

Mechanisms of beneficial colonisation of barley by fungal root endophytes

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Summary

Pathogenic fungal infections of barley can lead to costly crop losses. However, not all fungal infections are detrimental, and some are even beneficial. Beneficial root infections often involve symbiotic endophytic fungi. Benefits to barley and other plants infected with endophytic root fungi include an increase in seed yield, enhanced resistance to pathogens and improved stress tolerance. Here, we examine the mechanisms and outcomes of fungal endophyte colonisation of barley roots and briefly discuss reported benefits for the host. The most important factors that determine the nature of the relationship are the specific combination of partner genotypes and developmental stage, and the ecological and environmental setting. The full potential of these organisms is still to be determined and further studies are urgently required to develop specific beneficial root-endophyte associations, or combination of them, that are tailored to barley cultivars for maximum impact in agriculture.

Key words: Fungal endophytes, barley, symbiosis, pathogens, yield, stress-tolerance

Introduction

Many changes are taking place in the world of agriculture today, with global warming and land degradation bringing new stresses to crops, leading to a reduction of suitable growing locations and local food shortages. Over-use of agrochemicals in an effort to maintain yield under increasing stress is causing serious environmental damage and increasing economic costs. Beneficial fungal root endophytes have the potential to reduce chemical use, increase pathogen resistance and enhance stress tolerance while still maintaining yield. Realising this potential means that research in this field is critically important.

The full effects of a pathogenic infection on the host plant are sometimes not apparent until the disease is well established, and the negative impacts can be substantial. Endophytic fungal infections can have an equal, but beneficial impact on plant growth and survival. 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. The taxonomic range of fungal endophytes is huge, with foliar endophytes being particularly diverse (Arnold & Lutzoni, 2013; de Souza Leite et al., 2013). Root endophytes also belong to diverse taxa and can have a broad range of beneficial effects.

Several reviews have been published on fungal endophytes (Kogel et al., 2006; Schulz & Boyle, 2006; Mayerhofer et al., 2012). These reviews provide extensive coverage of endophytes in general, but there is a lack of a detailed synthesis of knowledge for the fungal endophytes of barley (*Hordeum vulgare* L.) roots in particular. The only review which specifically examines root endophytes (Mayerhofer et al., 2012) does not include the Basidiomycetes, which, as we will see, are important endophytes of barley. This paper addresses that gap and suggests where future research is required.

Endophytes and Barley

Benefits to barley and other plants infected with endophytic fungi include an increase in seed yield (Achatz et al., 2010), enhanced resistance to pathogens and herbivores (Cheplick & Faeth, 2009), and increased stress tolerance (Waller et al., 2005; Rodriguez et al., 2009). A beneficial host-endophyte association is a balanced antagonism, whereas a pathogenic association is imbalanced; the pathogenic infection results in disease (Wilson, 1995; Schulz et al., 1999).

Megatons of fungicides, pesticides, and fertilisers are applied to crops every year, incurring large economic costs and contributing to ecosystem degradation (Powell & Jutsum, 1993; Underwood, 2000). In 2000, the worldwide crop protection market was estimated to be about \$31 billion (Underwood, 2000). Further costs are incurred in testing varieties of barley to use as genetic resources for disease resistance breeding (Statkevičiūtė & Leistrumaitė, 2010; Knupfer et al., 2011). Beneficial fungal endophytes may have the potential to reduce these costs, and may even enable the production of crops on previously unsuitable or marginal sites.

Barley is the world's fourth most important crop, grown annually on 48 million hectares (CGIAR, 2012), and is often planted on stress-susceptible marginal land, so the potential of beneficial fungal root endophytes to improve barley crop performance requires extensive study. Endophyte colonisation can have neutral, positive or negative effects on the plant, depending on many variables (Tellenbach et al., 2011; Mayerhofer et al., 2012; Reininger et al., 2012).

