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Do endophytic fungi grow through their hosts systemically?

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ABSTRACT

Endophyte fungi are ubiquitous within vascular plants and recent evidence suggests that they have a number of effects on other organisms that attack those plants, such as insects and pathogens. Endophytes produce an array of metabolites in culture and it is possible that these fungi could be used in targeted programmes of application to plants, to provide a degree of pest protection. Such programmes would be most effective if the fungi grew systemically through their hosts. To date, evidence for systemic growth is equivocal and the aim of this study was to determine whether systemic growth occurs, through a detailed study of endophytes in one host plant species. We isolated a number of endophytes from the forb *Silene dioica* and examined fungal interactions in dual culture. We found very little evidence for any systemic growth within leaves and none within plants. Antagonistic interactions between fungi were extremely common, suggesting that any systemic effects of these fungi on other organisms are likely to be due to chemical movement, not fungal growth.

Introduction

Endophytic fungi are usually defined as those species that exist within the living tissues of plants, for some or all of their life cycle, without causing apparent harm to their hosts (Rodriguez et al., 2009). They have been isolated from almost every organ of every plant sampled (Rodriguez et al., 2009; Currie et al., 2014). Performing diverse roles, ranging from symbionts to latent pathogens, endophytes have been implicated in increasing environmental stress tolerance, improving plant vigour and decreasing herbivore attack (Arnold et al., 2003; Rodriguez et al., 2009; Gange et al., 2012).

Lodge et al. (1996) and Rodriguez et al. (2009) described the within-plant diversity of endophytic species as a mosaic of infections in a leaf. Most isolation studies have suggested that foliar endophyte occurrences are very small-scale and localised, with multiple species being found in single leaves, e.g. in tropical tree species (Arnold et al., 2000; Vaz et al., 2014), in soybean (Impullitti and Malvick, 2013) and in native forbs in the UK (Hodgson, 2010). However, these studies also frequently recovered the same fungal species from different leaves on the same plant, leading to the possibility that these fungi may exhibit systemic growth within their hosts.

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Systemic growth is well documented amongst endophytes of the Balansiaceae in grasses, but the evidence for systemic growth in forbs is equivocal. Observational studies such as those of Wearn et al. (2012) show a high degree of organ specificity, with different fungal communities occurring in roots and shoots of a range of perennial forbs. This appears to be corroborated by the infection experiments of Jaber and Vidal (2010) who infected roots of *Vicia faba* with *Acremonium strictum* and failed to recover the fungus from shoot tissue. Meanwhile, in leaf infection experiments involving *Cirsium arvense*, Gange et al. (2012) found very low recovery rates of inoculated fungi in new leaf tissue. Even in grasses, evidence of systemic growth of non-Balansiaceous endophytes is debatable, with Sánchez Márquez et al. (2012) stating that fungal growth through the plant and into seeds (potentially resulting in vertical transmission) is a very uncommon event.

However, recent evidence suggests that vertical transmission, from mother plant to offspring via the seeds, is more widespread than previously thought. The endophytic fungus *Undifilum oxytropis* occurs in species of locoweeds (*Astragalus* and *Oxytropis* sp.) and has been found to inhabit all tissues of the plants (Cook et al., 2009). It exhibits vertical transmission from one generation to another, which must indicate a degree of systemic growth, as the endophyte grows into seedlings from germinating seeds (Ralphs et al., 2011). Similarly, Hodgson et al. (2014) have recovered endophytes from seeds, cotyledons and first true leaves of six forb species when grown in sterile conditions, further suggesting that systemic growth occurs, at least in young plants. Quesada-Moraga et al. (2014) have also found evidence of vertical transmission of the entomopathogenic fungus *Beauvaria bassiana*, a species that has been recovered as an endophyte from a range of plants.

