# 4

# Crossing Frontiers: Endophytic Entomopathogenic Fungi for Biological Control of Plant Diseases

Lorena Barra-Bucarei, Andrés France and Paz Millas

## Abstract

World population growth has generated a demand for increased healthy food production and the development of sustainable agricultural technologies to replace environmentally damaging farming practices, including the overuse of pesticides. Biological control using entomopathogenic fungi, as natural pathogens of arthropod pests, is an alternative method to meet this objective. These fungi have been traditionally studied to control insects, but recent studies have begun to examine their activity as plant endophytes to protect plants against phytopathogens and improve other aspects of crop production. This chapter reviews the importance of these entomopathogenic fungi as endophytes in the context of biological control. Our studies focus on determining the ability of native strains of entomopathogenic fungi for endophytic colonisation and their potential application for the control of diseases in tomato. The chapter also discusses aspects to consider for their development as commercial biopesticides and suggests ways to make this control method available to producers of agricultural crops.

## 4.1 Introduction

Entomopathogenic fungi are a unique and highly specialised group of microbes that colonise and kill insects. They have several desirable traits making them potential

candidates for biopesticides (Lacey *et al.*, 2015). Some genera, such as *Beauveria* and *Metarhizium*, consist of species that are distributed worldwide, live in the soil and are insect pathogens. *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *M. anisopliae* (Metchnikoff) Sorokin (Ascomycota: Hypocreales), have been reported as natural and facultative endophytes of many plant species, including some agricultural crops. Endophytic fungi are able to invade plant tissues and live inside the plant during all or part of their life cycle (Wilson, 1995). Facultative entomopathogenic fungal endophytes have been the focus of much research because of their potential in the development of commercial products aimed at pest and/or biological control of plant diseases. Some authors have studied a dual effect, that is the ability of these fungi to act as insect pathogens and be antagonistic to phytopathogens at the same time (Ownley *et al.*, 2004).

It is important to consider that there are several biotic (Vicari *et al.*, 2002) and abiotic (Bultman and Bell, 2003) factors affecting the establishment of entomopathogenic fungi as endophytes. Among the biotic factors, those associated with the plant include host species/variety, age, tissue type and endophytic microbiota (Parsa *et al.*, 2013; Posada *et al.*, 2010). Studies conducted by Vega *et al.* (2008) determined that the presence of endophytic fungi in coffee plants can affect the establishment of another endophytic fungus, *B. bassiana*. Other aspects that influence establishment are related to the fungus, such as the strain, inoculum concentration (Parsa *et al.*, 2013; Vidal and Lara, 2015), and association with other microorganisms such as bacteria (Fisher and Petrini, 1987). Among abiotic factors, the type of substrate/soil where the plants grow has been shown to be important (Lingg and Donaldson, 1981; Pereira *et al.*, 1993). So too have other environmental factors, such as temperature, radiation and humidity, to which the plants are exposed, as well as their nutritional status (Vidal and Lara, 2015). The mode of application also affects establishment and determines persistence as an endophyte (Quesada-Moraga *et al.*, 2014).

There is a need to increase the number of studies on entomopathogenic fungi and also explore new areas, like field adaptations, effectiveness against insect and microbial diseases, biological mechanisms and compatibility with other biological agents. Therefore, this chapter aims to review the importance of entomopathogenic fungi in the context of sustainable agriculture and crop production, review their use as endophytes for biological control of pests and diseases, and evaluate their potential in the development of commercial biocontrol products.

## 4.2 Importance of Biological Control in the Context of Sustainable Agricultural Production

Growing world demand for food has intensified agricultural production, resulting in adverse effects on the environment and people's health (Tilman *et al.*, 2002)

including the indiscriminate use of pesticides. It is estimated that yield losses caused by pests (including invertebrates, pathogens and weeds) can vary from 27% to 42%; this range could increase from 48% to 83% if crops were not protected (Oerke and Dehne, 2004). A worldwide consensus has emerged about the necessity to develop new strategies for achieving sustainable agricultural production that does not compromise public health or the integrity of the environment. The use of chemical pesticides is creating environmental toxicity problems and expected control efficiency levels are often not met because the phytopathogen can develop different mechanisms of resistance to the chemical agents (Grille *et al.*, 2001; Leroux *et al.*, 2010). These chemicals also increase production costs. There is, therefore, a need to develop biological control as an alternative to reduce chemical inputs.

Biological control reduces pest populations using natural enemies and normally involves human intervention for its application (Hoffmann and Frodsham, 1993). Biological control agents are classified as predators, parasitoids and pathogens (pathogens are produced by microorganisms and cause diseases). Once identified, isolated and reproduced, biocontrol microorganisms can be applied in dilutions or released in a targeted manner on crops to reduce insect pests and diseases. These microorganisms can affect pests and diseases directly or indirectly and can reduce the damage below the economic threshold of the producer. Many microorganisms have been used as biopesticides because they offer a number of additional benefits beyond their intended use and known function (Glare *et al.*, 2012). Entomopathogenic fungi hold high potential in this group. More than a century ago, Louis Pasteur predicted the potential of entomopathogenic fungi for pest bioregulation (as parasites of insects harmful to plants). Since then, more than 700 fungal species have been identified as affecting insects (of different orders), and their use as biopesticides has increased over the last few decades (Shah *et al.*, 2009).

Mazón (2001) indicated that the most important entomopathogenic fungi with practical uses for pest control belong to *Metarhizium* and *Beauveria*. These genera are distributed worldwide, exhibit great metabolic and ecological versatility (Behie *et al.*, 2012; Lacey *et al.*, 2015), infect more than 200 insect species (Roberts and Hajek, 1992), have been extensively studied as biological control agents, and are commercially available (Lacey *et al.*, 2015). Entomopathogenic fungi parasitise insects and can cause their death. The traditional way to infect insects by entomopathogenic fungi is based on conidia inoculation of the insect cuticle. The germination tube penetrates the cuticle to the hemocele by mechanical and enzymatic action (Hajek and St. Leger, 1994). After invading the hemocele, the fungi have the ability to break through the host's cuticle once again and emerge outside where it can continue to develop saprophytically on the corpses, sporulating and converting them to new focal points of conidia dissemination (Meyling and Eilenberg, 2007). Insect pest control by entomopathogenic fungi is well known, but the same fungi have also been shown to protect the plant from damage caused by

pathogenic microorganisms (Arnold *et al.*, 2003; Ownley, 2010). Recently, attention has been centred on developing *B. bassiana* and *M. anisopliae* as inundative biopesticides (De Faria and Wraight, 2007). Licensed commercial products exist whose formulations contain conidia and/or mycelia that allow their application in the field (De Faria and Wraight, 2007; Lacey *et al.*, 2015). Furthermore, the durability of biological control has long been assumed to be higher than that of chemical control (Holt and Hochberg, 1997).

## 4.3 Entomopathogenic Fungal Endophytes

Endophytes are defined as microorganisms (fungi and bacteria) that colonise the inside of the plant without causing any apparent damage during part or all of their life cycle (De Bary, 1884; Wilson, 1995), that is, they can live asymptomatically inside plant tissues (Petrini, 1991; Hyde and Soytong, 2008). The term endophyte originated in the nineteenth century and has evolved significantly since its first use, because associations with different plant parts have been taken into account and the types of association with their hosts explored further (Carroll, 1988). Endophytes are associated with most plant species and have been found naturally in all ecosystems studied so far (Arnol *et al.*, 2000). Fungal endophytes mainly belong to the taxonomic divisions Ascomycota, Basidiomycota and Zygomycota. They are common in nature and can produce a huge diversity of secondary metabolites. These metabolites have high commercial value and can have multiple applications in the fields of human health, biotechnology and agriculture (Strobel and Daisy, 2003; Wang *et al.*, 2013).

These fungi are widely studied in agriculture because of the beneficial characteristics that are conferred to their hosts (Aly *et al.*, 2010; Murphy *et al.*, 2015; 2018). Endophytes accomplish a variety of symbiotic and ecological functions (Rodríguez *et al.*, 2009) that are translated into the promotion of plant growth, inhibition of pathogenic organisms, removal of soil contaminants, as well as increased tolerance to extreme conditions such as temperature, water availability, and salinity (Quesada-Moraga *et al.*, 2009; Ownley *et al.*, 2010; Kauppinen *et al.*, 2016). The host plant benefits in numerous ways from the interaction with the endophyte in exchange for its carbon-based resources (Herre *et al.*, 2007).

The range of hosts of different endophyte species varies, going from highly specific single species to those with multiple host species (Stone *et al.*, 2004; Sánchez-Márquez *et al.*, 2011). Endophyte transmission can be vertical (from one generation to the next) or horizontal (from one individual to another) (Quezada-Moraga *et al.*, 2014; Majumder *et al.*, 2016). They can remain in plant tissues for long periods of their life cycle, protecting its host from the attack of pathogens and avoiding the external environmental changes that could threaten their survival and biocontrol effectiveness (Card *et al.*, 2016).

For many years, research has focused on clavicipitaceous endophytes associated with pasture grasses (Johnson and Caradus, 2019, Chapter 15). However, there is growing interest in non-clavicipitaceous endophytes (many of which are usually horizontally transmitted), associated with different species that are widespread in nature and dominated by the ascomycetes in which several entomopathogenic fungal genera are found (Vega *et al.*, 2008; Lopez and Sword, 2015).