In contrast to the large number of beneficial fungal endophytes that have been recorded on various hosts, beneficial endophyte infection of barley roots has only been described with a few fungal species and most noticeably *Piriformospora indica*. Since its discovery in the Thar desert of north-west India in 1997 (Verma et al., 1998), the basidiomycete *P. indica* has become the model experimental organism for the study of fungal endophyte root colonisation (Oelmüller et al., 2009). *P. indica* belongs to the Sebaciales, an order of mostly endophytic fungi with extensive cryptic biodiversity (Oberwinkler et al., 2013). In barley, *P. indica* has been shown to increase yield between 5.3% and 11% (Achatz et al., 2010), enable salt tolerance (Waller et al., 2005; Baltruschat et al., 2008) and enhance pathogen resistance (Waller et al., 2008; Felle et al., 2009; Rahnamaeian et al., 2009). In other plant groups, such as orchids, *P. indica* may predominantly exist as a mycorrhizal partner (Schafer & Kogel, 2009).

P. indica, unlike many endophytes, can be easily cultured outside of a plant host (Singh et al., 2003; Oelmüller et al., 2009; Qiang et al., 2011). It is thus an ideal experimental subject, especially as it readily colonises the model angiosperms *Arabidopsis thaliana* and barley (Peskan-Berghofer et al., 2004; Stein et al., 2008; Oelmüller et al., 2009; Achatz et al., 2010; Qiang et al., 2012). Other fungal root endophytes have also shown promise as bio-control and bio-fertilizing organisms. For example, *Fusarium equiseti* (Macia-Vicente et al., 2008a) and *Sebacina vermifera* (Schafer & Kogel, 2009) have both been shown to reduce pathogen infection of barley by up to 80%.

This review will now examine the barley-endophyte relationship and the benefits conferred on barley by *P. indica* and other fungal root endophytes. It will overview what is known regarding colonisation and the benefits to the plant, including resistance to pathogens, yield enhancement, stress tolerance, and interactions with other organisms.

Colonisation

When plants are challenged by fungal pathogens, host factors control plant resistance and susceptibility through the complex signalling pathways that mediate plant disease resistance (Toyoda et al., 2002). Similarly, the nature of the relationship between barley and fungal root endophytes such as *Piriformospora indica* is controlled by a subtle physical and biochemical dialogue, with the final outcome dependent on the combination of genotype and developmental stage of each partner (Kogel et al., 2006).

The infection process starts with either germination and hyphal growth of spores present in soil or in the plant tissue, or is initiated by contact between the endophyte hyphal tip and the barley root hair. Hyphal apical dominance is abandoned and hyphal branching is triggered by fungal perception of the strigolactone 5-deoxy-strigol, followed by the formation of a pre-penetration apparatus (Genre et al., 2005). Strigolactones are a group of sesquiterpene lactones, previously isolated as seed germination stimulants for the parasitic weeds *Striga* and *Orobancha* (Akiyama et al., 2005). The endophyte must now colonise the root without triggering a full-blown defence response, so the relationship develops in a finely balanced way that does not result in pathogenicity or endophyte death. There is strong evidence for a balanced antagonism between the virulence of the colonising endophyte and the plant defence response (Maciá-Vicente et al., 2009). Recognition of the endophyte as a friendly intruder rather than a pathogen is realised by host receptor-kinase-mediated transmembrane signalling (Stracke et al., 2002). Fungal hyphae penetrate the root at the anticlinal interface of adjacent rhizodermal cell walls (Deshmukh et al., 2006).

The first hurdle to be overcome by the endophyte is to gain entry to the root cell. The cellulose, hemicellulose, pectin and lignin in the cell wall must be broken down by cellulolytic and ligninolytic enzymes, and endophytes use a variety of these enzymes (polyphenol oxidases, cellulases and laccases) to help with root cell penetration (Basiewicz et al., 2012). Direct endophyte contact with the barley root results in increased enzyme production, but in vitro cultures show a much reduced enzyme activity (Basiewicz et al., 2012), suggesting that living root contact is required to induce fungal enzyme production.

The barley root cell does make some attempt to arrest fungal proliferation. In *Blumeria graminis* f.sp. *hordei*-resistant barley strains, an increase in the secretion of building blocks for cell wall apposition and plasmodesmata blocking retards hypersensitive cell death in neighbouring cells, as early as 21 h after infection (An et al., 2006). In *P. indica* infection of barley roots, fungal colonisation and sporulation is always associated with limited cell death (Deshmukh et al., 2006). The infected cell may still die, but hypersensitive cell death of neighbouring cells is contained. The suppression of host defence reactions needed for successful infection is associated with the reduction of a cell death regulator protein of barley, resulting in a 50% increase in infection (Eichmann et al., 2006). Overexpression of the associated gene (BAX INHIBITOR-1) diminishes colonisation by *P. indica* (Imani et al., 2011). Older cells are more likely to undergo cell-death, and *P. indica* preferentially colonises the oldest root hairs of barley (Waller et al., 2005).

