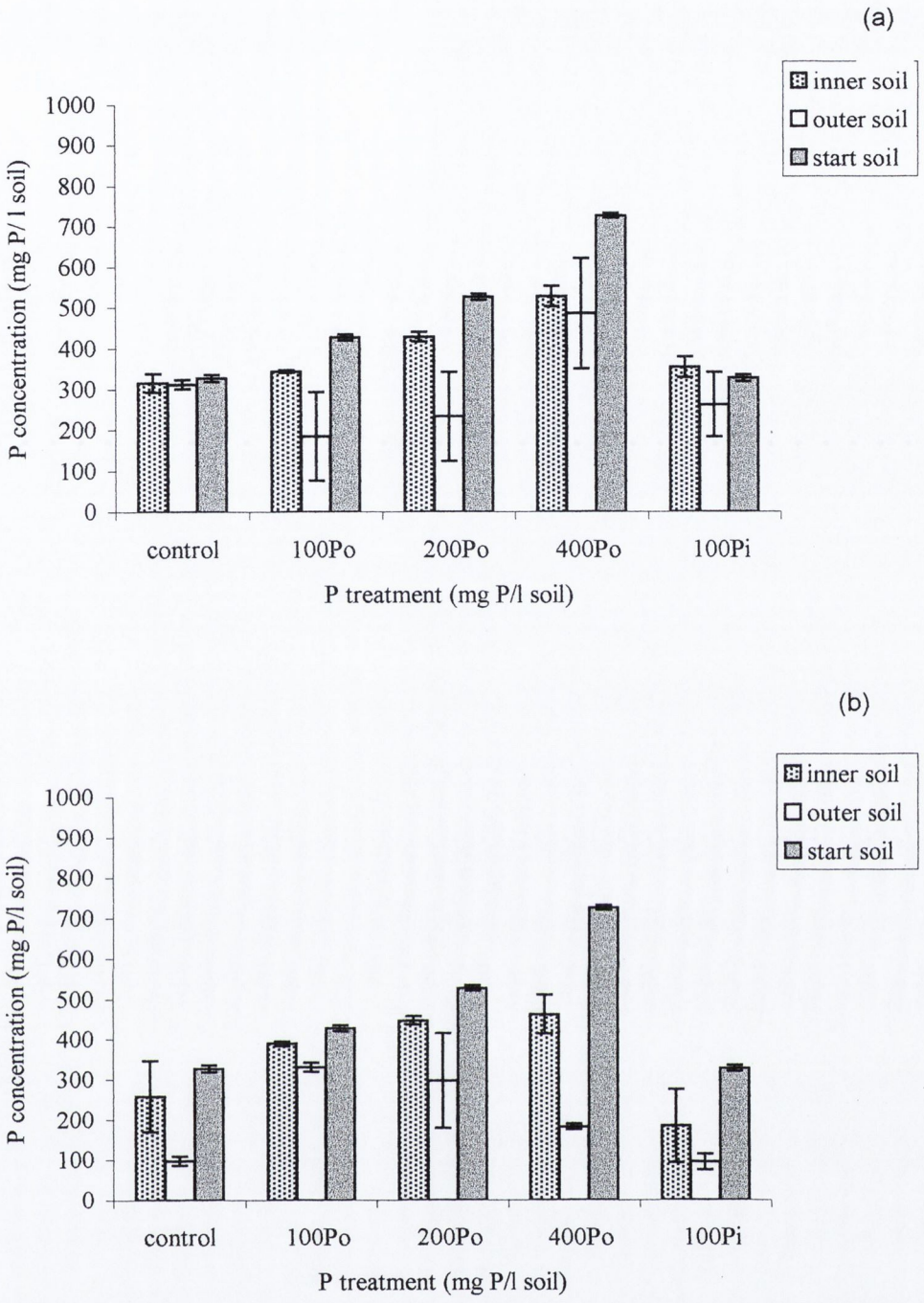


Fig 5.8: soil organic phosphate concentration for the control and P treatment soils of the *Agrostis stolonifera* experiment for (a) soil with no N treatment and (b) soil with N treatment. Each value represents the mean +/- SE of 3 seperate determinations.

the Po concentration was significant (Table 5.12). That is, the Po concentrations in the outer mycorrhizal zone have been depleted significantly more than in the inner root zone.

From Fig 5.8(a, b) there was a slight increase in the Po concentration of the postharvest soils with increasing levels of Po added to the soil at the start of the experiment. This significant affect of P treatment was examined Scheffe post hoc test (Table 5.9).

Table 5.9: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on postharvest soil Po concentration for the *Agrostis stolonifera* experiment.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control - 200Po	ns
control < 400Po	*
control - 100Pi	ns
100Po - 200Po	ns
100Po - 400Po	ns
100Po - 100Pi	ns
200Po - 400Po	ns
200Po - 100Pi	ns
400Po > 100Pi	**

The significant interaction between P treatment and the N treatment was examined using a Scheffe post hoc test (Table 5.10).

Table 5.10: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors N treatment and P treatment, that affected soil Po concentration for *Agrostis stolonifera* experiment. Soil without and with N application are indicated as –N and +N respectively.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-N, control - +N, control	ns
-N, 100Po - +N, 100Po	ns
-N, 200Po - +N, 200Po	ns
-N, 400Po - +N, 400Po	ns
-N, 100Pi - +N, 100Pi	ns
-N, control - -N, 100Po	ns
-N, control - -N, 200Po	ns
-N, control - -N, 400Po	ns
-N, control - -N, 100Pi	ns
+N, control - +N, 100Po	ns
+N, control - +N, 200Po	ns
+N, control - +N, 400Po	ns
+N, control - +N, 100Pi	ns

Table 5.10 indicates that there are no significant differences in soil Po at all combinations of P and N treatment.

Depletion of the soil Po concentration from that present in the start soil was evident (Fig 5.8 a, b). The percentage depletion of Po for each combination of P treatment and N treatment was calculated and the results were presented in Table 5.11.

Table 5.11: Percentage of soil Po depleted from the inner and outer soil for each combination of P treatment and N treatment. Soil without and with N was represented as –N and +N respectively. Each value represents the mean +/- SE (in brackets) of 3 separate determinations

	N treatment (mg N/l soil)			
	-N		**+N (68 mg N/ l soil)**	
P treatment (mg P/l soil)	**Inner soil**	**Outer soil**	**Inner soil**	**Outer soil**
Control	9.6 (5.8)	7.9 (2.0)	37.9 (36.5)	70.1 (3.3)
100Po	19.3 (0.8)	56.7 (25.3)	8.8 (1.3)	22.5 (2.5)
200Po	39.4 (20.9)	55.6 (20.7)	15.2 (2.0)	43.6 (22.4)
400Po	27.3 (3.5)	33.1 (18.7)	36.4 (6.6)	74.9 (1.0)
100Pi	6.8 (1.3)	67.9 (1.6)	71.5 (2.6)	71.0 (6.1)

A three way ANOVA was carried out to determine the affect of P treatment, N treatment and location of soil on the percentage of Po depleted form the soil. This ANOVA was presented in Appendix 5, Table 10.

There was significant affect of location of the soil on Po depletion (F ratio 9.26**). More Po was depleted from the outer soil. This is evident from Table 5.11 and Fig 5.8 (a, b). All other treatments and interactions were insignificant (Appendix 5, Table 10).

5.3.7 % Root length colonised by vesicular arbuscular mycorrhiza

The % root length colonisation (%RLC) by vesicular arbuscular mycorrhiza data was presented in Fig 5.9. A two way ANOVA was carried out to determine the affect of P treatment and N treatment on the %RLC by vesicular arbuscular mycorrhiza. This ANOVA was presented in Appendix 5, Table 11.

There was a significant P affect (F ratio 4.29**) on %RLC. There was also a significant P - N interaction (F ratio 4.03**). There was no significant affect of N treatment.

From Fig 5.9 there was a decrease in %RLC on the addition of Po to the soil. This was the case for plants with no N treatment. In N treated soil the addition of 100Po and 200Po resulted in an increase in %RLC. The 100Pi treatment had a %RLC between that of the control and Po treatments and was similar in N treated and untreated soil. This significant affect of P treatment on %RLC was examined using a Scheffe post hoc test (Table 5.12).

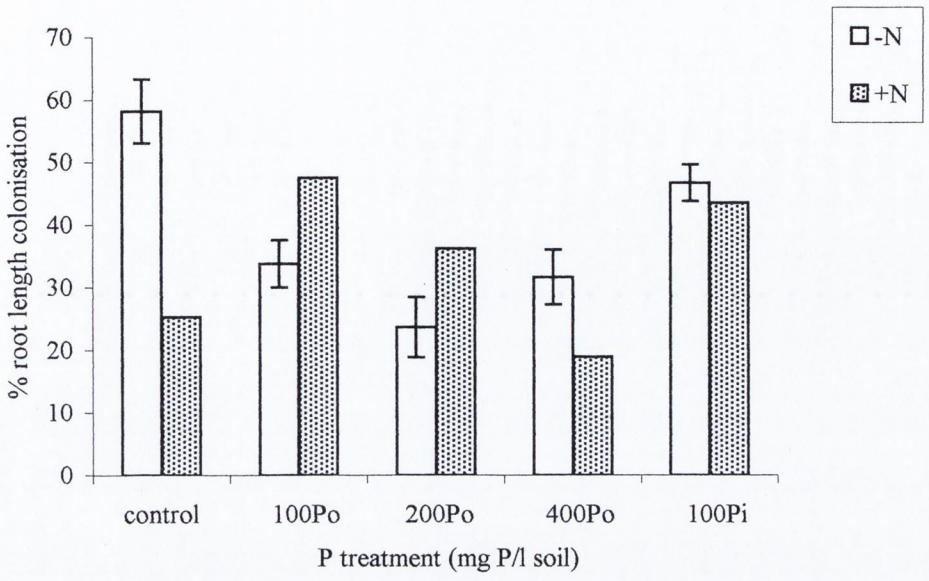


Fig 5.9: Percentage root length colonisation (%RLC) for the control and P treatment plants of *Agrostis stolonifera* for plants grown without (-N) and with (+N) N treatment.Each value represents the mean +/- SE of 3 separate determinations.

Table 5.12: Probability levels calculated from a Scheffe post hoc test that examined the affect of P treatment on %RLC for the *Agrostis stolonifera* experiment
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
control - 100Po	ns
control - 200Po	ns
control - 400Po	ns
control - 100Pi	ns
100Po - 200Po	ns
100Po - 400Po	ns
100Po - 100Pi	ns
200Po - 400Po	ns
200Po - 100Pi	ns
400Po < 100Pi	**

From Fig 5.9 as described above there were changes in %RLC with P treatment but because of large standard errors these changes were not significant.

The significant interaction between P treatment and N treatment was examined using a Scheffe post hoc test (Table 5.13).

Table 5.13: Probability levels, calculated from a Scheffe post hoc test, for combinations of interaction between the factors N treatment and P treatment, that affected %RLC for *Agrostis stolonifera* experiment. Soil without and with N application are indicated as –N and +N respectively.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Comparison	P value
-N, control - +N, control	ns
-N, 100Po - +N, 100Po	ns
-N, 200Po - +N, 200Po	ns
-N, 400Po - +N, 400Po	ns
-N, 100Pi - +N, 100Pi	ns
-N, control - -N, 100Po	ns
-N, control > -N, 200Po	**
-N, control - -N, 400Po	ns
-N, control - -N, 100Pi	ns
+N, control - +N, 100Po	ns
+N, control - +N, 200Po	ns
+N, control - +N, 400Po	ns
+N, control - +N, 100Pi	ns

From Table 5.13 only the 200Po treatment in soil with no N application results in a significant decrease in %RLC. This decrease does not occur with the 200Po treatment in soil with N application.

5.3.8 Correlations

Pearson Product Moment correlations was carried out between the variables Po depletion, shoot dry weight and shoot P concentration. The results are presented in Table 5.14.

Table 5.14: Pearson Product Moment correlations (r values) for the variables Po depletion, shoot dry weight and shoot P concentration and content for the Agrostis stolonifera experiment.
** $p < 0.01$

	Po depletion	Shoot dry weight	Shoot P concentration
Po depletion			
Shoot dry weight	0.495 **		
Shoot P concentration	0.496 **	0.527 **	
Shoot P content	0.448 *	0.967 ***	0.705 ***

There was a statistically significant positive correlation bewteen shoot dry weight and shoot P content and shoot P concentration. That is as the shoot dry weight increases so too does the shoot P content and the shoot P concentration. The shoot P concentration was positively correlated to the shoot P content.

There were also significant positive correlations between Po depletion and shoot dry weight, Po depletion and shoot P content and Po depletion and shoot P concentration. As the amount of Po depleted from the soil increased the shoot dry weight and P content and concentration also increased.

5.4: DISCUSSION

In order to examine the affect of increased nitrate in the soil on plant growth and use of Po by plants it was necessary to examine various factors. Nitrate does not only directly affect plant growth but also can affect other soil factors in the plant environment such as the mycorrhiza and soil and root enzymes. Therefore in this chapter the affect on nitrate on plant growth using dry weight, P content and P concentration was examined. But any affect of nitrate on soil phosphate concentrations, soil and root monoesterase enzymes and infection of roots by VAM was also examined. All these factors interact to provide the growth environment for the plant and changes in these factors will in turn affect plant growth.

5.4.1 Published work

The work that has been published relating to soil N and P nutrition relates to nitrogen and inorganic phosphate. Some of this work will however be discussed to identify trends and determine if these also occur in this study in relation to nitrogen and organic phosphate.

Lopez-Cantarero *et al.*, (1998) examined the affect of nitrate and inorganic phosphate addition, as nutrient solutions, to soil in which eggplant was growing. N was never applied alone but always in combination with Pi. N was applied at a rate of 150, 225 and 300 kg/hectare and P at a rate of 240 and 360 kg/hectare. They found that the highest P treatment resulted in an increase in leaf P concentration independent of N rate applied and increasing the N concentration applied to the soil resulted in an increase in leaf P concentration at all Pi levels employed. They concluded the rise in P concentration as P and N rates increased was due to the individual affects of the greater application of both nutrients and increased fertilisation.

Ameziane *et al.*, (1997) investigated the affects of nitrate and phosphate nutrition on the growth of the crop the Belgium endive. P was added as Pi and both N and P were supplied as nutrient solutions. Converting the concentration of N applied as a solution to g of N supplied to each plant resulted in a low N application rate of 0.02g N/plant and a high rate of 0.09g N/plant. N was never applied alone but always in combination with Pi and a control with no N or P addition was not used. Therefore it was not possible to determine if the addition of N to the plants at a rate of 0.2 g $N0_3$ per plant, a rate similar to that employed in this experiment, resulted in any change in plant dry weight or P content compared to plants receiving no additional N. However increasing the N applied from 0.02 g to 0.09 g $N0_3$ per plant both with low and high levels of Pi application resulted in an increase in shoot dry weight and P content. Increasing the concentration of N in the soil increased plant growth irrespective of Pi level in the soil.

Zoysa *et al.*, (1998) found that addition of NO_3^- at a rate of 200 mg N/kg soil to soil fertilised with rock phosphate resulted in an increase in shoot dry weight, but no increase in shoot P concentration compared to plants with no additional NO_3^-.

Ortas *et al.*, (1996) examined the affect of NO_3^- application and rate of Pi application on P uptake by sorghum. N was added in the form of calcium nitrate to yield a final concentration of 120 mg N/kg soil. N was added to all soils at the same rate and so it was not possible to investigate the affect of increasing soil N concentration. Increasing Pi concentration of the soil resulted in an increase in shoot dry weight. NO_3^- application caused the soil pH to increase and plant P content decreased as a consequence. It was proposed that this was because the solubility of inorganic P decreased as the pH increased.

Liu *et al.*, (2000) examined the affect of increasing rates of ammonium nitrate addition, (0, 70 and 140 mg N/kg soil) to soil with Pi addition. The addition of N resulted in an increase in shoot dry weight. Under conditions where N was sufficient in soil, P utilisation in biomass production was increased.

In the work discussed above increasing the concentration of NO_3^- in the soil resulted in increases in leaf P concentration (Lopez-Cantarero *et al.*, 1999), increases in dry weight (Ameziane *et al.*, 1997; Zoysa *et al.*, 1998) and leaf P content (Ameziane *et al.*, 1997). Only Ortas *et al.*, (1996) found a decrease in P content of the shoot with an increase in NO_3^-. Increases in NH_4^+concentration of the soil resulted in a rhizosphere pH decrease. Acidification of the rhizosphere soil resulted in increased availability and uptake of sparingly soluble nutrients such as P from rock phosphate (Ortas *et al.*, 1996). However the addition of NO_3^- to the soil resulted in an increase in soil pH and so decreased the availability of P from such Pi sources. Therefore in the work discussed above increases in plant growth with increases in nitrate application are not associated with increases in P availability due to falling pH. It is most likely as proposed by Lopez-Cantarero *et al.*, (1999) that increases in plant growth were due to the nitrate added simply increasing the N content of the soil and acting as a fertiliser in its own right. In the experiment discussed in this chapter no changes in shoot dry weight, P concentration or P content with the increase in nitrate concentration of the soil were observed. It is possible that the rate of 68 mg N/l soil (99 mg N/kg soil), a rate lower than that used by the above workers, was not high enough to result in any detectable change in plant growth or soil the parameters examined. Increasing the rate of nitrate added to the soil may result in increases in plant growth as seen with other workers. It is possible that the N concentration (7703 mg N/kg soil) in the PP1 soil was sufficient to support the growth of the *Agrostis* plants used in this experiment and increasing this concentration does not therefore result in any increase in plant growth. That is the plants are not N limited. Finally it is possible that at the increased N

concentration of the soil P is limiting on the plant growth. These possibilities will be discussed in more detail in the next section.

5.4.2 Information gained

The shoot dry weight of the *Agrostis* plants increased with increasing levels of Po and Pi added to the soil (Table5.2). This indicated that phosphate in the PP1 soil was limiting to plant growth. The 100Po treatment was insufficient to result in an increase in weight but the 200Po and 400Po and the 100Pi treatments resulted in dry weights significantly above that of the control plants. IHP was available for plant uptake and was being used by the plants as a source of P. This was as expected as data in Chapter 2, Section 2.3.3.4 also showed that IHP was used for plant growth. The dry weight that resulted from the 100Po treatment was not significantly different to that from the 100Pi treatment. The N treatment did not result in a significant increase in the shoot dry weight for the control or the P treatment plants (Table 5.2).

The shoot P concentration was also increased with increasing levels of Po added to the soil (Table 5.4). The 200Po and 400Po treatments resulted in a significant increase in shoot P concentration. The concentration in the 100Pi treatment plants was equal to that of the 100Po treatment plants. The amendment of the soil with N resulted in no significant affect on the P concentration in the shoots (Table 5.4).

The shoot P content followed the pattern of the shoot dry weight with increasing content with increasing levels of Po in the soil (Table 5.6). The 200Po, 400Po and 100Pi treatments resulted in significant increases in shoot P content. IHP was taken up by the plant and increased P accumulation in the shoot. The shoot P content of the 100Po treatment plants was not significantly different to that of the 100Pi treatment plants. There was no significant affect of increasing the level of N in the soil through the addition of nitrate (Table 5.4).

A statistically significant positive correlation was found between shoot dry weight and shoot P content and concentration (Table 5.22). Not only did the dry weight of the shoots increase with increasing levels of Po in the soil, but the concentration and content of P in these shoots and so the P uptake also increased.

As proposed by Jarrell and Beverly (1981) the dry weight, P content and P concentration were examined together. In the case of the Po treatments, 200Po and 400Po, there was a significant increase in dry weight, shoot P content and P concentration. When P as a growth limiting nutrient was supplied in the Po form, the rate of P accumulation increased more rapidly than the rate of dry matter accumulation. There was therefore an increase in the shoot P concentration as it was derived from the dry weight and P content data. When P was supplied as Pi there was an increase in the P content and the dry weight but no change in the P concentration. The rate of P accumulation increased but the rate of dry matter accumulation increased to the same extent.

There was therefore no change in the P concentration of the shoots. Rates of P accumulation were greater with a supply of Po.

The phosphorus use efficiency (PUE) is defined as the quantity (in grams) of shoot dry weight produced from 1mg of P (Sanginga *et al.*, 2000). Productivity of the nutrients allocated into shoots seems to be a more important determinant of nutrient productivity of a species than is the amount of nutrients allocated into the shoot. This has been clearly shown for P (Ryser *et al.*, 1997). Sangina *et al.*, (2000) examined the affect of P supply using superphosphate on P use efficiency of cowpea. They found a decrease in PUE with increasing amounts of P applied up to 60 kg P/ha. In the experiment in this chapter the PUE of the control and all Po treatments increased on the addition of nitrate to the soil, especially for the control soil (Table 5.8). The increased N in the soil increased the economical use of P by the plants. With N treatment a higher quantity of shoot dry weight was obtained using 1 mg P than without N. It was possible that the increased N improved the condition of the plant and its ability for efficient P use. There was however a decrease in PUE with increasing concentrations of Po in the soil. In soil with no N treatment the 100Po, 200Po and 400Po treatments resulted in a 46%, 73% and 70% decrease in PUE respectively. The decrease as a result of the 100Pi treatment at 40% was similar to that resulting from the 100Po treatment. Increasing the soil Po above 100Po increased P availability and afforded the plants greater luxury in the use of P. For N treated soil the decrease in PUE with increasing P in the soil was also evident. But this decrease was greater than in soil with no N treatment. The 100Po, 200Po and 400Po treatments resulted in a 72%, 78% and 81% decrease in PUE respectively. Again the decrease as a result of 100Pi addition at 78% was similar to that of the 100Po treatment. Increasing the concentration of N in the soil increased the ability of plants to use P efficiently but an increase in P increased P availability and decreased the need for economical use of P resources. Plants which use Po are more affected by the N level in the soil, than those that use Pi, in how efficiently they use their phosphorus.

