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Native fungal microorganisms enhance important agronomic traits in barley

by
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Abstract

Successfully addressing the challenge of providing future food security will require both improvements in crop yield as well as the cultivation of additional farmland. This may result in the steady increase of farming on marginal, arid, and semi-arid lands, especially in the developing world, leading in turn to greater biotic and abiotic stresses on crops. To enable crops to deal with these stresses, an ever-growing arsenal of chemicals will be needed to maintain acceptable yields, with consequent environmental damage and maybe even the loss of biodiversity. Any means of reducing these chemical crop inputs would be welcome, and a class of microorganisms called endophytes may provide part of the solution. We have isolated fungal endophyte strains from wild populations of Irish plants and carried out a number of experiments which assessed the effect of inoculating these endophytes onto barley (*Hordeum vulgare* L.) in a variety of stressful growing conditions. We have found that the endophytes induced improvements in important agronomic traits in nearly every situation, including 29% and 70% increases in grain yield and shoot biomass respectively in nutrient-stressed barley; 100% suppression of seed-borne barley diseases; 50% increase in both the number of shoots and grain yield in drought-stressed barley; and finally, a 600% increase in plant survival in multiply-stressed barley. These results suggest that the endophyte strains that we have isolated could provide the basis for the development of a commercially-viable biotechnological means of reducing chemical crop inputs, and we are currently working on such a project with industry partners.

Keywords

Barley, Endophytes, Agriculture, Biocontrol, Biofertilisation.

Introduction

Humanity faces a crisis in food security. It is estimated that we will need to produce 22% more food by the year 2050 to feed a growing world population. We will likely be able to meet part of this need by increases in food production through improvements in agricultural practices, breeding programmes, genetic modification and more efficient and increased use of chemical crop inputs. However, changes in growing conditions associated with global warming, such as increased heat and drought stress, may result in environmental degradation and loss of suitable crop growing land, therefore there is a need to increase the efficiency of nutrient utilisation by plants in order to face the agricultural land scarcity. What we need is a new 'Green Revolution' that does not rely on environmentally damaging chemicals, particularly fertilisers. Non chemical-based and more sustainable methods that can help crop plants to utilise lower nutrient inputs more efficiently and to resist biotic and abiotic stresses are urgently needed.

Beneficial fungal root endophytes have the potential to reduce chemical use, increase pathogen resistance and enhance stress tolerance while still maintaining yield. Fungal endophytes (hereafter endophytes) are non-mycorrhizal associates that spend most or all of their lives within plant tissues, often with no outward sign of their presence (Stone, Polishook, and White 2004; B. Schulz and Boyle 2006)[1]. Our work has focused on barley and examined the effects of native Irish and exotic endophytes on the growth, development and yield of barley grown under a variety of stressful conditions.

Barley (*Hordeum vulgare* L.) is the fourth most important global cereal crop, grown annually on 48 million hectares (CGIAR 2012)[2]. Benefits to barley and other plants colonised by endophytic fungi include an increase in seed yield (Achatz et al. 2010)[3], enhanced resistance to pathogens and herbivores (Cheplick and Faeth 2009)[4] and increased stress tolerance (Waller et al. 2005; Rodriguez et al. 2009)[5].

1 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.

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.

2 Consultative Group on International Agricultural Research. 2012. "Barley / CGIAR." *Barley Summary and World Crop*. http://www.cgiar.org/our-research/crop-factsheets/barley/.

3 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.

4 Cheplick, Gregory P., and Stanley Faeth. 2009. E*cology and Evolution of the Grass-Endophyte Symbiosis*. Illustrate. New York: Oxford University Press USA.

5 Waller, Frank, Beate Achatz, Helmut Baltruschat, József Fodor, Katja Becker, Marina Fischer,

Biofertilisation and biocontrol techniques using endophytic microorganisms may provide part of the solution to ensuring sustainable global food security. The apparent huge diversity and potential of endophytes, and particularly fungal endophytes, is little studied and research to date has focused on a few model organisms. This review will summarise our work to date with these microorganisms and contextualise the results in the light of potential uses and impacts for agriculture. Our objective was to examine the effects of endophyte inoculation on barley cultivars when grown under a variety of biotic and abiotic stresses, and to determine where and if the endophytes could improve important barley traits.