Once the endophyte has fully colonised the root cell, it will then move on to infect neighbouring cells or sporulate. Unlike mycorrhizal fungi (which sporulate outside the root), endophyte sporulation can occur inside or outside the barley roots (Schafer & Kogel, 2009), for example as chlamydospores, and this gives the fungus a long-term in planta residence potential, with spores able to remain dormant in plant tissue. Colonisation of further cells can then initiate from these spores in differentiated tissue (Deshmukh et al., 2006).

Although infection by the root endophyte may be entirely symptomless, gene expression studies indicate that infection by some fungal groups, such as *Sebacinales*, elicits a systemic resistance against leaf pathogenic fungi (Waller et al., 2008), and expression of these genes may provide a simple and reliable marker of colonisation. Roots colonised by *P. indica* show relatively low induction of defence-related genes, while other genes are differentially regulated, indicating a faster *P. indica*-dependent root development. Systemic gene expression analysis using mRNAs

has detected a *P. indica*-associated doubling in the expression of the pathogenesis-related gene HvPr17b and the molecular chaperone HvHsp70 (Waller et al., 2008).

Hormones and other metabolites are important signalling molecules in the plant system, and these too show marked changes in expression and activity in response to fungal endophyte infection (Schulz et al., 1999; Molitor & Kogel, 2009; Khatabi et al., 2012; Lahrmann & Zuccaro, 2012). Fungal-host interactions involve constant mutual antagonisms, often based on a coordinated response to the secondary metabolites the partners produce (Schulz et al., 1999). Endophyte colonisation success may ultimately depend on the nature of plant hormone signalling activity. In barley, the evasion and suppression of the host defences during early colonisation may be related to the perturbation of plant hormone balance and the secretion of fungal effectors such as lectins and other small proteins (Lahrmann & Zuccaro, 2012). Plant hormones are important factors for compatibility in plant root-*P. indica* associations, and might provide a first explanation for colonisation success in a wide range of higher plants. *P. indica* induces ethylene synthesis in barley, which suggests that ethylene signalling is required for symbiotic root colonisation (Molitor & Kogel, 2009; Khatabi et al., 2012). The hormones gibberellin, auxin and abscisic acid are up-regulated in response to *P. indica* infection, accompanied by a general suppression of the plant innate immune system, and these changes are significant factors of compatibility in the mutualistic association (Schäfer et al., 2009).

A significant increase in the phytohormones indoleacetic acid (IAA) and indole-3-lactate (ILA) is involved in the subsequent establishment of a biotrophic symbiosis (Hilbert et al., 2012), and might represent a compatibility factor in endophyte infection. The key player in this process is the *P. indica* gene *piTam1*, which is involved in tryptophan transamination. *P. indica* strains in which the *piTam1* gene was silenced were compromised in IAA and ILA production and displayed reduced colonization of barley roots in the biotrophic phase (Hilbert et al., 2012).

Outside of the endophyte-host system, infection potential from the rhizosphere associated microbial population is related to many factors, and soil type is the main determinant (Berg & Smalla, 2009). Soil fungi spore density and fungal development are positively correlated to high pH, high clay content and barley developmental stage (Black & Tinker, 1979). The ecological interactions are complex and covered elsewhere (Black & Tinker, 1979; Macia-Vicente et al., 2008b).

Once the endophyte is established, the infection may now develop as a beneficial symbiosis or a virulent pathogenesis, largely dependent on host and fungal genotypes, colonization density and the presence of competing endophyte strains (Tellenbach et al., 2011; Reininger et al., 2012). Environmental conditions can provoke a pathogenic lifestyle in the usually beneficial endophyte *P. indica*. Kaldorf et al. (2005) showed that *P. indica* infection of *Populus* seedlings resulted in reduced root growth and leaf necrosis when ammonium instead of nitrate was provided as the single nitrogen source during plant–fungus co-cultivation.