There was no significant affect of N treatment on the dry weight, P concentration or content of the shoots (Tables 5.2, 5.4, 5.6). This was not as expected. The addition of N usually stimulates uptake of P (Ortas *et al.*, 1996) and the dry matter production of plants is increased by increasing levels of soil nitrogen (Marschner, 1995). An increase in the N concentration of the soil was expected to result in an increase in plant growth and possibly P uptake, either due to N acting as a fertiliser in its own right to increase the nutrient level of the soil or, due to N increasing the amount of available P from the Po added to the soil.

It was possible that the P in the soil is limiting to plant growth even with the highest P treatment 400Po. Therefore there was no significant affect of the N treatment. This however was unlikely as the phosphorus use efficiency indicated that there was P in the soil above that required for plant growth, even with the 100Po treatment, allowing plants the uneconomical use of P. It was

possible that the concentration of N naturally present in the soil was not limiting to plant growth. Again this was unlikely as the phosphorus use efficiency of the control plants with no P treatment did increase on the addition of N to the soil, indicating an N deficiency in the soil. It was more likely that the rate of N addition used in this experiment, 68 mg P/l soil, was not large enough to result in any measurable affect on dry weight and P content.

Nitrate and ammonium are known to have an affect on the release of Pi from certain Pi sources due to pH changes in the soil. No such relationship between N and Po has been examined. The results from this experiment do not point to any interaction between N concentration and Po availability and plant use with the exception of the increased PUE with increased N in the soil. Increasing the Po concentration of the soil did result in increases in shoot dry weight, shoot P concentration and content. These increases showed a positive affect of the P treatment. Such increases in plant growth indicated the release of orthophosphate, from the IHP added, for plant uptake and that Po was acting as a source of available P for plant growth.

Soil acid monoesterase activity is attributed to plant roots and associated mycorrhizal and saprophytic fungi (Joner and Jakobsen, 1995). Soil acidic monoesterase levels were significantly affected by P treatment (Table 5.9). However it was the 100Pi treatment that resulted in an increase in enzyme activity compared to the control soil. This was unexpected, as orthophosphate is a competitive inhibitor of acid and alkaline phosphatase in soil (Joner and Jakobsen, 1995). As such the 100Pi treatment would be expected to result in a decrease in activity. Pi was added to the soil at 100 mg P/l soil which corresponds to 10 μmole PO_4^{3-}/g soil. Juma and Tabatabai (1978) found that at 10 μmole PO_4^{3-}/g soil inhibition of soil acidic monoesterase activity ranged from 21 to 42 percent. In Chapter 3 Section 3.3.2 no change in soil acidic monoesterase activity was found as a result of the 100Pi treatment. In this experiment an increase in soil acidic monoesterase activity was found as a result of increasing the orthophosphate concentration. The Pi added in the 100Pi treatment was possibly taken up rapidly by the plants for growth and monoesterase activity was stimulated to release more P for growth. A corresponding increase in activity for the Po treatment soils was not found despite the fact that Po was depleted for plant growth from these soils also.

None of the Po treatments resulted in a soil acidic monoesterase activity significantly different to that of the control. This was as expected as results in Chapter 3 Section 3.3.2 showed that the addition of IHP at 100Po and 400Po also did not significantly affect the soil acidic monoesterase activity. The inositol hexaphosphate (IHP) added to the soil is a form of monoester P and as such should provide a substrate for acid monoesterase. It would be expected that the addition of IHP, and therefore monoesterase substrate, to the soil would stimulate acidic monoesterase production and result in an increase in the acidic monoesterase activity. It was possible that there was sufficient levels of soil acidic monoesterase activity already present in

the soil to breakdown the IHP added to the soil. No increase in the activity of this soil enzyme with the addition of Po to the soil may also indicate that this enzyme was limiting in soil. One plant per pot with its associated mycorrhizal fungi was possibly insufficient to produce additional enzyme. It appears that soil acidic monoesterase does not make a large contribution to the mineralisation of Po for plant use.

There was no affect of the N treatment on the soil monoesterase activity (Table 5.9). Soil acidic monoesterase is produced mainly by mycorrhizal fungi in the soil. It was expected that the N addition might have stimulated fungal activity and increased populations resulting in an increase in phosphatase activity. This was not found to be the case. The application of nitrogen fertilisers with or with out P usually stimulates acid monoesterase activity (Harrison, 1987). N had no affect for the possible reasons put forward above in the discussion of plant dry weight and P content, that is N addition rates were not high enough to affect soil fungi or N was not limiting in the soil for the fungal population. Application of nitrogen in conjunction with a readily-usable carbon source may result in a marked increase in soil monoesterase activity (Harrison, 1987). It is therefore possible that despite the addition of N to the soil, a depletion in carbon reserves due to plant growth and soil microbial activity may have been responsible for the lack of response from the soil acidic monoesterase activity. There was also no difference in the activity between the inner and outer soil (Table 5.9). Soil exposed to roots and hyphae, the inner soil, and soil exposed to hyphae only, the outer soil, had equal levels of phosphatase activity. The soil monoesterase activity measured was therefore a product of the soil fungi and plants roots.

There was a significant affect of N treatment on root phosphatase activity (Table 5.11). The addition of nitrate to the soil resulted in an increased level of root phosphatase activity for all the P treatments. This would suggest that increasing the N concentration in the soil, increased the growth rate of the plants. An increase in the root phosphatase activity was necessary to mineralise Po and meet the increased demand for Pi to match this increased growth rate. Phosphatase activity indicates a potential to use Po (Fox and Comerford, 1992). The orthophosphate in the soil resulting from this mineralisation was removed by plant uptake and so did not build up to cause a reduction in phosphatase levels. This increase in activity was possibly not sufficient to significantly increase Po availability and therefore dry weight. A corresponding significant increase in dry weight of the shoots with increasing N concentration in the soil was not found. There was no such increase in root activity for the control plants on the addition of N, most likely because the P content of the control soil is limiting with or without N addition. There was also no correlation found between root phosphatase activity and shoot P concentration. Johnson *et al.*, (1999) found that increasing the level of ammonium nitrate in the soil resulted in a significant increase in root phosphatase activity. They suggested

that increased phosphatase activity implies greater phosphate limitation in plants following soil N enrichment. They however applied NH_4NO_3 over a minimum of 18 months at a rate of 140 kg N/hectare. Here a smaller N addition rate, 68 kg N/hectare, was used and over a shorter period. An increase in root phosphatase activity was still detected. Johnson *et al.*, (1999) also found no relationship between root surface phosphatase activity and shoot P concentration.

Root monoesterase activity displayed a trend of decreasing activity with increasing levels of Po added to the soil when no additional N was added to the soil. This decrease in root acidic monoesterase activity with increasing concentration of Po in the soil was also found in Chapter 3 Section 3.3.2. In soil treated with N there was no difference in root activity of plants grown under different P treatments. Therefore overall there was no significant affect of P treatment on root phosphatase activity (Table 5.11). The decrease in root activity in the soil not treated with N was possibly because the root phosphatase catalysed the breakdown of the IHP in excess of that required for plant growth, and orthophosphate levels rose and inhibited the root monoesterase activity. Orthophosphate is an inhibitor of root monesterase activity (Johnson *et al.*, 1999). Increasing plant growth was found with increasing levels of Po to the soil and it was most likely that the Po added as IHP was enzymatically broken to orthophosphate before uptake. The addition of N to the soil did not result in this decrease in activity. This was possibly because a slight insignificant increase in plant growth as a result of the additional N would require increased levels of orthophosphate uptake resulting in less orthophosphate in the soil to inhibit the root monoesterase activity.

Soil total phosphate concentration was depleted from that present in the start soil to that detected in the postharvest soil. For soil untreated and untreated with N Tp was depleted from the inner and outer soil from all the soils treated with Po and the Pi treated soil. Soil P depletion patterns were used as a measure of soil P mobilisation This depletion would indicate that soil total phosphate reserves were being drawn down due to plant P uptake. There was a significant affect of the location of the soil on the Tp remaining in the postharvest soil. The location of the soil refers to the inner root accessible soil and the outer hyphae only accessible soil. Greater depletion occurred in the outer soil. This implies that greater Tp was being taken up by the vesicular arbuscular mycorrhiza hyphae alone than by the roots and hyphae together.

The depletion in the Tp of the postharvest soil for the Po treatment soils was matched by depletion in the organic P concentration. The depletion in the Po reserves occurs in the inner and outer soils of the Po treated soils with and without N treatment. This depletion would indicate that phosphate from the organic P pool was being mobilised for plant uptake. Plants were drawing from the organic P pool for their growth. Any phosphate that the Pi pool may have contributed for the growth of the plants was too small to be detected. As with the Tp pool there was greater depletion in the outer soil, indicating the greater depletion of Po from the soil

as a result of vesicular arbuscular mycorrhizae. To further prove this, the % of organic phosphate depleted from the inner and outer postharvest soil was calculated (Table 5.17). There was significantly more Po depleted from the outer soil than from the inner soil (Table 5.18). The phosphate required for plant growth was being removed from the organic phosphate pool and mainly by the hyphae of the vesicular arbuscular mycorrhiza. This would indicate the importance of vesicular arbuscular mycorrhiza in nutrient uptake for these *Agrostis* plants. There was no significant affect of N application on the percentage of Po depleted from the soil (Table 5.18). The addition of N to the soil did not result in a change in the quantity of Po removed by plant growth. Significant positive correlations were also found between Po depleted from the soil and shoot dry weight and Po depletion and shoot P concentration and P content. This indicates that as increasing amounts of Po were depleted there was an increase in plant growth. This depletion in Po was due to the uptake of P by plants, increasing their biomass and P uptake of their shoots.

There was no significant depletion of the inorganic P concentration in the postharvest soil compared to the start soil for the control and Po treatment soils. There was also no Pi added to the pool already present through mineralisation of Po. The plants rapidly took up Po that was mineralised to Pi. There was a significant decrease in the Pi concentration of the inner and outer soil for the 100Pi treatment soil. The depletion occurred to equal extents in the inner and outer soil. Plant roots and mycorrhizal hyphae removed Pi to equal extents. This would indicate Pi was being removed from the soil by plant uptake only where additional Pi was added to the soil. In the control soil and Po treatment soils it was Po that was preferentially removed.

Mycorrhizae are important for optimal root function. The symbiotic association increases uptake of relatively immobile nutrients such as P. Mycorrhizal colonisation is a complex multi step process in which the mycorrhizal association is influenced not only by the host plant and AM fungi but also by soil and other environmental factors. If one of these factors is inhibitory to AM fungi the symbiosis will be inhibited even if the other factors are optimum. In this experiment the % root length colonisation by indigenous vesicular arbuscular mycorrhiza (VAM) was not affected by the N treatment (64 mg N/l soil equivalent to 99 mg N/kg soil) (Table 5.19). This was not as expected. It was thought that the %RLC would decrease with the addition of nitrate to the soil as generally additions of nitrate to the soil decrease VAM infection of roots. This is because the infectivity of mycorrhizal propagules such as spores or infected root fragments is reduced by nitrate (Haynes, 1986). It is possible that the rate of N addition to the soil was insufficient to result in such a decrease. However Ortas *et al.*, (1996) investigated the affect of nitrate, at a rate of 120 mg N/kg soil, on P uptake by sorghum plants with and without mycorrhizal inoculation. Although they did not have a control with no nitrate addition, the %RLC of plants where nitrate was present in the soil was substantial at an average of 66%.

Liu *et al.*, (2000) propose that as AM fungi have a high N requirement for the synthesis of protein and chitin, which are the main constituents of its cells walls, that colonisation would be reduced under any conditions in which N does not promote growth, for example extremely low or high fertility. An extremely high N level in the soil may directly repress AM fungi. Liu *et al.*, (2000) observed an observed an increase in %RLC with ammonium nitrate application at a rate of 70 mg kg/soil to corn growing in a sand soil mixture but a decrease was seen when the rate was increased to 140mg kg/soil. The VAM in the growing media was established through inoculation. The influence of N on mycorrhizal associations was not consistent. A similar rate of nitrate application was used in this chapter as by these two workers and no affect on nitrate was observed. It is possible that indigenous VAM in the soil react differently to nitrate than VAM inoculated into the soil.

As expected there was a decrease in %RLC with increasing concentration of Po in the soil (Table 5.19). Much experimental evidence shows that mycorrhizal colonisation is reduced in plants having a high level of phosphorus and that colonisation is often negatively correlated with soil P values. (Liu *et al.*, 2000). In this experiment P content of the shoots did increase with increasing levels of Po in the soil. And there was an increase in dry weight with increasing levels of Po in the soil indicating increased levels of available P through IHP. However despite a decrease in the %RLC with increasing levels of Po in the soil the VAM hyphae remained very important in the uptake of P for plants as was demonstrated by the greater depletion of Tp and Po in the outer soil of the hyphal compartment. A further discussion on the affect of organic P on %RLC and vesicular arbuscular mycorrhizae can be found in Chapter 2.

Sylvia and Neal (1990) demonstrated that P had a greater affect on root colonisation in N sufficient soils than in N deficient soils. However no significant interaction was found in this experiment to indicate that the affect of P on %RLC was influenced in any way by the addition of N to the soil.

5.5 SUMMARY

- Increasing the level of nitrogen in the soil did not result in a significant increase in plant growth as measured by the parameters, shoot dry weight, P concentration and P content. It is most likely that the rate of N addition at 84 mg P/ soil was insufficient to result in such increases.
- Increased levels of Po in the soil did result in an increase in shoot dry weight, P content and concentration indicating IHP was an available source of P for plant uptake.
- Increased nitrogen levels in the soil significantly increased root monoesterase activity. This implies increased potential for growth due to the addition of N to this N deficient soil and an increased need for orthophosphate which will result from the mineralisation of Po by the phosphatase. There was no significant affect of nitrogen treatment on the soil acidic monoesterase activity.
- Depletion of the soil total phosphate reserve was due to depletion from the organic phosphate pool. The major portion of this depletion was from the outer soil accessible by VAM hyphae only. The percentage of Po depleted from the soil was no significantly affected by the N treatment.
- There was no significant affect of nitrate application on the percentage root colonisation by vesicular arbuscular mycorrhiza.

CHAPTER 6: THE USE OF NUCLEAR MAGNETIC RESONANCE FOR THE IDENTIFICATION OF SOIL ORGANIC PHOSPHATE COMPOUNDS

6.1 Introduction

6.1.1 Objectives of Experiment

The objectives of the experiment described in this chapter were:

- to examine the range of P compounds in soil alkaline extracts using ^{31}P nuclear magnetic resonance (NMR).
- to assess the sensitivity of ^{31}P NMR to detect changes in types and quantities of different soil P species, resulting from short-term plant growth and uptake.

^{31}P NMR analysis was conducted using the control and P treatment soils, untreated with nitrogen, resulting from the experiment described in Chapter 5, The affect of nitrogen and organic phosphate on plant growth. This experimental soil consisted of a natural soil, collected from the PP1 site (for site description refer to chapter 1, section 1.3), containing no artificial growth media. This PP1 site soil was amended with inositol hexaphosphate (IHP) at increasing concentrations.

The forms of P and the quantities, present in the soil were analysed, both at the start of the experiment before plants were transplanted into the soil, and after a seven-week period during which *Agrostis stolonifera* was present.

6.1.2 The theory of nuclear magnetic resonance

^{31}P NMR spectroscopy is a useful tool for characterising the structural composition of P in alkali extracts of soils. NMR is a spectroscopic technique that exploits the magnetic property of the atomic nucleus. Atomic nuclei possess spin, which has a magnetic property. When a magnetic field is applied, the nuclei are induced into different orientations. These orientations have different energies and the energy difference between the two states is proportional to the magnitude of the applied magnetic field. Transitions between the two states may be induced by applying an oscillating magnetic field of suitable frequency. The resonance frequency of a nucleus is proportional to the local magnetic field experienced by the nucleus. The applied magnetic field is modified by the induced electronic current in the adjacent atoms and molecules for each nucleus. (McMurray, 1991). Thus different types of P compounds can be distinguished on the basis of the electronic environment around the various P nuclei, which determines the frequency of nuclear magnetic resonance (Makarov, 1997).

The change in frequency of the resonance is evaluated relative to a standard (85% H_3PO_4) and is termed a chemical shift (δ). Because of its small quantitative changes it is expressed in parts per million (ppm) (Makarov, 1997). Resonance signals which appear at higher field than H_3PO_4 are given positive values, signals at a lower field than H_3PO_4 are given negatitive values (Emsley

and Hall, 1976). The number of ester linkages and the presence or absence of direct C-P bonds, e.g. in phosphonates, are the major factors affecting resonance frequencies of P in soil organic matter. The quantitative detection and differentiation of monoester-P, diester-P, phosphonates, pyrophosphate, polyphosphates and inorganic P by ^{31}P NMR is well established. The NMR peak area is proportional to the relative amount of active nuclei giving that particular peak (Emsley and Hall, 1976) and intensities of signals can be determined by integration of peak areas (Sumann *et al.*, 1998). Differences in the molecular structure beyond the atoms neighbouring the ^{31}P exert a very weak affect on the character of NMR spectra. However along with the indicated classes of compounds it is also possible to identify some individual organic phosphates (Makarov, 1997).

6.1.3 Methods of extraction for ^{31}P NMR analysis

An ideal extractant should remove virtually all of the P from a soil sample without altering in any way the forms of P found in the soil. However the complex nature of soil Po may make it impossible for a single extractant to dissolve all P compounds. It also may not be possible to produce high quality spectra without some alteration of P compounds by hydrolysis. The extractant used will influence the concentration of P extracted and the forms of P seen in the NMR spectra and so may obscure changes in some P forms.

Workers have used various extractants. Most have used a rapid extraction method using 0.5 M NaOH (Gil-Sotres *et al.*, 1990; Tate and Newman, 1982; Hawkes *et al.*, 1984) which usually extracts less than 50% of the total phosphorus. Hinedi *et al.*, (1988) extracted soil P using a trichloroacetic acid and KOH sequential extraction procedure. Condron, *et al.*, (1985) used a sequential extraction procedure of 0.1 M NaOH, 0.5 M HCL, 0.2 M aqueous acetylacetone and 0.5 M NaOH. Emsley and Niazi (1983) used aqueous tetra-n-butyl-ammonium hydroxide.

In addition to the extraction of P these methods extract paramagnetic ions such as Mn and Fe which cause line broadening and distortion of the ^{31}P NMR spectra (Cade-Menun and Preston, 1996). Preston and Trofymow (1998), Adams and Byrne (1989), Adams (1990) and Condron *et al.*, (1996) used a chelating cation exchange resin, Chelex, to try to remove these interring ions. Chelax shows a high preference for Fe, Al and Ca. Bowman and Moir (1993) proposed the use of a NaOH-EDTA extractant to determine soil total Po. The EDTA chelates metal cations to increase the efficiency of P extraction by the NaOH which solubilises the Po. These workers did not however test this mixture as a NMR extractant solution.

Cade-Menun and Preston (1996) examined a range of different extraction procedures, including the 0.5 M NaOH : 0.1 M EDTA extractant of Bowman and Moir (1993), along with a 0.25 M NaOH extractant, a Chelex : deionised water mix and a Chelex : 0.25 M NaOH mix, to determine the one most efficient for ^{31}P NMR. NaOH + EDTA extracted the most P of all the

extractants, produced the greatest range of compounds and was the only method to show polyphosphate peaks. This was possibly because a greater amount of P was extracted or less hydrolysis occurred during extraction. Also the NaOH+EDTA mix did not cause hydrolysis of diesters which occurred with NaOH alone.

Robinson *et al.*, (1998) used the one step NaOH-EDTA solution to extract Po from soil for ^{31}P NMR analysis of P forms. They concluded that this procedure is a relatively mild extraction that causes minimal hydrolysis of Po. The chelating ability of EDTA increases the Po extracting efficiency of NaOH by breaking P containing organo-metal complexes. They showed that there was good agreement between total P determined by conventional digestion and ^{31}P nuclear magnetic resonance methods, which suggests that all the P in the extracts was detected by NMR using the NaOH + EDTA extraction.