Research review

The experimental cycle that we undertook consisted of five studies which examined the response of a barley cultivar to inoculation with endophytes when grown under temperature, nutrient, drought and pathogen stress, applied either singly or in combination (Experiments 1 – 5). Furthermore, we sequenced the nuclear ribosomal internal transcribed spacer (nrITS) region of the DNA recovered from the endophytes in order to characterise the phylogenetics of the endophytes (Experiment 6). From the combined results we hoped to gain important insight into the potential of this symbiosis for improving agronomic traits in barley crops.

Experiment 1. Biofertilisation under cold and nutrient stress

In our first experimental study (Murphy, Doohan, and Hodkinson 2014)[6], we assessed the efficacy of three model endophytes in barley varieties grown under low temperature stress with variable nutrient input. These endophytes - *Chaetomium globosum, Epicoccum nigrum and Piriformospora indica* - have previously been shown to have value as biocontrol and biofertilising organisms in barley, but have not been well tested at the low temperatures often encountered under Irish growing conditions.

Tobias Heier, et al. 2005. "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.

Rodriguez, R J, J F White, a E Arnold, and R S Redman. 2009. "Fungal Endophytes: Diversity and Functional Roles." *The New Phytologist* 182 (2) (January): 314–30.

6 Murphy, Brian R., Fiona M. Doohan, and Trevor R. Hodkinson. 2014. "Yield Increase Induced by the Fungal Root Endophyte Piriformospora Indica in Barley Grown at Low Temperature Is Nutrient Limited." Symbiosis 62 (January): 29-39.

Chaetomium globosum and *Epicoccum nigrum*, recovered from the roots of a field-grown barley cultivar (Propino), 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.

Seed from three cultivars of spring barley, 'Frontier', 'Propino' and 'Soldo', were inoculated with one of the three fungal root endophyte isolates and grown in low temperature under two different low nutrient input regimes. Five seeds of each barley cultivar were sown at 30 mm depth on top of 5 mm^2 endophyte culture plugs (*C. globosum, E. nigrum, P. indica*) or control pure agar plugs in 10 replicate pots for each treatment, giving 30 replicate plants per treatment (3 × endophyte inoculated and 1 × control). Pots were placed into two controlled environment chambers, which were programmed to produce a 9 hr photoperiod at a compost surface illumination of 210 $\mu mol.m^{-2}\ s^{-1}$, a constant temperature of 8°C and 70% relative humidity. The photoperiod was extended by 1.5 hr every 3 weeks until it reached 15 hr (at day 84). The temperature was raised to 13°C at day 70 and to 16°C at day 84. The temperature was therefore maintained at the acclimation temperature of 8°C for the first half of the growing period. The photoperiod was lengthened and temperature raised to speed up plant development. Half of the plants were given lower nutrient inputs (LO) and half were given higher (HI) nutrients; 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. However, the HI nutrient treatment is only a relative comparison with the LO, as this HI amount of nutrient input is less than a quarter of that normally recommended for barley crops.

Compared with the control, for the higher nutrient input treatment (HI) in *P. indica*-inoculated plants, flowering was earlier and grain dry weight significantly greater for all barley varieties by a mean of 22% (Table 1). Grain dry weight is the most important agronomic trait for barley growers so any increase in this parameter would be particularly beneficial. This suggests that treatments based on *P. indica* inoculation of barley crops may have the potential to extend the growing season in cooler climates and maintain yields with relatively low nutrient input. We also concluded that there is a minimum amount of nitrogen that is needed for the fungus to produce a beneficial effect on barley grown at low temperature. We demonstrated that any benefits for barley related to inoculation with *P. indica* was directly due to the environmental conditions, particularly low temperature and nutrient status, and this study represents an important contribution to the growing body of knowledge regarding the *Piriformospora indica*-barley symbiosis.

Treatment	*Nutrients*	*Flower days*	*No. tillers*	*No. grains*	*Dry wt. grains g*	*Dry wt. shoots g*	*Dry wt. roots g*
C. globosum	LO	116	10	131	3.66	4.75	4.88
	HI	121	10	160	4.30	4.80	6.72
E. nigrum	LO	112	9	128	3.79	4.39	4.55
	HI	119	9	130	3.50	4.87	8.13
P. indica	LO	109	9	126	3.66	4.29	4.88
	HI	**111***	11	168	***5.19****	6.29	6.35
ALL endophytes	LO	112	9	128	3.70	4.47	4.77
	HI	117	10	153	4.33	5.31	7.07
Control	LO	106	9	126	4.68	3.97	6.88
	HI	116	10	162	4.26	5.70	7.93
Endos / Control	LO	1.06	1.03	1.02	0.79	1.13	0.68
	HI	1.01	0.95	0.95	1.02	0.93	0.78

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 *. Table is adapted from the original Table 4 in Murphy et al. (2014) 'Yield increase induced by the fungal root endophyte *Piriformospora indica* in barley grown at low temperature is nutrient limited'. *Symbiosis* 62: 29-39.

