

A fitful fungus from a hot, arid climate increases grain yield in cool-cultivated barley

by

Brian R. Murphy

Fiona M. Doohan, Ph.D.¹

Trevor R. Hodkinson Ph.D²

Abstract

Purpose

The fungus *Piriformospora indica* was first isolated from plants growing in arid, hot desert conditions and has been shown to have significant potential as a biocontrol and biofertilising organism in barley under optimal growth conditions. However, it was not thought to be effective in plants grown in low temperatures and has consequently not been well tested in cold-stressed crops. This study sought to determine the effects of inoculating barley plants with this fungus in cool growth conditions with variable nutrient input.

Methods

Three barley varieties were inoculated with *P. indica* and two other fungal root endophytes, *Chaetomium globosum* and *Epicoccum nigrum*, in a controlled environment under low temperature stress with variable nutrient input, and measured growth, development and yield.

Results

With the higher nutrient input, the *P.indica*-inoculated plants flowered earlier and had 22% greater grain yield than the control. The other two endophytes, *C. globosum* and *E. nigrum*, conferred no significant benefits under either nutrient regime.

Conclusions

Piriformospora indica is easy to culture and propagate, and may have significant biofertilisation potential as a crop treatment for barley grown in cool climates

¹ UCD Earth Institute and School of Biology & Environmental Science, University College Dublin, Dublin 4, Ireland

² School of Natural Sciences & Trinity Centre for Biodiversity Research, Trinity College Dublin, College Green, Dublin 2, Ireland

provided that a threshold level of nitrogen is supplied. Such treatments may enable the profitable cultivation of barley in previously marginal sites and reduce the carbon footprint of barley through increased nitrogen use efficiency.

Keywords

Barley, biofertilisation, fungal root endophytes, low temperature and nutrient stress, symbiosis.

Introduction

Fungal root endophytes are plant associates that spend most or part of their lives within plant root tissue without inducing pathogenic symptoms (Stone, Polishook, and White 2004; Schulz and Boyle 2006; Weiss et al. 2011). They are known to colonize a wide variety of plants, including many important crops. Benefits to plants colonised by endophytic root fungi include increased yield (Achatz et al. 2010), enhanced resistance to pathogens and herbivores (Waller et al. 2008) and abiotic stress tolerance (Waller et al. 2005).

One particular fungal root endophyte, *Piriformospora indica*, discovered in the Thar desert of north-west India in 1997 (Verma et al. 1998), has been extensively studied (Ghahfarokhi & Goltapeh 2010; Qiang et al. 2011; Ansari et al. 2013; Unnikumar et al. 2013). Very few of these studies have examined the performance of *P. indica* in barley under cold growing conditions, probably due to the fact that the optimal growth temperature of ~35°C for this fungus (related to its provenance) has suggested that it may not perform well in the cooler climates in which barley is often grown. Studies focusing on similar crops in the field, such as wheat, showed no significant benefits for the crop plants deriving from colonization by *P. indica* (Serfling et al. 2007).

Barley is one of the world's most important cereal crops (Newton et al. 2011) and is often grown on poor, stress-prone land in cool climates, and is more cold-tolerant than most other cereal crops (Visioni et al. 2013). Obtaining acceptable yields in such conditions often requires high inputs of chemicals, with consequent economic and ecological costs. High chemical crop inputs may also be a major contributing factor to the carbon footprint of barley (Gan et al. 2012). Biofertilisation treatments using fungal root endophytes may help to reduce these costs while still maintaining yield (Murphy, Doohan, and Hodkinson

2013). While some studies (Achatz et al. 2010) showed that *P. indica* colonized plants to the same degree despite widely different nutrient input, others found a strong link between lower nitrogen input and greater colonisation of barley roots by *P. indica* (Lahrman et al. 2013). Two other fungal root endophytes, *Chaetomium globosum* and *Epicoccum nigrum*, have also been shown to increase yield and induce pathogen resistance in a range of plant species (Soytong & Ratanacherdchai 2005; Fávoro, Sebastianes, and Araújo 2012).

Our study examined how *P. indica*, *C. globosum* and *E. nigrum* interact with different barley cultivars at low growing temperature and with two different nutrient regimes.

Methods

The methods and results described in this paper are a summary of more detailed procedures and results, respectively, which are available on request.

