Symbiosis (2015) 65:1–7
DOI 10.1007/s13199-015-0314-6

Fungal root endophytes of a wild barley species increase yield in a nutrient-stressed barley cultivar

Brian R. Murphy · Fiona M. Doohan · Trevor R. Hodkinson

Received: 20 November 2014 / Accepted: 15 January 2015 / Published online: 26 January 2015

Abstract Overuse of chemical fertilisers in barley crops carries large economic and environmental costs and can lead to ecosystem degradation and loss of biodiversity. Methods of reducing chemical crop inputs using endophyte treatments have been demonstrated elsewhere. Here, we show that inoculation with six different fungal root endophytes isolated from wild populations of *Hordeum murinum* ssp. *murinum* increased grain yield in a nutrient-starved barley cultivar by up to 29 %. Furthermore, we also show that inoculation with the isolates induced increases of up to 70 % in shoot dry weight in the nutrient-starved barley. The greatest increases in grain yield and shoot dry weight were achieved under the lowest nutrient input. Several of the isolates may be new species, and one particularly effective isolate has previously been shown to completely suppress seed-borne infections of barley. Our results indicate that novel fungal root endophytes derived from a wild relative of barley may help to reduce fertiliser inputs while maintaining acceptable yields. If this potential can be realised in field crops it may result in more sustainable, economically cost-effective and environmentally friendly crop treatments and a reduction in chemical fertiliser use.

Keywords Barley · Biofertilisation · Fungal root endophytes · Nutrient stress · Symbiosis

B. R. Murphy (✉) · T. R. Hodkinson
School of Natural Sciences & Trinity Centre for Biodiversity Research, Trinity College Dublin, College Green, Dublin 2, Ireland
e-mail: murphb16@tcd.ie

F. M. Doohan
UCD Earth Institute and School of Biology & Environmental Science, University College Dublin, Dublin 4, Ireland

1 Introduction

Barley (*Hordeum vulgare* L.) is the world's fourth most important cereal crop, grown annually on 48 million hectares (CGIAR 2012), and like other food crops grown as monocultures, uses significant inputs of chemical fertilisers. Total global fertiliser consumption reached 180 mt in 2012 (FAO 2012), and according to one estimate, application of N-P-K fertilisers to barley crops alone worldwide will be over 4 mt in 2014 (Rosas 2011), which represents large economic and environmental costs, along with ecosystem degradation and potential losses in biodiversity (Dobermann and Nelson 2013). Ways of reducing these costs whilst still maintaining acceptable yields are required if sustainable agricultural practices are to be widely adopted. Inoculating barley with beneficial endophytic organisms may provide part of the solution.

Endophytes are microorganisms (bacteria, fungi and unicellular eukaryotes) which can live at least part of their life cycle inter- or intracellularly inside of plants usually without inducing pathogenic symptoms. This can include competent, facultative, obligate, opportunistic and passenger endophytes. Endophytes can have several functions and/or may change function during their lifecycle (Murphy et al. 2013). Fungal root endophytes (hereafter endophytes) have been shown to increase biomass in several globally important food crops, including cereals such as barley (Waqas et al. 2012). The model endophyte *Piriformospora indica* was first isolated from the roots of desert shrubs (*Prosopis juliflora* (Swartz) DC. and *Zizyphus nummularia* (Burm. fil.) Wt. & Arn.) in north-west India (Verma et al. 1998) and has been shown to increase grain yield in barley (Waller et al. 2005; Achatz et al. 2010; Murphy et al. 2014a, b). Although it has been shown to increase grain yield in cool-cultivated barley under some circumstances (Murphy et al. 2014a, b), it may not generally be suitable for use in cooler growing conditions. There is also the danger of releasing this organism as an invasive species into

foreign environments. Endophytes isolated from wild relatives of barley that are native to the crop growing area may be more reliable and safer to use.

Hordeum murinum subsp. *murinum* L. (*Hmm*), a wild relative of cultivated barley, is an annual grassy species and a ruderal of roadsides, rough grassland and waste places (Streeter et al. 2009; Stace 2010). This species generally grows in stressed environments (El-Shatnawi et al. 1999; Myrna Johnston et al. 2009; Murphy et al. 2014a), which may be due to symbiotically-conferred stress tolerance associated with endophyte inoculation (Rodriguez et al. 2008). We therefore hypothesised that endophytes isolated from *Hmm* could potentially benefit cultivated barley in low-nutrient conditions.