Research studies have reported that entomopathogenic fungi can act as endophytes of a large variety of plants, including ornamental, agricultural, silvacultural and medicinal plants. Various species of the genera *Acremonium*, *Beauveria*, *Cladosporium*, *Clonostachys*, *Fusarium*, *Metarhizium*, *Paecilomyces*, *Trichoderma* and *Verticillium* (Gómez-Vidal *et al.*, 2006; Vega *et al.*, 2008; Powell *et al.*, 2009; Akello and Sikora, 2012; Akutse *et al.*, 2013) have been reported as natural or facultative endophytes of different plant species. Of these genera, *Beauveria* and *Metarhizium* are explored further here.

*Beauveria bassiana* is the species of entomopathogens most reported as an endophyte; it is able to colonise different plant species in specific plant organs, such as leaves, stems, flowers, and roots (Behie *et al.*, 2015; Krell *et al.*, 2017). Studies by Wagner and Lewis (2000) demonstrated that *B. bassiana* penetrates maize leaves through natural openings or the cuticle, which is facilitated by the mechanical force exerted by the infection structure (Bidochka and Khachatourians, 1991), the enzymatic dissolution of the cuticle, or a combination of both (Ferron, 1978; Kolattukudy, 1984); these mechanisms are similar to those used by the fungus to penetrate insects. Wagner and Lewis (2000) also showed that hyphae grow through the spaces between the parenchyma cells without haustorium formation. The authors found hyphae in the xylem vessels and concluded that *B. bassiana* can grow from the epidermis to the vascular tissues and achieve a systemic colonisation of the whole plant. Thus, this species has the ability to colonise the plant apoplast, parenchyma and vascular elements (Wagner and Lewis, 2000; Gomez-Vidal *et al.*, 2006; Quesada-Moraga *et al.*, 2006).

The persistence of *B. bassiana* in several plants has also been evaluated and it can remain inside the plant for several months (Brownbridge *et al.*, 2012; Quesada-Moraga *et al.*, 2014). Studies conducted by Akello *et al.* (2007) demonstrated that *B. bassiana* persisted for at least 4 months inside *Musa* spp. plants (banana). Publications have shown the potential of this fungus as an endophyte to decrease insect pest populations of banana (Akello *et al.*, 2008), maize (Bing and Lewis, 1991; Wagner and Lewis, 2000), and tomato (Powell *et al.*, 2009). On the other hand, species of *Metarhizium* mainly colonise the root system (Liao *et al.*, 2014; Barelli *et al.*, 2016; Greenfield *et al.*, 2016).

Studies of entomopathogenic fungal endophytes (EFEs) suggest that several of these fungi have never given up their role as plant symbionts, and that insect pathogenicity is a derived adaptation that allowed certain species to have access to a

specialised source of nitrogen and other nutrients. The EFEs can substitute nutrients derived from insects with plant carbohydrates (Barelli *et al.*, 2016). For example, Behie *et al.* (2012) demonstrated that *Metarhizium robertsii* strains can translocate insect-derived nitrogen to plants. Furthermore, several studies have indicated that EFE applications do not negatively affect the yield of treated plants (Wagner and Lewis, 2000; Akello *et al.*, 2009; Tefera and Vidal, 2009), a desirable characteristic if they are to be used commercially.

EFEs can exhibit a systemic colonisation pattern in the whole plant (Quesada-Moraga *et al.*, 2006; Rodríguez *et al.*, 2009) or be located in a specific tissue/organ type such as leaves, flowers, roots, stems, bark and seeds (Tefera and Vidal, 2009; Behie *et al.*, 2015). Because of this property, *B. bassiana* and *M. anisopliae* have been artificially inoculated into plants using different techniques (Quesada-Moraga *et al.*, 2014) such as foliar spraying, stem injections, liquid applications to the soil, and seed treatment with conidial suspensions (Bing and Lewis, 1992; Quesada-Moraga *et al.*, 2006; Posada *et al.*, 2007; Terefa and Vidal, 2009).

The methodologies to study artificially inoculated EFEs usually include several steps. Reisolation of the plant endophyte requires rigorous surface sterilisation of the tissues. However, chemical product diffusion can affect the endophyte and result in underestimated colonisation rates (Ownley *et al.*, 2008; Lohse *et al.*, 2015). Furthermore, the techniques used, such as reisolation in different culture media, sometimes cannot differentiate between endophytic and epiphytic colonisations. For example, *B. bassiana* is horizontally transmitted and can simultaneously colonise external and internal tissues.

Morphological characters, taxonomic keys and molecular techniques are used to identify EFEs. In the first case, the fungus is identified when it is isolated from the tissues and grown in culture. For molecular techniques, the fungus can be identified when it is either inside the plant or grown in culture. The most widely used molecular techniques identify fungi by sequencing the nuclear ribosomal internal transcribed spacers 1 and 2 (nrITS1 and ITS2 regions) with universal primers (White, 1990). These primers work well for isolated fungi but exhibit difficulties when the fungus is inside the plant because the primers can amplify both plant and fungal DNA (Martin and Rygiewicz, 2005). Other regions that are used for identification of isolated EFE include: the intron sequences from β-tubulin (tubB), translation elongation factor 1-α (tefA) and γ-actin (actA) (Craven *et al.*, 2001), the largest and second largest subunits of ribosomal polymerase B (RPB1 and RPB2), and the nuclear intergenic region bloc (Rehner *et al.*, 2011). Intergenic specific markers (IGS) have also been designed to differentiate between species of the *M. anisopliae* complex (Kepler and Rehner, 2013). All these markers improve identification accuracy compared to that obtained with nrITS1 and ITS2 regions alone.

To detect the endophyte inside the plant it is possible to use specific primers for the target species (Garrido-Jurado *et al.*, 2016). It can be difficult to detect the endophyte

inoculated by foliar spraying due to the probability of contamination with epiphytic progagules (Clayton *et al.*, 2017). Genome sequencing can also be applied and this allows the identification and further functional characterisation of the endophyte. For example, it can help in elucidating the role of the endophyte in plant secondary metabolite production (Venugopalan and Srivastava, 2015), to determinate its usefulness as a biocontrol agent in the agricultural industry or evaluate its potential as an aromatic compound degrader in environmental biotechnology (Kwak *et al.*, 2012). In addition, genome comparisons with related microorganisms can help predict the benefits the endophyte receives from the plant (Hardoim *et al.*, 2015).

## 4.4 Entomopathogenic Fungal Endophytes for Plant Disease Control

Entomopathogenic fungi have an ability to become endophytes in different plant species (Jaber and Ownley, 2017). Together with their insect control activity, this ability presents an opportunity for biological pest control. Several researchers suggest that EFEs could be able to simultaneously control pests and diseases (Kim *et al.*, 2007; Ownley, 2008). Among the EFE genera reported with phytopathogenic activity, *Beauveria*, *Lecanicillium* and *Metarhizium* are further described here. *Beauveria bassiana* has been most often reported as an antagonistic endophyte to plant diseases, caused not only by fungi but also bacteria and viruses. This could be due to the great diversity of bioactive and antimicrobial metabolites that it is able to produce (Warner and Lewis, 2000; Parine *et al.*, 2010) including destruxins, oosporein, beauvericin, bassianolide, bassianin, beauveriolide, bassiacridin, cordycepin and ciclosporin (Susuki *et al.*, 1977; Logrieco *et al.*, 1998; Quesada-Moraga and Vey, 2004; Wang and Wang, 2017). Studies conducted by Feng *et al.* (2015) determined that the *B. bassiana* genome exhibits at least 45 groups of different secondary metabolite biosynthesis genes.

Jaber (2015) showed that *Beauveria bassiana* applied by foliar spraying can colonise grapevine (*Vitis vinifera*) leaves to protect them from the pathogen *Plasmopara viticola*. All the strains evaluated in the study, independent of the endophyte colonisation percentage reached, had an antagonistic effect against the pathogen. Several mechanisms might explain its antagonistic ability, including competition for niche or resources, antibiosis, parasitism and induced systemic resistance (Jaber, 2015). This endophyte has also been shown to control levels of *Xanthomonas axonopodis* pv. *malvacearum* in *Gossypium hirsutum* roots. The authors attribute this bacterial control to induced systemic resistance (Ownley *et al.*, 2008). In other studies, *G. hirsutum* seeds were inoculated with different *B. bassiana* conidium concentrations in loamy soil and exhibited a significant biocontrol level of the pathogen *Rhizoctonia solani* (Griffin, 2007).

Naturalis®, a commercial product based on *B. bassiana*, has showed good endophytic colonisation ability and antagonistic action against zucchini yellow mosaic virus (ZYMV) in *Cucurbita pepo* (gourds, pumpkins, squashes). This is one of the few studies reporting the use of EFEs against plant diseases caused by viruses and could be attributable to the ability of *B. bassiana* to systemically colonise the plant and inhibit or interfere with the systemic movement of ZYMV from cell to cell (Jaber and Salem, 2014). This finding could open an important line of research given the few biological tools currently available for virus control.