Experimental setup

Spring barley root samples from the cultivars 'Overture', 'Propino' and 'Sy Taberna' were collected prior to harvest from the Department of Agriculture, Forestry and the Marine (DAFM) trials site at Backweston, Co. Kildare, Ireland (53.348 N, 6.488 W). We isolated 33 individual fungi from the root pieces of the 16 barley plants sampled. Fourteen representative morphotypes were identified by using a combination of morphological and DNA character analysis. Two of the fungal species isolated have been reported as beneficial endophytes, *Chaetomium globosum* (Soytong & Ratanacherdchai 2005) and *Epicoccum nigrum* (Fávoro, Sebastianes, and Araújo 2012). These two species and a laboratory strain of *Piriformospora indica* (*P. indica*-DSM11827 from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), were chosen as experimental treatments for this study. Cultures for the experimental treatments were grown from single spores.

We used seeds of 3 spring barley cultivars, 'Frontier', 'Propino' and 'Soldo', for this experiment (Goldcrop Seeds, Cork, Ireland). Three barley cultivars were used in order to obtain more comprehensive results because they represent different growth, yield and disease resistance characteristics and have been successfully grown in low temperatures. 360 seeds were sown in 120 x 1 litre plant pots in a growth compost consisting of sterilised coarse vermiculite supplemented by water

absorbing polymer granules (Agricultural Polymers International Ltd.). Equal numbers of seeds were either inoculated with one of the three endophyte cultures (*C. globosum*, *E. nigrum*, *P. indica*) or not inoculated (the controls). Pots were placed into controlled environment chambers, with a 9 hour photoperiod at a compost surface illumination of $210 \mu\text{mol}\cdot\text{m}^{-2} \text{ s}^{-1}$, a constant temperature of 8°C and 70% relative humidity. After 70 days, the temperature was raised to 13°C , then raised to 16°C fourteen days later. The photoperiod was gradually extended until it reached 15 hours, 84 days after seed germination. The photoperiod was lengthened and temperature raised to speed up plant development.

Plant cultivation and processing

The plants were given a liquid fertiliser (Bayer Phostrogen®) at each watering after germination. This particular fertiliser was chosen for its relatively low nitrogen content. Half of the plants were given lower nutrient inputs (LO) and half given higher (HI) nutrients. The HI treatment contained the recommended input of fertiliser for hydroponically-grown plants. The nutrient content was calculated from the manufacturers analysis; for the HI nutrient treatments, the total nutrient input per pot was: ammoniacal N = 0.04728g, ureic N = 0.2836g, Total N = 0.3308g, P = 0.208g, K = 0.5292g, Mg = 0.0344g, S = 0.0714g, Ca = 0.0338g and traces of Boron, Copper, Iron, Manganese, Molybdenum and Zinc; for LO nutrient treatments, the total nutrient input per pot was halved for all elements. Development of the plants was monitored and recorded using recognised growth stages (Zadoks et al. 1974). Plants were grown for 147 days (21 weeks) from date of sowing, then harvested and processed. Plant dimensions and both fresh and dried plant parts (shoots, grains and roots) were counted and weighed. The nitrogen (N) and carbon (C) content of the grains was measured using an Elementar vario EL Cube. Approximately 7 mg of crushed and homogenised grain from each sample was used to determine proportions of total N and C. Pieces of root from each plant were incubated on agar culture medium to test for endophyte presence. Data analysis by ANOVA was performed using the Data Analysis modules provide by Microsoft Excel 2010®.

Results

Though there was very little difference in germination success, the *Piriformospora indica*-inoculated plants performed better for nearly all subsequent early growth and development parameters for both LO and HI nutrient input. A strong interaction was indicated between nutrient input and number of days to flowering (2-way

ANOVA, $F_{1,48} = 5.11$, $P = 0.028$), where the HI nutrient input *P. indica*-inoculated plants flowered 5 days earlier than the control (Table 1 and Figure 1). *Piriformospora indica*-inoculated plants matured 7 days earlier than the control for HI nutrient input, but 2 days later for LO.

Table 1. Mean values at harvest for 3 spring barley cultivars (Frontier, Propino and Soldo) inoculated with one of 3 fungal root endophytes, grown at low temperature under two nutrient regimes (LO = lower nutrient input and HI = higher nutrient input). All values are means per pot of 3 plants for each treatment ($n = 15$). Statistically significant differences of $P < 0.05$ (2-way ANOVA) between endophyte and control are indicated by *.