We isolated and cultured fungal root endophytes from wild populations of *Hmm* and tested the effects of inoculating these endophytes onto a barley cultivar growing under two different low-nutrient regimes.

2 Materials and methods

2.1 Endophyte collection

Whole plants of *Hmm* were collected from ten urban and suburban populations from within a ten km radius of a point centred at 53.39602 N, 6.21632 W (O 18636 39912) in June – July. Root samples were only collected from populations with greater than ten individuals, and a minimum of ten plants per population was collected. Roots were separated from whole plants and surface-sterilised in 5 % NaClO for 15 min then rinsed five times with sterile water. Ten root pieces of 5 mm length from each plant were inoculated onto culture plates of malt extract agar (Fluka 38954 modified MEA, Vegitone) and incubated in the dark at 25 C for 28 days. Dishes were inspected daily and those containing root pieces with surface fungal growth were discarded. Emergent endophytes were removed and subcultured on the same media in the dark at 25 C for a further 14 days. Ten endophytes were selected for the experiments; selection was based on rapid growth, the early appearance and quantity of spores, distinctive morphology and a lack of bacterial contaminants.

2.2 DNA analysis

For the DNA analysis, 20 mg of fungal material was scraped from the agar surface and placed into shaker tubes. DNA was extracted using a Qiagen DNeasy mini kit, following the Qiagen protocol, producing 200 μl of DNA extract for each isolate. PCR was carried out on the DNA extracts using the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) primers ITS1 and ITS4 (White et al. 1990). PCR products were purified using Exonuclease (New England Biolabs) and Shrimp Alkaline Phosphatase (ExoSAP; Roche). Purified PCR products underwent cycle sequencing using the reverse ITS4 primer (4 pmol) or forward ITS1 primer (4 pmol) in separate reactions with the ABI BigDye 3.1 kit (Foster City, CA). The products were further purified using a BigDye XTerminator purification kit and protocol. DNA was sequenced using an Applied Biosystems 3130xL Genetic Analyzer. The isolate sequences were compared with GenBank accessions using the Basic Local Alignment Search Tool (BLAST), and the isolates identified using morphological and DNA characters. The nearest named matches from BLAST were investigated using internet searches for any reported human or plant toxicity and for any extant patents for utilisation of the organisms.

2.3 Experimental procedure

Pure cultures of the isolates were prepared from single spores, following Murphy et al. (2014a, b). The barley cultivar 'Propino' (supplied as untreated seeds by Goldcrop Seeds, Cork, Ireland) was selected for the experiment as it is an established British cultivar (Cross: (Quench×NFC Tipple)) which is widely grown in Ireland. It is suitable for both malting and feed, with very high yield potential and good resistance to both *Rhynchosporium* and net blotch (*Pyrenophora teres*).

Seeds were surface-sterilised by soaking in 5 % NaClO for 15 min, rinsing three times with 70 % ethanol and then rinsing five times with pure water. The growth compost consisted of sterilised coarse vermiculite to which 1.43 g P4 broadleaf water absorbing polymer granules (Agricultural Polymers International Ltd.) per 1.5 l of vermiculite were added. The compost was dry mixed, moistened with tap water and placed into 1.5 l washed and sterilised plastic pots.

For each inoculation treatment (including a control), 50 seeds of barley, five per pot, were sown at 30 mm depth and inoculated with one of the 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 2 h at 35 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 h 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 2 h at 21 days from date of sowing and to 17 h at day 42. The temperature was raised by 2 C at day 21 and by a further 2 C at day 42.

Three agar-filled and covered culture dishes containing five sterilised seeds of barley were kept in the growth chambers during the experimental period to monitor any seed-produced

endophyte growth. Further, agar-filled covered culture dishes containing five split sterilised and unsterilised seeds were incubated in the dark at 25 C.