*Lecanicillum lecanii* (= *Verticillium lecanni*) is another EFE that has been reported as an antagonist to plant diseases. Studies by Benhamou and Brodeur (2001) determined that it can colonise the root of *Cucumis sativus* (cucumber) and significantly reduce the incidence and severity of the oomycete pathogen *Pythium ultimum*. Hirano *et al.* (2008) later demonstrated that inoculating *C. sativus* cv. Hokushin plants with *L. lecanii* induces systemic resistance against the *P. ultimum*. Few papers report the use of *Metarhizium* spp. as an endophytic mycopathogen. However, the bean endophyte *M. robertsii* was shown to exhibit an antagonistic effect against *Fusarium* spp. in both *in vitro* and *in vivo* assays (Sasan and Bidochka, 2013).

The ability of EFEs to act antagonisticly to phytopathogens could be due to different mechanisms, however, knowledge in this field is still limited. They are attributed with direct mechanisms, such as competition and parasitism, and indirect mechanisms, such as antibiosis (production of primary and secondary metabolites, enzymes, or volatile compounds), resistance induction (Arnold *et al.*, 2003; Herre *et al.*, 2007) or a combination of the abovementioned mechanisms (Vega *et al.*, 2009; Ownley *et al.*, 2010), as outlined below:

1. Competition between the endophyte and pathogen inside the plant can be for space and/or food. The EFEs are able to successfully colonise different tissues and use available sources of food provided by the plant, which can reduce the probability of colonisation by pathogens (Ownley *et al.*, 2004). Early inoculation of the endophyte could be advantageous because, by being the first to colonise the plant, it depletes the plant's resources, thus leaving less available for the pathogen (especially carbon sources). The plant's development is not significantly affected by the endophyte and it can improve growth by several mechanisms related to nutrition or stress resistance (Behie and Bidochka, 2014).
2. Mycoparasitism is an antagonistic interaction between a fungal parasite and fungal host that usually involves the action of extracellular lytic enzymes that break down the host's cell walls (Sharma, 2011). Although there are several studies about the action of EFEs as mycoparasites, they only show results at the *in vitro* level (Fenice and Gooday, 2006) and little is known about their action in plants as endophytes.

3. Antibiosis is the direct action of antibiotics or toxic metabolites produced by microorganisms on another microorganism that is sensitive to them. Boucias and Pendland (1998) suggest that opportunistic microorganisms, such as phytopathogens, could be kept at bay by the antimicrobial substances produced by EFEs. For example, Beauvericin is a well-known mycotoxin produced by EFEs such as *B. bassiana*, *Paecilomyces fumosoroseus*, and *Fusarium* spp. (Bernardini *et al.*, 1975). This metabolite has high crop protection potential because it exhibits important antibacterial, antifungal, and antiviral activities (Xu *et al.*, 2010; Wang and Xu, 2012). Bassianolide is another metabolite with antibiotic activity that has been reported in *B. bassiana* and *L. lecanii* (Suzuki *et al.*, 1977). This metabolite plays a significant role in virulence of *B. bassiana* against insects (Xu *et al.*, 2009) and currently little is known about its toxicity in animals and plants. On the other hand, *Metarhizium* produce destruxins (Roberts and Leger, 2004) and they have been successfully evaluated to control the pathogens *Fusarium oxysporum* and *Cladosporium herbarum* (Ravindran *et al.*, 2014).
4. Systemic resistance can be an important antagonistic mechanism against phytopathogens and could be triggered in the plant by the presence of non-pathogenic fungi (Kavroulakis *et al.*, 2007) such as endophytes (Shoresh *et al.*, 2010). Plants that are endophytically colonised by fungi can generate a response more rapidly, which can be associated with its ability to produce secondary metabolites that act as elicitors of phytoalexin production (Gao *et al.*, 2010). In systemic resistance, the endophyte and the phytopathogen do not need to be in direct contact for the plant to react with a rapid expression of defence-related genes (Ownley *et al.*, 2010). There are two main types of induced resistance: systemic acquired resistance (SAR) and induced systemic resistance (ISR), which are differentiated on the basis of the nature of the elicitor and their regulatory pathways. SAR is induced by pathogen infection or chemical compounds, is dependent on salicylic acid and is associated with the accumulation of pathogenesis-related (PR) proteins. ISR, in contrast, is induced by other non-pathogenic microorganisms including endophytes and rhizobacteria. It is generally independent of salicylic acid but dependent on jasmonate and ethylene and is not associated with the accumulation of PR proteins (Pieterse *et al.*, 1998). However, some studies show that some rhizobacteria, including some of endophytic strains, that elicit ISR may be dependent on salicylic acid and independent of jasmonate or ethylene, in contrast to the model proposed by Pieterse *et al.* (1998) (Ryu *et al.*, 2003). Moreover, some strains of bacteria and fungal endophytes that induce ISR were associated with the expression of PR genes (Ryu *et al.*, 2003; Kavroulakis *et al.*, 2007).

The following section reports data on the endophytic colonisation ability in tomato of six native strains of *B. bassiana*, *M. anisopliae* and *M. robertsii* that form part of the Chilean Collection of Microbial Genetic Resources (CChRGM) and their antagonistic effects (*in vitro*) against *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *F. oxysporum*.

## 4.5 Materials and Methods

### 4.5.1 Fungal Isolates

Six native strains of *B. bassiana* and *M. anisopliae* were used in the assays (Table 4.1). These were isolated using the methodology described by France *et al.* (2000). Fungal conidia were sown on Petri dishes with 100% potato dextrose agar (PDA, Disfco™) and 150 mg $L^{-1}$ chloramphenicol to prevent the growth of contaminating bacteria. The dishes were incubated in the dark at 25 ± 2°C for 10 d. Viability of the harvested conidia was determined according to the methodology described by Moore *et al.* (1995). They were suspended in test tubes with 10 ml sterile distilled water and 0.01% (v/v) Tween 80% (Difco™), and dilutions were performed until a concentration of $1 \times 10^6$ conidia $ml^{-1}$ was reached. The concentration of conidia was determined with a Neubauer counting chamber (BOECO, Germany), and the inocula were used for all the experiments. The pathogens were used at concentrations of $1 \times 10^6$ conidia $ml^{-1}$.

### 4.5.2 Plant Inoculation and Endophytic Colonisation

Ten-day old samples of *Beauveria* and *Metarhizium* were removed from the surface of Petri dishes under sterile conditions, sown in 100 ml test tubes containing 15 ml PDA, and inoculated with a solution of $1 \times 10^6$ conidia $ml^{-1}$ (Griffin, 2007). One test tube was left without fungal inocula as a control. Test tubes were incubated in the dark at 25 ± 2°C for 4 d. Once growth of the fungi was observed in each tube, 20 ml of substrate was added, which consisted of a mixture of perlite, peat, compost, and vermiculite (2:2:2:1) sterilised twice in an autoclave at 120°C and 115 psi for 1 h.

Tomato plants (var. Limachino; an INIA traditional cultivar) were disinfected according to a protocol adapted from Griffin (2007). Seeds were submerged in a 95% ethanol solution for 1 min and then in 1.5% NaOCl for 3 min; they were washed three times for 1 min in sterile distilled water. A control to demonstrate disinfection was made following the protocol of Ownley *et al.* (2008). Two seeds were planted for each test tube and, after the emergence, one plant per tube was left.

The tubes were incubated for 30 d in growth chambers at 25 ± 2°C with 12 h photoperiod and arranged in a randomised design with three replications. Plants were then removed from the tubes, washed with tap water and two cuts were made to separate roots, stems and leaves. The separate plant parts were subjected to disinfection with 70% ethanol for 2 min, 1.5% NaOCl for 5 min, and rinsed three times

**Table 4.1** Fungal strains selected for use in colonisation and radial growth studies.

| Bank code | Species | Collection location in Chile | Origin |
|---|---|---|---|
| RGM 673 | *Metarhizium anisopliae* | Futaleufú, Los Lagos Region | Natural pasture soil |
| RGM 677 | *Metarhizium anisopliae* | Puyehe, Los Lagos Region | Natural pasture soil |
| JFD1 23 | *Metarhizium robertsii* | Robinson Crusoe, Valparaíso Region | Natural pasture soil |
| RGM 1237 | *Beauveria bassiana* | Pozo Almonte, Tarapacá | Arable soil, *Citrullus lanatus* cultivated |
| JFD1 49 | *Beauveria bassiana* | Robinson Crusoe, Valparaíso Region | Arable soil in national park |
| JFD2 1 | *Beauveria bassiana* | Robinson Crusoe, Valparaíso Region | Native forest soil |
| RGM 2519 | *Botrytis cinerea* | Colín, Maule Region | Tomato plant |
| RGM 1630 | *Fusarium oxysporum* | San Ignacio, Biobío Region | Arable vegetable soil |
| RGM 1420 | *Sclerotinia sclerotiurum* | Chillán, Biobío Region | Arable vegetable soil |

for 1 min with sterile distilled water. The parts were left to dry on sterile absorbent paper. A disinfection control was included using the same procedure used for the seeds. Once sterilisation was done, five samples were cut from each of the plant part (roots, stem and leaves), resulting in a total of 15 parts per plant. Root and stem samples were 10 mm long, whereas leaves were cut in 6 mm discs. The cuttings were distributed in the Petri dishes with Noble agar medium and incubated in the dark at 25 ± 2°C for 30 d. The percentage of endophytic colonisation was evaluated.

