Endophytic microorganisms for biocontrol of the phytopathogenic fungus *Botrytis cinerea*

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† Dedicated to the Dr. James R. Hanson in Memoriam

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Abstract:

Botrytis cinerea is the most widely studied necrotrophic phytopathogenic fungus. It causes economic losses that are difficult to calculate due to the large number of hosts. While there are a wide array of fungicides on the market to control this phytopathogen, they are not considered sustainable in terms of the environment and human health. The search for new alternatives to control this phytopathogen has led to the use of endophytic microorganisms as biological control agents. Endophytic bacteria and endophytic fungi have been isolated from different plant species and some have proven effective in inhibiting *B. cinerea*. Furthermore, a significant number of fungistatic or fungicidal metabolites which could be used as alternative complementary chemical controls have been isolated from these fungi and bacteria. In this review, in addition to the metabolites which have shown fungicide activity against this phytopathogen, the different genera and species of endophytic bacteria and fungi are also considered. These have been isolated from various plant species and have displayed antagonistic activity against *B. cinerea*.

Keywords: antifungal, biological control agents, endophytic fungus and bacteria**,** grey mould disease.

Abbreviations

BCAs	biological control agents
CFU	colony forming unit
EC50	half maximal effective concentration
IC50	half maximal inhibitory concentration
ISR	inducing systemic resistance
MIC	minimal inhibitory concentration
SAR	systemic acquired resistance
VOCs	volatile organic compounds

Introduction

Plant diseases and pests are the main factors responsible for food loss around the world (Parnell et al. 2016). At least 20-40% of these losses are caused by pathogenic infections and they account for losses of $40 billion a year worldwide (Syed Ab Rahman et al. 2018). *Botrytis cinerea* (figure 1) is the second most important plant pathogen in the world and it is therefore one of the most extensively studied necrotrophic fungal phytopathogen (Dean et al. 2012; Williamson et al. 2007). It causes diseases known as "grey mould" which are responsible for economic losses that are difficult to calculate due to its wide range of hosts (Dean et al. 2012). While there are fungicides to combat *B. cinerea*, their use is not considered sustainable due to their adverse effects on human health and the environment. Moreover, frequent applications increase the risk of the fungus developing resistance, *B. cinerea* is considered a high-risk pathogen in terms of resistance to fungicides and this is a limiting factor in terms of its chemical control (Williamson et al. 2007; Rodríguez et al. 2014; Haidar et al. 2016; Lu et al. 2016). Despite this fact, fungicides are still the most common method used to control gray mold and account for 10% of the cost of the world fungicide market. It is estimated that over €1 billion is spent annually worldwide to control this phytopathogen (Dean et al. 2012). Therefore, the development of methods that are complementary to chemical control, such as the use of non-pathogenic microorganisms as biological control agents (BCAs), are increasingly considered to be promising alternatives (Haidar et al. 2016). Although in the last decade the search for new BCAs to combat *B. cinerea* has increased, the corresponding efficiency studies are conducted under controlled laboratory or greenhouse conditions and eventually most fail in the field (Haidar et al. 2016; Nicot et al. 2016). Hence, the number of BCAs marketed as fungicides to combat *B. cinerea* is still very small (Haidar et al. 2016). There is therefore a need to search for new microorganisms or

their metabolites that are able to control *B. cinerea.* This search offers a promising opportunity to prevent food loss caused by this fungus and to improve agricultural productivity. This review summarizes the different genera and species of endophytic microorganisms which have been isolated from various plant species and show to have biocontrol capacity against *B. cinerea,* as well as the secondary metabolites that have been isolated from endophytic microorganisms and characterized as having antifungal activity against this phytopathogen.

Fig 1 Infection by *Botrytis cinerea*

1. Biological control by microorganisms

The United States and European Union are the main consumers of chemical fungicides worldwide. However, since 2011 the use of these chemical agents has declined, mainly in the USA, perhaps due to environmental protection and consumer health regulations (Carbú et al. 2016). The major crop protection companies have been investing in the field of biocontrol in response to legal restrictions and consumer demand for pesticide-free foods (Romanazzi et al. 2016). In 2011 the global biocontrol market was worth a reported US$2.1 billion and it was influenced by the growing demand for organic products (Velivelli et al. 2014).