The seedlings were thinned to three plants per pot when the last seedling in each pot had reached Zadoks stage 10 (first leaf unfolded) (Zadoks et al. 1974). The final planting density was 106 plants m^2, which is within the normally recommended field planting density for barley (Kirby and Faris 1972). Plants were given a liquid fertiliser (Bayer Phostrogen®) at each watering after germination. 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 plant was: ammoniacal N=0.013 g, ureic N=0.078 g, Total N=0.09 g, P=0.057 g, K=0.146 g, Mg=0.01 g, S=0.02 g, Ca=0.01 g and traces of Boron, Copper, Iron, Manganese, Molybdenum and Zinc; for LO nutrient treatments, the total nutrient input per plant was halved for all elements. The HI treatment contained the recommended input of fertiliser for hydroponically-grown plants (2 g Bayer Phostrogen® per 5 l water, see http://www.phostrogen.co.uk/gardenerscorner/guides). However, even for the HI treatment the amounts of N, P and K were much less than the recommended inputs (lower by 18, 44 and 42 % respectively) for barley growing on low-nutrient soils (http://www.teagasc.ie/crops/winter/fertilisers/winter_cereals_fertiliser_requirements.pdf). To ensure healthy growth, the plants were sprayed with plain water once a week and the compost washed through with plain water monthly to remove any accumulation of nutrient salts. The growing compost was kept above 40 % moisture content and the total water input was 6.1 l per plant.

2.4 Data analysis

The number of days to reach selected Zadoks stages (Zadoks et al. 1974) was recorded for each plant. Plants were grown for 98 days (14 weeks) from date of sowing, by which time over 75 % of plants had reached full maturity, then harvested and processed over a period of 2 days. Pots were selected for processing in random order. All of the grains had at least reached the soft dough stage (Zadoks stage 85). Measurements were made for each plant of fresh and dry weights of grains, shoots and roots; mean height of heads to tip of highest grain; number of heads; number of tillers and number of grains per head. All plant parts were separately dried in ovens for 3 days at 65 C before dry weights were measured.

At the end of the experiment and before drying the roots, four 5 mm pieces of mid-section root from each plant were surface-sterilised and incubated on half-strength MEA at 25 C in the dark to test for endophyte presence.

In order to determine if the endophyte isolates could be transmitted vertically and/or horizontally, we sowed 30 surface-sterilised seeds from plants inoculated with endophyte isolates E4 and E6 into fresh compost, and 30 new seeds into the compost which contained the plants previously inoculated with these two isolates. Seedlings were harvested at Zadoks growth stage 12 (second leaf 50 % emerged) and the roots processed as before to check for emergent endophytes.

Data analysis by Student's t-test was performed using the Data Analysis modules provided by Microsoft Excel 2010®.

3 Results

The nrITS sequences obtained from analysis of the endophyte isolates were used to search for similar sequences in GenBank using the BLAST search tool. Closest matches ranged from 93.8 to 100 % identity, with a mean pairwise identity match of 97.3 % (Table 1). A criterion of less than 97 % match was used to indicate possible new species, and four of the endophytes were identified as new species. The search results indicated that the ten endophyte isolates represented five different fungal orders (Capnodiales, Chaetothryiales, Eurotiales, Hypocreales and Pleosporales). The generic identity obtained from GenBank was confirmed by morphological examination. There were extant patents for four of the endophytes but none of these patents were related to use as biofertilisation agents, and four endophytes were reported to have human or plant toxicity (Table 1). All ITS sequences were deposited in GenBank (Accession numbers KM492834-KM492843).

All sterilised control seeds on agar in the growth chambers had no external fungal growth after 7 days, and all germinated, indicating that surface sterilisation was successful. The control surface-sterilised split seeds produced mycelia (less than 10 mm diameter) of only one fungus, probably *Pyrenophora teres,* attached to the seed after 7 days but the split unsterilised seeds had produced at least 4–5 different types of fungal growth.