### 4.5.3 *In Vitro* Antifungal Activity

Mycelium discs (3-d old, 5 mm diameter) from the pure *Beauveria* and *Metarhizium* cultures were placed 1.5 cm from the edge of a 9 cm Petri dish containing PDA medium (20 ml). Discs with each of pathogen were placed equidistantly opposite the endophyte and incubated in the dark at 25 ± 2°C for 10 d. Five replicate dishes per treatment were placed in a completely randomised design. At the same time, control dishes (from 3-d old *B. cinerea* culture) containing only pathogen discs were prepared. When the pathogen colonised the whole dish, the percentage of radial growth inhibition of the pathogen (PRGIP) was determined using equation (4.1). The radius of the colonies was measured (mm) in each of the treatments using a digital caliper.

$$PRGIP = [(R1 - R2)/R1] \times 100 \quad (4.1)$$

where R1 is the radius of the pathogen colony (mm) and R2 is the radius of the pathogen colony against the endophyte (mm).

## 4.6 Results and Discussion

Our study demonstrated that the six evaluated strains can be established as endophytes in tomato leaves, stems and roots, by inoculating plants in soil. Epiphytic colonisations could not be detected in the disinfection control samples, which demonstrates that all the observed mycelium growth came from internal tissues. Means of the colonisation percentages fluctuated between 0% and 100% in the leaves, and 0% and 40% in stems and roots (Figure 4.1). Furthermore, four strains demonstrated a localised pattern of colonisation in leaves, two in stems and two could colonise both leaves and roots. The colonisation of the different plant parts indicates that the EFEs move within the whole plant. Roots and stem colonisation was much lower than leaf colonisation. The reason for this difference could be explained by differences in microbial and physiological conditions in the different plant parts, according to Terefa and Vidal (2009). The EFEs can be adapted to particular conditions present in a given plant tissue/organ.

The native strains of endophytic *Beauveria* and *Metarhizium* demonstrated significant levels of radial growth inhibition of the pathogen. The pathogen (control) was able to totally colonise the dish (attained a 75 mm radius) at 7 d post-inoculation in the case of *B. cinerea*, 7 d for *F. oxysporum* and 5 d for *S. sclerotiorum*. The PRGIP fluctuated for *B. cinerea*, *F. oxysporum* and *S. sclerotiorum*: 26%–34%; 37%–47% and 26%–30%, respectively (Figure 4.2). These results might be explained by the ability of these fungi to produce metabolites with antimicrobial activities (Ownley *et al.*, 2010). When we observed the advancing hyphal zone under the microscope mycoparasitism mechanisms were not detected; this suggests that the evaluated strains have no ability to parasitise the phytopathogens evaluated.

Our results provide evidence of endophytic colonisation of tomato by native strains of *B. bassiana*, *M. anisopliae* and *M. robertsii*. These EFEs can colonise some part plants more efficiently than others, which might consequently influence the level of plant protection against different phytopathogens. All the evaluated strains showed some level of inhibition of pathogens and hence have potential as innovative management tools against plant diseases. Research is required to assess the antifungal effect of the EFEs on plants under laboratory and field conditions against different types of phytopathogens. Despite highly promising laboratory results, a number of challenges need also be addressed before the EFEs can be commercially exploited for crop protection as outlined below.

### 4.6.1 Commercial Potential of EFEs in Disease and Pest Biocontrol

The current use of chemical pesticides is not sustainable because of their negative impact on the environment and people's health (Levitan *et al.*, 1995; Kim *et al.*, 2017). Chemical pesticides are broad spectrum and affect non-target organisms, including predators and parasites as well as humans (Kumar, 2015). This has

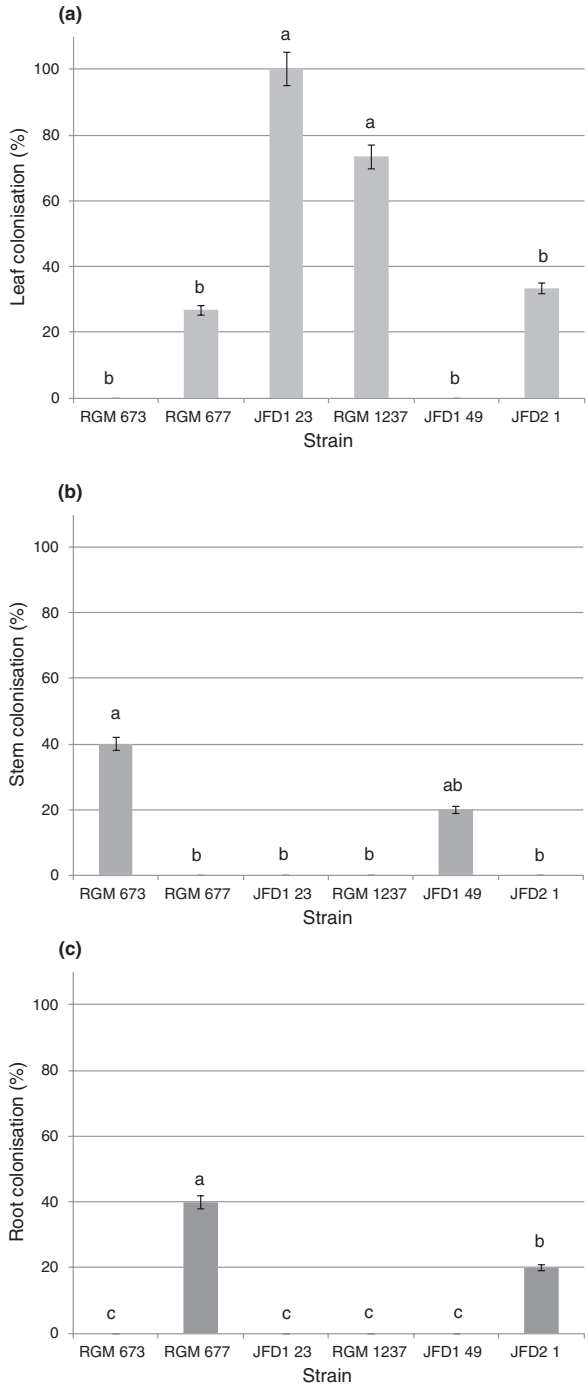


**Figure 4.1** Percentage colonisation of tomato by *Beauveria bassiana*, *Metarhizium anisopliae* and *M. robertsii*: (a) leaves, (b) stems and (c) roots. Different letters over the bars indicate significant differences according to Fisher's least significant difference (LSD) test. Each bar represents the mean (± SE). Endophyte strain codes are given in Table 4.1.

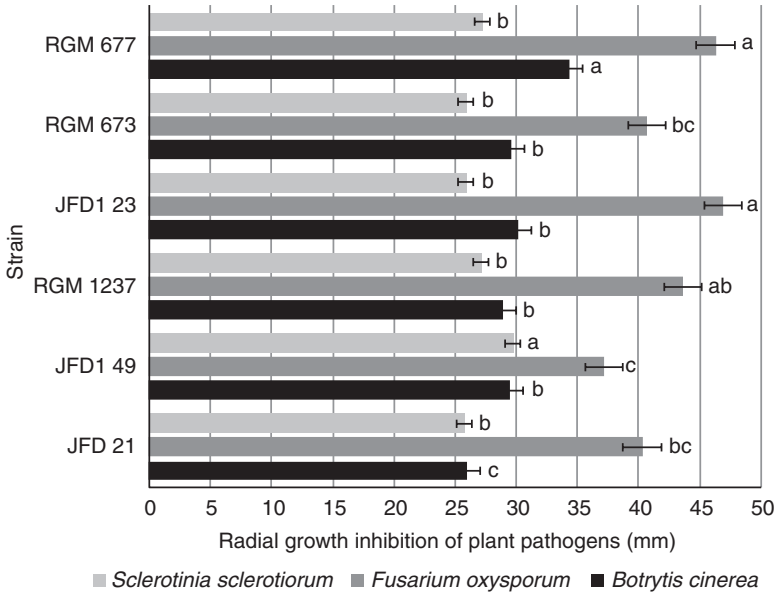


**Figure 4.2** Radial growth inhibition of the plant pathogens *Botrytis cinerea, Fusarium oxysporum and Sclerotinia sclerotiorum* (%) by six native strains of endophytic *Beauveria bassiana, Metarhizium anisopliae* and *M. robertsii* in tomato. Different letters over the bars indicate significant differences according to Fisher's least significant difference (LSD) test. Each bar represents the mean (± SE). Endophyte codes are given in Table 4.1.

resulted in a more restricted use of agrochemicals in many countries. Biopesticides are a sustainable alternative and the incorporation of biopesticides in different agricultural systems has significantly increased over time. Biopesticides are highly specific affecting only the targeted pest or closely related pests, without harm to humans, other beneficial organisms or members of agricultural landscape ecosystems. Thus, many institutions worldwide have directed actions to increase research and development in this field, aimed at developing strategies for promoting their use.

Entomopathogenic fungi have been widely studied for their ability to control insect pests, and they are currently active ingredients of several commercial products (De Faria and Wraight, 2007). More than 170 products have been developed to control pests, which use approximately 12 fungal species (Lacey *et al.*, 2015). Soper and Ward (1981) suggest that a number of aspects associated with their biology need to be considered (including different types of mycelial propagules, conidiospore and blastospores; high pathogenicity to the target pest). Several aspects associated with their mass production also need to be considered (rapid growth, asexual reproduction, easy culture in different media, production of large amounts of conidia).