Fungal root endophytes are often intimately associated with particular bacterial companions (Sharma et al., 2008), and both *P. indica* and *S. vermifera* have species-specific associations with bacteria. In fact, it is nearly always the case that both partners are found together (Sharma et al., 2008; Schafer & Kogel, 2009). This raises the question as to whether it is the association or the individual partners that confer the beneficial effects on plants. Schafer & Kogel (2009) showed that inoculating barley with the bacteria alone (*Rhizobium radiobacter*) gave similar effects as *P. indica* inoculation.

Resistance to Pathogens

Fungal root endophytes can inhibit the colonisation potential of the most damaging barley pathogens. Plant protection associated with endophyte presence may result from an indirect effect, with the endophyte inducing plant defence responses that protect against disease.

Many antifungal and antibacterial agents from various endophytes have been described (Joseph & Priya, 2011; Favarro et al., 2012; Liang et al., 2012; Mousa & Raizada, 2013), but details regarding the beneficial interactions and mechanisms involved are often not well understood. Although research has focussed on a few well characterised fungal root endophytes, there may be many others that have the potential to inhibit barley pathogens. Macia-Vicente et al. (2008a) tested 73 endophyte isolates belonging to diverse genera, and all showed some inhibition of the pathogen *G. graminis* in barley roots. More experimental work with barley is needed to fully describe and utilise the potential and diversity of these agents.

A number of mechanisms have been implicated in endophyte-induced pathogen resistance in barley. Even though it is clear that *P. indica* requires host cell death to proliferate (Deshmukh et al., 2006), the *P. indica* induced programmed cell death - where cell death is restricted to fully colonised barley root cells - is different from the hypersensitive cell death response in pathogen defence. The limited cell death associated with *P. indica* infection allows for *P. indica*-induced pathogen resistance without causing disease symptoms in the barley.

The production of reactive oxygen species is a prerequisite for successful fungal development and pathogenesis of necrotrophic infections (Waller et al., 2005), and pathogen resistance related antioxidant capacity is enhanced in *P. indica* infected barley (Waller et al., 2005; Harrach et al., 2013). Changes in the root surface pH are a feature of *P. indica* colonisation, resulting in a long term response of a change in leaf surface pH upon *B. graminis* infection (Felle et al., 2009).

Variable gene expression may occur in response to fungal pathogen infection, depending on the presence or absence of a beneficial endophyte. The changes in hormone homeostasis are accompanied with a general suppression of the plant innate immune system.

Barley does produce antifungal and herbicidal substances of its own, and these are enhanced by the presence of the beneficial endophyte. Concentrations of defence related plant secondary metabolites such as phenylpropanoids and oligomeric proanthocyanidins can increase significantly in the roots of barley infected with several different endophyte species (Schulz et al., 1999).

Yield Enhancement

Piriformospora indica infection of barley grown in a glasshouse increased shoot fresh weight by up to 65% after only 4 weeks of mutualistic symbiosis (Waller et al., 2005), but in field colonised barley, increases in plant biomass due to endophyte treatment were only 10% (Schafer et al., 2009), probably due to other stresses not encountered in the glasshouse.

Grain yield effects due to fungal root endophyte infection also vary depending on the experimental environment. The increases in barley grain yield due to root colonisation by *P. indica* are apparent even under very different nutrient regimes (Achatz et al., 2010), due to accelerated growth of barley plants early in development and earlier maturation of barley heads. Achatz et al. (2010) showed that higher grain yield was induced by *P. indica* infection independent of markedly different phosphate and nitrogen fertilisation levels. An endophyte induced relative increase in root biomass over shoot biomass has been demonstrated in some grass species (Czarnoleski et al., 2012), including rice (Redman et al., 2011). No equivalent pattern has been reported in barley studies, though nearly all barley yield related experiments have shown an overall increase in plant biomass due to beneficial endophyte infection (Waller et al., 2005; Schafer et al., 2009).