When the harvest measurements were compared, we found that the plants with the HI nutrient input had the greater mean values for all parameters but root dry weight (Fig. 1 and Table 2). The greatest difference was shown in the shoot dry weight, where the HI nutrient input plants were a mean of 53 % greater than the LO (P<0.001). The mean grain dry weight for the HI treatments was 35 % greater than the LO (P<0.001). Compared with the control, all of the endophyte-inoculated plants had a greater mean number grains per plant for both HI and LO treatments (Table 3). Overall, the LO treatment produced the greatest number of significant differences compared with the control – 22 versus 7 for the HI treatments. The parameters that had higher values for the endophyte-inoculated plants over the control for both treatments combined were (in order of greatest difference): mean grain dry weight, mean number of grains and mean shoot dry weight. The mean number of mature heads, mean height and

Table 1 Nearest BLAST search matches for ITS sequences obtained from ten endophyte isolates derived from wild populations of wall barley. Patents/Toxicity refers to known (+) or absence (−) of patents and human or plant toxicity

Isolate	Nearest BLAST Match	% Identical	Patents/Toxicity	GenBank Accession
E1	*Paecilomyces marquandii* JQ013003 [1]	98.6		KM492834
E2	*Viridispora alata* JF832678 [2]	94		KM492835
E3	*Cladosporium* sp. GQ169491 [3]	99.6		KM492836
E4	*Penicillium brevicompactum* EU587331 [1]	94.8	+/+	KM492837
E5	*Pyrenochaeta unguis-hominis* JX966641 [4]	98		KM492838
E6	Uncultured *Metarhizium* KC797571 [2]	95.5	+/–	KM492839
E7	Uncultured fungus FJ820798 (*Penicillium*?)	93.8		KM492840
E8	*Exophiala oligosperma* JN655630 [5]	100		KM492841
E9	*Penicillium brevicompactum* FJ884117 [1]	99.6	+/+	KM492842
E10	*Penicillium* sp. FR822844 [1]	99.4		KM492843

Fungal orders (superscript numbers on Nearest BLAST match): 1 = Eurotiales, 2 = Hypocreales, 3 = Capnodiales, 4 = Pleosporales, 5 = Chaetothyriales

mean root dry weight each had only one significantly greater value than the control.

Plants that were each inoculated with one of six endophytes (E2, E3, E4, E5, E6 and E7) had significantly greater mean shoot dry weight than the control for the LO nutrient treatments (a range of single Student's t-tests, three at $P<0.01$ and three at $P<0.05$) but none of the HI nutrient treatments inoculated with the endophytes had a greater mean shoot dry weight than the control. Three of the endophyte isolates (E4, E6 and E5) induced both significantly greater shoot dry weight and mean grain dry weight than the control for the LO nutrient treatments, with E4 having 70 % greater mean shoot dry weight ($P<0.01$) and E6 having 29 % greater mean grain dry weight ($P<0.01$). The endophyte isolate E4 had the greatest number of significantly higher parameter values than the control.

The mean grain dry weight was the parameter with the most number of significantly greater values than the control (three for the LO treatment and five for the HI), and the mean

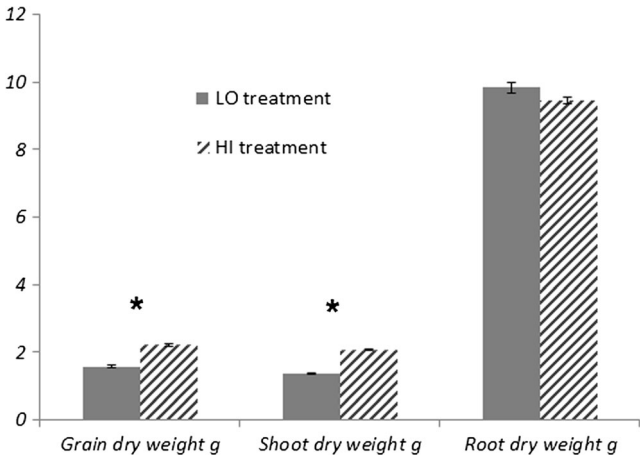

Fig. 1 Comparison of grain, shoot and root mean dry weights±S.E. between the LO and HI nutrient treatments. Statistically significant differences with $P<0.01$are marked with *

number of mature heads and mean height had the least number of significantly greater values than the control (one each for the LO treatment). Only one of the endophyte isolates (E1) produced no significant differences for any parameter compared with the control.

There were minor symptoms of stress or disease on all of the mature plants, with symptoms affecting from 30 – 90 % of leaf and stem tissue. Most of the stress symptoms were probably related to nutrient deficiency, consisting of chlorotic spots and leaf browning. There were no visible signs of disease at the seedling stage and the few disease symptoms that did appear matched those usually seen in plants infected with *Ramularia collo-cygni* (although some of the plants could also have been infected with the spot form of net-blotch).