However, as *P. indica* was isolated from the roots of shrubs growing in the Thar desert of India, there may be some cause for concern regarding the introduction of an alien species into non-native environments. Consequently, we decided to focus on endophytes derived from wild Irish plant populations for the remainder of our experiments. The following studies detailed in this review used the endophytes isolated and selected from a range of over 150 strains recovered from the roots of ten wild Irish populations of wall barley (*Hordeum murinum* ssp. *murinum* L.). The populations were within a ten km radius of a point centered at 53.39602N, 6.21632W (O 18636 39912) in June – July 2013.

Experiment 2. Biocontrol of pathogens

For our next experiment (Murphy, Doohan, and Hodkinson 2014a)[7], ten different endophytes were selected and inoculated onto untreated seeds of a barley cultivar. Fifteen unblemished and surface-sterilised seeds of the barley cultivar 'Propino' were surface-sown on separate culture dishes of five different media and inoculated with 250 μl of each individual endophyte or a combination of all ten. Culture dishes were incubated at 25°C for 21 days.

The co-inoculant of all ten isolates as well as two individual isolates completely suppressed the development of seed-borne fungal infections on germinated and ungerminated seed. We also found that one of the endophytes, 040901(3), completely suppressed the growth of the most serious barley pathogen, *Gaeumannomyces graminis* var. *tritici,* when co-inoculated onto an agar substrate (Figure 1).

The results have important implications because the seed-borne infections that emerged from control seeds with no inoculant are some of the most devastating pathogens of barley. The variation in endophyte response would have implications for any future development and use of some of these endophytes as barley crop inoculants, emphasising the need to carefully match the inoculant to the environment if these organisms are to be deployed with the greatest success. To our knowledge, this is the first time that fungal root endophytes isolated from roots of *any* wild *Hordeum* species have been shown to control vertically transmitted infections in a barley cultivar. All of the sampling sites were characterised by a high

7 Murphy, Brian R, Fiona M Doohan, and Trevor R Hodkinson. 2014a. "Persistent Fungal Root Endophytes Isolated from a Wild Barley Species Suppress Seed- Borne Infections in a Barley Cultivar." *Biocontrol*. doi:10.1007/s10526-014-9642-3.

soil salinity (mean = 1.37 bars), high soil pH (mean = 7.7) and low soil moisture content (mean = 10.7%), with four sites having no measurable soil moisture, suggesting that the endophytes may be most effective as a biocontrol inoculant for barley crops growing on similar soils.

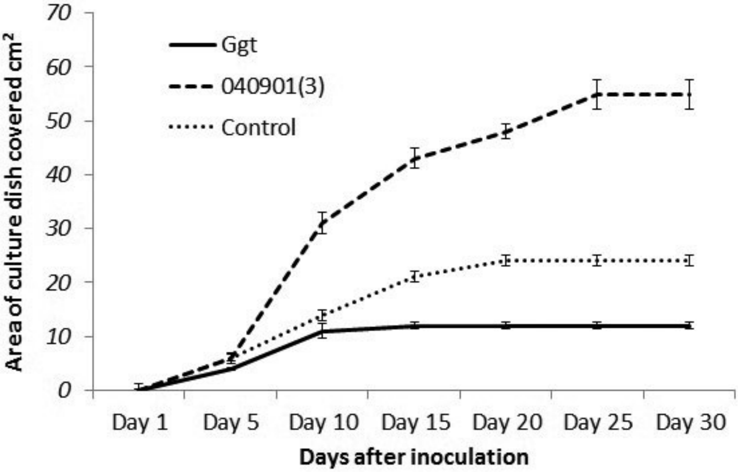

Fig 1. Mean proportion ± S.E. of 900 mm diameter culture dish covered by the endophyte isolate 040901(3) and the barley pathogen *Gaeumannomyces graminis* var. *tritici* (Ggt) over time when co-inoculated onto Sabouraud growth medium (n=5). The isolate 040901(3) had significantly greater mean culture dish cover than Ggt (one-way ANOVA; $F1,8 = 59.85$, $P < 0.01$) from day 10 to day 30. 'Control' is the area of culture plate covered when Ggt was cultured alone. Figure is adapted from the original Figure 1 in Murphy et al. (2014) 'Persistent fungal root endophytes isolated from a wild barley species suppress seed-borne infections in a barley cultivar'. *Biocontrol* 60: 281-292.