There is also a desire to develop the use of the fungi as plant endophytes instead of derived biopesticides. Their action as living endophytes could help address several of the commercial deficiencies they have exhibited as biopesticides, as they act of from inside the plant against multiple factors, including plant pathogens. However, there are several aspects that should be considered to assess the potential of EFEs for commercial development which are outlined below.

1. **Type of symbiosis, host specificity and multifunctionality.** The fact that EFEs are facultative plant colonisers gives them an advantage from the commercial point of view. This is because they could remain as saprophytes in the soil after their application and enter the plant when needed. Alternatively, they can be applied as a seed dressing and colonise the germinating seed (Murphy *et al.*, 2019, Chapter 18). Many of the published studies clearly show the low specificity of EFEs to the host plant. For example, *B. bassiana* has been reported as an endophyte in more than 30 plant species. This characteristic can be commercially advantageous because the endophyte could be used in a variety of crops. Furthermore, EFEs are known to perform multiple functions to protect plants against biotic (pests and diseases) and abiotic (such as high temperatures and water deficit) stresses, as well as promote growth (Quesada-Moraga *et al.*, 2009; Ownley *et al.*, 2010; Elena *et al.*, 2011). This characteristic provides an element of differentiation regarding the use of chemical products developed for plant protection.
2. **Dispersal, type of colonisation and persistence.** Entomopathogenic fungi are dispersed in the air and soil by living infected hosts that migrate and die in a site other than where they were infected (Hajek, 1997; Feng *et al.*, 2004). In addition, dispersal in localised plant parts can occur through the rain (Bruck and Lewis, 2002). Alternative crop inoculation methods are therefore required that do not rely on insects. EFEs can exhibit localised or systemic colonisation patterns. For example, *B. bassiana* strains applied to the roots have been reisolated from the leaves. Some species prefer certain plant parts, such as *Metarhrizium* spp., which prefers roots as an endophyte (Behie *et al.*, 2015), and *B. bassiana*, which prefers aerial plant parts. However, the differential preference in plant organs would not be a limiting factor for the commercial development of these fungi, because of the endophyte's ability to execute its antagonistic action through indirect mechanisms that act independently of the colonisation site (Jaber and Vidal, 2010).

   There are reports that *B. bassiana* persisted for more than 9 months inside *Pinus radiata* plants under controlled conditions (Brownbridge *et al.*, 2012). Mantzoukas *et al.* (2015) also reported that *B. bassiana*, *M. robertsii* and *Isaria fumosorosea* persisted 30 days after being inoculated by foliar pulverisation in *Sorghum bicolor* under field conditions. This level of persistence presents advantages of endophytes over chemical products that usually do not persist for over 10 days.

3. **Crop application.** Entomopathogenic fungi have been known to colonise plants after being inoculated by different methods, such as foliar spraying, root drenching and seed immersion in fungal solutions. This approach requires high conidial concentrations and an appropriate formulation, which allows the EFE to efficiently carry out its action. The efficiency of these applications is diminished because conidia normally spend a significant amount of time on the plant surface where they are exposed to suboptimal temperatures, UV radiation and low humidity that can affect their germination and viability (Fargues *et al.*, 1996; Vega *et al.*, 2012). Given this, EFEs could have an advantage in commercial development because it would be sufficient for only some of the conidia to grow and enter the plant to trigger a biocontrol action. Thus, low volumes of inocula could be used. Endophytic foliar spraying could be used to control diseases, such as powdery mildew, or EFEs could be applied to seeds before sowing (Taylor and Harman, 1990). In such situations their action starts at germination and could be used to protect the plants from seedling diseases such as damping off. Soil EFE applications would allow application of treatments to places where chemical products cannot reach. Furthermore, they could help manage saprophytes by competing with soil fungi such as *Sclerotinia* spp., *Phytophthoptora* spp. and *Rhizoctonia solani*. Moreover, endophytes could provide additional benefits to the plant by, for example, promoting growth.
4. **Compatibility with chemical treatments and the plant microbiome.** More studies are required to determine the different susceptibility levels of EFEs to chemical fungicides and insecticides. Combining these fungi with other products could produce higher control effectiveness levels (Gurulingappa *et al.*, 2011). Furthermore, little is known about the compatibility and competition with other endophytes existing in the plant. The plant microbiome could, for example, promote or hamper the establishment and persistence of the inoculated endophytes (Posada *et al.*, 2007; Jaber and Enkerli, 2016).
5. **Field studies, application and widespread use.** Many of the evaluations related to the ability of endophytic colonisation are carried out under controlled laboratory/growth conditions. Although this provides important scientific validity, it does not necessarily guarantee similar action under field conditions. The same occurs when evaluating the antagonistic effect against phytopathogens or pests because most studies have been conducted *in vitro*; very few studies involve plants and even fewer include plants under field conditions.

   Research is also required to optimise application and widespread use/adoption by the farming community. Fermentation and formulation of endophyte treatments need optimisation for commercial use to maximise endophytic colonisation in the different plant tissues, as well as increasing their persistence. The use of endophyte mixtures can be a strategy to increase the

variety of action modes and expand the range of controlled pests and pathogens. Currently, there are few reports of compatible consortia involving endophytes and other biological agents to simultaneously control pests and disease, but this approach is likely to increase in the future (Karthiba *et al.*, 2010).

6. **Safety and registrations.** An advantage of entomopathogenic fungi over pesticides is that they are usually safe for humans. There is also no evidence that they have a negative impact on the environment (Vega *et al.*, 2008) and some, such as *B. bassiana*, have also been reported as natural endophytes of some cultivated and wild plants. Given that the same entomopathogenic fungi are already used in commercial products to control insects, the registration process as antagonistic endophytes of phytopathogens should be facilitated. This could be an opportunity for products, such as Naturalis® and BotaniGard®, which are being adapted for potential application as entomopathogenic and phytopathogenic endophytes (Powell *et al.*, 2009). Both products are widely commercialised in America and Europe. Naturalis® is used for biological control of the whitefly and has been tested as an endophyte to control *Tuta absoluta* and *Planococcus ficus* (Rondot and Reineke, 2016).

## 4.7 Conclusions

EFEs have high potential for the biological control of pests and diseases by acting from inside the plants instead of the outside. EFEs can also provide additional benefits to plants in terms of yield improvement and stress tolerance. There are social and environmental benefits associated with the use of these technologies compared to the application of synthetic chemical pesticides as they present fewer problems for human health and less pollution of soil and water. There have been very few research studies focused on the antagonistic activity of EFEs against plant-pathogenic microorganisms. We have demonstrated their ability *in vitro* to be antagonistic against different phytopathogens. This suggests great potential to control diseases in the whole plant. To take advantage of this potential, more research is required in which endophytes are used against plant pathogens, ideally under field conditions in order to increase the external validity of these assays. It is necessary to expand the number of applied studies aimed at increasing their compatibility and maximise their effectiveness when trying to incorporate them in different agricultural production systems.

The increase in the supply of bioinsecticides formulated on the basis of entomopathogenic fungi presents an opportunity to commercially develop EFEs as biofungicides. It should be possible to overcome registration hurdles associated with the use of these fungi because they include the same active ingredients, and already registered products, to those already commercialised in different markets.

Thus, EFEs represent an exciting development for more sustainable plant production by contributing to both pest and disease management in agriculture.

## Acknowledgements

We are grateful to the organisers of the international conference in Trinity College Dublin 'SUSTAIN: Endophytes for a growing world' for recognising the value of our work and inviting us to contribute to the knowledge of this interesting discipline.

## References

Aly, A. H., Debbab, A., Kjer, J. and Proksch, P. (2010). Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Diversity*, **41**, 1–16.

Akello, J. and Sikora, R. (2012). Systemic acropedal influence of endophyte seed treatment on *Acyrthosiphon pisum* and *Aphis fabae* offspring development and reproductive fitness. *Biological Control*, **61**, 215–221.

Akello, J., Dubois, T., Coyne, D. and Hillnhutter, C. (2007). *Beauveria bassiana* as an endophyte in tissue-cultured banana plants: a novel way to combat the banana weevil *Cosmopolites sordidus*. In *III International Symposium on Banana: ISHS-ProMusa Symposium on Recent Advances in Banana Crop Protection for Sustainable Production and Improved Livelihoods*, ed. D. Jones and I. Van den Bergh. *Acta Horticulturae*, 828, 129–138.

Akello, J., Dubois, T., Coyne, D. and Kyamanywa, S. (2008). Endophytic *Beauveria bassiana* in banana (*Musa spp.)* reduces banana weevil (*Cosmopolites sordidus*) fitness and damage. *Crop Protection*, **27**, 1437–1441.

Akello, J., Dubois, T., Coyne, D. and Kyamanywa, S. (2009). The effects of *Beauveria bassiana* dose and exposure duration on colonization and growth of tissue cultured banana (*Musa sp.)* plants. *Biological Control*, **49**, 6–10.

Akutse, K. S., Maniania, N. K., Fiaboe, K. K. M., Van den Berg, J. and Ekesi, S. (2013). Endophytic colonization of *Vicia faba* and *Phaseolus vulgaris* (Fabaceae) by fungal pathogens and their effects on the life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae). *Fungal Ecology*, **6**, 293–301.