6.1.4 ^{31}P NMR research

NMR has been widely used in the study of P forms in soils from various environments and locations. Newman and Tate (1980, 1982) used NMR to examine P forms in New Zealand top-soils and in a climosequence of soils extracted with 0.5 M NaOH. Hawkes *et al.*, (1984) examined different forms of P in NaOH extracts of soils, from long-term field experiments where Pi fertiliser had been added to plots over an average of 100 years, using NMR. Condron and Goh (1985) examined the nature of soil P from Pi fertilised and grazed pasture as revealed by ^{31}P NMR. Adams (1990) employed NMR to examine P compounds in extracts of mountain ash soil. Condron *et al.*, (1990) used ^{31}P NMR to examine affect of moisture status and vegetation on soil Po while Gil-Sortres *et al.*, (1990) used the technique to characterise the phosphorus fractions in surface horizons of soils from Spain. Escudey *et al.*, (1997) used ^{31}P NMR to analyse the nature of P in Pi fertilised and unfertilised samples of a P enriched volcanic soil of Chile. Hinedi *et al.*, (1988) monitored the mineralisation of phosphorus in sludge-amended soils by ^{31}P NMR. Preston and Trofymow (1998) used ^{31}P NMR to characterise soil P in forest soils on Vancouver Island and detect the affects of climate and disturbance on soil P. Pant *et al.*, (1999) identified soil Po by ^{31}P NMR and determined the affect of different cropping systems on the various P compounds. Preston and Trofymow (2000) used ^{31}P NMR to characterise soil P in coastal forest chronosequences and to examine the affects of climate and harvesting disturbance on the soil P in Vancouver Island. Turrion *et al.*, (2001) evaluated the influence of annual precipitation and P fertilisation on the forms of soil Po of forest soils in western Spain using ^{31}P NMR

Characterisation of organic phosphate in Irish soils using ^{31}P NMR was first carried out by Power (1992, Ph.D thesis). These soils were all grassland, fertilised, agricultural soils. Power, Jeffrey and Tunney (1995) published a section of this ^{31}P NMR analysis work relating to a soil selected from a long-term grazing experiment at Johnstown Castle, Co. Wexford. The author of

this thesis is the second worker to use ^{31}P NMR to examine organic P compounds in Irish soils. To date, no studies have been carried out to determine if ^{31}P NMR is a suitable technique to detect changes in soil Po due to the uptake of P by plants over a short period. The work described in this chapter attempts to address this problem.

6.2 Methods

Several variations of the following method, including omitting the EDTA and changing centrifugation speeds, varying rotary evaporation conditions and filtration methods, were tested. The omission of the EDTA and the filtration resulted in line broadening and distortion of the peaks. The method described here produced the best quality spectra.

To reduce changes in P forms due to drying, moist soil samples were sieved and stored at 4°C until analysed as recommended by Pant *et al.*, 1999.The use of dry soils may mask the detection of the more labile Po forms due to their hydrolysis during the drying process. A 1:1 mixture of 0.5 M NaOH and 0.1 M EDTA as recommended by Cade-Menun and Preston (1996) was the extractant used. Rather than using ultrasonification the soils were shaken over a period of 16 hours. In order to reduce the suspended particulate matter which interferes with NMR analysis and results in poor quality spectra, the supernatant obtained following centrifugation was filtered through a 0.45 um Millipore filter (Condron *et al.*, 1990).

6.2.1 Soils

Soils for ^{31}P NMR analysis were sampled from the experiment described in Chapter 5, The affect of nitrogen and organic phosphate on plant growth, in which *Agrostis stolonifera* was grown.

This Chapter 5 experiment was based on the PP1 site soil and had a control (no P addition) and 4 P treatments, where phosphate was added at 3 rates, 100, 200, 400 mg P/l soil in the form of Po and 1 rate, 100 mg P/l soil in the form of Pi (Table 6.1). For the purpose of the results and discussion these Po treatments are referred to as 100Po, 200Po and 400Po respectively. The Pi treatment is referred to as 100Pi.

Table 6.1: Experimental layout for the pot experiment from which NMR soil samples were obtained.

P Treatments	Concentration P added	Source of P
Control (PP1 soil)	no P addition	
100Po (PP1 soil)	100 mg P/l soil	IHP
200Po (PP1 soil)	200 mg P/l soil	IHP
400Po (PP1 soil)	400 mg P/l soil	IHP
100Pi (PP1 soil)	100 mg P/l soil	Pi fertiliser

Organic phosphate was added in the form of phytic acid (myo-inositol hexakis [dihydrogen phosphate], Sigma Chemicals) which was added to the soil as a powder and was thoroughly mixed with soil. Inorganic phosphate was added in the form of commercially available Super phosphate fertiliser.

The 'growth bag' method was used in this experiment in order to distinguish between contributions of roots and of mycorrhizal hyphae to the uptake of P by plants. (further details on

the growth bag method: Chapter 2, sections 2.1.2.1 and 2.2.3). The plants were harvested after 9 weeks. On harvesting the plants the inner and outer soil was separated and stored separately.

In relation to ^{31}P NMR analysis only the soil, resulting from the experiment described in Chapter 5, with no nitrogen amendment was analysed.

6.2.2 Extraction of P from soil

To prepare soil extracts for NMR analysis 0.5 g soil (fresh weight) was extracted in 20 mls of a 1:1 solution of 0.5 M NaOH: 0.1 M $EDTANa_2$ (BDH Chemicals) in 50 ml centrifuge tubes. Soils were shaken on a reciprocating shaker for 16 hours and then centrifuged at 10000 g for 40 minutes (Sanyo Harrier centrifuge 15/80). The supernatant was removed and filtered through a 45 μm Millipore filter (Whatman Filter Papers). The filtered supernatant was reduced using a rotary evaporator at 40 °C to 2 ml (Labo Roto 300 rotary evaporator). 0.6 ml of this evaporated sample was transferred to a 5 mm NMR tube for analysis.

6.2.3 Running conditions for samples

^{31}P NMR analysis was carried out on a Brucker DPX400 spectrometer at 162 MHz, with a standard proton decoupled phosphorus acquisition program. All samples were externally referenced to 85% orthophosphoric acid, which was set at 0.0 ppm.

6.2.4 Peak identification

Peaks were identified by chemical shift as described by Hawkes *et al.*, (1984) and were quoted relative to H_3PO_4. Shifts were quoted in the dimensionless unit of ppm. Peak areas were integrated and expressed as percentages of total spectral area using the manual integration facility of the Brucker NMR program.

In order to ensure the purity of the inositol hexaphosphate (myo-inositol hexakis [dihydrogen phosphate], Sigma Chemicals) used throughout this thesis 0.0051 g of this compound was dissolved in 1 ml 0.5 M NaOH for ^{31}P NMR analysis. The resulting ^{31}P NMR spectrum is given in Appendix 2. No inorganic orthophosphate was found to be present in this inositol phosphate.

6.3 RESULTS

Extractions of soils for ^{31}P NMR analysis was carried before the planting of *Agrostis,* referred to as the start soil, and after the harvest of the *Agrostis*, referred to as the post harvest soil. The post harvest soil was split into inner and outer soil, relating to soil from inside the growth bag and outside the growth bag respectively. Soil samples examined by ^{31}P NMR are referred to as start soil, outer soil and inner soils in the results and discussion section.

This experiment consisted of a control soil and several P treatments. In the case of the control, PP1 site soil was used and no P was added to the soil. This was for

(1) the identification of Po compounds present naturally in the soil and

(2) to determine if changes in these compounds could be observed following the growth of *Agrostis* in the soil for a short period.

For the P treatments 100Po, 200Po and 400Po, Po was added in the form of inositol hexaphosphate, a Po compound naturally dominant in soil. A Pi treatment with 100Pi with Pi in the form of superphosphate was included. These treatments were designed in order to examine if ^{31}P NMR could detect

(1) increasing levels of Po in the soil due to IHP addition

(2) changes in the soil P compounds and their quantities due the presence of *Agrostis s.* in the soil and uptake of P for growth

Peaks were identified by chemical shift as described by Hawkes *et al.,* (1984). Slight variation in chemical shift can occur due to variation in final sample volume and NaOH concentration, leading to differences in sample pH, which in turn can affect the chemical shift (Adams and Byrne, 1989). Signals were not sufficiently resolved to allow the peak in the monoester region be differentiated into more specific compounds such as inositol phosphate.

6.3.1 Description of the ^{31}P NMR spectra

The ^{31}P NMR spectra obtained for the alkali extracts of the control soil were presented in Fig 6.1 (a, b, c). In the case of the start soil (Fig 6.1 a), signals may be assigned to orthophosphate monoesters (4.6 –5.5 ppm), orthophosphate diesters (-0.1 ppm) and pyrophosphate (-4.4 ppm). The monoester peak dominated the spectrum. The main difference between this and the spectra for the post harvest inner and outer soil of the control pots (Fig 6.1 b, c) was the presence of a peak indicating inorganic orthophosphate (5.7 ppm) in the postharvest soils. The orthophosphate monoester peak remained the dominant peak and orthophosphate diester and pyrophosphate were present in both the inner and outer soils. There was little difference in the signal size of the diester and pyrophosphate peak in any of the three spectra.

The spectra for the 100Po treatment start soil (Fig 6.2 a) was similar to that for the control start soil. Peaks indicate orthophosphate monoesters, orthophosphate diesters and pyrophosphate.

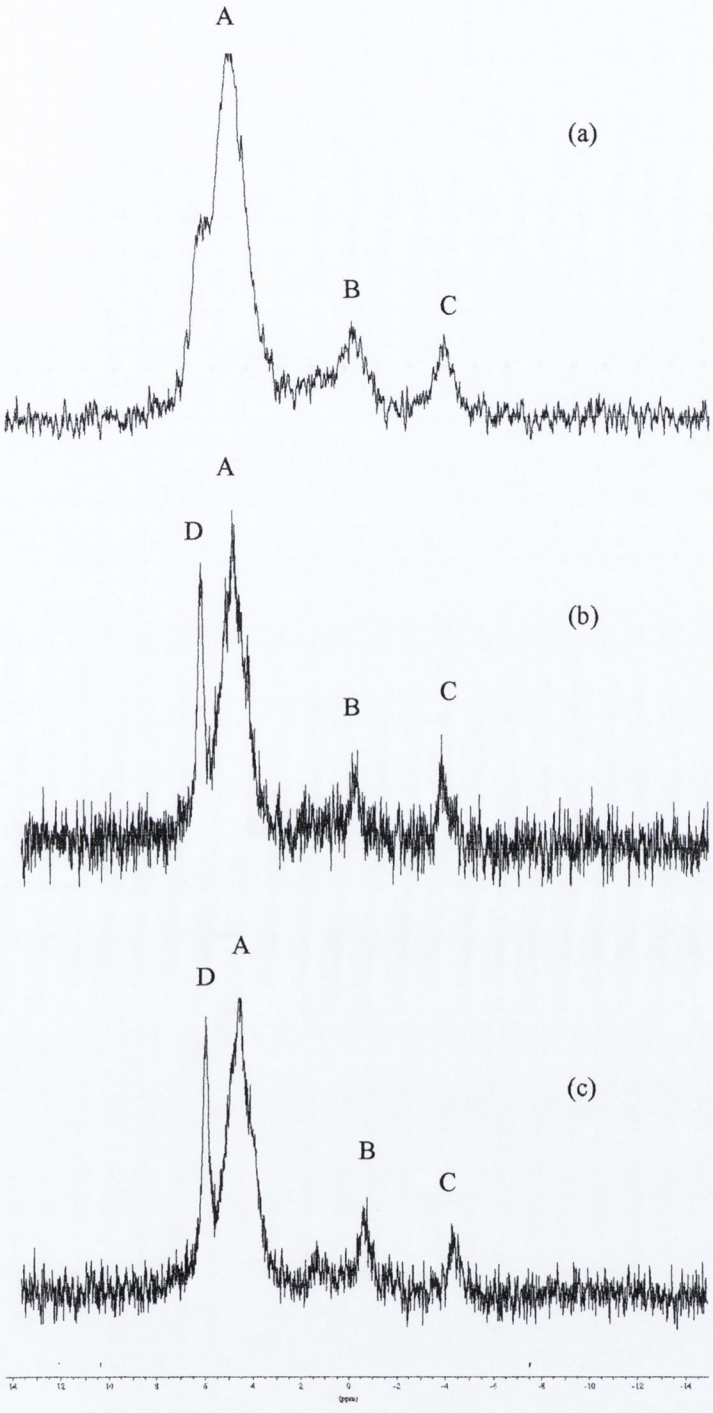


Fig 6.1: ^{31}P NMR spectra of alkali extracts of (a) control start soil (b+c) control post harvest soil (b) outer soil (c) inner soil

A = orthophosphate monoesters, B = orthophosphate diester,

C= pyrophosphate, D = inorganic orthophosphate

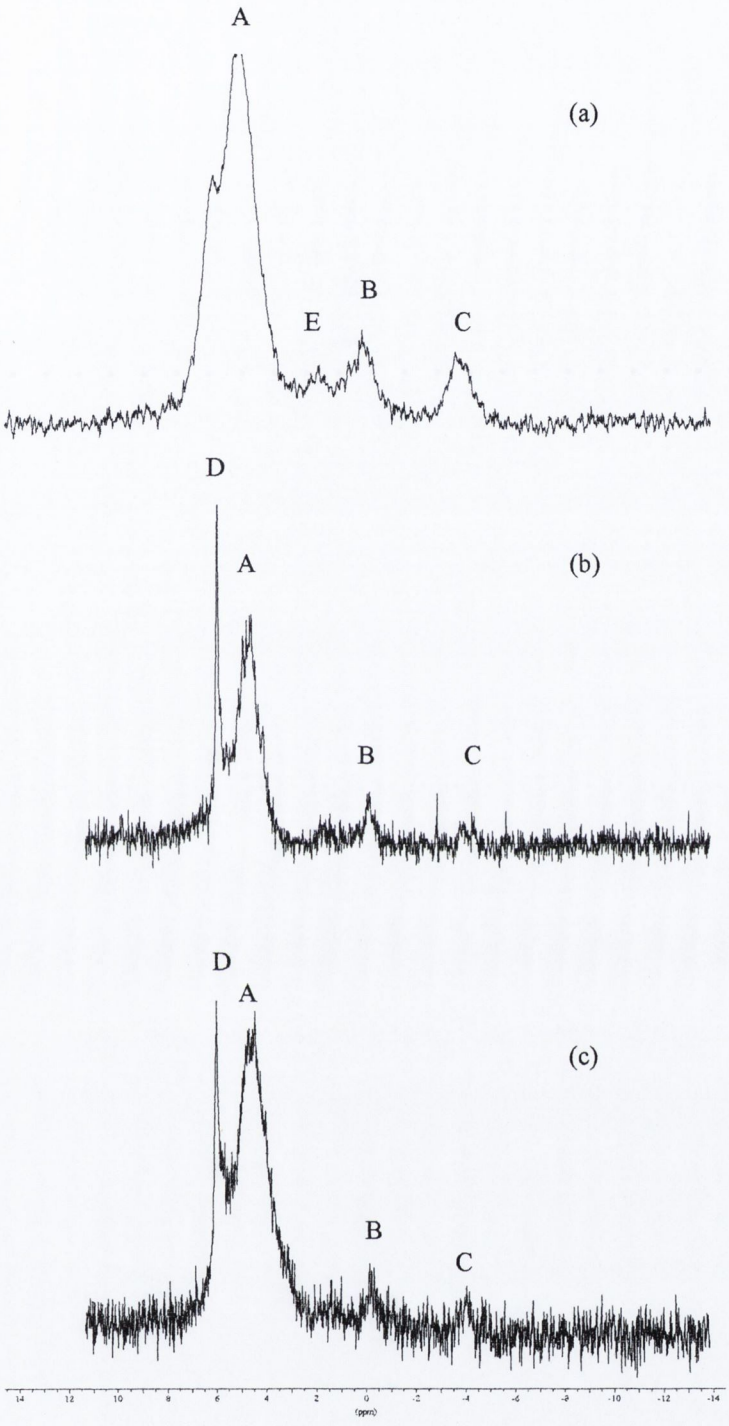


Fig 6.2: ^{31}P NMR spectra of alkali extracts of (a) 100Po start soil (b+c) 100Po post harvest soil (b) outer soil (c) inner soil

A = orthophosphate monoesters, B = orthophosphate diester, C = pyrophosphate, D = inorganic orthophosphate, E = teichoic acid

The monoester was the largest peak with the diester and pyrophosphate similar in size. There was a small peak at 1.6 ppm, which was assigned to teichoic acid by Condron *et al.*, (1990). The 100Po outer and inner post harvest soils (Fig 6.2 b, c) had a sharp peak for orthophosphate ions. A dominant orthophosphate monoesters peak and diester and pyrophosphate peaks, similar in size, were also detected.

The 200Po start treatment soil (Fig 6.3 a) had a spectrum similar to those described above. The spectrum was dominated by the monoester peak with smaller peaks at the region allocated to diesters and pyrophosphate. The teichoic acid peak at 1.5 ppm was visible. Unlike the control and 100Po start soils described however there was a small signal for orthophosphate ions. The 200Po inner and outer soils exhibited the same peaks as the start soil with the exception that there was no teichoic acid peak at 1.6ppm (Fig 6.3 b, c). The orthophosphate peaks while still smaller than the monoester peaks had considerably increased in size compared to that for the start soil. The diester and pyrophosphate peaks were again similar in size.

The NMR spectra for the 400Po start soil was presented in (Fig 6.4 a). As well as the teichoic acid peak that was detected in the 100Po and 200Po start soils there was a large peak for inorganic orthophosphate. As with all other samples there was a large monoester peak and peaks indicating diester and pyrophosphate phosphorus. The 400Po inner and outer soils (Fig 6.4 b, c) had dominant inorganic orthophosphate peaks. The monoester peak was the second largest peak and there were smaller peaks for diester P and pyrophosphate.

The monoester peak dominates the spectrum for the 100Pi start soil (Fig 6.5 a). There were smaller orthophosphate, diester and pyrophosphate peaks. The latter two peaks were quite broad. The 100Pi outer soil (Fig 6.5 b) had a large orthophosphate peak and monoester peak. There were smaller peaks corresponding to diester P and pyrophosphate, with the diester being the larger of the two. The spectrum for the 100Pi inner soil (Fig 6.5 c) was similar to that of the start soil. There was a smaller orthophosphate peak and a dominant monoester peak. Diester P and pyrophosphate are detected with pyrophosphate being the larger of the two.

All samples had peaks at the monoester, diester and pyrophosphate regions. Orthophosphate was also detected in the majority of the samples with the exception of the lowest P start soils – the control and 100Po treatment soils. The teichoic acid peak at 1.5 ppm was present only in the start soils only where Po was added to the soil, that is the 100Po, 200Po and 400Po treatments.

6.3.2 Peak Areas

Peak areas were integrated and expressed as percentages of total spectral area. For the purpose of integration the spectral area is defined as from +12 ppm to –12 ppm. Calculation of area for smaller peaks will be subject to greater error than for the more dominant compounds due to background noise.