The use of microorganisms or their metabolites to control plant disease has received greater attention, with some exceptions and when they have no negative effects on human or animal health, and are environmentally friendly. Unlike their chemical counterparts, in general, they do not affect other beneficial organisms (Ritika and Utpal 2014; Parnell et al. 2016; Syed Ab Rahman et al. 2018). Although biological methods to control plant pathogens have been under study for more than 70 years, biocontrol products account for a mere 3.5% of the global pesticide market which is still dominated by synthetic pesticides (Carbú et al. 2016; Parnell et al. 2016).

The fungi biocontrol market is dominated by bacteria-based and fungi-based products accounting for approximately 85% of the available products. The remaining 15% is made up of products based on viruses, predators and other organisms (e.g. protozoa, nematodes) (Glare et al. 2012).

During biological control, BCAs can inhibit pathogens directly either by mediating physical contact or by means of very specific mechanisms for combating the pathogen (hyperparasitism, predation, etc.). They may act indirectly by means that do not target a specific type of pathogen (stimulation of plant defenses, competition by substrates, etc.) or they may act by mixed-path antagonism (antibiotics, lytic enzymes, etc.) which are mutually compatible and can act simultaneously or synergistically (Bardin et al. 2015). However, BCAs effectiveness depends on factors such as climate variation, ecological competition, the intrinsic traits of the BCAs, the exertion of selection pressure and the quality of the product as it is formulated. Moreover, the traits of the pathogen such as its genetic diversity and ability to evolve in response to selection pressures must also be taken into account (Bardin et al. 2015).

The bacteria which are used as BCAs have been isolated mainly from the root zone, although some have also been isolated from other plant-related environments such

as the endosphere, the phyllosphere and the espermosphere (Lazarovits et al. 2014). Bacteria exert their control mechanism mainly through competition for the niche, the production of allelochemicals and the induction of resistance pathways in plants, (Compant et al. 2005; Lazarovits et al. 2014). Fungi, like bacteria, act as biocontrols through various mechanisms such as antibiosis, competition, parasitism, predation and stimulation of plant defense mechanisms (Lazarovits et al. 2014).

1.1 Endophytic microorganisms as biological control agents

Of the nearly 300,000 species of higher plants that exist today, each can host several species of endophytic microorganisms (Ryan et al. 2008; Aly et al. 2010; Senthilkumar et al. 2011). However, only a few of these plants have been thoroughly studied in terms of their endophytic microbiota despite the fact that endophytic microorganisms are a potential source of new natural products for use in medicine, biotechnology, industry and agriculture (Ryan et al. 2008; Senthilkumar et al. 2011).

Endophytes are microorganisms that are found within plant tissues during at least part of their life cycle. They do not cause disease under any known circumstances and are generally considered as organisms that have beneficial effects on their host (Ludwig-Müller 2015; Cocq et al. 2017). The fact that endophytic microorganisms are able to colonize an ecological niche similar to that of some phytopathogens means that they have potential as biocontrol agents. However, their effectiveness depends on many factors including host specificity and colonization patterns, population dynamics, the ability to move within host tissue, the ability to induce systemic resistance, the physical structure of the soil, environmental conditions and the growth phase and physiological state of the plant (Ryan et al. 2008; Senthilkumar et al. 2011; Eljounaidi et al. 2016; Eun and Mee

2016). The success of endophytic microorganisms as BCAs is linked to all of these factors.

Because of the administration and establishment of microorganisms in plants is difficult, the use of endophytes generates a greater expectation since, due to their life cycle, this could help to overcome the difficulties of delivery and survival in the plant (Lazarovits et al. 2014; Busby et al. 2016; O´Brien 2017). The benefits of endophytic microbiota for host plants include their ability to act as biocontrol agents through mechanisms such as competition for a niche or substrate, hyperparasitism, predation, allelochemical production (antibiotics, lytic enzymes, siderophores) and by inducing systemic resistance in plants (ISR) (Compant et al. 2005). Mechanisms such as parasitism and competition for substrates are likely to be less effective than antibiosis and ISR as biological control strategies in endophytes (Card et al. 2016). In addition to acting directly on the pathogen, endophytic microorganisms can stimulate the growth of the host plant through various mechanisms such as biological nitrogen fixation, solubilization of minerals, production of phytohormones and others (Van et al. 2014).