Experiment 3. Yield and biomass increases under nutrient stress

Having determined that the endophytes isolated from the wild populations of *H. murinum* could be used for biocontrol purposes, we decided to examine whether they could also act as biofertilisation agents (Murphy, Doohan, and Hodkinson 2015)[8]. Here, we grew the barley plants under two different nutrient regimes, high (HI) and low (LO). The HI nutrient

8 Murphy, Brian R, Fiona M Doohan, and Trevor R Hodkinson. 2015. "Fungal root endophytes of a wild barley species increase yield in a nutrient-stressed barley cultivar." *Symbiosis*. doi: 10.1007/s13199-015-0314-6.

input treatment was only approximately 18%, 44% and 42% of that recommended for a barley crop for nitrogen, phosphorous and potassium respectively; the LO nutrient input was half that of HI. For each inoculation treatment (including a control), fifty seeds of barley, five per pot, were sown at 30 mm depth and inoculated with one of ten endophytes. The inoculant solution was prepared by mixing 10 mg of spores and/or mycelium from each fungal culture with 5 ml of pure water and stirring with a magnetic bar for 1 hr at 25°C, and 250 μl of the solution was directly inoculated onto each seed. Control seeds were inoculated as described above with 250 μl pure water without any fungal inoculum. The environmental settings were programmed to produce a 13 hr photoperiod at a compost surface illumination of 210 $\mu mol.m^{-2} s^{-1}$, a photoperiod temperature of 15°C, a dark period temperature of 8°C and a constant 70% relative humidity. The photoperiod was extended by two hours at 21 days from date of sowing and to 17 hr at day 42. The temperature was raised by 2°C at day 21 and by a further 2°C at day 42.

We found that inoculation with six different endophytes increased grain yield for both the HI and LO nutrient input by up to 29%. Furthermore, we also showed that inoculation with the isolates induced increases of up to 70% in shoot dry weight of the barley. The greatest increases in grain yield and shoot dry weights were achieved under the lowest nutrient input (LO) (Table 2).

The most important result from a growers' point of view was that the endophytes induced a greater improvement in mean grain dry weight than any other yield parameter. The environmental characteristics of our plant sampling sites may hint at the mechanisms responsible for endophyte-induced increases in grain yield and shoot biomass. The plants at these sites were healthy and growing strongly despite the shallow, alkaline, salty and dry soil. The plants would benefit from any increase in root associated nutrient acquisition efficiency. The endophytes may enhance phosphorous and nitrogen uptake in particular, as has been shown elsewhere (Vohnik et al. 2005; Yadav et al. 2010)[9]. The endophyte isolate E6 had a closest DNA

9 Vohnik M, Albrechtova J, Vosatka M. 2005. "The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in Rhododendron cv. Azurro." *Symbiosis* 40:87–96.

Yadav, Vikas, Manoj Kumar, Deepak Kumar Deep, Hemant Kumar, Ruby Sharma, Takshashila Tripathi, Narendra Tuteja, Ajay Kumar Saxena, and Atul Kumar Johri. 2010. "A Phosphate Transporter from the Root Endophytic Fungus Piriformospora Indica Plays a Role match with a *Metarhizium* sp., and this normally nematophagous species has been shown to transfer insect-derived nitrogen to plants (Behie and Bidochka 2014)[10]. The confined root conditions in the very well drained compost with the few nutrients that we used is similar to conditions in the endophyte source soils and the same endophyte-induced mechanisms may have been triggered.

	Number of grains % vs Control		Dry shoot weight % vs Control		Grain Yield % vs Control	
Isolate	**HI**	**LO**	**HI**	**LO**	**HI**	**LO**
E1	97	117	91	118	105	104
E2	97	113	84	127*	100	91
E3	101	136**	102	143*	107	115
E4	124*	163**	103	170**	125**	117**
E5	108	148**	88	134**	108	127**
E6	116	167**	98	139**	124**	129**
E7	124	107	100	111*	120**	96
E8	119	110	103	98	115*	88
E9	106	104	95	102	111	104
E10	125*	109	106	100	117**	88

Table 2. Comparison of the harvest parameters from the barley cultivar 'Propino' between plants inoculated with one of ten endophytes and controls, grown under two nutrient regimes, 'HI' and 'LO'. Figures are mean percentage differences per plant (n=15). ** indicates a statistically significant difference of $P < 0.01$ (ANOVA), * indicates $P < 0.05$. Table is adapted from the original Table 3 in Murphy et al. (2015) 'Fungal root endophytes of a wild barley species increase yield in a nutrient-stressed barley cultivar'. *Symbiosis* 65: 1-7.