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.

When we tested for the vertical transmission potential of the endophytes, we found that only one root piece (3 %) produced the emergent endophyte E4. For the horizontal transmission test, three root pieces (10 %) produced emergents of E4. The endophyte isolate E6 did not emerge from any root pieces.

4 Discussion

We have shown that inoculation with fungal root endophytes derived from *Hordeum murinum* subsp. *murinum* L. significantly increased grain yield, shoot dry weight and number of grains per plant in the barley cultivar 'Propino' grown in nutrient-starved conditions (Table 3). Further, the increases in these parameters were greatest with the lowest nutrient regime. Nearly all of the ten endophyte isolates produced some improvement in at least one barley trait, with only one

Table 2 Mean harvest measurements per plant for the barley cultivar 'Propino' inoculated with one of 10 different fungal root endophytes and grown under 2 nutrient regimes, 'HI' and 'LO'

Treatment	Nutrients	No. of mature heads	No. of grains	Height at maturity mm	Dry shoot weight g	Dry root weight g	Dry grain weight g
E1	LO	1.26±0.1	28.40±3.3	57.33±3.9	1.32±0.13	11.15±0.68	1.56±0.07
	HI	1.60±0.3	35.00±2.3	63.00±2.7	1.96±0.09	9.34±0.32	2.08±0.08
E2	LO	1.66±0.2	27.46±1.8	61.60±3.6*	1.42±0.09*	9.96±0.44	1.36±0.04
	HI	1.33±0.3	34.73±3.3	61.86±3.6	1.82±0.07	9.69±0.44	1.98±0.15
E3	LO	1.20±0.3	33.00±1.8**	58.80±3.9	1.60±0.11*	9.63±0.47	1.72±0.09
	HI	1.60±0.2	36.13±3.9	63.40±4.4	2.20±0.07	9.66±0.41	2.12±0.15
E4	LO	1.60±0.2	39.60±3.9**	59.33±4.3	1.90±0.21**	9.29±0.54	1.76±0.02**
	HI	1.73±0.2	44.40±3.2*	61.73±4.4	2.22±0.15	9.39±0.63	2.49±0.08**
E5	LO	1.33±0.3	35.80±2.8**	58.66±4.0	1.50±0.15**	11.75±0.31*	1.90±0.07**
	HI	1.40±0.2	38.93±2.6	64.20±4.2	1.90±0.07	9.28±0.40	2.14±0.08
E6	LO	1.60±0.2	40.40±2.0**	60.06±4.7	1.56±0.09**	10.05±0.27	1.94±0.04**
	HI	1.80±0.3	41.53±3.2	63.13±4.6	2.12±1.0	9.69±0.35	2.46±0.07**
E7	LO	1.73±0.2	26.00±2.5	50.93±3.6	1.24±0.02*	9.40±0.51	1.44±0.11
	HI	2.00±0.1	44.53±3.3	59.27±5.1	2.16±0.12	9.71±0.31	2.38±0.04**
E8	LO	1.40±0.1	26.73±2.7	51.20±3.2	1.1±0.04	9.23±0.52	1.32±0.09
	HI	1.93±0.2	42.73±3.3	61.33±4.5	2.22±0.09	8.63±0.30	2.28±0.09*
E9	LO	2.00±0.1*	25.20±2.3	52.46±3.2	1.14±0.01	8.96±0.32	1.56±0.05
	HI	1.73±0.2	37.93±2.7	59.27±4.4	2.06±0.13	9.27±0.33	2.2±0.07
E10	LO	1.73±0.2	26.40±2.3	46.26±4.2	1.12±0.02	8.47±0.32*	1.32±0.06
	HI	2.06±0.3	45.06±2.4*	61.93±4.6	2.28±0.06	9.85±0.39	2.32±0.07**
Control	LO	1.60±0.1	24.26±2.4	51.33±2.6	1.12±0.05	10.11±0.63	1.5±0.07
	HI	1.80±0.2	35.93±2.5	57.73±4.2	2.16±0.2	9.58±0.43	1.99±0.08
Means	LO	1.56	30.3	55.2	1.37	9.82	1.58
	HI	1.73	39.7	61.5	2.1	9.46	2.13

* indicates a statistically significant difference between treatment and control of $p<0.05$ (ANOVA), ** indicates $p<0.01$ ($n=15$)

producing no measurable benefit. 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.