Inoculation of plants with endophytic microorganisms can inhibit disease symptoms caused by insects, viruses, bacteria, nematodes and fungi (Eun and Mee 2016). In the initial stages, the interaction between endophytic microorganisms and their host plant promotes an immune response by the plant. However, these endophytic microorganisms are able to overcome this response and successfully colonize the plant, acting as an immune stimulant or a natural vaccination (Hardoim et al. 2015). Endophytic microorganisms also have the capacity to synthesize a wide range of bioactive chemical compounds that plants use as to defend themselves against pathogens (Nair and Padmavathy 2014). Pathogens can induce endophytic microorganisms to synthesize these antimicrobial compounds. Moreover, endophytes have an influence on the secondary

metabolism of their host plant (Combés et al. 2012; Hardoim et al. 2015). Products obtained from endophytic microorganisms include antibiotics, immunosuppressants, anticancer agents, antioxidants and other biologically active substances (Zhang et al. 2006; Dutta et al. 2014). These compounds belong to various structural groups such as terpenoids, steroids, phenols, coumarins and others (Ludwig-Müller 2015).

2. Endophytic microorganisms for the biological control of *B. cinerea*

B. cinerea is a phytopathogenic fungus that affects the flowers, leaves, buds, seeds and fruits of numerous crops around the world. Infection occurs either through direct penetration or through wounds following pruning or harvesting. Although the most noticeable effects of the infection are observed in mature or senescent tissue, the fungus can invade the plant at early stages of cultivation and remains dormant until the conditions are propitious. Consequently, the serious damage may be caused after the harvest of apparently healthy crops (Williamson et al. 2007; Özer and Bayraktar 2014). *B. cinerea* is difficult to control because it has several modes of attack, multiple hosts, high genetic variability, and it can survive for long periods of time either as mycelia or conidia. Its management depends mainly on synthetic fungicides whose frequent application increases the risk of resistance (Williamson et al. 2007; Rodríguez et al. 2014; Haidar et al. 2016; Lu et al. 2016). Despite this fact, fungicides are still the most common method used to control this phytopathogen. The global market for these products is estimated at US$15-25 million (Elad and Stewart 2007). In addition to *B. cinerea* resistance to synthetic fungicides, the negative effects that these products have on health and the environment has stimulated the search for new strategies to control this phytopathogen (Rodríguez et al. 2014). Biocontrol offers an alternative or an attractive complement since biological control agents are considered to be less harmful to the environment. Their

multiple and complex modes of action reduce the risk of resistance (Elad and Stewart 2007). Rhizosphere microorganisms have played a key role in biological control insofar as the rhizosphere is the first line of defense protecting root systems from pathogen attacks (Suprapta 2012). A growing number of endophytic microorganisms are being considered in the search for new biological control agents since they colonize the same ecological niche in plants as pathogens and can be found in roots, stems, leaves, fruits and seeds (Ryan et al. 2008; Bulgarelli et al. 2013; Chebotar et al. 2015; Santoyo et al. 2016).

The main modes of action of bacterial antagonists and other microorganisms against *B. cinerea* involve competition for space and nutrients, antibiosis, production of lytic enzymes, interference with pathogen growth and activity, the induction of host plant resistance and the production of volatile organic compounds (Haidar et al. 2016). Knowledge of endophytic microorganisms and their metabolites that are active against *B. cinerea* has become a fundamental tool in the search for new alternatives for the control of this phytopathogen that is the cause of great food losses. Since some studies suggest that *B. cinerea* has the potential to change its lifes cycle under appropriate conditions and shift from classic necrotrophic behavior to facultative endophytic behavior (Van et al. 2014), the use of antagonists with this same lifestyle is seen as an effective tool for the control of this phytopathogen.