Indeed, the best evidence for systemic endophytic growth in mature plants comes from those fungi that are latent pathogens of insects or plants. For example, Quesada-Moraga et al. (2006) found *B. bassiana* spread through *Papaver somniferum* after application of a conidial suspension to a single leaf. Gurulingappa et al. (2010) recorded systemic growth of *B. bassiana*, *Lecanicillium lecanii* and *Aspergillus parasiticus* in cotton (*Gossypium hirsutum*), wheat (*Triticum aestivum*), bean (*Phaseolus vulgaris*), corn (*Zea mays*), tomato (*Lycopersicum esculentum*), and pumpkin (*Cucurbita maxima*). Meanwhile, Batta (2013) also found translocation of *Metarhizium anisopliae*, from sprayed applications to single leaves of oilseed rape (*Brassica napus*). Systemic growth in the endophytic phase is also well known for latent plant pathogens, such as *Botrytis cinerea* in lettuce (Sowley et al., 2010).

In forbs, non-pathogenic endophytes can produce an array of secondary metabolites, the ecological roles of which are mostly unknown (Kusari et al., 2012; Verma et al., 2014). As many of these chemicals have biological activity, the potential exists for certain fungi to be used in plant protection programmes against pests or pathogens (Suryanarayanan, 2013). Infection experiments suggest that endophytes may increase plant resistance to insects (McGee, 2002; Jallow et al., 2004; Jaber and Vidal, 2010; Gange et al., 2012) but application of endophytes to plants is likely to be more effective if growth of the fungi is systemic within the host. Much of the chemical production within leaves is likely to arise from fungal–fungal interactions (Schulz et al., 2002) since it is noticeable that negative associations between the occurrence of fungal species within the same plant hosts are common (Gange et al., 2007) and can lead to dramatic effects on insect herbivores (Gange et al., 2012). The probability of such antagonistic interactions leading to anti-herbivore effects will be greatly enhanced if systemic fungal growth occurs.

The primary objective of this study was to test if the fungal species isolated from different parts of a host plant were separate infections or extended individuals, by examining whether the interactions between isolates of the same species were antagonistic. Molecular techniques can be used to address this question, but the dual culture method (based on Glass et al., 2000; Diaz et al., 2013) is effective and gave us the advantage of enabling large numbers of pairwise interactions to be tested and to measure the strength of these interactions. We hypothesised that there is no systemic growth of foliar fungal endophytes in host plants, but instead the leaf is a mosaic of individual fungal colonies, the result of separate instances of horizontal infection from aerial spores. Endophytes from the same leaf and from different leaves on the same plant were tested in these competition experiments, since hyphae from samples of the same individual merge when resources become limited (Moore et al., 2011), whereas different individuals, even of the same species, exhibit antagonistic reactions (for example Demirci et al., 2011; Santamaria et al., 2011; Gruber and Seidl-Seiboth, 2012; Jonkers, 2012).

Given that endophytic fungal species antagonism seems to be common (Gange et al., 2007), the form such antagonism might take was also considered to be significant, as fungal species can compete by outgrowing their competitors, producing compounds that affect competitors' growth and survival or directly parasitizing the rival's mycelia (Mejia et al., 2008; Rodriguez Estrada et al., 2012). Thus our second objective was to examine the inter-specific interactions in fungi from the same host plant. We hypothesised that many of these interactions would be characterised by reduced growth of one species, resulting from metabolite production (Demirci et al., 2011).

Materials and methods

Isolation and identification of endophytes

Our host plant system was *Silene dioica* (Caryophyllaceae), a semi-evergreen, usually perennial, herbaceous forb with creeping rhizomes that is locally common and native to the British Isles (Rose, 1991). It is one of the few non-mycorrhizal forbs outside the Brassicaceae, and, as Eschen et al. (2010) found that the foliar endophytic fungal community composition in *C. arvense* is affected by mycorrhizal colonisation, using a non-mycorrhizal plant as a subject removed the potential for confounding factors arising from this source.

Three asymptomatic leaf samples were collected from twenty healthy plants of *S. dioica* (sixty leaves in total) located in Chertsey, Surrey, England (51° 23′ N, 0° 31′ W) in Oct. 2012. Leaves are produced in pairs along the stem, and samples were taken from one leaf of a pair, separated by one or two nodes. All leaves and plants were of approximately the same