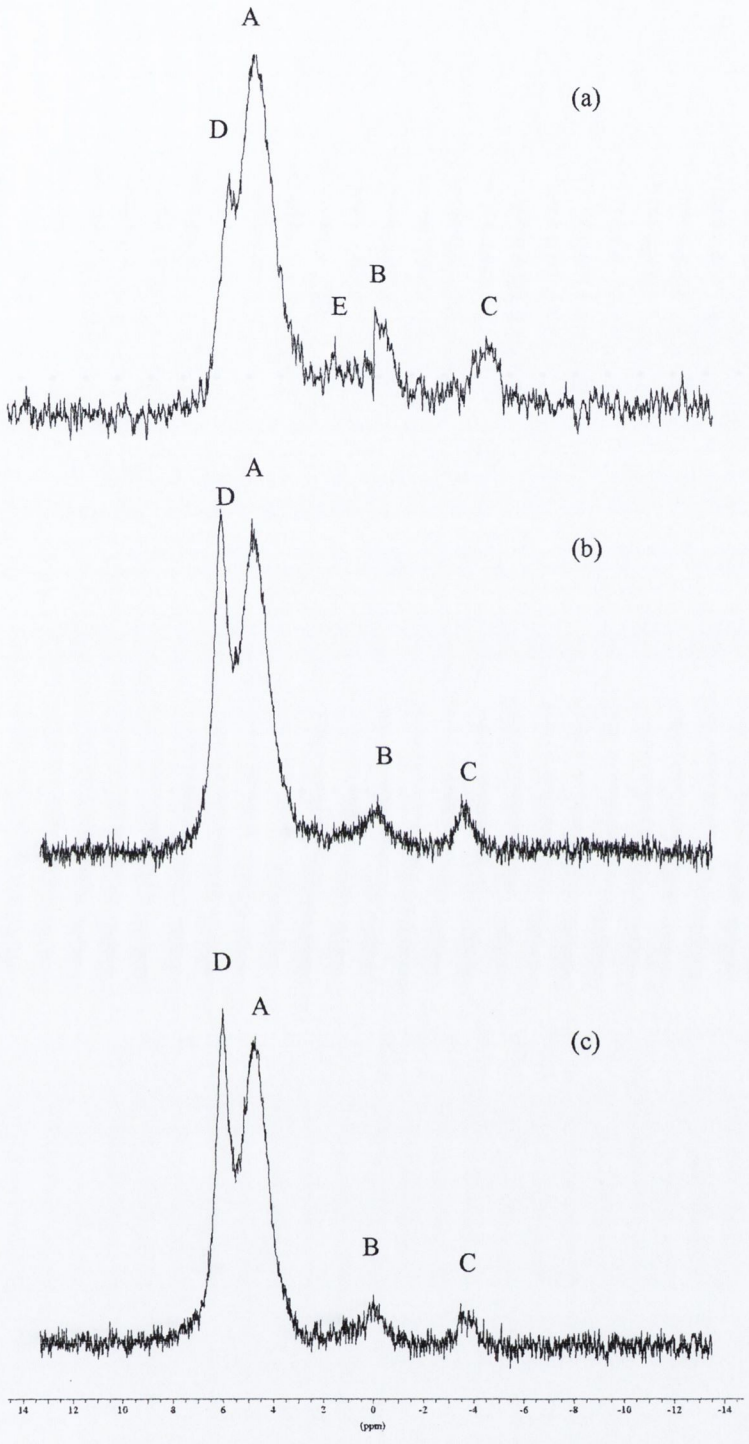


Fig 6.3: ^{31}P NMR spectra of alkali extracts of (a) 200Po start soil (b+c) 200Po post harvest soil (b)outer soil (c) inner soil

A = orthophosphate monoesters, B = orthophosphate diesters

C = pyrophosphate, D = inorganic orthophosphate, E = teichoic acid

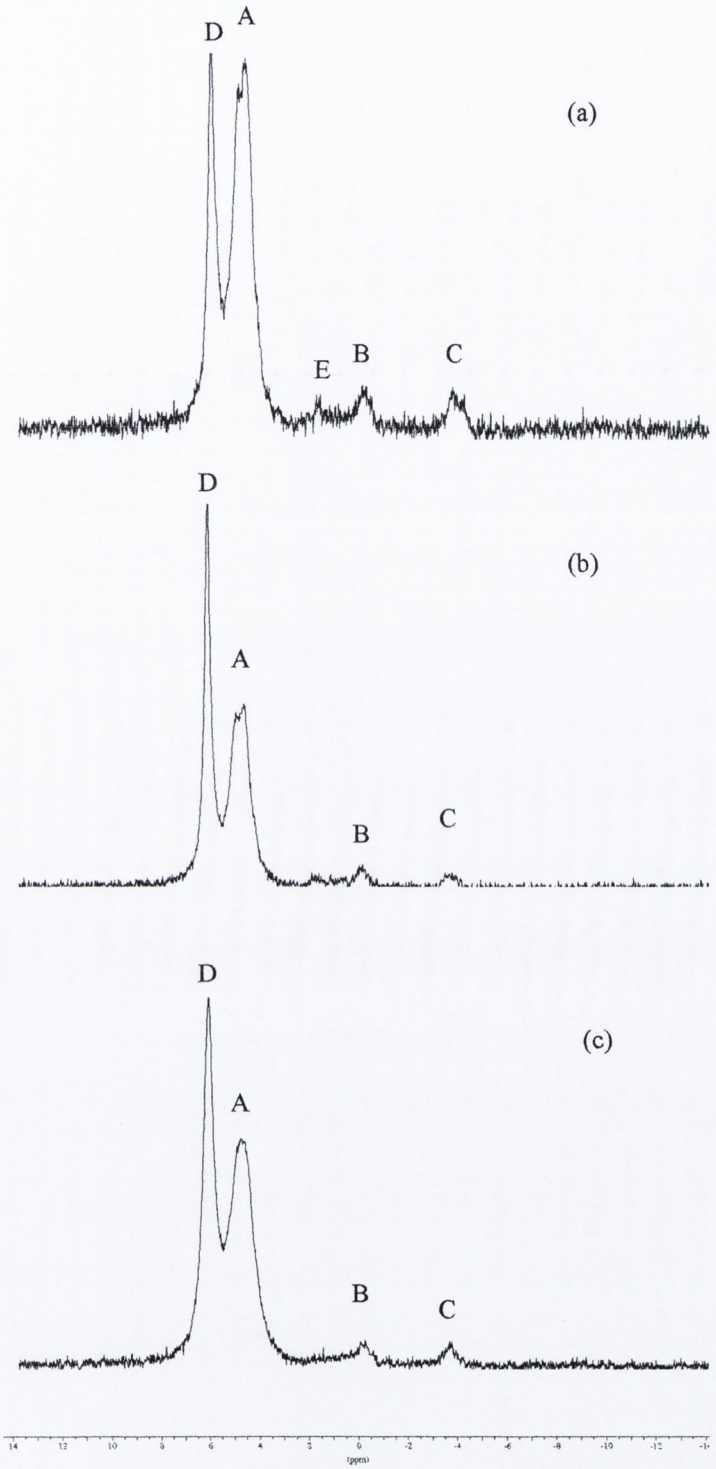


Fig 6.4: ^{31}P NMR spectra for alkali extracts of (a) 400Po start soil (b+c) 400Po post harvest soil (b) outer soil (c) inner soil

A = orthophosphate monoesters, B = orthophosphate diesters,

C = pyrophosphate, D = inorganic orthophosphate, E = teichoic acid

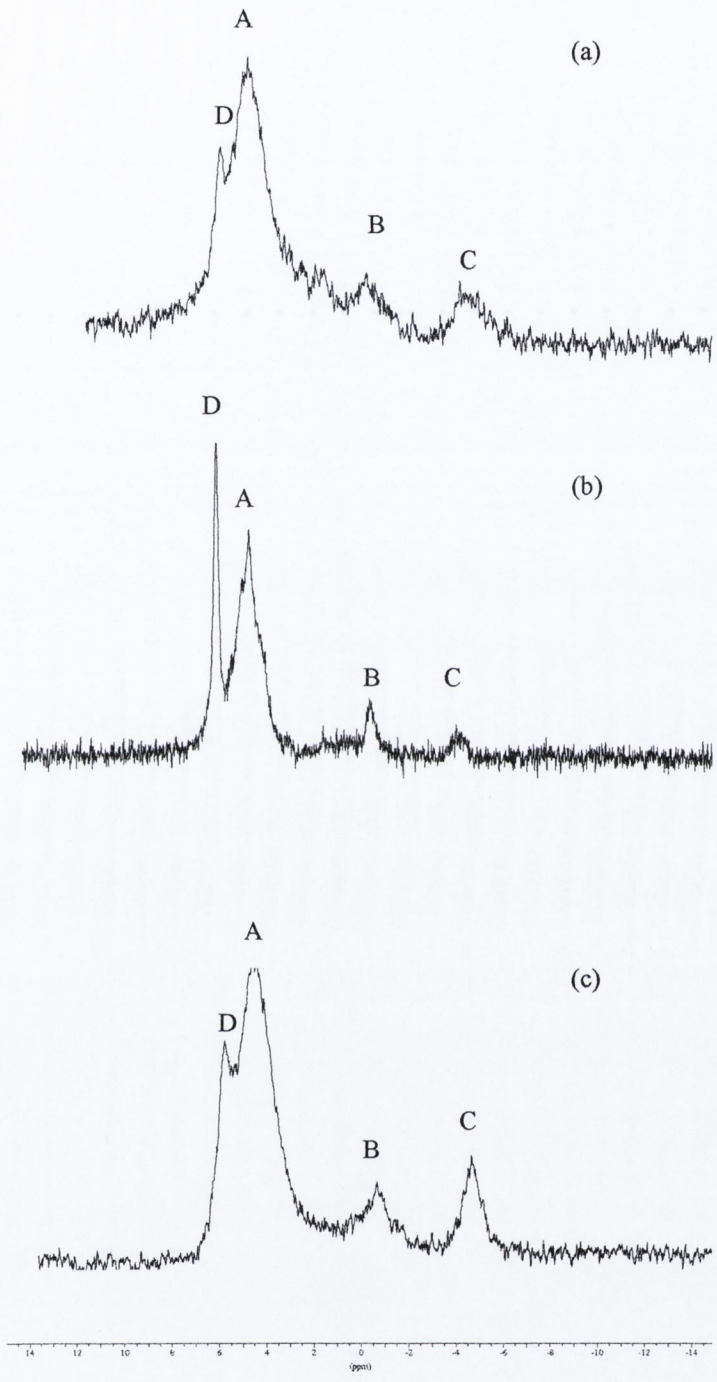


Fig 6.5: ^{31}P NMR spectra of alkali extracts of (a) 100Pi start soil (b+c) 100Pi post harvest soil (b) outer soil (c) inner soil

A = orthophosphate monoesters, B = orthophosphate diesters, C = pyrophosphate, D = inorganic orthophosphate, E = teichoic acid

The peak areas for each P species expressed as a % of the total spectral area are presented in Table 6.2.

From Table 6.2 the control start soil contained 62% monoester P and much lower proportions of diester P and pyrophosphate, 9% and 5% respectively. For the control inner and outer soil there was a drop in the monoester level to 42 and 41% respectively. There was also a drop in the diester (4 ppm inner and outer) and pyrophosphate (3 ppm inner; 5 ppm outer) levels. Inorganic orthophosphate at 13% of the spectral area for both the inner and outer soil was the second most prominent compound next to monoester P. The reduction in the amounts of the monoester, diester and pyrophosphate compounds may be due the uptake of P by plants. Some of these Po compounds may also have been converted to orthophosphate accounting for its appearance.

Table 6.2: Proportion (% of total spectral area) of various P compounds in alkali extracts of soils as determined by ^{31}P NMR. The chemical shifts of the P compounds are given in parentheses.
S. S. = start soil O.S. = outer soil I.S. = inner soil

Soil	P compounds (% of total spectral area)				
	Inorganic orthophosphate (5. 7 ppm)	Orthophosphate monoesters (4. 6 – 5.0 ppm)	Orthophosphate diesters (- 0.1 ppm)	Pyrophosphate (-4.4 ppm)	Teichoic Acid (1.5 – 2.0 ppm)
Control S.S.	-------	62	9	5	-------
Control O.S.	13	41	4	5	-------
Control I.S.	13	42	4	3	-------
100Po S.S.	-------	66	7	6	4
100Po O.S.	12	35	3	4	-------
100Po I.S.	12	43	3	2	-------
200Po S.S.	15	50	7	6	2
200Po O.S.	29	50	3	3	-------
200Po I.S.	31	46	3	1	-------
400Po S.S.	25	47	3	3	1
400Po O.S.	35	39	2	2	-------
400Po I.S.	40	46	2	1	-------
100Pi S.S.	13	53	5	5	-------
100Pi O.S.	26	52	5	2	-------
100Pi I.S.	15	51	7	8	-------

The 100Po start soil showed similar levels of orthophosphate diester (7%) and pyrophosphate (6%) as the control start soil. There was a slight increase in the quantity of orthophosphate monoester (66%). There was in addition a small quantity (4%) of teichoic acid with a chemical shift at 1.5 ppm. There was no teichoic acid peak in the inner or outer soil for the 100Po treatment. There was a considerable drop in the monophosphate P (43%, inner soil; 35% outer soil) from that detected in the start soil. There was also a drop in the diester (3% inner and outer soil) and the pyrophosphate (2% and 4% inner and outer soil) when comparing the post harvest soils to the start soil for the 100Po treatment. As with the control soil the reduction in the various Po compounds may be due to plant uptake and partly due to the conversion of some Po forms to Pi. (Table 6.2)

The 200Po start soil had a lower quantity of monoester P (50%) but similar levels of diester (7%) and pyrophosphate (6%) when compared to either the control or 100Po start soil. It also contained inorganic orthophosphate (15%) unlike the control and 100Po start soils. This would suggest that the monoester P was being mineralised, before any plants were present in the soil, to form orthophosphate ions as the 200Po treatment soil is exactly the same as the control soil with the exception of the addition of 200Po in the form of IHP. No Pi had been added. The level of teichoic acid had decreased from 4% in the 100Po treatment soil to 2% in the 200Po treatment soil. There was little difference in the monoester presence of the postharvest (46% inner; 50% outer) soils when compared to the start soil. There was however a decrease in the diester (3% inner and outer) and pyrophosphate (1% inner; 3% outer) levels. There was increase in the orthophosphate ions from 15% in the start soil to 31% and 29% in the inner and outer soils respectively. As there was no difference in the monoester presence in the start and post harvest soils it was possibly the diester and pyrophosphate species were converting to Pi. There were however insufficient levels of these two compounds to account for the total increase in orthophosphate. It was possible therefore that this Pi entered the soil through dead *Agrostis* material entering the soil and bacterial P release during microbial population turnover. It is also possible that the diester P and the pyrophosphate were taken up for plant use resulting in a decrease of these compounds.

The pattern for the 400Po treatment was similar to that discussed for the 200Po treatment. The 400Po treatment contained the highest percentage of orthophosphate of any of the start soils (25%). The monoester P of the 400Po start soil at 47% was similar to that of the 200Po start soil (50%). Therefore it was possible that monoester P mineralised to form Pi while in storage. The orthophosphate diester (3%) and pyrophosphate (3%) were at lower levels than in any of the other start soils. This would indicate some breakdown of these compounds. The unidentified P compound was present at 1% of the total spectral area. The 400Po postharvest soils had a similar level of monoester (46% inner; 39% outer) as the start soil yet a higher level of orthophosphate. There was little change between the postharvest soil diester P (2% inner; 2% outer) and pyrophosphate (1% inner; 25 outer) and the start soil levels for these compounds. As further Po mineralisation was unlikely to be the reason for the large increase in Pi levels it again suggests that dead plant material falling on the soil was responsible for the Pi increase. The small depletion in the Po compounds may have been due to plant growth and uptake.

The 100Pi soil was the same as the control soil with the exception that 100Pi was added. Orthophosphate monoester (53%), diester P (5%) and pyrophosphate (5%) were present in the 100Pi start soil. Inorganic orthophosphate (13%) was present as expected as it was added to the start soil. The monoester content of the postharvest soils was similar to that of the start soil (51% inner; 52% outer). The diester orthophosphate levels were also similar (7% inner; 5%

outer) There was a slight decrease in the pyrophosphate content of the outer soil to 2% but an increase in that of the inner soil to 8%. There was a slight increase in the inorganic orthophosphate content of the postharvest soils (15% inner; 26% outer) compared to the start soil. Again this suggests plant material falling on the soil increased the Pi content of the soil through mineralisation as there was little change in Po levels to account for the increase in the Pi content of the post harvest soils.

As the amount of Po added to the soil in the form of IHP increased, there was no corresponding increase in the % area of monoester P compounds, where the inositol would be located, in the ^{31}P NMR spectra. There was a slight increase from the control to the 100Po treatment, but then a decrease for the higher Po treatments of 200Po and 400Po. At these higher levels it would appear the additional Po was being mineralised into inorganic orthophosphate without any plants being present. This may have occurred while the samples were in storage awaiting NMR analysis. It is also possible that the extraction procedure did not extract all of the organic P in the soil and underestimated the monoester pool.

The control and 100Po treatment soils showed substantial decreases in the presence of orthophosphate monoester and smaller decreases in diester P and pyrophosphate that could be due to the uptake of these Po compounds by plants following their mineralisation to inorganic orthophosphate. Pi is thought to be the only form of P directly taken up by plants. In the case of the higher Po treatment soils, the 200Po and 400Po treatments, small depletions in the post harvest soils were evident in the diester P and pyrophosphate compounds. This may have been due to plant uptake. There was no change in the monoester percentage. There was however a large increase in the inorganic orthophosphate which can only be accounted for by the breakdown of plant material in the soil forming Pi or the return of P to the soil by bacterial turnover. This was probably providing the P used by the plants.

There was little difference in the pattern of change between the inner and outer soil P quantities. It was expected that a larger decrease in P compounds would occur in the inner soil as this is where the plant roots and mycorrhizal hyphae are located. The outer soil is inaccessible to the roots, with only mycorrihzal hyphae present, and therefore it was expected to show less of a decrease in P content. Where decrease occurred it was to the same extent in the inner and outer soil. This would indicate that the phosphorus compounds are being accessed by mycorrhiza in the outer soil, which transport the P to the plant roots, and plant roots and mycorrhiza in the inner soil. Pyrophosphate is the only exception to this. The decrease in this compound is always greater in the inner soil. Pyrophosphate is mainly depleted by the roots in the inner compartment.

6.3.3 Correlation of soil P forms

The percentage area for the compounds calculated by integration of peak areas were correlated against each other using Pearson Product Moment correlation analysis. The results are presented in Table 6.3.

Table 6.3: Pearson Product Moment correlations (r values) using % area of total spectrum for each compound for P compounds detected by ^{31}P NMR analysis.
(** $p < 0.01$, * $p < 0.05$)

	(1)	(2)	(3)
Pyrophosphate (1)			
Inorganic orthophosphate (2)	-0.759 **		
Monoesters (3)	0.484	0.093	
Diesters (4)	0.729 **	-0.643 **	0.748 **

Pyrophosphate was negatively correlated with inorganic orthophosphate. That is as pyrophosphate content decreased, inorganic orthophosphate increased suggesting that mineralisation of this Po forms contributed to Pi content of the soil. Pyrophosphate was positively correlated with orthophosphate diester. This suggests that pyro P was a diester hydrolysis product. There was a significant negative correlation between diester P and inorganic orthophosphate. A decrease in diester P possibly by mineralisation was adding to the Pi pool. There was also a significant positive correlation between the two most prominent Po types, the monoester P and the diester P. As monoester P increased so too did diester P.

6.4 DISCUSSION

In this study a NaOH-EDTA extraction procedure was employed as it has been shown to be an efficient method (Cade-Menun and Preston, 1996), equally affective for all forms of P without serious distortion (Escudey *et al.*, 1997). There is good agreement between NMR and chemical analysis – NMR is detecting all forms of P present and is not causing serious distortions (Hawkes *et al.*, 1984). Extractants greatly affect the results of ^{31}P MNR analysis as they influence the concentration of P extracted and the forms of P seen in the NMR spectra. This makes it difficult to compare results from studies using different extractants. For this reason the results from this study will mainly be compared to those studies that also used a NaOH extraction.

The forms of P detected in the NMR spectra from this experiment were inorganic orthophosphate, orthophosphate monoester, orthophosphate diester and pyrophosphate and teichoic acid. Monoester P was the dominant compound in all the soils. This was as expected as monoester P, and in particular the monoester compound inositol phosphate, is one of the most dominant types of organic P in soil (Stevenson and Cole, 1999). When present orthophosphate was the second most dominant. Similar levels of pyrophosphate and diester P were detected. Teichoic acid was only present in the start soils of the 100Po, 200Po and 400Po treatments. Overall there was a higher level of Po in the soil. Dominance of organic forms of P was expected as the Po content of this PP1 soil is higher than the inorganic P content, both with and without Po amendment. The fine structure in the monoester region of the spectra was not resolved sufficiently for identification of individual P species, such as inositol hexaphosphate that would be present in the monoester fraction.

Orthophosphate monoesters are known to include inositol phosphate, mononucleotides and sugar P such as pentose and hexose sugars. Inositol phosphates which can constitute up to 60% of the total soil Po (Dalal, 1977; Newman and Tate, 1982) and are derived from plant, animal and microbial residues (Anderson, 1980). The inositol is composed largely of inositol penta- and hexa- phosphates (Hawkes *et al.*, 1984). In contrast to the single phosphate moiety of monoesters such as sugar phosphates, inositol phosphates carry a high charge density which allows strong interactions with soil minerals and precipitation as Al- Fe- and Ca-salts of low solubility (Adams and Byrne, 1989; Sumann *et al.*, 1998; Robinson *et al.*, 1988). Inositol phosphates are therefore much less labile, with a high stability against microbial and enzymatic attack (Sumann *et al.*, 1998), tending to accumulate in soil and having longer residence times (Tate and Newman, 1982).

Diester P includes phospholipids and nucleic acids (Newman and Tate, 1980) and is considered to be a labile fraction (Sumann *et al.*, 1998). Diester P in soils is largely microbially derived (Tate and Newman, 1982) as the major Po structure in microorganisms is diester P whereas,

plants contribute primarily to monoester P in soil (Sumann *et al.*, 1998). The modest amount of diester P produced by plants is readily decomposed whereas diester P produced by microbes is more likely to be stabilised on mineral surfaces possibly due to closer proximity of microbes to those surfaces (Sumann *et al.*, 1998). Tate and Newman (1982) suggested that choline phosphoglycerides are at least partly responsible for diester peak as other diesters such as RNA degrade rapidly in alkali and are unlikely to contribute to the NMR signal.

Pyrophosphate has previously been found in alkali extracts of soil but it is not known whether it occurs in an inorganic form or as an ester which undergoes hydrolysis under alkaline conditions (Condron *et al.*, 1985). A report of pyro P mineral in a natural rock has been made (Rouse *et al.*, 1988). Power (1992) suggested that pyrophosphate may be a hydrolysis product of polyphosphates which are themselves the result of hydrolysis of longer chain poly P compounds. Polyphosphate is due to microbial activity as P is accumulated by microbes in this form.