Treatment	Nutrients	Days to flower	Height cm	Fresh wt. grains g	Fresh wt. shoots g	Dry wt. grains g	Dry wt. shoots g
<i>C. globosum</i>	LO	116	77.1	9.34	19.81*	3.66	4.75
	HI	121	92.3	11.38	23.40	4.30	4.80
<i>E. nigrum</i>	LO	112	73.6	10.10	18.74	3.79	4.39
	HI	119	91.8	9.05	22.03	3.50	4.87
<i>P. indica</i>	LO	109	74.9	9.42	17.64	3.66	4.29
	HI	111*	82.3	13.02	25.01	5.19*	6.29
ALL endophytes	LO	112	75.2	9.62	18.73	3.70	4.47
	HI	117	88.8	11.15	23.48	4.33	5.31
Control	LO	106	68.0	9.36	15.27	4.68	3.97
	HI	116	94.3	11.50	24.41	4.26	5.70
Endos / Control	LO	1.06	1.11	1.03	1.23	0.79	1.13
	HI	1.01	0.94	0.97	0.96	1.02	0.93

When the final harvest characteristics of the plants were analysed (Table 1), it was found that, compared with the *P. indica* treatment, control plants had the greatest mean dry weight of grains with LO nutrient input. However, under the HI nutrient input regime, the *P. indica*-inoculated plants performed better in almost all respects compared with all other treatments. For HI nutrient input, comparison of harvest parameters for *P. indica* treatments and controls indicated

a strong interaction between nutrient input and grain dry weight (2-way ANOVA, $F_{1,48} = 7.59$, $P = 0.008$), where *P. indica* inoculated plants had 22% greater grain dry weight than the control (ANOVA, $F_{1,24} = 4.75$, $P = 0.039$) (Figure 2). Shoot dry weight for the *P.indica*-inoculated plants was also greater than the other treatments, though not significantly. The nitrogen and carbon content of the grains did not differ significantly between treatments or cultivars. All of the root pieces from the endophyte inoculated plants produced growth from root endophytes at the end of the experiment, which matched the morphology of the original inoculants.

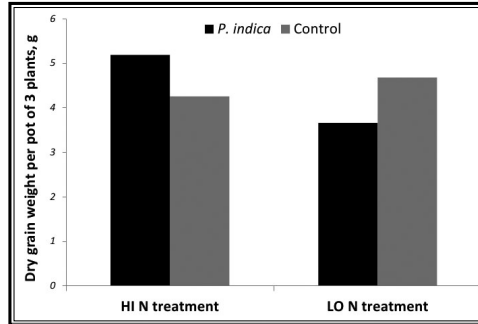


Figure 1: With the higher nitrogen (HI N) input, cold-stressed barley plants inoculated with *Piriformospora indica* flowered 5 days earlier than the control.

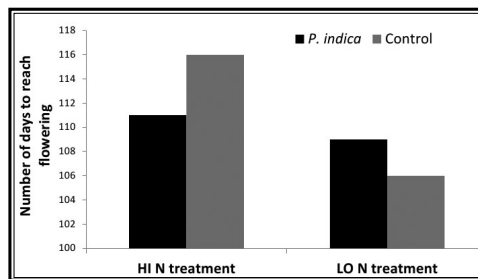


Figure 2: With the higher nitrogen (HI N) input, dry grain weight was 22% greater than the control in cold-stressed barley plants inoculated with *Piriformospora indica*.

Discussion

This study has shown that the improvements in time taken to reach flowering and maturity, and the grain dry weight at harvest due to colonization by the fungal root endophyte *Piriformospora indica* in low temperature stressed barley are positively related to nutrient input. The 22% increase in grain yield represents

a massive economic benefit when projected onto agricultural scales. The shorter time required to reach flowering and maturity indicates that treatments based on *P. indica* inoculation of barley crops may even have the potential to extend the growing season in cooler climates. These results suggest that *P. indica*, despite its origin in hot desert conditions –and contrary to earlier reports (Serfling et al. 2007) - may persist and have significant beneficial effects in barley grown in cool temperate conditions, provided adequate nutrients are supplied. We found similar effects as other studies regarding *P. indica*-induced earlier flowering and higher biomass (Achatz et al. 2010), but only for the HI nutrient input.