Experiment 4. Improved resistance to drought stress

Though barley is a relatively drought-tolerant crop (Li et al. 2007)[11], the

in Phosphate Transport to the Host Plant." *The Journal of Biological Chemistry* 285 (34) (August 20): 26532–44.

10 Behie, Scott W, and Michael J Bidochka. 2014. "Ubiquity of Insect-Derived Nitrogen Transfer to Plants by Endophytic Insect-Pathogenic Fungi: An Additional Branch of the Soil Nitrogen Cycle." *Applied and Environmental Microbiology* 80 (5) (March): 1553–60.

11 Li, Chengdao, Guoping Zhang, and Reg Lance. 2007. "Recent Advances in Breeding Barley

incidence and severity of drought events can have devastatingly negative impacts on its growth and yield (Filek et al. 2014)[12]. These extreme drought events may increase in the future due to global warming (IPCC 2014)[13] and predicted increases in drought and temperature-related stresses are expected to reduce crop productivity (Larson, 2013)[14]. Successfully addressing the challenge of providing future food security will require both improvements in crop yield as well as the cultivation of additional farmland. This may result in the steady increase of farming on marginal, arid, and semi-arid lands, especially in the developing world, with consequent extra stresses on crops, including heat and drought-related stress. The key risks associated with these stresses will be reduced crop productivity, with strong adverse effects on regional, national, and household livelihood and food security (IPCC 2014)[15]. This is especially true with drought stress.

While breeding programmes and genetic modification can produce barley cultivars with much improved drought tolerance (Li et al. 2007; Cattivelli et al. 2008)[16], supplementary techniques and practices using microorganisms may help to alleviate the worst effects of drought (Coleman-Derr & Tringe 2014)[17]. In the next experiment, twenty five seeds of barley, in 5 pots containing 5 seeds each, were sown at 30 mm depth in a soil-based compost and inoculated with one of five endophytes. The inoculant solution was

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for Drought and Saline Stress Tolerance." In *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops*, edited by Matthew A. Jenks, Paul M. Hasegawa, and Shri Mohan Jain, 817. Dordrecht: Springer Science & Business Media.

12 Filek, M., M. Łabanowska, J. Kościelniak, J. Biesaga-Kościelniak, M. Kurdziel, I. Szarejko, and H. Hartikainen. 2014. "Characterization of Barley Leaf Tolerance to Drought Stress by Chlorophyll Fluorescence and Electron Paramagnetic Resonance Studies." *Journal of Agronomy and Crop Science.* 2014. (April 16): n/a–n/a. doi:10.1111/jac.12063.

13 IPCC. 2014. "Summary for policymakers." In: Climate Change 2014: Impacts, adaptation, and vulnerability. Part A: Global and sectoral aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. 2014. "Biotechnological Potential of Plant-Associated Endophytic Fungi: Hope versus Hype." *Trends in Biotechnology* (April 3): 1–7.

14 Larson, C. 2013. "Losing arable land, China faces stark choice: adapt or go hungry." *Science* 339, 644–645.

15 IPCC, "Summary for policymakers", 1–7.

16 Li et al., "Recent Advances in Breeding Barley", 817.

Cattivelli, Luigi, Fulvia Rizza, Franz-W. Badeck, Elisabetta Mazzucotelli, Anna M. Mastrangelo, Enrico Francia, Caterina Marè, Alessandro Tondelli, and A. Michele Stanca. 2008. "Drought Tolerance Improvement in Crop Plants: An Integrated View from Breeding to Genomics." *Field Crops Research* 105 (1-2) (January): 1–14.