The P compounds identified in this experiment were similar to those obtained in other studies of alkali extracts. Table 6.4 presents a summary of the P compounds identified in different types of soil by workers using ^{31}P NMR analysis.

Power (1992) conducted ^{31}P NMR analysis on alkaline extracts of Irish P sufficient grassland soils. These soils were agricultural soils with a high nutrient turnover and cropping rate. She detected a large range of Po compounds including phosphonate, teichoic acid, phospholipid, diester P, monoester P, inorganic orthophosphate, pyrophosphate and polyphosphate. This was the first record of teichoic acid in an agricultural soil. In work presented by Power, Jeffrey and Tunney (1995) on a specific soil selected from a long term grazing experiment at Johnston Castle, Co. Wexford phosphonate, monoester P, teichoic acid, diester P and pyrophosphate were detected.

The work presented in this chapter is the second analysis of an Irish soil using ^{31}P NMR. In contrast to the agricultural soil of Power (1992) the PP1 site soil analysed in this chapter was a semi-natural grassland soil, uncropped, with no fertilisation and little grazing except by occasional deer. In this moderately fertile soil a simpler situation, in relation to soil Po compounds, was uncovered by ^{31}P NMR. Unlike the soils of Power (1992) no phosphonate, phospholipid or polyphosphate P was detected. Accumulation of phosphonates in soil has been considered to be due to the cells of protozoa, in which up to 15% of the total P may be in phosphonate form. Power (1992) concluded that the detection of phosphonate under intensive grassland conditions suggests that protozoa played a significant role in nutrient turnover under Irish grassland conditions. As no phosphonate was detected in the PP1 soil protozoa may not be so important in the P cycle in more natural unmanaged grassland soil. Power (1992) suggested

Table 6.4: P compounds identified by a number of workers using ^{31}P NMR spectroscopy of soil extracts.

Vegetation/ Soil Type	Inorganic orthophosphate	Monoester P	Diester P	Phosphonate	Teichoic Acid	Pyrophosphate	Polyphosphate	Reference:
Unfertilised grassland soil	22%)	48%	14%	7%		4%		Hawkes *et al.*, 1984
Volcanic soil, origin Chile	Y	Y	Y					Escudey *et al.*, 1997
Grassland soil		70%		Y		1%	Y	Tate and Newman, 1982
Forest soil, origin Canada	Y	Y	Y	Y		Y		Preston and Trofymow, 1988
Wetland soil Origin Florida	Y	50%	Y					Robinson *et al.*, 1998
Grassland soil, origin New Zealand	28%	50%	10%	2%		<1%	4%	Tate and Newman, 1980
Semi-natural grassland soil Origin Phoenix Park, Dublin	Y	Y	Y		Y	Y		Carroll, 2001

the high levels of phospholipid in her soils, especially those soils receiving slurrry applications, were of microbial origin. The application of slurry may have stimulated the bacteria. In the more natural PP1 soil there was possibly a lower bacterial population which did not result in a detectable phospholipid presence. Polyphosphate is present in soil due to microbial activity as P is accumulated by microbes in this form. The highly fertilised agricultural soil of Power (1992) especially with slurry application would have stimulated microbial activity and so a high presence of polyphosphate would be expected. In the more natural PP1 soil a lower nutrient input would result in a lower microbial presence and less polyphosphate. The differences in the range of Po compounds, as detected by ^{31}P NMR, between the Irish agricultural, managed soil and the Irish semi-natural grassland soil were related to differences in Po compounds associated with microbial activity and protozoa. The semi-natural soil possibly had a lower overall microbial activity as a result of a lower nutrient input, which in turn lead to a smaller range of Po compounds.

The range of P compounds found in this experiment, inorganic orthophosphate, monoester P, diester P and pyrophosphate, was similar to that found by these other workers. And in common with much of the previous research (Hawkes *et al.*, 1984; Newman and Tate, 1982; Robinson *et al.*, 1998; Newman and Tate, 1980) monoester P was found to be the most prominent P form in all soils. This is probably due to its tendency to build up in soil as discussed previously. Inorganic P was the second most prominent fraction. This was also the case with work carried out by Hawkes *et al.*, (1984) and Adams and Byrne (1989). Diester P is usually found to be less than 20% of the P detected (Newman and Tate, 1980; Tate and Newman, 1982; Adams and Byrne, 1989; Hawkes *et al.*, 1984). This was also the case in this experiment with an average of 4% diester P in the soil extracts. Diester P compounds (nucleic acids, phospholipids) are readily mineralised in soil which would account for the small quantities found in these extracts (Condron *et al.*, 1985). Pyrophosphate is believed to be involved in biological P cycling in soil (Condron *et al.*, 1985). Pyrophosphate varied from 1 to 6 % in this work. The quantity of pyrophosphate detected by other workers varies. Hawkes *et al.*, (1984) detected this compound at 4%. Adams and Byrne (1989) detected 10% and claimed this was more than usually detected. Tate and Newman (1982) detected <1% pyrophosphate and Condron *et al.*, (1985) found up to 7% in their extracts. Therefore the level detected in this work was in line with that found by other workers.

Many workers have detected phosphonates (Newman and Tate, 1980; Tate and Newman, 1982; Hawkes *et al.*, 1984; Preston and Trofymow, 1998; Sumann *et al.*, 1998; Mahieu *et al.*, 2000) and polyphosphates (Tate and Newman 1982; Newman and Tate, 1980). No traces of these compounds were found in any of the samples examined in this experiment. Escudey *et al.*, (1997) and Robinson *et al.*, (1998) also found no trace of these compounds in any of their

extracts. Polyphosphates are short-lived intermediates in the P cycle and it is proposed by Sumann *et al.*, (1998) that they can be synthesised my microorganisms under conditions of nutrient imbalance (Sumann *et al.*, 1998). It is possibly due to their short life that they were not found in this study or due to a low presence of microorganisms in the soil. Phosphonates are thought to be of microbial origin the product of protozoan metabolism (Sumann *et al.*, 1998). Hawkes *et al.*, (1984) concluded that the presence of phosphonates is a result of reduced decomposing activity due to low pH or cool moist conditions. This was also put forward by Mahieu *et al.*, (2000). Neither, low pH or cool moist conditions were present in this experiment, nor any other obvious conditions that would slow microbial activity. However Sumann *et al.*, (1998) suggest that phosphonates are also present in microbially active soils with slightly acid to slightly alkaline pH. As they are derived from protozoa the phosphonates may be part of cells that lysed because of drying. No dried soils were used for NMR in this experiment. There appears to be no definite criteria for the presence of phosphonates.

A small peak at 1.5 to 2.0 ppm was found in the start soil extracts of the 100Po, 200Po and 400Po treatments. Adams and Byrne (1989) and Hawkes *et al.*, (1984) also detected a small peak at this chemical shift (1.9 / 2.3 ppm) but failed to identify it. Condron *et al.*, (1990) assigned this range in the region between mono and diesters that had been unknown to teichoic acids. Teichoic acids are diester structures and are derived only from cell walls of gram positive bacteria (Condron *et al.*, 1990), where they can account for 85% of the total P found in the bacterial cell walls, and may function as a reserve P source (Condron *et al.*, 1990). Teichoic acids consist of chains of glycerol or sugar molecules linked by phosphate and attached to the mureine of gram positive bacteria. Naturally occurring teichoic acid is not a single compound but a complex the composition of which changes with environmental conditions and nutritional status of the bacterial population (Condron *et al.*, 1990). Its occurrence indicates an important role for bacteria in P transformations. As it only occurred in start soils where IHP was added and not in the control soil or the soil with Pi amendment it would suggest that the IHP caused a flush of bacterial activity resulting in the presence of teichoic acid. It is available for uptake by plants, after hydrolysis to orthophosphate, and therefore is not present in the postharvest soil. The initial flush in bacterial numbers with the addition of IHP may not have lasted. A subsequent reduction in numbers would also explain the lack of teichoic acid in the postharvest soil and the lack of phospholipid and polyphosphate that may be expected with a bacterial flush.

There was appreciable overlap between the Pi and monoester peaks making analysis difficult. This has been noted by others (Gil- Sotres *et al.*, 1990; Condron *et al.*, 1985; Robinson *et al.*, 1998). However a quantitative estimate of the relative proportions of these three types of P can be determined by integrating the area under the curve for each type of P and ratioing this value to the total integrated area (Robinson *et al.*, 1998). Soil was extracted with NaOH-EDTA both

before the transplanting of the *Agrostis stolonifera* into it and after the harvest of the plants. This was in order to determine if, any changes in the forms of P compounds and their quantities, due to plant growth and uptake could be detected. A range of P compounds commonly detected in soils was found. The appearance of orthophosphate was detected in some soils, and changing levels of the P compounds is evident from Table 6.2, despite the short growth period used in this experiment. This would indicate that NMR is suitable for detecting changing levels of the various P compounds even after a short period. It is then necessary to interpret these changing levels with respect to soil P cycling or plant uptake. Forms of P extracted from whole soil by NaOH have not yet been linked to uptake of P (Mahieu *et al.*, 2000).

Inositol hexaphosphate was added to the soil. This compound would be detected in the monoester region. However an increase in the monoester P content of the start soil with increasing levels of IHP added was not detected with the exception of a small increase in the 100Po treatment. It would appear that some mineralisation of the monoester P fraction occurred at the higher Po treatments, 200Po and 400Po, start soils before the transplanting of the *Agrostis* or during storage of the soil before NMR analysis. Orthophosphate was present in the start soils for these treatments which was not present in the control start soil.

Depletion of the monoester P fraction, the diester P and pyrophosphate occurred in the control and 100Po treatments following the growth of the *Agrostis*. Diesters are a relatively available pool of Po that are decreased or disappear with disturbance or cultivation (Preston and Trofymow, 1998; Robinson *et al.*, 1988). The depletion of the diesters is most likely due to plant uptake. It is likely that the pyrophosphate was also used for plant growth. Monoester is the Po species most resistant to mineralisation (Makarov *et al.*, 1997). However a reduction in the level of this fraction occurred. The mineralisation of this monoester could be responsible for the increasing levels of inorganic orthophosphate, which is plant available P.

There was also small depletion in the diester and pyrophosphate fractions in the 200Po and 400Po treatment soil. Again such depletions were probably due to plant P uptake. An increase in the inorganic P was detected in these soils but there is no corresponding decrease in monoester P to account for this. While mineralization of microbially derived diester P could be replenishing the concentration of native Pi (Tate and Newman, 1982) there was insufficient decrease in this Po compound to account for the large increase in Pi. It was possible that the presence of the plants in the soil was responsible for this increase in Pi. Plant debris resulting from the senescence of parts of the *Agrostis* may be entering the soil and being mineralised to Pi. It was also possible that some root exudates, such as citric acid, from the *Agrostis* were solubilising some of the inorganic P compounds in the soil. This would result in an increase in orthophosphate P content in the soil.

Overall, a decrease in the diester P fraction was found as expected, as this is a very labile Po compound, as is the pyrophosphate (Makarov *et al.*, 1997). Orthophosphate monoester was reduced to 40% - 50% from 60%, either through mineralisation to Pi and by plant uptake (control, 100Po soil) or by mineralisation before the addition of plants during the storage of the soil (200Po, 400Po) but never below this level. It is also impossible to distinguish hydrolysis in soil from any hydrolysis that may have occurred during extraction. Findings from several studies on the affects of long term cultivation on levels of Po in soils have indicated decreases in Po contents under cultivation due to the mineralisation of soil Po which occurs during long term rotational cropping, and to the removal of P by crops (Condron *et al.*, 1990). Decreases in Po were detected here using ^{31}P NMR analysis of alkali extracts of soils from a short-term pot experiment. Not only were decreases possibly resulting from mineralisation and plant uptake detected, but also increases in Pi illustrating the cycling of P soil.

Hawkes *et al.*, (1984) for their grassland soil found monoester P to be dominant in their unfertilised plots, but fertilised plots were dominated by Pi (79%). Escudey *et al.*, (1997) observed an increase in monoester and diester P as a result of Pi fertilisation. Soil Po increased as a result of adding super P fertiliser to pasture soils in work by Condron *et al.*, (1985). In this experiment in the soil fertilised with super P orthophosphate monoesters still dominated but there was no increase in their quantity. While there was an increase in the Pi content of the 100Pi treatment soil compared to the control soil as expected, the rate of 100Pi was insufficient to increase the Pi level above that of the monoester P. There was little change in the amount of diester and pyrophosphate in the Pi fertilised soil compared to the control. Levels of the Po compounds in the start and postharvest soil were similar. Changes occurred in the Pi content that are possibly the results of plant material returning to the soil and plants are obtaining their P from this dynamic pool.

Fig 6.6 presents a proposed scheme linking the proposed origins and fate of different organic P compounds in soil. Correlations were carried out between the different compounds detected. Negative correlations between orthophosphate and pyrophosphate and orthophosphate and diester P indicate, orthophosphate was contributed to by the mineralisation of these two Po compounds. This was in keeping with the fact that diester P and pyrophosphate are readily labile Po forms. Pyrophosphate was positively correlated with orthophosphate diester. This suggested that pyro P is a diester hydrolysis product. This relationship was also observes by Power (1992). There was also a significant correlation between monoester P and the diester P. These were the two most prominent Po types and an increase in one of these compounds was associated with an increase in the other. Diester P in soils is largely microbially derived (Tate and Newman, 1982) whereas plants contribute primarily to monoester P in soil (Sumann et al 1998). The addition of monoester to the soil through plant senescence possibly results in an

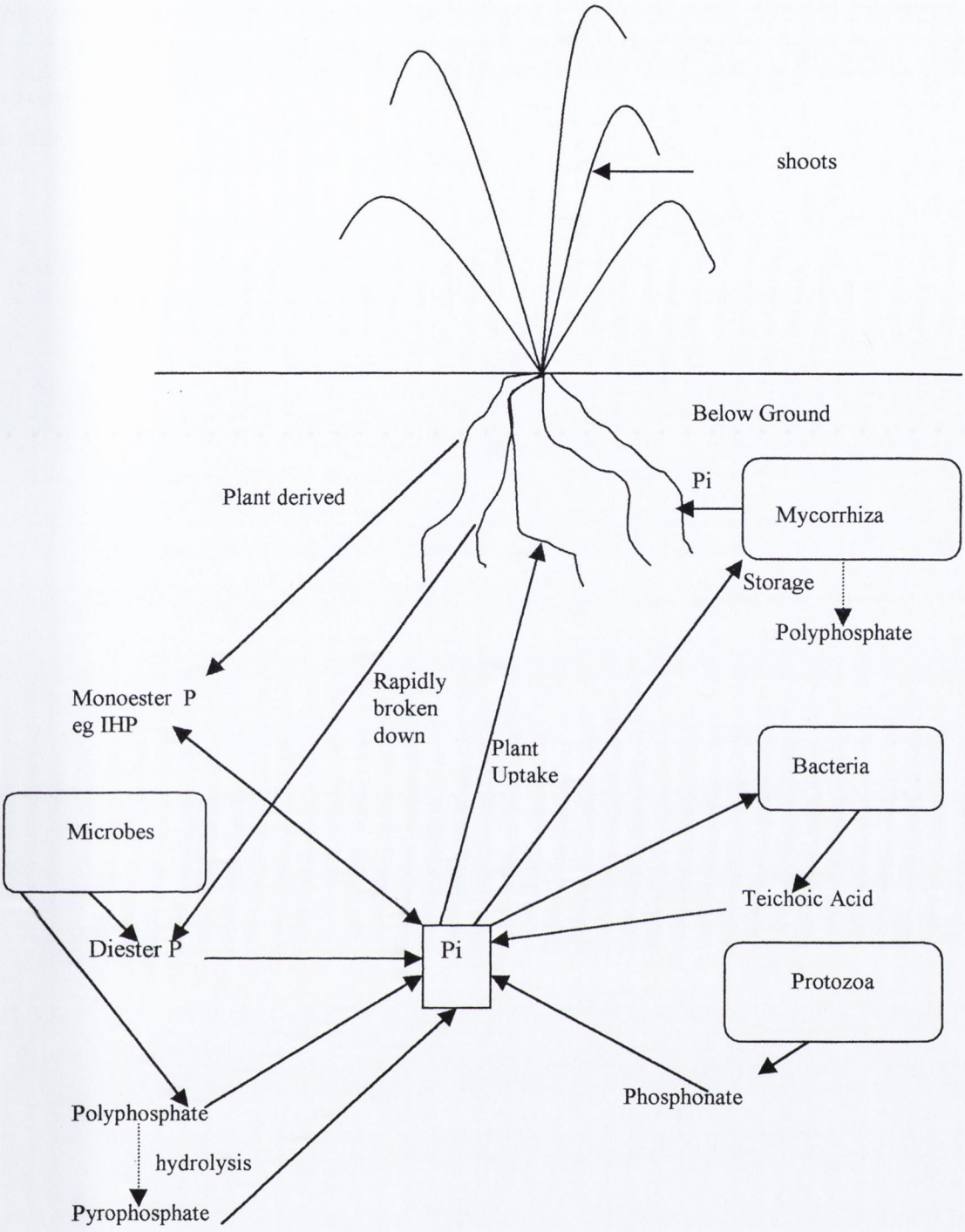


Fig 6.6: Proposed scheme for the origin and fate of organic P compounds in the soil.

increase in bacteria to mineralise this plant material. The increase in bacterial presence results in an increase in diester P.

6.5 SUMMARY

- ^{31}P NMR using a NaOH-EDTA extraction procedure is a suitable method to detect changes in levels of soil P compounds after a short period of plant growth.
- The range of Po compounds detected by ^{31}P NMR in extracts from a semi-natural grassland soil was: inorganic orthophosphate, orthophosphate monoesters, orthophosphate diesters, pyrophosphate and teichoic acid. There were no unidentified peaks.
- As expected orthophosphate monoesters were the most prominent compound in the soil.
- Diester P and pyrophosphate were consistently depleted from soil following plant growth, most likely due to plant uptake of these labile P compounds.
- Depletion of monoester P was the result of uptake by plants and mineralisation to increase the Pi pool present in soil.
- Diester P and pyrophosphate were negatively correlated to inorganic orthophosphate indicating that these Po forms are mineralised and available for plant uptake.

CHAPTER 7

The aim of this work was examine the availability of soil organic phosphate for plant growth. This was done through amendment of soil with inositol hexaphosphate, the most abundant Po compound found in soils. A semi-natural grassland soil with its indigenous population of microorganisms was used and experiments were conducted with plant species growing naturally in this soil in the field. Plants do not grow in isolation and their nutrition is influenced by factors present in the soil such as fungi and bacteria. It is necessary to look at the plant environment as a whole, remembering such interacting factors. Therefore in order to maintain a balanced outlook on Po nutrition of plants, several aspects of the plant system were examined in this thesis. The role of vesicular arbuscular mycorrhizae, soil bacteria, phosphatase enzymes, and nitrogen on the Po nutrition of plants were examined. The technique nuclear magnetic resonance was used for the identification of soil Po compounds. The following is a brief synopsis of the significant findings from each chapter to draws the different sections together.

IHP was an available source of soil P for plant use even at low concentrations. Plants were able to access this P source for growth through VAM hyphae. The infection of plant roots by VAM fungi was of benefit to plant growth at low soil Po concentrations. Plant P uptake was shown to be from the Po pool but from the area of soil accessible to VAM hyphae only, indicating the importance of these fungi to the Po nutrition of plants.

Increasing the concentration of Po in the soil did not affect the percentage root length infected by VAM hyphae for the grasses *Dactylis glomerata* and *Agrostis stolonifera* but there was a decrease in root infection for the herb *Plantago lanceolata*. The reaction is therefore possibly species dependent.

IHP amendments to the soil decreased the root acidic monoesterase activity, indicating plant P deficiency was alleviated by these amendments. Root acidic monoesterase is likely to be the most important enzyme with respect to IHP breakdown for uptake by plants.

Soil diesterase had the highest activity in the PP1 site soil followed by the acidic monoesterase and then the alkaline monoesterase . None of these enzymes reacted to IHP amendments in the soil.

VAM fungi were not involved in root acidic monoesterase production for the grasses *Dactylis glomerata* and *Agrostis stolonifera*. For the herb *Plantago lanceolata*, VAM are indicated as having some influence. VAM fungi were not involved in the production of soil phosphatase enzymes.

There was no affect of increased soil Po concentration on soil bacteria numbers. Streptomycin sensitive bacteria were in competition with *Dactylis glomerata* plants for available soil P. These

bacteria were not involved in the production of soil or root acidic monoesterase activity. Streptomycin sensitive bacteria were not therefore involved in the hydrolysis of soil Po to orthophosphate, the form of P taken up by plants, in the PP1 soil.

The rate of N application used in this experiment was insufficient to result in a significant increase in plant growth. There was also no significant affect of N treatment on the %RLC by VAM fungi. While N treatment did not significantly affect soil acidic monoesterase activity, it did increase root acidic monoesterase activity. This implies an increased potential for plant growth because of the increased N supply, which would require an increase in Po breakdown to orthophosphate. In this N limited soil the N addition did increase the biomass production but not significantly.