The results clearly demonstrate a positive relationship between total nutrient input and the beneficial effects due to *P. indica* colonization of barley grown in low temperature. The fertiliser used to feed the plants has a relatively small nitrogen content (14%) and relatively high phosphorous (10%) and potassium (27%) contents. Most agricultural fertilisers would normally contain a much higher proportion of nitrogen and lower proportions of phosphorous and potassium, so even at the HI nutrient input the amount of nitrogen available to the plants was relatively low. As it has been shown that *P. indica* colonization only produces beneficial effects for the barley with a higher nutrient input, it would seem that there is minimum amount of nitrogen that is needed for the fungus to produce a beneficial effect on barley grown at low temperature (this threshold amount lies somewhere between a total nitrogen input of 0.055g and 0.11g per plant, equivalent to between 4.1×10^{-4} and 8.2×10^{-4} g N per plant per day). Fungal endophytes must rely to a great extent on the nutrients they can obtain from the host plant, particularly for endophytes such as *P. indica* which have only a few nutrient scavenging structures external to the plant root (Deshmukh et al. 2006). The endophyte must compete with the host for available nutrients. With the lower nutrient input, the increased grain yield for the control plants relative to the endophyte treated plants indicates that the plant is outcompeting the endophyte and is sequestering most of the available nitrogen at the expense of the endophyte.

With low levels of nitrogen entering the system, the metabolic interdependence between host metabolism and fungal nutrient uptake (Lahrman et al. 2013) combined with low temperatures may lead to reduced optimal metabolic processes in the endophyte (Sherameti et al. 2005). Low levels of nitrogen and reduced metabolic efficiency may also compromise the ability of the fungus to manufacture the amino acid tryptophan, which has been shown to be a key factor in the establishment of the barley-*P. indica* symbiosis (Hilbert et al. 2012). At low

temperature, *P. indica* can only increase barley grain yield above a certain threshold level of available nitrogen. With nitrogen input below the threshold level, *P. indica* colonization is slightly detrimental for barley yield, which contradicts the widely-held belief that endophytes, and particularly *P. indica*, are always beneficial, or at least neutral, for the host. Even though nitrogen is strongly implicated as the limiting element for *P. indica* efficacy, a role for deficiencies of phosphorous and potassium (or even a micronutrient) cannot be ruled out.

In a recent review (Murphy, Doohan, and Hodkinson 2013) evidence is presented to support the view that endophytes can be either a 'friend or foe' to barley, depending on prevailing circumstances, and the current results seem to support that position. The neutral response of the plants to the proven biocontrol fungi *C. globosum* and *E. nigrum* also supports the conclusion that the particular combination of determining factors (e.g. genotypes, environment) necessary to promote benefits to the plants was not present for these two endophytes.

Higher yield associated with *P.indica* colonization in stressed plants is partly due to the endophyte-induced increase in antioxidant activity (Waller et al. 2005; Ansari et al. 2013; Harrach et al. 2013). *Piriformospora indica* associated stimulation of antioxidant activity in cold-stressed plants may be a contributory factor to the increase in grain yield shown in the current study, but only when the endophyte receives enough nitrogen to function optimally.

Some of the results from this study which contradict the findings from previous work may be directly due to the environmental conditions, particularly low temperature and nutrient status, and represent an important contribution to the growing body of knowledge regarding the *Piriformospora indica*-barley symbiosis. Further experiments, including field testing, which would involve fungal root endophyte colonization of barley using these organisms and others will give new insight into these results. Any further development of fungal root endophytes as inoculants for barley would have to demonstrate their ability to persist in the plant over the long term under field conditions. They would also have to demonstrate that they represent reliable alternatives to chemical treatments. The discovery of previously unrealised benefits associated with these fungi holds great future promise for developing economically and ecologically viable crop treatments for barley.

Conclusions

Reducing agricultural inputs of nitrogen is of critical importance in the development of future strategies for more sustainable and environmentally friendly farming and these results suggest that tailoring the nutrient and endophyte treatment combination may provide part of the solution. Gan et al. (2012) make the point that the key to lowering the carbon footprint of barley is to increase grain yield, reduce nitrogen inputs and improve nitrogen use efficiency. Discovering and developing potentially beneficial endophyte treatments through experimentation such as ours may make a significant contribution towards reducing the carbon footprint of barley.

We have determined that under low temperature and nutrient stressed growing conditions, there is a threshold level of nitrogen input above which *P. indica* colonization will be beneficial for barley. Future global climate change will result in local alterations in growing conditions, and the contribution of fungal root endophytes in enabling successful cultivation of barley in previously unsuitable situations may become crucial.