17 Coleman-Derr, Devin, and Susannah G Tringe. 2014. "Building the Crops of Tomorrow: Advantages of Symbiont-Based Approaches to Improving Abiotic Stress Tolerance." *Frontiers in Microbiology* 5:283. doi:10.3389/fmicb.2014.00283.

prepared by mixing 10 mg of each fungal culture with 5 ml of sterile ultra-pure water and stirring with a magnetic bar for 1 hr at 25°C. 250 μl of the solution was directly inoculated onto each seed. For the controls, the seeds were inoculated with 250 μl of pure water. Soil moisture was measured daily and soil moisture content at a depth of 50 mm was maintained at 45% ± 10% (representing field capacity) from germination until 14 days after germination by watering with tap water. After this period, half of the plants were given lower water inputs (drought stressed; DS) and half were given higher (no deficit; ND) water input. For the ND treatments, the soil moisture content was maintained at approximately 45% as before; for DS treatments, pots were only watered when the soil moisture content was between 10% and 15% and the plants showed visible signs of stress (colour change, wilting), when the pots were watered until soil moisture content was again at field capacity (~ 45%). Total water input for the ND treatment was 6.39 litres per pot compared to 3.47 for the DS treatment. Results from our experiment showed that all five endophyte strains induced significant improvements in agronomic traits for a severely drought-stressed barley cultivar grown in a controlled environment, including number of tillers (shoots), grain yield and shoot biomass (unpublished, under review). The increase in shoot and grain weights induced by the endophytes were

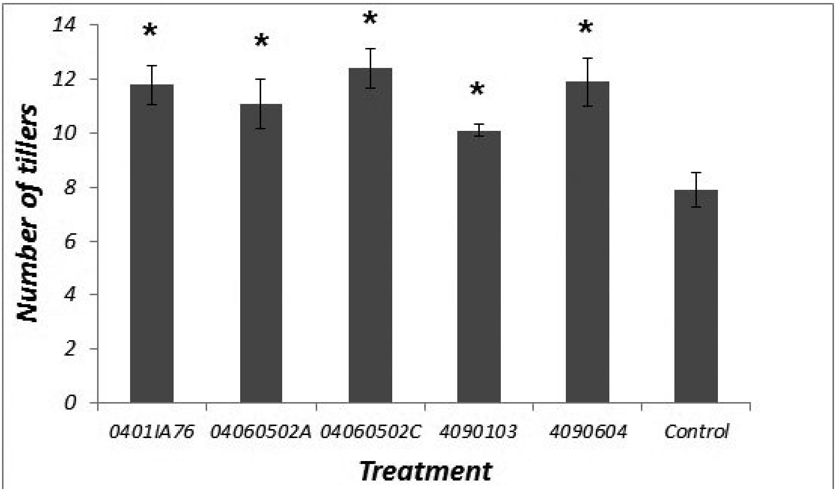

Fig 2. Number of tillers ± S.E. per plant for a drought-stressed (DS) barley cultivar for endophyte and control treatments. Significant differences (P < 0.05) between endophyte and control treatments are indicated with *.

directly correlated with the number of tillers, suggesting that tillering was the main driver of increases in both grain and shoot yield (Figure 2).

As these growth studies were conducted using soil-based compost the results may translate to the field and suggest that some of these endophytes have potential as barley inoculants in arid growing conditions.

Experiment 5. Enhanced survival under multiple stresses

Biotic and abiotic stresses such as extreme temperatures, low water availability, low nutrient availability and pathogenic infections are frequently simultaneously encountered by plants in both natural and agricultural systems (Langridge, Paltridge, and Fincher 2006)[18]. For example, high temperature and water stress are often co-associated with periods of drought. Abiotic stresses alone are estimated to reduce global crop yields by over a half of that possible under optimal growing conditions (Boyer 1982)[19]. Plants activate a specific and unique stress response when subjected to a combination of multiple stresses so current techniques for developing and testing stress-tolerant plants by imposing each stress individually may be inadequate.

While previous studies have examined the effects of one or two simultaneous stresses on barley, our final experiment in this cycle compared the performance of barley when inoculated with five endophytes, either individually or in combination, grown in optimal conditions (OC) and under a combined drought, heat, nutrient and pathogen stress (MS). Fifteen plants for each endophyte treatment, plus a control, were grown for 90 days in a controlled environment at a temperature of 33°C and only watered when the soil moisture content was below 15%. Furthermore, plants were given less than 10% of normal barley crop nutrients and infected with the serious barley pathogen “take-all”. We found a greater endophyte-induced improvement in important agronomic traits in the MS plants compared with the OC plants (unpublished, under review). For the MS plants only 13% of the controls survived to the end of the experiment compared with 80% of the endophyte treatments. In MS plants, the

18 Langridge, Peter, Nick Paltridge, and Geoff Fincher. 2006. “Functional Genomics of Abiotic Stress Tolerance in Cereals.” *Briefings in Functional Genomics & Proteomics* 4 (4) (February): 343–54.
19 Boyer JS. 1982. “Plant productivity and environment.” *Science* 218: 443–448.

endophytes induced increases in the number of tillers (shoots) and root and shoot biomass (Figure 3).