Nuclear magnetic resonance was employed to identify the Po compounds present in the PP1 soil and to assess the suitability of this technique for assessing changes in types and quantities of Po compounds resulting from plant P uptake over a short period. Orthophosphate monoester P was the most prominent compound in the soil extracts. Inorganic orthophosphate when present, was the second most prominent, followed by pyrophosphate, teichoic acid and diester P in smaller quantities. To quantify amounts of Po compounds present peak areas were integrated and expressed as percentages of total spectral area. Diester P and pyrophosphate were consistently depleted from soil following plant growth, most likely due to plant uptake of these labile P compounds. It was concluded that this method was more suited to the qualitative rather than quantitative analysis of soil Po compounds because changing levels of particular Po compounds could have been due to either plant P uptake or mineralisation. For example, depletion of the monoester P pool was evident, but there were also increases in the Pi pool.

Areas for further experimental exploration

Following on from the information obtained in this thesis there are some areas of plant Po nutrition that require further examination.

(1) The chemical extractions that are currently used to measure the available P within soil underestimate the availability of Po to plants. A fractionation scheme needs to be developed which will account for the Po as an available pool.

(2) In this work the IHP added to the soil was a commercially available calcium salt. In soil IHP forms complexes of low solubility with polyvalent cations, such as Fe and Al in acidic soils and Ca in calcareous soils. These insoluble complexes are undoubtedly less available to microorganisms and degradative enzymes than are the more soluble forms. (Stevenson and Cole, 1999). It is unknown if chelating agents such as citric acid could free the IHP from these complexes leaving it available for breakdown by phosphatases.

(3) High inorganic P levels in the soil have been linked to the inhibition of vesicular arbuscular mycorrhizal infection of roots. It is possible therefore that the Po in soil builds up because the amount of Po that can be transported into the plant through VAM hyphae is reduced. It would be of interest to determine if increased numbers of VAM fungi in soil significantly decreased Po build up in the soil and if this was affected by the species of VAM fungus present.

Appendix 1

Methods used to determine the soil basic characteristics

Soil pH

A small quantity of soil was placed into a 100ml beaker (approximately 1/3 full).

An equal volume of distilled deionised water was added and stirred to a slurry with a glass rod. The mixture was allowed to equilibrate for 15 minutes. The pH was measured with a Jenway pH Meter 3310.

Loss on Ignition

Loss on ignition was carried out to determine the % organic matter in the soil

1g of oven dried soil was weighed into a weighed crucible. This was ignited at 500°C for 8 hours. The samples were allowed to cool in a dessicator and were then re-weighed. The loss as a percentage of the original weight was calculated.

Bulk density

Bulk density was calculated from the LOI from the method of Jeffrey (1970) Journal of Ecology, 58: 297-299

Total, inorganic and organic P levels in soil

Total P was measured by the ignition method using 1 M H_2SO_4 (BDH chemicals) as the extracting solution. 2 g of dried soil was weighed out into a crucible and ignited at 550°C for 1 hour in a furnace (Thermolyne furnace Type 6000). The ignited sample was transferred to a 100 ml conical flask and 50 ml of 1 M H_2SO_4 was added. The mixture was heated at 90°C for one hour. The cooled solutions were diluted with 50 ml of distilled water, filtered through Whatman no. 1 filter paper and made up to 100 ml in a volumetric flask. The solution was then analysed for P as described in section 2.2.10.

Inorganic P was measured using the method described above with the exception of the ignition step. The two grams of dried soil was transferred directly into the 100 ml conical flask containing 50 ml H_2SO_4 and heated at 90°C for one hour. Organic P was measured as the difference between total P and inorganic P.

Particle size

The particle size composition of a soil is the separation of the mineral particles into mutually exclusive size-classes, generally sand, silt and clay. Each fraction was expressed as a percentage of the total mineral matter by weight. Particle size analysis was determined using a Bouyoucos Hydrometer, calibrated directly in g of material in suspension. The hydrometer method depends on Stokes Law where it is possible to predict which size class of particle is in suspension after a given time of settlement

Soils with > 10% organic matter required pre-treatment with hydrogen peroxide (H_2O_2) to destroy it as organic matter acts as a flocculating agent. Samples (50 g) of air dried soil were transferred to a 1 litre measuring cyclinder and 400 ml tap water and 25 ml Calgon added. The soil was dispersed thoroughly using a mixer. The volume was made up to 1 litre with tap water at room temperature. The mix was stirred for 1 minute and timings commenced for readings with the hydrometer. The hydrometer was inserted 20 seconds before reading. Readings were taken at 4 minutes 48 seconds and at 5 hours values referred to as A and B respectively. Reading A includes silt and clay and at reading B only clay is suspended. Percentage sand, silt and clay were calculated using the following equations:

$$\text{Clay} = B \times 100/50$$

$$\text{Silt} + \text{Clay} = A \times 100/50$$

$$\text{Silt} = (\text{Silt} + \text{Clay}) - \text{clay}$$

$$\text{Sand} = 100 - (\text{Silt} + \text{clay})$$

Appendix 2

Table 1: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Tp concentration** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1/Tp).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	44.5578	44.5578	4344.8	<0.0001
Pl	3	1.91504	0.638347	62.245	<0.0001***
Bl	1	0.0116352	0.0116352	1.1345	0.2948 ns
Pl*Bl	3	0.0228994	0.00763314	0.7443	0.5336 ns
Ara	1	0.00309226	0.00309226	0.30152	0.5867 ns
Pl*Ara	3	0.160564	0.0535215	5.2188	0.0048 **
Bl*Ara	1	0.00689219	0.00689219	0.67205	0.4184 ns
Pl*Bl*Ara	3	0.0135275	0.00450918	0.43969	0.7262 ns
Error	32	0.328174	0.0102554		
Total	47	2.46183			

Table 2: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Pi concentration** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1/Pi).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	203.183	203.183	3414.9	<0.0001
Pl	3	4.2123	1.4041	23.599	<0.0001***
Bl	1	0.130341	0.130341	2.1907	0.1486 ns
Pl*Bl	3	0.260871	0.0869569	1.4615	0.2435 ns
Ara	1	0.0708895	0.0708895	1.1915	0.2832 ns
Pl*Ara	3	0.096595	0.0321983	0.54116	0.6576 ns
Bl*Ara	1	0.000203799	0.000203799	0.0034253	0.9537 ns
Pl*Bl*Ara	3	0.0105258	0.00350861	0.05897	0.9809 ns
Error	32	1.90394	0.0594982		
Total	47	6.68567			

Table 3: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Po concentration** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (Log Po).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	3.23284	3.23284	227.89	<0.0001
Pl	3	0.844319	0.28144	19.84	<0.0001***
Bl	1	0.0027091	0.0027091	0.19097	0.6650 ns
Pl*Bl	3	0.0717168	0.0239056	1.6852	0.1898 ns
Ara	1	0.00165593	0.00165593	0.11673	0.7348 ns
Pl*Ara	3	0.219951	0.073317	5.1684	0.0050**
Bl*Ara	1	0.018231	0.018231	1.2852	0.2654 ns
Pl*Bl*Ara	3	0.0210043	0.00700144	0.49356	0.6893 ns
Error	32	0.453942	0.0141857		
Total	47	1.63353			

Table 4: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **% Root Length Colonisation** for the *Dactylis g.*. experiment. To obtain normal data, values were transformed before ANOVA was performed (√ %RLC).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	516.875	516.875	370.13	<0.0001
Pl	3	15.2296	5.07655	3.6353	0.0248*
Bl	1	28.1414	28.1414	20.152	0.0001***
Pl*Bl	2	1.02614	0.513068	0.36741	0.6958 ns
Error	28	39.1007	1.39645		
Total	34	177.125			

Table 5: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil acidic monoesterase** activity for the *Dactylis g.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	2.51525	2.51525	538.22	<0.0001
Pl	3	0.0494804	0.0164935	3.5293	0.0266*
Bl	1	0.00301151	0.00301151	0.64441	0.4284 ns
Pl*Bl	3	0.0275793	0.0091931	1.9672	0.1402 ns
Ara	1	0.0000765175	0.0000765175	0.016373	0.8990 ns
Pl*Ara	3	0.00760396	0.00253465	0.54237	0.6570 ns
Bl*Ara	1	0.0126952	0.0126952	2.7166	0.1097 ns
Pl*Bl*Ara	3	0.000909347	0.000303116	0.064862	0.9780 ns
Error	30	0.140198	0.00467328		
Total	45	0.247688			

Table 6: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root acidic mononesterase** activity for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1/ monoesterase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	4.98352	4.98352	94.351	<0.0001
Pl	3	0.976691	0.325564	6.1638	0.0077**
Bl	1	0.0171911	0.0171911	0.32547	0.5781 ns
Pl*Bl	3	0.051281	0.0170937	0.32363	0.8083 ns
Error	13	0.686647	0.052819		
Total	20	1.80321			

Table 7: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot dry weight** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (√ shoot dry weight).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	4.33245	4.33245	126.16	<0.0001
Pl	3	0.87187	0.290623	8.4631	0.0013***
Bl	1	0.0508277	0.0508277	1.4801	0.2414 ns
Pl*Bl	3	0.168112	0.0560373	1.6318	0.2215 ns
Error	16	0.549439	0.0343399		
Total	23	1.64025			

Table 8: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P concentration** for the *Dactylis g.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	74.3956	74.3956	104.26	<0.0001
Pl	3	7.1805	2.3935	3.3542	0.0506*
Bl	1	0.0342354	0.0342354	0.047977	0.8310 ns
Pl*Bl	3	0.307833	0.102611	0.1438	0.9313 ns
Error	10	7.13584	0.713584		
Total	17	14.6664			

Table 9: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P content** for the *Dactylis g.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	8.38337	8.38337	35.58	<0.0001
Pl	3	3.96921	1.32307	5.6153	0.0080**
Bl	1	1.06157	1.06157	4.5055	0.0497 ns
Pl*Bl	3	1.7837	0.594565	2.5234	0.0945 ns
Error	16	3.76989	0.235618		
Total	23	10.5844			

Table 10: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Tp concentration** for the *Plantago l.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1/Tp).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	51.7828	51.7828	4975	<0.0001
Pl	3	2.19164	0.730546	70.187 <	<0.0001***
Bl	1	0.0308635	0.0308635	2.9652	0.0947 ns
Pl*Bl	3	0.0303526	0.0101175	0.97203	0.4180 ns
Ara	1	0.00359522	0.00359522	0.34541	0.5608 ns
Pl*Ara	3	0.0181929	0.00606429	0.58262	0.6307 ns
Bl*Ara	1	0.00642982	0.00642982	0.61774	0.4377 ns
Pl*Bl*Ara	3	0.0232792	0.00775972	0.74551	0.5329 ns
Error	32	0.333076	0.0104086		
Total	47	2.63743			

Table 11: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Pi concentration** for the *Plantago l.* experiment. To obtain normal data, values were transformed before ANOVA was performed ($\sqrt{}$ Pi).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	21.6375	21.6375	7919.3	<0.0001
Pl	3	0.15563	0.0518765	18.987	<0.0001***
Bl	1	0.00530143	0.00530143	1.9403	0.1732 ns
Pl*Bl	3	0.00824814	0.00274938	1.0063	0.4027 ns
Ara	1	0.00360085	0.00360085	1.3179	0.2595 ns
Pl*Ara	3	0.0134215	0.00447382	1.6374	0.2002 ns
Bl*Ara	1	0.0000127954	0.0000127954	0.0046831	0.9459 ns
Pl*Bl*Ara	3	0.00861528	0.00287176	1.0511	0.3835 ns
Error	32	0.0874324	0.00273226		
Total	47	0.282262			

Table 12: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Po concentration** for the *Plantago l.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log Po).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	3.84994	3.84994	355.56	<0.0001
Pl	3	1.04348	0.347826	32.123	<0.0001***
Bl	1	0.00487062	0.00487062	0.44983	0.5072 ns
Pl*Bl	3	0.012388	0.00412932	0.38136	0.7671 ns
Ara	1	0.000021086	0.000021086	0.0019474	0.9651 ns
Pl*Ara	3	0.00873657	0.00291219	0.26895	0.8473 ns
Bl*Ara	1	0.00183955	0.00183955	0.16989	0.6830 ns
Pl*Bl*Ara	3	0.0127662	0.00425539	0.39301	0.7589 ns
Error	32	0.34649	0.0108278		
Total	47	1.43059			

Table 13: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **% Root Length Colonisation** for the *Plantago l.* experiment. To obtain normal data, values were transformed before ANOVA was performed (√ %RLC).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	1092.5	1092.5	333.56	<0.0001
Bl	1	85.1322	85.1322	25.992	<0.0001***
Pl	3	36.1964	12.0655	3.6838	0.0236*
Bl*Pl	3	5.21498	1.73833	0.53074	0.6649 ns
Error	28	91.7074	3.27526		
Total	35	259.496			

Table 14: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil acidic monoesterase** activity for the *Plantago l.* experiment. To obtain normal data, values were transformed before ANOVA was performed (√ soil phosphatase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	12.7949	12.7949	14603	< 0.0001
Pl	3	0.0276823	0.00922743	10.532	<0.0001***
Bl	1	0.00356468	0.00356468	4.0685	0.0521 ns
Pl*Bl	3	0.00214434	0.000714782	0.8158	0.4947 ns
Ara	1	0.000171015	0.000171015	0.19518	0.6616 ns
Pl*Ara	3	0.0000835843	0.0000278614	0.031799	0.9922 ns
Bl*Ara	1	0.000066466	0.000066466	0.07586	0.7848 ns
Pl*Bl*Ara	3	0.00331756	0.00110585	1.2621	0.3039 ns
Error	32	0.0280375	0.000876172		
Total	47	0.0650674			

Table 15: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root acidic monoesterase** activity for the *Plantago l.*. experiment. To obtain normal data, values were transformed before ANOVA was performed (log root phosphatase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	3.17279	3.17279	193.52	<0.0001
Pl	3	3.59671	1.1989	73.124	<0.0001***
Bl	1	0.168468	0.168468	10.275	0.0064**
Pl*Bl	3	0.725774	0.241925	14.756	0.0001***
Error	14	0.229537	0.0163955		
Total	21	5.11794			

Table 16: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot dry weight** for the *Plantago l.*. experiment. To obtain normal data, values were transformed before ANOVA was performed (log shoot dry weight).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	27.0622	27.0622	1568.5	<0.0001
Pl	3	8.24903	2.74968	159.37	<0.0001***
Bl	1	0.069529	0.069529	4.0298	0.0619 ns
Pl*Bl	3	2.02917	0.676389	39.202	<0.0001***
Error	16	0.276061	0.0172538		
Total	23	10.6238			

Table 17: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P concentration** for the *Plantago l.*. experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	45.5201	45.5201	964.72	<0.0001
Pl	3	6.80944	2.26981	48.105	<0.0001***
Bl	1	1.73121	1.73121	36.69	0.0002***
Pl*Bl	3	0.394347	0.131449	2.7858	0.1019 ns
Error	9	0.424661	0.0471846		
Total	16	8.94544			

Table 18: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P content** for the *Plantago l.*. experiment. To obtain normal data, values were transformed before ANOVA was performed ($\sqrt{}$ shoot P content).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	5.58191	5.58191	315.06	<0.0001
Pl	3	2.67923	0.893078	50.408	<0.0001***
Bl	1	0.0142989	0.0142989	0.80707	0.3823 ns
Pl*Bl	3	0.51362	0.171207	9.6633	0.0007***
Error	16	0.283474	0.0177171		
Total	23	3.49063			

Table 19: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Tp concentration** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1/Tp).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	48.7263	48.7263	25903	<0.0001
Pl	4	1.82145	0.455362	242.07	<0.0001***
Bl	1	0.00201051	0.00201051	1.0688	0.3079 ns
Pl*Bl	4	0.00508905	0.00127226	0.67633	0.6127 ns
Ara	1	0.000719103	0.000719103	0.38227	0.5402 ns
Pl*Ara	4	0.0027996	0.0006999	0.37206	0.8270 ns
Bl*Ara	1	0.0000747613	0.0000747613	0.039743	0.8431 ns
Pl*Bl*Ara	4	0.00505218	0.00126304	0.67143	0.6160 ns
Error	37	0.0696021	0.00188114		
Total	56	1.93446			

Table 20: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Pi concentration** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed ($\sqrt{}$ Pi).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	25.96	25.96	64993	<0.0001
Pl	4	0.145423	0.0363558	91.019	<0.0001***
Bl	1	0.000686175	0.000686175	1.7179	0.1978 ns
Pl*Bl	4	0.000716771	0.000179193	0.44862	0.7727 ns
Ara	1	0.000146778	0.000146778	0.36747	0.5480 ns
Pl*Ara	4	0.00325191	0.000812977	2.0353	0.1089 ns
Bl*Ara	1	0.00102699	0.00102699	2.5711	0.1171 ns
Pl*Bl*Ara	4	0.00198004	0.000495009	1.2393	0.3107 ns
Error	38	0.0151783	0.00039943		
Total	57	0.171051			

Table 21: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Po concentration** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed ($\sqrt{}$ Po).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	37.5333	37.5333	28302	< 0.0001
Pl	4	1.01469	0.253672	191.28	<0.0001***
Bl	1	0.000655649	0.000655649	0.49439	0.4864 ns
Pl*Bl	4	0.00782812	0.00195703	1.4757	0.2293 ns
Ara	1	0.000635998	0.000635998	0.47958	0.4929 ns
Pl*Ara	4	0.00691127	0.00172782	1.3029	0.2869 ns
Bl*Ara	1	0.000581637	0.000581637	0.43858	0.5119 ns
Pl*Bl*Ara	4	0.00347678	0.000869195	0.65542	0.6268 ns
Error	37	0.0490682	0.00132617		
Total	56	1.08933			

Table 22: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **% Root Length Colonisation** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed (√ %RLC).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	707.655	707.655	459.74	<0.0001
Pl	3	36.1289	12.043	7.8239	0.0004***
Bl	1	21.5456	21.5456	13.997	0.0006***
Pl*Bl	3	4.42451	1.47484	0.95815	0.4227 ns
Error	37	56.9525	1.53926		
Total	44	115.345			

Table 23: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil acidic monoesterase** activity for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	1.56982	1.56982	4718.3	<0.0001
Pl	4	0.0104337	0.00260843	7.8399	0.0001***
Bl	1	0.0000568575	0.0000568575	0.17089	0.6816 ns
Pl*Bl	4	0.000698505	0.000174626	0.52486	0.7180 ns
Ara	1	0.00212338	0.00212338	6.382	0.0158*
Pl* Ara	4	0.00183304	0.000458259	1.3773	0.2600 ns
Bl* Ara	1	0.000233358	0.000233358	0.70138	0.4076 ns
Pl*Bl*Ara	4	0.00204767	0.000511917	1.5386	0.2106 ns
Error	38	0.012643	0.000332712		
Total	57	0.0299261			

Table 24: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root acidic monoesterase** activity for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log root phosphatase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	42.1887	42.1887	1176.7	<0.0001
Pl	4	1.14467	0.286168	7.9819	<0.0001***
Bl	1	0.0282298	0.0282298	0.7874	0.3805 ns
Pl*Bl	3	0.084817	0.0282723	0.78858	0.5078 ns
Error	38	1.36238	0.035852		
Total	46	2.65198			

Table 25: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot dry weight** for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	19.1183	19.1183	261.9	<0.0001
Pl	4	0.807814	0.201954	2.7666	0.0614 ns
Bl	1	1.06202	1.06202	14.549	0.0014***
Pl*Bl	3	0.128889	0.042963	0.58855	0.6308 ns
Error	17	1.24096	0.0729977		
Total	25	8.22941			

Table 26: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root dry weight** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log root dry weight).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	5.8447	5.8447	114.93	<0.0001
Pl	3	0.223763	0.0745877	1.4667	0.2635 ns
Bl	1	0.800918	0.800918	15.749	0.0012***
Pl*Bl	3	0.0425634	0.0141878	0.27899	0.8397 ns
Error	15	0.762812	0.0508542		
Total	22	1.92248			

Table 27: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	75.5659	75.5659	1388.9	<0.0001
Pl	4	0.821705	0.205426	3.7757	0.0256*
Bl	1	0.0372514	0.0372514	0.68468	0.4209 ns
Pl*Bl	3	0.380673	0.126891	2.3323	0.1154 ns
Error	15	0.816104	0.0544069		
Total	23	4.30593			

Table 28: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root P concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	14.551	14.551	683.38	<0.0001
Pl	3	1.61761	0.539202	25.323	<0.0001***
Bl	1	0.00126548	0.00126548	0.059433	0.8107 ns
Pl*Bl	3	0.0259397	0.00864656	0.40608	0.7508 ns
Error	15	0.319389	0.0212926		
Total	22	2.04543			

Table 29: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **total plant P content** for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	90.9402	90.9402	378.81	<0.0001
Bl	1	5.20335	5.20335	21.675	0.0003 ***
Pl	4	6.63928	1.65982	6.914	0.0023**
Bl*Pl	3	0.912303	0.304101	1.2667	0.3214 ns
Error	15	3.60097	0.240065		
Total	23	26.8151			