Acknowledgements

The authors would like to thank: Goldcrop Seeds, Cork, Ireland for the generous supply of barley seeds, and for advice on suitable cultivars to use; Josephine Brennan of the Department of Agriculture, Fisheries and the Marine at the Backweston crop trials site for providing plant material; Mark Kavanagh of the Centre for the Environment in Trinity College Dublin for expert advice and help in elemental analyses; Helena Murphy for proofreading and the de-cluttering of technical terms. Trinity College Dublin provided financial support through a PhD studentship grant.

References

- Achatz, Beate, Sibylle Rüden, Diana Andrade, Elke Neumann, Jörn Pons-Kühnemann, Karl-Heinz Kogel, Philipp Franken, and Frank Waller. 2010. "Root Colonization by *Piriformospora Indica* Enhances Grain Yield in Barley under Diverse Nutrient Regimes by Accelerating Plant Development." *Plant and Soil* 333 (1-2) (March 6): 59–70.
- Ansari, Mohammad Wahid, Dipesh Kumar Trivedi, Ranjan Kumar Sahoo, Sarvajeet Singh Gill, and Narendra Tuteja. 2013. "A Critical Review on Fungi Mediated Plant Responses with Special Emphasis to *Piriformospora Indica* on Improved Production and Protection of Crops." *Plant Physiology and Biochemistry : PPB / Société Française de Physiologie Végétale* 70 (September): 403–10.
- Deshmukh, Sachin, Ralph Hüchelhoven, Patrick Schäfer, Jafargholi Imani, Monica Sharma, Michael Weiss, Frank Waller, and Karl-Heinz Kogel. 2006. "The Root Endophytic Fungus *Piriformospora Indica* Requires Host Cell Death for Proliferation during Mutualistic Symbiosis with Barley." *Proceedings of the National Academy of Sciences of the United States of America* 103 (49) (December 5): 18450–7.
- Fávoro, Léia Cecilia De Lima, Fernanda Luiza De Souza Sebastianes, and Welington Luiz Araújo. 2012. "*Epicoccum Nigrum* P16, a Sugarcane Endophyte, Produces Antifungal Compounds and Induces Root Growth." *PloS One* 7 (6) (January): e36826.
- Gan, Yantai, Chang Liang, William May, Sukhdev S. Malhi, Junyi Niu, and Xiaoyu Wang. 2012. "Carbon Footprint of Spring Barley in Relation to Preceding Oilseeds and N Fertilization." *The International Journal of Life Cycle Assessment* 17 (5) (February 15): 635–645.
- Ghahfarokhi, R.M. and Goltapeh, M.E.*. 2010. "Potential of the Root Endophytic Fungus *Piriformospora Indica* ; *Sebacina Vermifera* and *Trichoderma* Species in Biocontrol of Take-All Disease of Wheat *Gaeumannomyces Graminis* Var . *Tritici* in Vitro." *Journal of Agricultural Technology* 6 (1): 11–18.
- Harrach, Borbála Dorottya, Helmut Baltruschat, Balazs Barna, Jozsef Fodor, and Karl-Heinz Kogel. 2013. "The Mutualistic Fungus *Piriformospora Indica* Protects Barley Roots from a Loss of Antioxidant Capacity Caused by the Necrotrophic Pathogen *Fusarium Culmorum*." *Molecular Plant-Microbe Interactions : MPMI* (February 13).
- Hilbert, Magdalena, Lars M Voll, Yi Ding, Jörg Hofmann, Monica Sharma, and Alga Zuccaro. 2012. "Indole Derivative Production by the Root Endophyte *Piriformospora Indica* Is Not Required for Growth Promotion but for Biotrophic Colonization of Barley Roots." *The New Phytologist* 196 (2) (October): 520–34.
- Lahrman, Urs, Yi Ding, Aline Banhara, Magnus Rath, Mohammad R Hajirezaei, Stefanie Döhlemann, Nicolaus von Wirén, Martin Parniske, and Alga Zuccaro. 2013a. "Host-Related Metabolic Cues Affect Colonization Strategies of a Root Endophyte." *Proceedings of the National Academy of Sciences of the United States of America* 110 (34) (August 20): 13965–70.
- Murphy, B. R., F. M. Doohan, and T. R. Hodkinson. 2013. "Fungal Endophytes of

Barley Roots." *The Journal of Agricultural Science* (June 13): 1–14.