Abiotic Stress Tolerance

Piriformospora indica is beneficial in protecting the barley plant against many different biotic and abiotic stresses (Schafer et al., 2009). Some barley species (e.g. *Hordeum spontaneum*) are adapted to marginal and stress prone environments (Willcox, 2005), and these environments are often characterised by gradually increasing salinity. Therefore, salt tolerance in derived modern barley varieties is an important factor for the grower considering planting barley as a risk aversion crop on saline soils. Saline soils can inhibit the activity of most soil fungi (Dixon et al., 1993) and may lead to reduced competition for a more salt-tolerant beneficial root endophyte.

Metabolic heat efflux, salt induced lipid changes, and fatty acid desaturation are all associated with salt stress in plants (Criddle et al., 1989; Ahmad et al., 2013). Barley root colonisation by *P. indica* attenuated all of these factors in the leaves of a salt sensitive cultivar, and still increased plant growth (Baltruschat et al., 2008). Several studies have found that endophyte-mediated salt tolerance was associated with a strong increase in antioxidants (Criddle et al., 1989; Baltruschat et al., 2008).

Studies using other plant species have examined a broader range of abiotic stresses (Rodriguez et al., 2008, 2009; Redman et al., 2011), but more work needs to be done on endophyte-induced cold and drought tolerance in barley, which may extend the growing season.

Synthesis and Conclusions

Fungal root endophyte infections are often beneficial to the host, and much of the evidence examined here shows that barley can particularly benefit from endophyte infection. But endophytes can also be detrimental, and this review reveals contradictory study results regarding the effects of fungal root endophytes on the barley plant.

Clearly, prevailing circumstances dictate the nature of the plant-endophyte relationship. Even if an endophyte is never pathogenic, it is not always beneficial, and is often neutral in effect. Despite the potential benefits of benign fungal root endophyte infection, there will always be a cost to the plant, with the fungus needing to extract at least some nutrients to survive. Gorischek et al. (2013) showed that endophyte infection of *Elymus virginicus* (a grass species related to *Triticum*) can shift host resources towards the preferred method of endophyte transmission (for example, through an increase in infected seeds). Martin et al. (2013) found that a reduction in xylem endophytic fungi was associated with increased resistance to Dutch elm disease; a clear trade-off between resistance to all fungal infections and the potential benefits related to endophyte infection. More work with the barley-endophyte relationship will reveal if these effects are significant in barley.

In situations where there may be many fungal organisms in the rhizosphere, the competition for root living space will inevitably result in contingent temporal outcomes. At different times, the same fungus can be either a winner or loser, depending on the mix of competing organisms and the developmental stages of both host and fungi. Likewise, the same fungus may turn out to be predominantly beneficial or pathogenic. The antibiosis displayed *in vitro* by putative beneficial fungal root endophytes may be partly a result of the unnatural experimental conditions, and may not translate to a complex natural ecology.

The greatest advantages to the barley plant resulting from the colonisation of root tissue by a beneficial fungal endophyte seem to be obtained in abiotically stressed environments. In these situations, endophyte colonisation does seem to confer consistent and predictable benefits on the barley plant. An up-regulation of antioxidants and endophyte associated lipid changes in the plant cell membrane enhance plant tolerance of the stress, and increase growth and yield over non-infected plants. Salt stress in particular may inhibit the activity of other potentially pathogenic fungi and lead to a relaxation of competitive pressure, enabling the full effects of the beneficial endophyte to be realised.

The relationship between fungal root endophytes and barley is based on a finely balanced and complex dialogue between the partners, with the final effect on the plant uncertain. The ability of

beneficial endophytic fungi to improve barley crop yields, increase pathogen resistance and enhance abiotic stress tolerance depends on many variables: plant and fungal species or genotype, developmental stage of both partners, intermicrobial competition, and the biotic and abiotic environmental stresses. Each new study reveals an increasing diversity of beneficial fungal root endophytes, and the full potential of these organisms is still to be determined. There have been reports of beneficial endophytes isolated from wild barley populations (Clement et al., 1997; Dugan et al., 2002), but there is a need for more study of wild barley species to fully elucidate the potential of discovered endophytes for improving cultivated barley performance. It is likely that there are ideal fungal partners for each variety of barley but there is also the potential to develop 'universal' partners that can benefit all. If we can discover these ideal fungal partners, then we may yet see a farmer harvesting a healthy crop of chemical-free salt marsh barley in winter.

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