Table 30: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Tp concentration** for the *Plantago l.* hyphal exploration experiment. To obtain normal data, values were transformed before ANOVA was performed (log Tp).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	0.28281	0.28281	3026.3	<0.0001
Pl	1	0.00353655	0.00353655	37.844	<0.0001***
Bl	1	0.000449974	0.000449974	4.8151	0.0433*
Pl*Bl	1	0.000329604	0.000329604	3.5271	0.0787 ns
Ara	1	0.00461	0.00461	49.331	<0.0001***
Pl*Ara	1	0.0021155	0.0021155	22.638	0.0002***
Bl*Ara	1	0.000215309	0.000215309	2.304	0.1486 ns
Pl*Bl*Ara	1	0.000157364	0.000157364	1.6839	0.2128 ns
Error	16	0.0014952	0.00009345		
Total	23	0.0129095			

Table 31: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Pi concentration** for the *Plantago l.* hyphal exploration experiment. To obtain normal data, values were transformed before ANOVA was performed (log Pi).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	4.86971	4.86971	7119.6	<0.0001
Pl	1	0.000419154	0.000419154	0.61281	0.4452 ns
Bl	1	0.000223339	0.000223339	0.32653	0.5757 ns
Pl*Bl	1	0.000447594	0.000447594	0.65439	0.4304 ns
Ara	1	0.000929158	0.000929158	1.3584	0.2609 ns
Pl*Ara	1	0.000540636	0.000540636	0.79042	0.3871 ns
Bnl*Ara	1	0.0000576714	0.0000576714	0.084317	0.7753 ns
Pl*Bnl*Ara	1	0.000115942	0.000115942	0.16951	0.6860 ns
Error	16	0.0109438	0.000683986		
Total	23	0.0136773			

Table 32: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Po concentration** for the *Plantago l.* hyphal exploration experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	10.1767	10.1767	260.45	<0.0001
Pl	1	0.0110854	0.0110854	0.28371	0.6016 ns
Bl	1	0.00334648	0.00334648	0.085646	0.7735 ns
Pl*Bl	1	0.00270938	0.00270938	0.06934	0.7957 ns
Ara	1	0.0124033	0.0124033	0.31743	0.5810 ns
Pl*Ara	1	0.00618246	0.00618246	0.15823	0.6961 ns
Bl*Ara	1	0.00027744	0.00027744	0.0071004	0.9339 ns
Pl*Bl*Ara	1	0.000743707	0.000743707	0.019033	0.8920 ns
Error	16	0.625178	0.0390736		
Total	23	0.661926			

Table 33: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **% Root Length Colonisation** for the *Plantago l.* hyphal exploration experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	33920.3	33920.3	860.56	<0.0001
Pl	1	12	12	0.30444	0.5962 ns
Bl	1	13872	13872	351.93	<0.0001***
Pl*Bl	1	320.333	320.333	8.1268	0.0215*
Error	8	315.333	39.4167		
Total	11	14519.7			

Table 34: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil hyphal content** for the *Plantago l.* hyphal exploration experiment. To obtain normal data, values were transformed before ANOVA was performed (log soil hyphal content).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	3.09528	3.09528	212.04	<0.0001
Pl	1	0.0240461	0.0240461	1.6472	0.2176 ns
Bl	1	0.0720618	0.0720618	4.9365	0.0411*
Pl*Bl	1	0.00502322	0.00502322	0.34411	0.5657 ns
Ara	1	0.209995	0.209995	14.385	0.0016***
Pl*Ara	1	0.0059971	0.0059971	0.41082	0.5306 ns
Bl*Ara	1	0.0157681	0.0157681	1.0802	0.3141 ns
Pl*Bl*Ara	1	0.0114051	0.0114051	0.78128	0.3898 ns
Error	16	0.233566	0.0145979		
Total	23	0.577863			

Table 35: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot dry weight** for the *Plantago l.* hyphal exploration experiment. To obtain normal data, values were transformed before ANOVA was performed (log shoot dry weight).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	31.5108	31.5108	652.69	<0.0001
Pl	1	0.0425642	0.0425642	0.88164	0.3752 ns
Bl	1	1.43791	1.43791	29.784	0.0006***
Pl*Bl	1	0.0243265	0.0243265	0.50388	0.4980 ns
Error	8	0.386227	0.0482784		
Total	11	1.89103			

Appendix 3

Table 1: ANOVA examining the effect of P treatment (Pl) and Streptomycin treatment (Stp) on **soil Tp concentration** for the *Dactylis g.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	38.7831	38.7831	1882.2	<0.0001
Pl	4	2.34359	0.585897	28.435	<0.0001***
Stp	1	0.000582865	0.000582865	0.028288	0.8682 ns
Pl*Stp	4	0.0123642	0.00309104	0.15001	0.9607 ns
Error	19	0.391493	0.0206049		
Total	28	2.7586			

Table 2: ANOVA examining the effect of P treatment (Pl), and Streptomycin treatment (Stp) on **soil Pi concentration** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1/Pi).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	164.999	164.999	1900.5	<0.0001
Pl	4	8.01632	2.00408	23.084	<0.0001***
Stp	1	0.0925126	0.0925126	1.0656	0.3143 ns
Pl*Stp	4	0.162258	0.0405644	0.46724	0.7591 ns
Error	20	1.73635	0.0868173		
Total	29	10.0074			

Table 3: ANOVA examining the effect of P treatment (Pl), and Streptomycin treatment (Stp) on **soil Po concentration** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1/Po).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	75.2818	75.2818	2366.1	<0.0001
Pl	4	7.00033	1.75008	55.005	<0.0001***
Stp	1	0.00625185	0.00625185	0.19649	0.6626 ns
Pl*Stp	4	0.0965644	0.0241411	0.75875	0.5648 ns
Error	19	0.604523	0.031817		
Total	28	7.68908			

Table 4: ANOVA examining the effect of P treatment (Pl), and Streptomycin treatment (Stp) on **soil acidic monoesterase activity** for the *Dactylis g.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	28.4179	28.4179	2506	<0.0001
Pl	4	0.0792515	0.0198129	1.7472	0.1793 ns
Stp	1	0.031143	0.031143	2.7463	0.1131 ns
Pl*Stp	4	0.0169195	0.00422986	0.373	0.8251 ns
Error	20	0.2268	0.01134		
Total	29	0.354114			

Table 5: ANOVA examining the effect of P treatment (Pl), and Streptomycin treatment (Stp) on **root acidic monoesterase activity** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1/root acidic monoesterase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	11.4053	11.4053	494.15	<0.0001
Pl	4	0.908607	0.227152	9.8418	0.0003***
Stp	1	0.323862	0.323862	14.032	0.0016***
Pl*Stp	4	0.379925	0.0949813	4.1152	0.0164**
Error	17	0.392367	0.0230804		
Total	26	1.97667			

Table 6: ANOVA examining the effect of P treatment (Pl), and Streptomycin treatment (Stp) on **shoot dry weight** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log shoot dry weight).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	19.7039	19.7039	228.09	<0.0001
Pl	4	11.773	2.94325	34.07	<0.0001***
Stp	1	1.88082	1.88082	21.772	0.0002***
Pl*Stp	4	2.26821	0.567053	6.564	0.0017***
Error	19	1.64138	0.0863882		
Total	28	17.7694			

Table 7: ANOVA examining the effect of P treatment (Pl), and Streptomycin treatment (Stp) on **shoot P concentration** for the *Dactylis g.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	100.932	100.932	1310.4	<0.0001
Pl	4	20.177	5.04426	65.488	<0.0001***
Stp	1	4.3026	4.3026	55.859	<0.0001***
Pl*Stp	4	2.42411	0.606027	7.8679	0.0006***
Error	19	1.46348	0.0770254		
Total	28	28.6184			

Table 8: ANOVA examining the effect of P treatment (Pl), and Streptomycin treatment (Stp) on **shoot P content** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log shoot P content).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	11.5566	11.5566	151.84	<0.0001
Pl	4	22.5766	5.64416	74.156	<0.0001***
Stp	1	0.947241	0.947241	12.445	0.0022**
Pl*Stp	4	1.64738	0.411846	5.411	0.0044**
Error	19	1.44613	0.0761121		
Total	28	26.7181			

Table 9: ANOVA examining the effect of P treatment (Pl), and Streptomycin treatment (Stp) on **soil bacterial numbers** for the *Dactylis g.* experiment.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05^{ns}$ (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	60.5373	60.5373	35187	<0.0001
Pl	4	0.155031	0.0387579	22.528	0.0001***
Stp	1	0.091073	0.091073	52.935	<0.0001***
Pl*Stp	4	0.0961121	0.024028	13.966	0.0007***
Error	9	0.0154841	0.00172046		
Total	18	0.411544			

Table 10: ANOVA examining the effect of P treatment (Pl), and Streptomycin treatment (Stp) on **% root length colonisation** for the *Dactylis g.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05^{ns}$ (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	50244.5	50244.5	121.76	<0.0001
Stp	1	237.62	237.62	0.57585	0.4517 ns
Error	48	19806.9	412.643		
Total	49	20044.5			

Appendix 4

Table 1: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil acidic monoesterase activity** for the *Agrostis s.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	1.56104	1.56104	767.92	<0.0001
Pl	2	0.00633654	0.00316827	1.5586	0.2310 ns
Bl	1	0.00151079	0.00151079	0.7432	0.3972 ns
Pl*Bl	2	0.00597098	0.00298549	1.4687	0.2502 ns
Ara	1	0.00577058	0.00577058	2.8387	0.1050 ns
Pl*Ara	2	0.000483635	0.000241818	0.11896	0.8884 ns
Bnl*Ara	1	0.000586344	0.000586344	0.28844	0.5962 ns
Pl*Bl*Ara	2	0.00174107	0.000870534	0.42824	0.6565 ns
Error	24	0.0487873	0.0020328		
Total	35	0.0711872			

Table 2: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil alkaline monoesterase activity** for the *Agrostis s.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	0.353113	0.353113	745.55	<0.0001
Pl	2	0.00307151	0.00153576	3.2426	0.0567 ns
Bl	1	0.0017612	0.0017612	3.7186	0.0657 ns
Pl*Bl	2	0.00256763	0.00128381	2.7106	0.0868 ns
Ara	1	0.000643468	0.000643468	1.3586	0.2552 ns
Pl*Ara	2	0.00177324	0.00088662	1.872	0.1756 ns
Bl*Ara	1	0.000831361	0.000831361	1.7553	0.1977 ns
Pl*Bl*Ara	2	0.000517387	0.000258694	0.5462	0.5862 ns
Error	24	0.011367	0.000473625		
Total	35	0.0225328			

Table 3: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil diesterase activity** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log soil diesterase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	0.00193553	0.00193553	0.31138	0.5820
Pl	2	0.0481893	0.0240946	3.8763	0.0348 *
Bl	1	0.000000759153	0.00000075	0.00012213	0.9913 ns
Pl*Bl	2	0.071357	0.0356785	5.7399	0.0092**
Ara	1	0.0000461316	0.0000461316	0.0074216	0.9321 ns
Pl*Ara	2	0.00927512	0.00463756	0.74608	0.4849 ns
Bl*Ara	1	0.00138604	0.00138604	0.22298	0.6410 ns
Pl*Bl*Ara	2	0.0215312	0.0107656	1.732	0.1983 ns
Error	24	0.149181	0.00621587		
Total	35	0.300966			

Table 4: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root acidic monoesterase activity** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed ($1/\sqrt{}$ root acidic monoesterase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	24.547	24.547	271.52	<0.0001
Pl	2	0.12773	0.0638651	0.70643	0.5128 ns
Bl	1	0.000013879	0.000013879	0.00015352	0.9903 ns
Pl*Bl	2	0.00449409	0.00224704	0.024855	0.9755 ns
Error	12	1.08487	0.0904055		
Total	17	1.2171			

Table 5: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot dry weight** for the *Agrostis s.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	9.0287	9.0287	88.665	<0.0001
Pl	2	3.82626	1.91313	18.788	0.0002***
Bl	1	0.00315748	0.00315748	0.031007	0.8632 ns
Pl*Bl	2	0.065823	0.0329115	0.3232	0.7299 ns
Error	12	1.22196	0.10183		
Total	17	5.1172			

Table 6: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root dry weight** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed ($1/\sqrt{}$ root dry weight).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	277.895	277.895	497.95	<0.0001
Pl	2	573.832	286.916	514.11	<0.0001***
Bl	1	202.769	202.769	363.33	<0.0001***
Pl*Bl	2	320.054	160.027	286.74	<0.0001***
Error	9	5.02276	0.558084		
Total	14	750.401			

Table 7: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	20.3371	20.3371	345.99	<0.0001
Trt	2	0.430673	0.215336	3.6634	0.0441*
Bl	1	0.00875913	0.00875913	0.14901	0.7036 ns
Trt*Bl	2	0.0461096	0.0230548	0.39222	0.6806 ns
Error	20	1.17561	0.0587803		
Total	25	1.65272			

Table 8: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root P concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	4.48564	4.48564	204.06	<0.0001
Pl	2	0.15647	0.0782349	3.5591	0.0784 ns
Bl	1	0.0707267	0.0707267	3.2176	0.1106 ns
Pl*Bl	1	0.0776418	0.0776418	3.5322	0.0970 ns
Error	8	0.175852	0.0219814		
Total	12	0.439954			

Table 9: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P content** for the *Agrostis s.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	6.87275	6.87275	52.785	<0.0001
Pl	2	2.87299	1.43649	11.033	0.0019***
Bl	1	0.00000198381	0.00000198381	0.000015236	0.9969 ns
Pl*Bl	2	0.0244176	0.0122088	0.093768	0.9112 ns
Error	12	1.56242	0.130202		
Total	17	4.45983			

Table 10: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root P content** for the *Agrostis s.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	0.445116	0.445116	38.852	0.0002
Pl	2	0.0593525	0.0296762	2.5903	0.1293 ns
Bl	1	0.0000481673	0.0000481673	0.0042043	0.9497 ns
Pl*Bl	1	0.000855036	0.000855036	0.074632	0.7909 ns
Error	9	0.103111	0.0114567		
Total	13	0.1729			

Table 11: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Tp concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	19.1444	19.1444	3078	<0.0001
Pl	2	0.0418445	0.0209223	3.3639	0.0550*
Bl	1	0.0066309	0.0066309	1.0661	0.3141 ns
Pl*Bl	2	0.00311269	0.00155635	0.25023	0.7810 ns
Ara	1	0.000921359	0.000921359	0.14814	0.7044 ns
Pl*Ara	2	0.00524711	0.00262355	0.42182	0.6616 ns
Bl*Ara	1	0.000229335	0.000229335	0.036873	0.8497 ns
Pl*Bl*Ara	1	0.00060483	0.00060483	0.097245	0.7584 ns
Error	20	0.124393	0.00621965		
Total	30	0.21773			

Table 12: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Pi concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	3.97196	3.97196	3637.6	<0.0001
Pl	2	0.00400283	0.00200142	1.8329	0.1846 ns
Bl	1	0.000000275104	0.0000002751	0.00025194	0.9875 ns
Pl*Bl	2	0.00333865	0.00166932	1.5288	0.2400 ns
Ara	1	0.00000641063	0.00000641063	0.0058709	0.9396 ns
Pl*Ara	2	0.000782355	0.000391177	0.35825	0.7031 ns
Bl*Ara	1	0.00263388	0.00263388	2.4121	0.1353 ns
Pl*Bl*Ara	2	0.000487952	0.000243976	0.22344	0.8016 ns
Error	21	0.0229305	0.00109193		
Total	32	0.0344185			

Table 13: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Po concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	5.57871	5.57871	820.81	<0.0001
Pl	2	0.0203139	0.010157	1.4944	0.2509 ns
Bl	1	0.00363389	0.00363389	0.53466	0.4741 ns
Pl*Bl	2	0.00305244	0.00152622	0.22456	0.8011 ns
Ara	1	0.00000490511	0.00000490511	0.0007217	0.9789 ns
Pl*Ara	2	0.00594565	0.00297283	0.4374	0.6524 ns
Bnl*Ara	1	0.000201379	0.000201379	0.029629	0.8653 ns
Pl*Bl*Ara	1	0.00337362	0.00337362	0.49637	0.4901 ns
Error	18	0.122339	0.00679663		
Total	28	0.190472			

Table 14: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil acidic monoesterase activity** for the *Holcus l.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1/soil acidic monoesterase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	315.751	315.751	2920.6	<0.0001
Pl	2	0.683302	0.341651	3.1601	0.0622 ns
Bl	1	0.000699887	0.000699887	0.0064737	0.9366 ns
Pl*Bl	2	0.877804	0.438902	4.0597	0.0316*
Ara	1	1.06454	1.06454	9.8466	0.0048**
Pl*Ara	2	0.420053	0.210026	1.9427	0.1671 ns
Bnl*Ara	1	0.261382	0.261382	2.4177	0.1342 ns
Pl*Bl*Ara	2	0.078461	0.0392305	0.36287	0.6998 ns
Error	22	2.37847	0.108112		
Total	33	5.91329			

Table 15: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil alkaline monoesterase activity** for the *Holcus l.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	0.150932	0.150932	329.1	<0.0001
Pl	2	0.000479807	0.000239903	0.5231	0.5993 ns
Bl	1	0.00011664	0.00011664	0.25433	0.6186 ns
Pl*Bl	2	0.00216185	0.00108092	2.3569	0.1163 ns
Ara	1	0.00309507	0.00309507	6.7487	0.0158**
Pl*Ara	2	0.00124566	0.000622831	1.3581	0.2762 ns
Bnl*Ara	1	0.000402671	0.000402671	0.87801	0.3581 ns
Pl*Bl*Ara	2	0.0018055	0.000902751	1.9684	0.1616 ns
Error	24	0.0110068	0.000458616		
Total	35	0.020314			

Table 16: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil diesterase activity** for the *Holcus l.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	37.2834	37.2834	927.58	<0.0001
Pl	2	0.112429	0.0562146	1.3986	0.2664 ns
Bl	1	0.000667688	0.000667688	0.016612	0.8985 ns
Pl*Bl	2	0.0319739	0.0159869	0.39774	0.6762 ns
Ara	1	0.146357	0.146357	3.6412	0.0684 ns
Pl* Ara	2	0.108467	0.0542337	1.3493	0.2784 ns
Bl* Ara	1	0.00615736	0.00615736	0.15319	0.6990 ns
Pl*Bl* Ara	2	0.090772	0.045386	1.1292	0.3399 ns
Error	24	0.964662	0.0401943		
Total	35	1.46149			

Table 17: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root acidic monoesterase activity** for the *Holcus l.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1og root acidic monoesterase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	0.93107	0.93107	79.551	<0.0001
Pl	2	0.148098	0.0740489	6.3267	0.0148**
Bl	1	0.0168179	0.0168179	1.4369	0.2558 ns
Pl*Bl	2	0.0634344	0.0317172	2.7099	0.1104 ns
Error	11	0.128745	0.0117041		
Total	16	0.380834			

Table 18: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot dry weight** for the *Holcus l.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ns (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	17.0645	17.0645	253.06	<0.0001
Pl	2	8.37114	4.18557	62.071	<0.0001***
Bl	1	0.183618	0.183618	2.723	0.1248 ns
Pl*Bl	2	0.618308	0.309154	4.5847	0.0332*
Error	12	0.809182	0.0674319		
Total	17	9.98225			

Table 19: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root dry weight** for the *Holcus l.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ns (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	21.414	21.414	81.754	<0.0001
Pl	2	10.5653	5.28263	20.168	0.0002***
Bl	1	0.0180698	0.0180698	0.068986	0.7977 ns
Pl*Bl	2	0.0315121	0.015756	0.060153	0.9419 ns
Error	11	2.88127	0.261933		
Total	16	13.7132			

Table 20: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P concentration**for the *Holcus l.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ns (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	14.4457	14.4457	879.23	<0.0001
Pl	2	0.375271	0.187635	11.42	0.0004***
Bl	1	0.0569294	0.0569294	3.465	0.0761 ns
Pl*Bl	2	0.0551906	0.0275953	1.6796	0.2095 ns
Error	22	0.36146	0.01643		
Total	27	1.03568			

Table 21: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root P concentration** for the *Holcus l.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ns (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	3.62645	3.62645	399.54	<0.0001
Pl	2	0.134547	0.0672736	7.4118	0.0187*
Bl	1	0.00221459	0.00221459	0.24399	0.6365 ns
Pl*Bl	1	0.000132421	0.000132421	0.014589	0.9073 ns
Error	7	0.0635358	0.00907655		
Total	11	0.201054			

Table 22: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P content** for the *Holcus l.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	9.44938	9.44938	757.39	< 0.0001
Pl	2	5.41849	2.70925	217.15	<0.0001***
Bl	1	0.000638345	0.000638345	0.051165	0.8249 ns
Pl*Bl	2	0.0905084	0.0452542	3.6272	0.0586 ns
Error	12	0.149715	0.0124762		
Total	17	5.65935			

Table 23: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root P content** for the *Holcus l.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	6.88605	6.88605	87.106	<0.0001
Pl	2	2.16045	1.08023	13.664	0.0019***
Bl	1	0.0331445	0.0331445	0.41926	0.5335 ns
Pl*Bl	1	0.0218065	0.0218065	0.27584	0.6121 ns
Error	9	0.711486	0.079054		
Total	13	3.24413			