Newton, Adrian Clive, Andrew J. Flavell, Timothy S. George, Philip Leat, Barry Mullholland, Luke Ramsay, Cesar Revoredo-Giha, et al. 2011. "Crops That Feed the World 4. Barley: A Resilient Crop? Strengths and Weaknesses in the Context of Food Security." *Food Security* 3 (2) (June 1): 141–178.

Qiang, Xiaoyu, Michael Weiss, Karl-Heinz Kogel, and Patrick Schäfer. 2011. "*Piriformospora Indica*-a Mutualistic Basidiomycete with an Exceptionally Large Plant Host Range." *Molecular Plant Pathology* 13 (5): 508–518.

Schulz, Barbara, and Christine Boyle. 2006. "What Are Endophytes?" In *Microbial Root Endophytes*, edited by B.J.E. Schulz, C.J.C. Boyle, and Th.N. Sieber, 9:1–14. Berlin: Springer-Verlag.

Serfling, Albrecht, Stefan G R Wirsal, Volker Lind, and Holger B Deising. 2007. "Performance of the Biocontrol Fungus *Piriformospora Indica* on Wheat Under Greenhouse and Field Conditions." *Phytopathology* 97 (4) (April): 523–31.

Sherameti, Irena, Bationa Shahollari, Yvonne Venus, Lothar Altschmied, Ajit Varma, and Ralf Oelmüller. 2005. "The Endophytic Fungus *Piriformospora Indica* Stimulates the Expression of Nitrate Reductase and the Starch-Degrading Enzyme Glucan-Water Dikinase in Tobacco and Arabidopsis Roots through a Homeodomain Transcription Factor That Binds to a Conserved Motif in." *The Journal of Biological Chemistry* 280 (28) (July 15): 26241–7.

Soytong, Kasem, and Kanchalika Ratanacherdchai. 2005. "Application of Mycofungicide to Control Late Blight of Potato." *Journal of Agricultural Technology* April: 19–32.

Stone, Jeffrey K, Jon D Polishook, and James F White. 2004. "Endophytic Fungi." In *Biodiversity of Fungi: Inventory and Monitoring Methods*, edited by Gregory M. Mueller, Gerald F. Bills, and Mercedes S. Foster, 241–270. Amsterdam: Elsevier Inc.

Unnikumar, K. R., K. Sowjanya Sree, and Ajit Varma. 2013. "*Piriformospora Indica*: A Versatile Root Endophytic Symbiont." *Symbiosis* (May) (June 11).

Verma, Savita, Ajit Varma, Karl-heinz Rexer, Annette Hassel, Gerhard Kost, Prakash Bisen, Britta Bütchorn, and Philipp Franken. 1998. "*Piriformospora Indica*, Gen . et Sp . Nov ., a New Root-Colonizing Fungus." *Proceedings of the National Academy of Sciences of the United States of America* 90 (5): 896–903.

Visioni, Andrea, Alessandro Tondelli, Enrico Francia, Alexander Pswarayi, Marcos Malosetti, Joanne Russell, William Thomas, et al. 2013. "Genome-Wide Association Mapping of Frost Tolerance in Barley (*Hordeum Vulgare* L.)." *BMC Genomics* 14 (1) (January): 424.

Waller, Frank, Beate Achatz, Helmut Baltruschat, József Fodor, Katja Becker, Marina Fischer, Tobias Heier, et al. 2005a. "The Endophytic Fungus *Piriformospora Indica* Reprograms Barley to Salt-Stress Tolerance, Disease Resistance, and Higher Yield." *Proceedings of the National Academy of Sciences of the United States of America* 102 (38) (September 20): 13386–91.

Waller, Frank, Krishnendu Mukherjee, Sachin D Deshmukh, Beate Achatz, Monica

Sharma, Patrick Schäfer, and Karl-Heinz Kogel. 2008. "Systemic and Local Modulation of Plant Responses by *Piriformospora Indica* and Related Sebaciniales Species." *Journal of Plant Physiology* 165 (1) (January): 60–70.

Weiss, Michael, Zuzana Sýkorová, Sigisfredo Garnica, Kai Riess, Florent Martos, Cornelia Krause, Franz Oberwinkler, Robert Bauer, and Dirk Redecker. 2011. "Sebaciniales Everywhere: Previously Overlooked Ubiquitous Fungal Endophytes." *PloS One* 6 (2) (January): e16793.