Arnold, A., Maynard, Z., Gilbert, G., Coley, P. and Kursar, T. (2000). Are tropical endophytic fungi hyper diverse? *Ecology Letters*, **3**, 267–274.

Arnold, A., Mejía, L., Kyllo, D. *et al.* (2003). Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 15649–15654.

Barelli, L., Moonjely, S., Behie, S. W. and Bidochka, M. J. (2016). Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi. *Plant Molecular Biology*, **90**, 657–664.

Behie, S. W. and Bidochka, M. J. (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**, 1553–1560.

Behie, S. W., Zelisko, P. M. and Bidochka, M. J. (2012). Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. *Science*, **336**, 1576–1577.

Behie, S. W., Jones, S. J. and Bidochka, M. J. (2015). Plant tissue localization of the endophytic insect pathogenic fungi *Metarhizium* and *Beauveria*. *Fungal Ecology*, **13**, 112–119.

Benhamou, N. and Brodeur, J. (2001). Pre-inoculation of Ri T-DNA transformed cucumber roots with the mycoparasite, *Verticillium lecanii*, induces host defense reactions against *Pythium ultimum* infection. *Physiological and Molecular Plant Pathology*, **58**, 133–146.

Bernardini, M., Carilli, A., Pacioni, G. and Santurbano, B. (1975). Isolation of beauvericin from *Paecilomyces fumoso-roseus*. *Phytochemistry*, **14**, 1865.

Bidochka, M. and Khachatourians, C. (1991). The implication of metabolic acids produced by *Beauveria bassiana* in pathogenesis of the migratory grasshopper, *Melanoplus sanguinipes*. *Journal of Invertebrate Pathology*, **58**, 106–117.

Bing, L. and Lewis, L. (1992). Endophytic *Beauveria bassiana* in corn, the influence of the plant growth stage and *Ostrimia nubilalis*. *Biocontrol Science and Technology*, **2**, 39–47.

Boucias, D. G. and Pendland, J. C. (1998). Entomopathogenic fungi: fungi imperfecti. In *Principles of Insect Pathology*, ed. D. Boucias and Jacquelyn C. Pendland. Boston, MA: Springer, pp. 321–364.

Brownbridge, M., Reay, S. D., Nelson, T. L. and Glare, T. R. (2012). Persistence of *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte following inoculation of radiata pine seed and seedlings. *Biological Control*, **61**, 194–200.

Bruck, D. J. and Lewis, L. C. (2002). Rainfall and crop residue effects on soil dispersion and *Beauveria bassiana* spread to corn. *Applied Soil Ecology*, **20**, 183–190.

Bultman, T. L. and Bell, G. D. (2003). Interaction between fungal endophytes and environmental stressors influences plant resistance to insects. *Oikos*, **103**, 182–190.

Card, S., Johnson, L., Teasdale, S. and Caradus, J. (2016). Deciphering endophyte behaviour: the link between endophyte biology and efficacious biological control agents. *FEMS Microbiology Ecology*, **92**, fiw114.

Carroll, G. (1988). Fungal endophytes in stems and leaves: from latent pathogens to mutualistic symbiont. *Ecology*, **69**, 2–9.

Clayton, W., Eaton, C. J., Dupont, P.-Y. *et al.* (2017). Analysis of simple sequence repeat (SSR) structure and sequence within *Epichloë* endophyte genomes reveals impacts on gene structure and insights into ancestral hybridization events. *PLoS One* **12**, e0183748.

Craven, K. D., Hsiau, P. T. W., Leuchtmann, A., Hollin, W. and Schardl, C. L. (2001). Multigene phylogeny of Epichloë species, fungal symbionts of grasses. *Annals of the Missouri Botanical Garden*, **88**, 14–34.

De Bary, A. (1884). *Vergleichende Morphologie und Biologie der Pilze, Mycetozoen und Bacterien*. Leipzig: Wilhelm Engelmann.

De Faria, M. R. and Wraight, S. P. (2007). Mycoinsecticides and mycoacaricides: a comprehensive

list with worldwide coverage and international classification of formulation types. *Biological Control*, **43**, 237–256.

Elena, G. J., Beatriz, P. J., Alejandro, P. and Lecuona, R. E. (2011). *Metarhizium anisopliae* (Metschnikoff) Sorokin promotes growth and has endophytic activity in tomato plants. *Advances in Biological Research*, **5**, 22–27.

Fargues, J., Goettel, M. S., Smits, N. *et al.* (1996). Variability in susceptibility to simulated sunlight of conidia among isolates of entomopathogenic Hyphomycetes. *Mycopathologia*, **135**, 171–181.

Feng, M. G., Chen, C. and Chen, B. (2004). Wide dispersal of aphid pathogenic Entomophthorales among aphids relies upon migratory alates. *Environmental Microbiology*, **6**, 510–516.

Feng, P., Shang, Y., Cen, K. and Wang, C. (2015). Fungal biosynthesis of the bibenzoquinone oosporein to evade insect immunity. *Proceedings of the National Academy of Sciences of the United States of America*, **112**, 11365–11370.

Fenice, M. and Gooday, G. W. (2006). Mycoparasitic actions against fungi and oomycetes by a strain (CCFEE 5003) of the fungus *Lecanicillium muscarium* isolated in Continental Antarctica. *Annals of Microbiology*, **56**, 1.

Ferron, P. (1978). Biological control of insect pests by entomogenous fungi. *Annual Review of Entomology*, **23**, 409–442.

Fisher, P. J. and Petrini, O. (1987). Location of fungal endophytes in tissues of *Suaeda fruticosa*: a preliminary study. *Transactions of the British Mycological Society*, **89**, 246–249.

France, I., Gerding. G., Gerding, M. and Sandoval, A. (2000). Patogenicidad de una colección de cepas nativas de *Metarhizium* spp. y *Beauveria* spp. en *Aegorhinus superciliosus*, *Asynonychus cervinus* y *Otiorhynchus sulcatus*. *Agricultura Técnica*, **60**, 205–215.

Gao, F. K., Dai, C. C. and Liu, X. Z. (2010). Mechanisms of fungal endophytes in plant protection against pathogens. *African Journal of Microbiology Research*, **4**, 1346–1351.

Garrido-Jurado, I., Landa, B. B. and Quesada-Moraga, E. (2016). Detection and quantification of the entomopathogenic fungal endophyte *Beauveria bassiana* in plants by nested and quantitative PCR. *Microbial-Based Biopesticides: Methods and Protocols*, pp. 161–166.

Glare, T., Caradus, J., Gelernter, W. *et al.* (2012). Have biopesticides come of age? *Trends in Biotechnology*, **30**, 250–258.

Greenfield, M., Gómez-Jiménez, M. I., Ortiz, V. *et al.* (2016). *Beauveria bassiana* and *Metarhizium anisopliae* endophytically colonize cassava roots following soil drench inoculation. *Biological Control*, **95**, 40–48.

Griffin, M. R. (2007). *Beauveria bassiana*, a cotton endophyte with biocontrol activity against seedling disease. PhD Dissertation. The University of Tennessee, Knoxville, TN.

Grille, G., Pascal, C., Franco, J. and Basso, C. (2001). Distribución espacial de *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae) en plantas de tomate. *Boletín de Sanidad Vegetal. Plagas*, **27**, 475–488.

Gómez-Vidal, S., Lopez-Lorca L., Jansson, H. and Salinas, J. (2006). Endophytic colonization of date palm (*Phoenix dactylifera* L.) leaves by entomopathogenic fungi. *Micron*, **37**, 624–632.

Gurulingappa, P., McGee, P. A. and Sword, G. (2011). Endophytic *Lecanicillium*

*lecanii and Beauveria bassiana* reduce the survival and fecundity of *Aphis gossypii* following contact with conidia and secondary metabolites. *Crop Protection*, **30**, 349–353.

Hajek, A. and St. Leger, F. (1994). Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology*, **39**, 293–322.

Hajek, A. E. (1997). Ecology of terrestrial fungal entomopathogens. In *Advances in Microbial Ecology*, Vol. 15, ed. J. G. Jones. Boston, MA: Springer, pp. 193–249.

Hamil R., Higgens C., Boaz H. and Gorman M. (1969). The structure of beauvericin, a new depsipeptide antibiotic toxic to *Artemia salina*. *Tetrahedron Letters*, **49**, 4255–4258.

Hardoim, P. R., Van Overbeek, L. S., Berg, G. *et al.* (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews*, **79**, 293–320.

Herre, E. A., Mejía, L. C., Kyllo, D. A. *et al.* (2007). Ecological implications of antipathogen effects of tropical fungal endophytes and mycorrhizae. *Ecology*, **88**, 550–558.

Hirano, E., Koike, M., Aiuchi, D. and Tani, M. (2008). Pre-inoculation of cucumber roots with *Verticillium lecanii* (*Lecanicillium muscarium*) induces resistance to powdery mildew. *Research Bulletin of Obihiro University*, **29**, 82–94.

Holt, R. D. and Hochberg, M. E. (1997). When is biological control evolutionarily stable (or is it)? *Ecology*, **78**, 1673–1683.