Three of the endophyte isolates (E4, E5 and E6) performed significantly better than the others, and these were isolated from plants of *Hordeum murinum* growing on the same site. Each of these endophyte treatments significantly increased the number of grains, shoot dry weight and grain dry weight for the LO nutrient input. The greater improvement in these traits associated with the lowest nutrient input contrasts with the findings of Murphy et al. (2014a, b) who found, in an experiment with similar HI and LO nutrient treatments, that an increase in grain dry weight induced by the model endophyte *Piriformospora indica* was only apparent with the HI nutrient input. The endophytes we have isolated and tested gave greater improvements than *P. indica* in important barley traits in the most nutrient-stressed plants.

These results suggest that crop inoculants using selected endophytes would have the most beneficial impacts on very low nutrient sites, allowing growers to reduce fertiliser inputs while still maintaining acceptable yields. The reduction in economic and environmental costs could potentially be great, particularly for subsistence farmers in poorer parts of the world. Some barley traits (for instance, kernel weight) are primarily genetically determined and thus more stable as compared to other traits (such as seed number) (Borras et al. 2003). An already complex trait, such as seed yield, depends on many environmental variables, such as planting density (Diepenbrock 2000). Growers are more interested in crop yield per area, whereas in a controlled environment study such as ours we can more easily measure results per plant or pot. When translated to the number of plants per square metre then our planting density was representative of that usually recommended, thus enabling interpretive translation to field conditions.

Water run-off and nutrient leaching were not recorded here, but Broschat (1995) suggests that nutrient loss due to leaching from pot-grown plants can be high, depending on compost composition. If we use these nutrient losses through leaching as a guide to the real amount of nutrients available to the plants then it is clear that the plants were even more nutrient-starved than we suspected, particularly in such a free draining compost as vermiculite.

Table 3 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'

	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
Means	112	127	97	124	113	106
Control	100	100	100	100	100	100

Figures are mean percentage differences per plant ($n=15$). ** indicates a statistically significant difference of $p<0.01$ (ANOVA), * indicates $p<0.05$

The environmental characteristics of our plant sampling sites may hint at the mechanisms responsible for endophyte-induced increases in grain yield and shoot biomass. Murphy et al. (2014a) report that plants at this site 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). The isolate E6 had a closest BLAST match with a *Metarhizium* sp., and this normally nematophagous species has been shown to transfer insect-derived nitrogen to plants (Behie and Bidochka 2014). 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. The exact same strain of one of the three most effective endophyte isolates (E4) has also been shown to supress seed-borne infection in barley (Murphy et al. 2014a), indicating that suppression of yield-reducing infections may be part of the mechanism or a contributory factor by which grain yield is increased.

Substantially more research is needed to identify the mechanisms responsible for the endophyte-induced benefits to barley that we observed in our study. 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. As already discussed, the suppression of normally detrimental seed-borne infections by the endophyte 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, 2008; Waller et al. 2008; Schäfer et al. 2009; Felle et al. 2009). Identification of the bioactive compounds involved in endophyte competence would also prove fruitful in elucidating the symbiosis.

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 et al. 2014), but curiosity 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 in the field 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 et al. 2014).

We do not know how these endophytes will perform in the 'ecological marketplace'. Some endophyte spores may remain in the soil after harvest and root endophytes may even be vertically transmitted (Barrow et al. 2004). In our study, there was very little evidence for the vertical transmission of the endophytes, with only one out of thirty root pieces producing an emergent isolate, though a recent study has shown stronger indications for this method of transmission in endophytes (Hodgson et al. 2014).

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 and other agricultural crops.

Acknowledgments We thank: Goldcrop Seeds, Cork, Ireland for the generous supply of barley seeds, and for advice on suitable cultivars to use; Helena Murphy for proof reading and the de-cluttering of technical terms; laboratory technicians at Trinity College Dublin for providing supplies and technical support; Aude Perdereau for assistance with plant processing. Trinity College Dublin provided financial support through a PhD studentship grant.

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