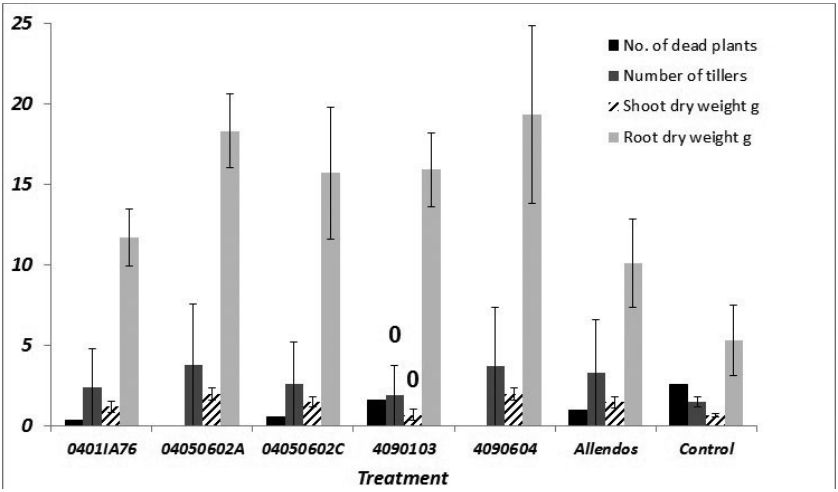

Fig 3. Mean harvest values per plant ± S.E. for barley grown under multiple stresses (MS). All values for endophyte treatments, except those marked with '0', are significantly greater (or less for the number of dead plants) ($P < 0.05$) than the control. 'Allendos' treatment is a co-inoculant of all 5 endophytes.

The combined endophyte inoculant (AllEndos) was the treatment that gave the greatest improvement for all harvest traits (number of dead plants, plant height, number of tillers, shoot dry weight, root dry weight) in the multiply-stressed plants ($P < 0.01$ for every trait). The results demonstrate potential for these endophytes as barley inoculants in similarly multiply-stressed farming environments. To our knowledge, this is the first experiment which has examined the effect of inoculating endophytes from a congeneric wild relative of barley onto abiotically and biotically stressed barley.

Experiment 6. Endophyte identities and diversity

In recent years, genetic studies of endophytic organisms isolated from particular ecosystems and plant systems has led to an increasing level of awareness of the high phylogenetic diversity within fungal endophytes. Investigations focusing on the endophyte communities inhabiting roots are extremely limited and, to our knowledge, no previous studies have examined the genetic diversity of fungal root endophytes isolated from a wild relative of a major cereal crop. An understanding of their genetic diversity is essential in order to fully comprehend the phylogenetic relationships to known taxa. We carried out the first ever ecological and phylogenetic survey of the culturable fungal root endophytes of a wild barley species, some of which have significantly improved agronomic traits in cultivated barley, as detailed in the experiments above (unpublished, under review). DNA was extracted and sequenced from the fungal root endophytes that were isolated from the ten populations of wall barley (*Hordeum murinum*), and 112 taxa of fungi were identified based on nuclear ribosomal internal transcribed spacer (nrITS) sequences. The mean pairwise similarity in GenBank was only 92%, with 21% of sequences returning no significant match. Only 30% of sequences could be confidently assigned specific rank. For sequences assigned a matching taxon, we found representatives from 8 orders, 18 genera, 12 families and only 12 species (Table 3).

These results suggest a high proportion of novel fungi, with 28% of sequences not assigned to a fungal order. Extrapolating from this, the study highlights the largely unknown, untapped and potentially useful resource of crop wild relatives and endophytes in general.

Discussion

The comprehensive cycle of experiments that we have completed clearly demonstrates the great potential of these endophytes for agriculture. Results show that novel symbiotic associations between barley and fungal root endophytes significantly increase yield and biomass in barley grown under nutrient, drought, heat and pathogen stress, and also suppress the development of seed-borne pathogenic infections of barley. If these endophytes can be successfully developed and utilised as crop inoculants, then they may play a significant role in increasing global food production and alleviating environmental damage and biodiversity loss.

The challenge now is to transfer this research from a controlled environment to the field. There are many challenging issues involved in achieving a reliable and sustainable strategy for realising the full potential of endophytic fungi (Kusari, Singh, and Jayabaskaran 2014)[20], but curiosity

20 Kusari, Souvik, Satpal Singh, and Chelliah Jayabaskaran. 2014. "Biotechnological Potential of Plant-Associated Endophytic Fungi: Hope versus Hype." *Trends in Biotechnology* (April 3): 1–7.