Hoffmann, M. and Frodsham, A. (1993). *Los Enemigos Naturales de las Plagas de Insectos Vegetales*. Ithaca, New York: Extensión Cooperativa de la Universidad de Cornell.

Hu, Q., Li, F. and Zhang, Y. (2016). Risks of mycotoxins from mycoinsecticides to humans. *BioMed Research International*, **2016**, ID 3194321, 1–13.

Hyde, K. D. and Soytong, K. (2008). The fungal endophyte dilemma. *Fungal Diversity*, **33**, e173.

Jaber, L. R. (2015). Grapevine leaf tissue colonization by the fungal entomopathogen *Beauveria bassiana s. l.* and its effect against downy mildew. *BioControl*, **60**, 103–112.

Jaber, L. R. and Enkerli, J. (2016). Effect of seed treatment duration on growth and colonization of *Vicia faba* by endophytic *Beauveria bassiana* and *Metarhizium brunneum*. *Biological Control*, **103**, 187–195.

Jaber, L. R. and Ownley, B. H. (2017). Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? *Biological Control*, **116**, 35–45.

Jaber, L. R. and Salem, N. M. (2014). Endophytic colonisation of squash by the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) for managing Zucchini yellow mosaic virus in cucurbits. *Biocontrol Science and Technology*, **24**, 1096–1109.

Jaber, L. R. and Vidal, S. (2010). Fungal endophyte negative effects on herbivory are enhanced on intact plants and maintained in a subsequent generation. *Ecological Entomology*, **35**, 25–36.

Karthiba, L., Saveetha, K., Suresh, S. *et al.* (2010). PGPR and entomopathogenic fungus bioformulation for the synchronous management of leaffolder pest and sheath blight disease of rice. *Pest Management Science*, **66**, 555–564.

Kauppinen M., Saikkonen K., Helander M., Pirttilä A. M. and Wäli P. R. (2016). *Epichloë* grass endophytes in

sustainable agriculture. *Nature Plants*, **2**, 15224.

Kavroulakis, N., Ntougias, S., Zervakis, G. I. *et al.* (2007). Role of ethylene in the protection of tomato plants against soil-borne fungal pathogens conferred by an endophytic *Fusarium solani* strain. *Journal of Experimental Botany*, **58**, 3853–3864.

Kepler, R. M. and Rehner, S. A. (2013). Genome-assisted development of nuclear intergenic sequence markers for entomopathogenic fungi of the *Metarhizium anisopliae* species complex. *Molecular Ecology Resources*, **13**, 210–217.

Kim, J. J., Goettel, M. S. and Gillespie, D. R. (2007). Potential of *Lecanicillium* species for dual microbial control of aphids and the cucumber powdery mildew fungus, *Sphaerotheca fuliginea*. *Biological Control*, **40**, 327–332.

Kim, K. H., Kabir, E. and Jahan, S. A. (2017). Exposure to pesticides and the associated human health effects. *Science of The Total Environment*, **575**, 525–535.

Kolattukudy, P. (1984). Biochemistry and function of cutin and suberin. *Canadian Journal of Botany*, **62**, 2918–2933.

Kumar, V. (2015). A review on efficacy of biopesticides to control the agricultural insect's pest. *International Journal of Agricultural Science Research*, **4**, 168–179.

Krell, V., Jakobs-Schoenwandt, D., Vidal, S. and Patel, A. V. (2017). Encapsulation of *Metarhizium brunneum* enhances endophytism in tomato plants. *Biological Control*, **116**, 62–73.

Kwak, M. J., Song, J. Y., Kim, S. Y. *et al.* (2012). Complete genome sequence of the endophytic bacterium *Burkholderia* sp. strain KJ006. *Journal of Bacteriology*, **194**, 4432–4433.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I. *et al.* (2015). Insect pathogens as biological control agents: back to the future. *Journal of Invertebrate Pathology*, **132**, 1–41.

Lahiri, A., Douglas, G. C., Murphy, B. R. and Hodkinson, T. R. (2019). *In vitro* methods for plant–microbe interaction and biocontrol studies in european ash (*Fraxinus excelsior* L.). In *Endophytes for a Growing World*, ed. T. R. Hodkinson, F. M. Doohan, M. J. Saunders and B. R. Murphy. Cambridge: Cambridge University Press, Chapter 15.

Leroux, P., Gredt, M., Leroch, M. and Walker, A. S. (2010). Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. *Applied and Environmental Microbiology*, **76**, 6615–6630.

Levitan, L., Merwin, I. and Kovach, J. (1995). Assessing the relative environmental impacts of agricultural pesticides: the quest for a holistic method. *Agriculture, Ecosystems & Environment*, **55**, 153–168.

Liao, X., O'Brien, T. R., Fang, W. and Leger, R. J. S. (2014). The plant beneficial effects of *Metarhizium* species correlate with their association with roots. *Applied Microbiology and Biotechnology*, **98**, 7089–7096.

Lingg A. and Donaldson M. (1981). Biotic and abiotic factors affecting stability of *B. bassiana* conidia in soil. *Journal of Invertebrate Pathology*, **38**, 191–200.

Logrieco A., Moretti A., Castella G. *et al.* (1998). Beauvericin production by *Fusarium* species. *Applied Environmental Microbiology*, **64**, 3084–3088.

Lohse, R., Jakobs-Schönwandt, D., Vidal, S. and Patel, A. V. (2015). Evaluation

of new fermentation and formulation strategies for a high endophytic establishment of *Beauveria bassiana* in oilseed rape plants. *Biological Control*, **88**, 26–36.

Lopez, D. C. and Sword, G. A. (2015). The endophytic fungal entomopathogens *Beauveria bassiana* and *Purpureocillium lilacinum* enhance the growth of cultivated cotton (*Gossypium hirsutum*) and negatively affect survival of the cotton bollworm (*Helicoverpa zea*). *Biological Control*, **89**, 53–60.

Majumder, D., Kangjam, B., Devi, K. J. *et al.* (2016). Endophytes: an emerging tool for plant disease management. In *The Handbook of Microbial Bioresources*, ed. V. K. Gupta, G. D. Sharma, M. G. Tuohy and R. Gaur. Oxfordshire: CABI, pp. 179–192.

Mantzoukas, S., Chondrogiannis, C. and Grammatikopoulos, G. (2015). Effects of three endophytic entomopathogens on sweet sorghum and on the larvae of the stalk borer *Sesamia nonagrioides*. *Entomologia Experimentalis et Applicata*, **154**, 78–87.

Martin, K. J. and Rygiewicz, P. T. (2005). Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology*, **5**, 28.

Mazón, A. (2001). Producción, uso y control de calidad de hongos entomopatógenos en Nicaragua. *Manejo Integrado de plagas, CATIE, Costa Rica*, **63**, 95–103.

Meyling, N. V. and Eilenberg, J. (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biological Control*, **43**, 145–155.

Moore D., Bateman R. P., Carey M. and Prior C. (1995). Long-term storage of *Metarhizium flavoviride* conidia in oil formulations for the control of locusts and grasshoppers. *Biocontrol Science and Technology*, **5**, 193–200.

Murphy, B. R., Martin Nieto, L., Doohan, F. M. and Hodkinson, T. R. (2015). Fungal endophytes enhance agronomically important traits in severely drought-stressed barley. *Journal of Agronomy and Crop Science*, **201**, 419–427.

Murphy, B. R., Doohan, F. M., Hodkinson, T. R. (2018). From concept to commerce: developing a successful fungal endophyte inoculant for agricultural crops. *Journal of Fungi*, **4**, 24.

Murphy, B. R., Doohan F. M and Hodkinson, T. R. (2019). Prospecting crop wild relatives for beneficial endophytes. In *Endophytes for a Growing World*, ed. T. R. Hodkinson, F. M. Doohan, M. J. Saunders and B. R. Murphy. Cambridge: Cambridge University Press, Chapter 18.

Oerke, E. C. and Dehne, H. W. (2004). Safeguarding production: losses in major crops and the role of crop protection. *Crop Protection*, **23**, 275–285.

Ownley, B. H., Pereira, R. M., Klingeman, W. E., Quigley, N. B. and Leckie, B. M. (2004). *Beauveria bassiana*, a dual purpose biocontrol organism, with activity against insect pests and plant pathogens. In *Emerging Concepts in Plant Health* Management, ed. R. T. Lartey and A. J. Caesar. Kerala, India: Research Signposts, pp. 255–269.

Ownley, B. H., Griffin, M. R., Klingeman, W. E. *et al.* (2008). *Beauveria bassiana*: endophytic colonization and plant disease control. *Journal of Invertebrate Pathology*, **98**, 267–270.

Ownley, B., Gwinn, K. and Vega, F. (2010). Endophytic fungal entomopathogens with activity against plant

pathogens: ecology and evolution. *Biocontrol*, **55**, 113–128.

Parine, N. R., Devinder, K., Khan, P. A. A. and Varaprasad, B. (2010). Antifungal efficacy of secondary metabolites from entomopathogenic fungi *Beauveria bassiana*. *Journal of Pharmacy Research*, **3**, 855–856.

Parsa, S., Ortiz, V. and Vega, F. E. (2013). Establishing fungal entomopathogens as endophytes: towards endophytic biological control. *JoVE*, **74**, e50360.