Table 24: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Tp concentration** for the *Holcus l.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	20.9424	20.9424	3785.8	<0.0001
Pl	2	0.0292305	0.0146152	2.642	0.0927 ns
Bl	1	0.000314747	0.000314747	0.056898	0.8136 ns
Pl*Bl	2	0.0198486	0.00992429	1.794	0.1888 ns
Ara	1	0.0017507	0.0017507	0.31648	0.5792 ns
Pl*Ara	2	0.00162999	0.000814995	0.14733	0.8638 ns
Bl*Ara	1	0.00290065	0.00290065	0.52436	0.4763 ns
Pl*Bl*Ara	2	0.00431927	0.00215964	0.3904	0.6812 ns
Error	23	0.127232	0.00553182		
Total	34	0.190795			

Table 25: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Pi concentration** for the *Holcus l.* experiment. Data was normal, no transformation necessary ($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ns (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	4.42082	4.42082	7381.8	<0.0001
Pl	2	0.000516387	0.000258193	0.43113	0.6547 ns
Bl	1	0.00000531717	0.00000531717	0.0088785	0.9257 ns
Pl*Bl	2	0.00166828	0.000834138	1.3928	0.2677 ns
Ara	1	0.00230997	0.00230997	3.8571	0.0612 ns
Pl*Ara	2	0.00288449	0.00144225	2.4082	0.1114 ns
Bl*Ara	1	0.00000000530962	0.00000000	0.0000088659	0.9976 ns
Pl*Bl*Ara	2	0.000796837	0.000398418	0.66527	0.5234 ns
Error	24	0.0143732	0.000598882		
Total	35	0.0225545			

Table 26: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Po concentration** for the *Holcus l.* experiment. Data was normal, no transformation necessary ($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ns (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	6.25002	6.25002	1186.7	<0.0001
Pl	2	0.0339321	0.0169661	3.2213	0.0584*
Bl	1	0.000418824	0.000418824	0.079521	0.7805 ns
Pl*Bl	2	0.0323056	0.0161528	3.0669	0.0660 ns
Ara	1	0.00799185	0.00799185	1.5174	0.2305 ns
Pl*Ara	2	0.00216811	0.00108406	0.20583	0.8155 ns
Bl*Ara	1	0.00285843	0.00285843	0.54272	0.4688 ns
Pl*Bl*Ara	2	0.00308189	0.00154095	0.29258	0.7491 ns
Error	23	0.121137	0.00526682		
Total	34	0.207938			

Table 27: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil acidic monoesterase activity** for the *Dactylis g.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ns (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	5.18264	5.18264	5407.8	<0.0001
Pl	2	0.00116629	0.000583144	0.60848	0.5524 ns
Bl	1	0.00133844	0.00133844	1.3966	0.2489 ns
Pl*Bl	2	0.0186673	0.00933364	9.7391	0.0008***
Ara	1	0.00459165	0.00459165	4.7911	0.0386*
Pl*Ara	2	0.000118631	0.0000593153	0.061892	0.9401 ns
Bl*Ara	1	0.00236077	0.00236077	2.4633	0.1296 ns
Pl*Bl*Ara	2	0.00195476	0.00097738	1.0198	0.3758 ns
Error	24	0.0230007	0.000958364		
Total	35	0.0531985			

Table 28: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil alkaine monoesterase activity** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log soil alkaline monoesterase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	0.221825	0.221825	503.63	<0.0001
Pl	2	0.00125717	0.000628585	1.4271	0.2596 ns
Bl	1	0.0000180625	0.0000180625	0.041009	0.8412 ns
Pl*Bl	2	0.00184535	0.000922676	2.0948	0.1450 ns
Ara	1	0.00962034	0.00962034	21.842	<0.0001***
Pl*Ara	2	0.00368394	0.00184197	4.182	0.0277*
Bl*Ara	1	0.0000605803	0.0000605803	0.13754	0.7140 ns
Pl*Bl*Ara	2	0.000883347	0.000441674	1.0028	0.3817 ns
Error	24	0.0105708	0.000440451		
Total	35	0.0279396			

Table 29: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil diesterase activity** for the *Dactylis g.* experiment Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	89.1057	89.1057	5341.2	<0.0001
Pl	2	0.0906343	0.0453172	2.7164	0.0864 ns
Bl	1	0.0254221	0.0254221	1.5239	0.2290 ns
Pl*Bl	2	0.132466	0.0662331	3.9702	0.0324*
Ara	1	0.07177	0.07177	4.3021	0.0490*
Pl*Ara	2	0.0815465	0.0407733	2.444	0.1081 ns
Bl*Ara	1	0.0302098	0.0302098	1.8108	0.1910 ns
Pl*Bl*Ara	2	0.00782302	0.00391151	0.23446	0.7928 ns
Error	24	0.400385	0.0166827		
Total	35	0.840257			

Table 30: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) **on root acidic monoesterase activity** for the *Dactylis g.* experiment Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	3.20416	3.20416	709.92	<0.0001
Pl	2	0.992209	0.496105	109.92	<0.0001***
Bl	1	0.00777089	0.00777089	1.7217	0.2140 ns
Pl*Bl	2	0.0292837	0.0146419	3.2441	0.0748 ns
Error	12	0.0541608	0.0045134		
Total	17	1.08342			

Table 31: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot dry weight** for the *Dactylis g.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	8.93799	8.93799	107.68	<0.0001
Pl	2	3.65845	1.82922	22.038	<0.0001***
Bl	1	0.0307024	0.0307024	0.36989	0.5544 ns
Pl*Bl	2	0.421893	0.210946	2.5414	0.1202 ns
Error	12	0.996046	0.0830038		
Total	17	5.10709			

Table 32: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root dry weight** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed ($\sqrt{}$ root dry weight).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	5.80494	5.80494	176.93	<0.0001
Pl	2	1.88206	0.941031	28.682	<0.0001***
Bl	1	0.0000655183	0.0000655183	0.001997	0.9651 ns
Pl*Bl	2	0.21803	0.109015	3.3227	0.0711 ns
Error	12	0.393707	0.0328089		
Total	17	2.49386			

Table 33: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P concentration** for the *Dactylis g.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	30.6758	30.6758	827.55	<0.0001
Pl	2	1.92136	0.960682	25.916	<0.0001***
Bl	1	0.4679	0.4679	12.623	0.0020**
Pl*Bl	2	1.49692	0.748459	20.191	<0.0001***
Error	20	0.741367	0.0370684		
Total	25	4.81879			

Table 34: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root P concentration** for the *Dactylis g.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	4.58321	4.58321	257.49	<0.0001
Pl	2	0.291351	0.145675	8.1843	0.0193*
Bl	1	0.0148999	0.0148999	0.8371	0.3955 ns
Pl*Bl	1	0.0196438	0.0196438	1.1036	0.3339 ns
Error	6	0.106797	0.0177995		
Total	10	0.435311			

Table 35: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **shoot P content** for the *Dactylis g.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	10.0223	10.0223	349.58	<0.0001
Pl	2	5.0046	2.5023	87.279	<0.0001***
Bl	1	0.0251223	0.0251223	0.87626	0.3677 ns
Pl*Bnl	2	0.0860299	0.043015	1.5003	0.2621 ns
Error	12	0.34404	0.02867		
Total	17	5.45979			

Table 36: ANOVA examining the effect of P treatment (Pl) and Benlate treatment (Bl) on **root P content** for the *Dactylis g.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	0.72059	0.72059	46.216	<0.0001
Pl	2	0.401552	0.200776	12.877	0.0017***
Bl	1	0.0000846748	0.0000846748	0.0054307	0.9427 ns
Pl*Bl	1	0.0203541	0.0203541	1.3054	0.2798 ns
Error	10	0.155918	0.0155918		
Total	14	0.679765			

Table 37: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Tp concentration** for the *Dactylis g.*. experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	21.3089	21.3089	5846.7	<0.0001
Pl	2	0.0545519	0.027276	7.4839	0.0030**
Bl	1	0.0030265	0.0030265	0.8304	0.3712 ns
Pl*Bl	2	0.000696605	0.000348302	0.095566	0.9092 ns
Ara	1	0.00476507	0.00476507	1.3074	0.2641 ns
Pl*Ara	2	0.000871245	0.000435623	0.11952	0.8879 ns
Bl*Ara	1	0.0000622624	0.0000622624	0.017083	0.8971 ns
Pl*Bl*Ara	2	0.00582917	0.00291458	0.7997	0.4611 ns
Error	24	0.0874708	0.00364462		
Total	35	0.157274			

Table 38: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Pi concentration** for the *Dactylis g.* experiment. To obtain normal data, values were transformed before ANOVA was performed (1/soil Pi concentration).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	263.012	263.012	2363.5	<0.0001
Pl	2	0.182092	0.0910458	0.81816	0.4532 ns
Bl	1	0.0026117	0.0026117	0.023469	0.8795 ns
Pl*Bl	2	0.518011	0.259005	2.3275	0.1192 ns
Ara	1	0.00366112	0.00366112	0.0329	0.8576 ns
Pl*Ara	2	0.104147	0.0520734	0.46794	0.6319 ns
Bl*Ara	1	0.157944	0.157944	1.4193	0.2452 ns
Pl*Bl*Ara	2	0.0543083	0.0271542	0.24401	0.7854 ns
Error	24	2.67076	0.111281		
Total	35	3.69353			

Table 39: ANOVA examining the effect of P treatment (Pl), Benlate treatment (Bl) and location of soil (Ara) on **soil Po concentration** for the *Dactylis g.* experiment. Data was normal, no transformation necessary
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	5.57424	5.57424	843.42	<0.0001
Pl	2	0.0441231	0.0220616	3.3381	0.0526*
Bl	1	0.0031398	0.0031398	0.47507	0.4973 ns
Pl*Bl	2	0.0107384	0.0053692	0.8124	0.4556 ns
Ara	1	0.00366353	0.00366353	0.55432	0.4638 ns
Pl*Ara	2	0.00395543	0.00197771	0.29924	0.7441 ns
Bl*Ara	1	0.00560841	0.00560841	0.84859	0.3661 ns
Pl*Bl*Ara	2	0.0132317	0.00661584	1.001	0.3823 ns
Error	24	0.158618	0.00660907		
Total	35	0.243078			

Appendix 5

Table 1: ANOVA examining the effect of P treatment (Pl), and Nitrogen treatment (N) on **shoot dry weight** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log shoot dry weight).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	24.6589	24.6589	167.49	<0.0001
Pl	4	7.11411	1.77853	12.08	<0.0001***
N	1	0.00753799	0.00753799	0.051201	0.8233 ns
Pl*N	4	0.59589	0.148972	1.0119	0.4249 ns
Error	20	2.94447	0.147223		
Total	29	10.662			

Table 2: ANOVA examining the effect of P treatment (Pl), and Nitrogen treatment (N) on **shoot P concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	25.726	25.726	170.23	<0.0001
Pl	4	2.83343	0.708359	4.6873	0.0255*
N	1	0.655156	0.655156	4.3353	0.0670 ns
Pl*N	4	0.671317	0.167829	1.1105	0.4089 ns
Error	9	1.36011	0.151123		
Total	18	5.85487			

Table 3: ANOVA examining the effect of P treatment (Pl), and Nitrogen treatment (N) on **shoot P content** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log shoot P content).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	27.7269	27.7269	188.33	<0.0001
Pl	4	14.8042	3.70105	25.139	<0.0001***
N	1	0.406556	0.406556	2.7615	0.1122 ns
Pl*N	4	0.535499	0.133875	0.90933	0.4775 ns
Error	20	2.94447	0.147223		
Total	29	18.6907			

Table 4: ANOVA examining the effect of P treatment (Pl) and Nitrogen treatment (N) on **phosphorus use efficiency** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log phosphorus use efficiency).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	0.0924317	0.0924317	infinity	<0.0001
Pl	4	1.55975	0.389938	infinity	<0.0001
N	1	0.299523	0.299523	infinity	<0.0001
Pl*N	4	0.174158	0.0435395	infinity	<0.0001
Error	20	0 0			
Total	29	2.03343			

Table 5: ANOVA examining the effect of P treatment (Pl), Nitrogen treatment (N) and location of soil (Ara) on **soil acidic monoesterase activity** for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	52.8701	52.8701	1146.6	<0.0001
Pl	4	0.491752	0.122938	2.6662	0.0461*
N	1	0.0215288	0.0215288	0.4669	0.4984 ns
Pl*N	4	0.44818	0.112045	2.4299	0.0633 ns
Ara	1	0.0923404	0.0923404	2.0026	0.1648 ns
Pl*Ara	4	0.128337	0.0320842	0.69581	0.5993 ns
Nl*Ara	1	0.00312838	0.00312838	0.067845	0.7958 ns
Pl*Nl*Ara	4	0.0925153	0.0231288	0.5016	0.7347 ns
Error	40	1.84442	0.0461105		
Total	59	3.1222			

Table 6: ANOVA examining the effect of P treatment (Pl) and Nitrogen treatment (N) on **root acidic monoesterase activity** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log root acidic monoesterase activity).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	0.230138	0.230138	4.7964	0.0412
Pl	4	0.274438	0.0686094	1.4299	0.2625 ns
N	1	0.579684	0.579684	12.081	0.0025**
Pl*N	4	0.2683	0.0670751	1.3979	0.2725 ns
Error	19	0.911644	0.0479813		
Total	28	2.09594			

Table 7: ANOVA examining the effect of P treatment (Pl), Nitrogen treatment (N) and location of soil (Ara) on **soil Tp concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	38.1068	38.1068	1284.5	<0.0001
Pl	4	0.720022	0.180005	6.0676	0.0007***
N	1	0.112719	0.112719	3.7995	0.0583 ns
Pl*N	4	0.59669	0.149173	5.0283	0.0022**
Ara	1	0.324458	0.324458	10.937	0.0020**
Pl*Ara	4	0.0101689	0.00254223	0.085694	0.9864 ns
Nl*Ara	1	0.0287066	0.0287066	0.96764	0.3312 ns
Pl*Nl*Ara	4	0.151842	0.0379604	1.2796	0.2941 ns
Error	40	1.18666	0.0296665		
Total	59	3.13126			

Table 8: ANOVA examining the effect of P treatment (Pl), Nitrogen treatment (N) and location of soil (Ara) on **soil Pi concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	7.43157	7.43157	7187.2	<0.0001
Pl	4	0.0160945	0.00402363	3.8913	0.0692 ns
N	1	0.00000836267	0.00000836267	0.0080877	0.9288 ns
Pl*N	4	0.00342261	0.000855652	0.82752	0.5156 ns
Ara	1	0.00431802	0.00431802	4.176	0.0576 ns
Pl*Ara	4	0.00398092	0.00099523	0.96251	0.4386 ns
N*Ara	1	0.000963203	0.000963203	0.93153	0.3403 ns
Pl*N*Ara	4	0.00584311	0.00146078	1.4127	0.2473 ns
Error	40	0.0413599	0.001034		
Total	59	0.0759907			

Table 9: ANOVA examining the effect of P treatment (Pl), Nitrogen treatment (N) and location of soil (Ara) on **soil Po concentration** for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	11.8817	11.8817	388.18	<0.0001
Pl	4	0.577135	0.144284	4.7138	0.0033**
N	1	0.114669	0.114669	3.7463	0.0600 ns
Pl*N	4	0.523744	0.130936	4.2777	0.0056 **
Ara	1	0.403637	0.403637	13.187	0.0008***
Pl*Ara	4	0.0242473	0.00606183	0.19804	0.9379 ns
N*Ara	1	0.0401865	0.0401865	1.3129	0.2587 ns
Pl*N*Ara	4	0.112459	0.0281147	0.91852	0.4627 ns
Error	40	1.22434	0.0306086		
Total	59	3.02042			

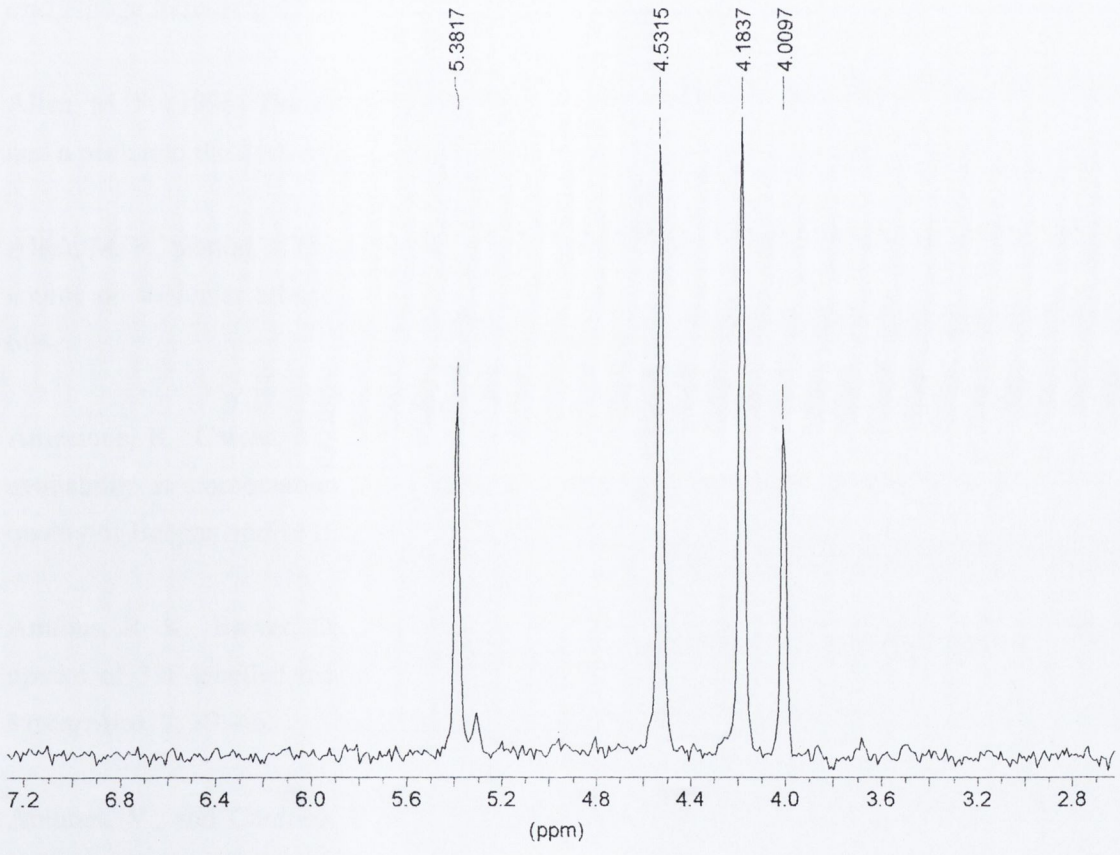
Table 10: ANOVA examining the effect of P treatment (Pl), Nitrogen treatment (N) and location of soil (Ara) on **% of Po depleted from the soil** for the *Agrostis s.* experiment. To obtain normal data, values were transformed before ANOVA was performed (log %Po depleted).
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	106.207	106.207	814.9	<0.0001
Pl	4	1.20097	0.300242	2.3037	0.0798 ns
N	1	0.393985	0.393985	3.023	0.0917 ns
Pl*N	4	1.53935	0.384838	2.9528	0.0549 ns
Ara	1	1.20658	1.20658	9.2578	0.0047**
Pl*Ara	4	0.197322	0.0493306	0.3785	0.8223 ns
N*Ara	1	0.0425757	0.0425757	0.32667	0.5716 ns
Pl*N*Ara	4	0.769775	0.192444	1.4766	0.2324 ns
Error	32	4.1706	0.130331		
Total	51	9.52754			

Table 11: ANOVA examining the effect of P treatment (Pl) and Nitrogen treatment (N) on **% root length colonisation** for the *Agrostis s.* experiment. Data was normal, no transformation necessary.
($P \leq 0.001$ *** $P \leq 0.01$ ** $P \leq 0.05$ * $P > 0.05$ ^{ns} (not statistically significant))

Source	**df**	**Sums of Squares**	**Mean Square**	**F-ratio**	**Prob**
Const	1	83603.6	83603.6	379.95	<0.0001
Pl	4	3784.22	946.054	4.2995	0.0044**
N	1	285.708	285.708	1.2984	0.2596 ns
Pl*N	4	3544.09	886.024	4.0267	0.0063**
Error	53	11662.1	220.039		
Total	62	19907.4			

Appendix 6: ^{31}P NMR spectrum of an alkali (0.5 M NaOH) solution of inositol hexaphosphate (myo-inositol hexakis [dihydrogen phosphate], Sigma Chemicals). Spectrum shows there is no inorganic orthophosphate present in the sample. The four peaks are characteristic of inositol hexaphosphate which contains six P atoms. These six P atoms appear as four peaks in the ^{31}P NMR spectrum because there are two sets of two atoms in the same chemical environment, accounting for two peaks and the remaining 2 atoms are in different environments and are indicated by separate peaks. (Dr. J. O'Brien personal communication)



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