Fungal order	Family	Genus	Species*	Number of species
Capnodiales	Davidiellaceae	*Cladosporium*	sp.	4
	Leotiomycetidae	*Leptodontidium*	sp.	2
Chaetothryales	Herpotrichiellaceae	*Exophiala*	*oligosperma*	4
			sp.	2
Eurotiales	Trichocomaceae	*Paecilomyces*	*marquandii*	2
			sp.	2
	Trichocomaceae	*Penicillium*	*brevicompactum*	6
			chrysogenum	1
			glabrum	2
			sp.	18
		Uncertain	sp.	2
Hypocreales	Nectriaceae	*Fusarium*	*avenaceum*	1
			tricinctum	3
			sp.	1
	Clavicipitaceae	*Metarhizium*	*anisopliae*	1
			sp.	2
Incertae sedis	Incertae sedis	*Cyclothyrium*	sp.	1
	Pleosporaceae	*Epicoccum*	*nigrum*	1
Magnaporthale	Magnaporthaceae	*Gaeumannomyce*	sp.	1
Pleosporales	Pleosporaceae	*Alternaria*	*tenuissima*	1
	Leptosphaeriaceae	*Coniothyrium*	sp.	1
	Montagnulaceae	*Dendrothyrium*	sp.	1
	Phaeosphaeriaceae	*Ophiosphaerella*	sp.	2
	Incertae sedis	*Phoma*	sp.	1
	Incertae sedis	*Pyrenochaeta*	*unguis-hominis*	3
			sp.	3
	Phaeosphaeriaceae	*Vrystaatia*	sp.	1
		Uncertain	sp.	2
Sordariales	Chaetomiaceae	*Chaetomium*	sp.	1
Xylariales	Incertae sedis	*Microdochium*	*bolleyi*	3
			sp.	6
Uncultured			sp.	8
Uncultured – No Match			sp.	23

*Listed as species (sp.) when a sequence could be assigned with confidence to a genus but not to a species within that genus.

Table 3. Taxonomic summary of 112 fungal root endophyte isolates derived from ten Irish populations of *Hordeum murinum.*

driven research may be more effectively developed for biotechnological purposes if we can more closely fit the symbiotic partners to the growing conditions. The endophyte isolates that we have shown to improve important barley traits suggest great promise for several reasons. Firstly, the plants from which the endophytes were isolated were healthy and growing strongly despite the poor growing conditions, and secondly, the endophytes are derived from congeneric plants which may make them more suited as inoculants in the cultivated relatives of wall barley. But field conditions are very different to a controlled environment. The transient nature and shifting lifestyles of plant-microbe interactions make any extrapolation of results from 'pot to plot' difficult to justify (Nelissen, Moloney, and Inzé 2014)[21]. We still do not know how these endophytes will perform in the 'ecological marketplace', but the field trials that we have planned will provide the answer.

Future directions

Substantially more research is needed to identify the mechanisms responsible for the endophyte-induced benefits to barley that we observed in our studies. It is unlikely that just one mechanism is involved, and there may be multiple dimensions to the interactions involved. One major question that needs to be addressed is whether the grain yield increase is directly induced by the endophyte or by the induction of endogenous plant mechanisms. The suppression of normally detrimental seed-borne infections by the endophytes may release the plant from pathogen pressure allowing better growth and yield. Much of the work already done with the model endophyte *Piriformospora indica* suggests that induction of plant defences or mechanisms associated with greater nutrient use efficiency may be involved (Sherameti et al. 2005; Sherameti et al. 2008; Waller et al. 2008; Felle et al. 2009)[22]. Identification of the bioactive compounds involved in endophyte competence would also prove fruitful in elucidating the symbiosis.

21 Nelissen, Hilde, Maurice Moloney, and Dirk Inzé. 2014. "Translational Research: From Pot to Plot." *Plant Biotechnology Journal* 12 (3) (April): 277–85.

22 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.

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Immediate areas of research which will be critical in determining the usefulness of these organisms as inoculants for field barley crops will involve investigations into how best to develop a commercial product, the maintenance or loss of fungal competence over time and the most effective inoculant delivery methods. Perhaps most important of all will be to determine if endophyte inoculants can offer a safe and viable economic alternative or supplement to traditional chemical crop treatments. When the potential of these fascinating organisms has been fully elucidated and with grower and public buy-in, they may make a significant and important contribution to the sustainable cultivation of barely.

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