Pereira, R., Stimac, J. and Alves, S. (1993). Soil antagonism affecting the dose- response of workers of the red imported fire ant *Solenopsis invicta*, to *Beauveria bassiana* conidia. *Journal of Invertebrate Pathology*, **61**, 156–161.

Petrini, O. (1991). Fungal endophytes of tree leaves. In *Microbial Ecology of Leaves*, ed. J. H. Andrews and S. S. Hirano. New York, NY: Springer, pp. 179–197.

Pieterse, C. M., Van Wees, S. C., Van Pelt, J. A. *et al.* (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *The Plant Cell*, **10**, 1571–1580.

Posada, F., Aime, M. C., Peterson, S. W., Rehner, S. A. and Vega, F. E. (2007). Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). *Mycological Research*, **111**, 748–757.

Posada, F. J., Chaves, F. C., Gianfagna, T. J., Pava-Ripoll, M. and Hebbar, P. (2010). Establishment of the fungal entomopathogen *Beauveria bassiana* as an endophyte in cocoa pods (*Theobroma cacao* L.). *Revista UDCA Actualidad & Divulgación Científica*, **13**, 71–78.

Powell, W. A., Klingeman, W. E., Ownley, B. H. and Gwinn, K. D. (2009). Evidence of endophytic *Beauveria bassiana* in seed-treated tomato plants acting as a systemic entomopathogen to larval *Helicoverpa zea* (Lepidoptera: Noctuidae). *Journal of Entomological Science*, **44**, 391–396.

Quesada-Moraga, E. and Vey, A. (2004). Bassiacridin, a protein toxic for locusts secreted by the entomopathogenic fungus *Beauveria bassiana*. *Mycological Research*, **108**, 441–452.

Quesada-Moraga, E., Ruiz-García, A. and Santiago-Alvarez, C. (2006). Laboratory evaluation of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against puparia and adults of *Ceratitis capitata* (Diptera: Tephritidae). *Journal of Economic Entomology*, **99**, 1955–1966.

Quesada-Moraga, E., Muñoz-Ledesma, F. J. and Santiago-Alvarez, C. (2009). Systemic protection of *Papaver somniferum* L. against *Iraella luteipes* (Hymenoptera: Cynipidae) by an endophytic strain of *Beauveria bassiana* (Ascomycota: Hypocreales). *Environmental Entomology*, **38**, 723–730.

Quesada-Moraga, E., López-Díaz, C. and Landa, B. B. (2014). The hidden habit of the entomopathogenic fungus *Beauveria bassiana*: first demonstration of vertical plant transmission. *PloS One*, **9**, e89278.

Ravindran, K., Chitra, S., Wilson, A. and Sivaramakrishnan, S. (2014). Evaluation of antifungal activity of *Metarhizium anisopliae* against plant phytopathogenic fungi. In *Microbial Diversity and Biotechnology in Food Security*, ed. R. N. Kharwar, R. S. Upadhyay, N. K. Dubey and R. Raghuwanshi. India: Springer, pp. 251–255.

Rehner, S. A., Minnis, A. M., Sung, G.-H., Luangsa-ard, J. J., Devotto, L. and

Humber, R. A. (2011). Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. *Mycologia*, **103**, 1055–1073.

Roberts, D. W. and Hajek, A. E. (1992). Entomopathogenic fungi as bioinsecticides. In *Frontiers in Industrial Mycology*, ed. G. Leatham. New York: Chapman and Hall, pp. 144–159.

Roberts, D. W. and Leger, R. J. S. (2004). *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. *Advances in Applied Microbiology*, **54**, 1–70.

Rodríguez, R., White Jr, J., Arnold, A. and Redman, R. (2009). Fungal endophytes: diversity and functional roles. *New Phytologist*, **182**, 314–330.

Rondot, Y. and Reineke, A. (2016). Endophytic *Beauveria bassiana* in grapevine *Vitis vinifera* (L.) reduces infestation with piercing-sucking insects. *Biological Control*, **116**, 82–89.

Ryu, C. M., Hu, C. H., Reddy, M. S. and Kloepper, J. W. (2003). Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae*. *New Phytologist*, **160**, 413–420.

Sánchez Márquez, S., Bills, G. F., Herrero, N. and Zabalgogeazcoa, I. (2011). Non-systemic fungal endophytes of grasses. *Fungal Ecology*, **5**, 289–297.

Sasan, R. K. and Bidochka, M. J. (2012). The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *American Journal of Botany*, **99**, 101–107.

Sasan, R. K. and Bidochka, M. J. (2013). Antagonism of the endophytic insect pathogenic fungus *Metarhizium robertsii* against the bean plant pathogen *Fusarium solani* f. sp. *phaseoli*. *Canadian Journal of Plant Pathology*, **35**, 288–293.

Shah, F. A., Ansari, M. A., Watkins, J. *et al.* (2009). Influence of commercial fungicides on the germination, growth and virulence of four species of entomopathogenic fungi. *Biocontrol Science and Technology*, **19**, 743–753.

Sharma, P. (2011). Complexity of '*Trichoderma-fusarium*' interaction and manifestation of biological control. *Australian Journal of Crop Science*, **5**, 1027–1038.

Shoresh, M., Harman, G. E. and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annual Review of Phytopathology*, **48**, 21–43.

Soper, R. S. and Ward, M. G. (1981). Production, formulation and application of fungi for insect control. In *Biological Control in Crop Production*, BARC Symposium No. 5, ed. G. C. Papavizas. Montclair, NJ: Allanheld, Osmun, pp. 161–180.

Stone, J., Polishook, J. and White, J. (2004). Endophytic fungi. In *Biodiversity of Fungi, Inventory and Monitoring Methods*, ed. G. M. Mueller, G. F. Bills and M. S. Foster. Amsterdam: Elsevier, pp. 241–270.

Strobel, G. and Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews*, **67**, 491–502.

Susuki, A., Kanaoka, M., Isogai, A. *et al.* (1977). Bassianolide, a new insecticidal cyclodepsipeptide from *Beauveria bassiana* and *Verticillium lecanni*. *Tetrahedrom*, **25**, 2167–2170.

Tefera, T. and Vidal, S. (2009). Effect of inoculation method and plant growth medium on endophytic colonization

of sorghum by the entomopathogenic fungus *Beauveria bassiana*. *Biocontrol*, **4**, 663–669.

Taylor, A. G. and Harman, G. E. (1990). Concepts and technologies of selected seed treatments. *Annual Review of Phytopathology*, **28**, 321–339.

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R. and Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature*, **418**, 671–677.

Vega, F., Posada, F., Aime, M. *et al.* (2008). Entomopathogenic fungal endophytes. *Biological Control*, **46**, 72–82.

Vega, F. E., Goettel, M. S., Blackwell, M. *et al.* (2009). Fungal entomopathogens: new insights on their ecology. *Fungal Ecology*, **2**, 149–159.

Vega, F. E., Meyling, N. V., Luangsaard, J. J. and Blackwell, M. (2012). Fungal entomopathogens. *Insect Pathology*, **2**, 171–220.

Venugopalan, A. and Srivastava, S. (2015). Endophytes as *in vitro* production platforms of high value plant secondary metabolites. *Biotechnology Advances*, **33**, 873–887.

Vicari, M., Hatcher, P. and Ayres, P. (2002). Combined effect of foliar and mycorrhizal endophytes on an insect herbivore. *Ecology*, **83**, 2452–2464.

Vidal, S. and Jaber, L. R. (2015). Entomopathogenic fungi as endophytes: plant–endophyte–herbivore interactions and prospects for use in biological control. *Current Science*, **109**, 46–54.

Vining, L., Kelleher, W. and Schwarting, A. (1962). Oosporein production by a strain of *Beauveria bassiana* originally identified as *Amanita muscaria*. *Canadian Journal of Microbiology*, **8**, 931–933.

Wagner, B. L. and Lewis, L. C., (2000). Colonization of corn, *Zea mays*, by the entomopathogenic fungus *Beauveria bassiana*. *Applied and Environmental Microbiology* **66**, 3468–3473.

Wang, C. and Wang, S. (2017). Insect pathogenic fungi: genomics, molecular interactions, and genetic improvements. *Annual Review of Entomology*, **62**, 73–90.

Wang, Q. and Xu, L. (2012). Beauvericin, a bioactive compound produced by fungi: a short review. *Molecules*, **17**, 2367–2377.

Wang, X., Radwan, M. M., Taráwneh, A. H. *et al.* (2013). Antifungal activity against plant pathogens of metabolites from the endophytic fungus *Cladosporium cladosporioides*. *Journal of Agricultural and Food Chemistry*, **61**, 4551–4555.

White, T. J., Bruns, T. D., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*, ed. M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White. New York: Academic Press, pp. 315–322.

Wilson, D. (1995). Endophyte: the evolution of a term, and clarification of its use and definition. *Oikos*, **73**, 274–276.

Xu, Y., Orozco, R., Wijeratne, E. K. *et al.* (2009). Biosynthesis of the cyclooligomer depsipeptide bassianolide, an insecticidal virulence factor of *Beauveria bassiana*. *Fungal Genetics and Biology*, **46**, 353–364.

Xu, L., Wang, J., Zhao, J. *et al.* (2010). Beauvericin from the endophytic fungus, *Fusarium redolens*, isolated from *Dioscorea zingiberensis* and its antibacterial activity. *Natural Product Communications*, **5**, 811–814.