Today there are commercial biopesticides on the market to combat *B. cinerea* that contain microorganisms as their active ingredient. These microorganisms have various modes of action that are summarized in Table 1. Since it has recently been found that *B. cinerea* is an endophyte at a certain stage of its life cycle, research on microorganisms that share this same niche is considered a new option in the search for new biological control agents against grey mould (Dean et al. 2012; Haidar et al. 2016).

The following microorganisms are among those that are the active ingredient of products that are currently marketed as fungicides against *B. cinerea*: *Aureobasidium pullulans, Bacillus amyloliquefaciens, B. subtilis, B. megaterium, Pantoea agglomerans, Pseudomonas syringae, Streptomyces griseoviridis, Streptomyces lydicus, Chlonostachys rosea, Gliocladium catenulatum, Trichoderma atroviride, T. harzianum, T. polysporum* and *Ulocladium oudemansii* (Haidar et al. 2016; Nicot et al. 2016).

A wide variety of endophytic microorganisms have been isolated from different plant species with potential for the biological control of *B. cinerea*, although more detailed studies of the interactions between these microorganisms, *B. cinerea*, their host plant and the remaining microbiota are needed before they can be successfully used in agriculture.

Table1. Commercial pesticides with microorganisms as an active ingredient.

Commercial name	**Microorganism composition**	**Mode of action**
Botector	*Aureobasidium pullulans* strains 14940/14941	Competitive exclusion
Double Nickel 55WDG/LC™	*Bacillus amyloliquefaciens*	Antimicrobial
Serenade ® Max	*B. subtilis* QST 713	Antimicrobial, Sparking of plant defenses
Companion	*B. subtilis* GB03	Antibiosis (iturins), Induced Systemic Resistance (ISR)
Bio Arc	*B. megaterium*	Enzymatic action
Endofine	*Chlonostachys rosea*	Competition
Prestop	*Gliocladium catenulatum* J1446	Competition, hyperparasitism
Bio-save	*Pseudomonas syringae* ESC-10	Competition
Mycostop	*Streptomyces griseoviridis* K61	Competition
Actinovate	*S. lydicus* WYCD108	Competition, antibiosis
Sentinel	*Trichoderma atroviride* LC52	Competitive exclusion
BinabTF	*T. harzianum* + *T. polysporum*	Antibiosis, Systemic acquired resistance (SAR)
Supresivit	*T. harzianum*	Competition
Botryzen	*Ulocladium novo-zealandiae*	Competitive exclusion

Adapted from Nicot et al. (2016)

In addition, taking into account the general rule that a single strain of endophyte can produce multiple bioactive compounds (Zhang et al. 2006), the isolation of new strains of endophytes from different plant species may lead to the discovery of new bioactive molecules to combat gray mold.

3. Biocontrol of *B. cinerea* by endophytic bacteria

Various studies have been conducted to assess the potential of endophytic bacteria for the biocontrol of *B. cinerea*. These bacteria have been isolated from different plant species leading to the identification of new microorganisms that can inhibit the growth of this fungus (Table 2).

Trotel-Aziz et al. (2008) isolated two bacterial strains identified as *Pantoea agglomerans* PTA-AF1 and *Pseudomonas fluorescens* PTA-CT2 from the leaves and stems of *Vitis vinifera* L.cv. Chardonnay. Leaves immersed in a solution of bacteria were inoculated with a conidial suspension of *B. cinerea* after a needle-prick wound. Disease development was measured as the average diameter of lesions formed 7 days after inoculation and the protection percentage was defined as reduction in lesion diameter relative to the control. The strain *P. agglomerans* PTA-AF1 and *P. fluorescens* PTA-CT2 showed protection percentages in leaf assays of 61% and 87% respectively, exhibiting an apparent antagonistic effect against *B. cinerea*. The bacterial strains identified as *Lysisnibacillus* sp. 3Y22, *Nocardioides* sp. 3Y27, *Brevibacills* sp. 3Y41, *Stenotrophomonas* sp. 3T7, *Bacillus* sp. 3R1, *Bacillus* sp. 3R4 and *Lysisnibacillus* sp. 3Y25 isolated from three-year-old *Vitis vinifera* plants cv. Corvina also inhibited *B. cinerea.* Four-day-old plugs of *B. cinerea* were placed in the centre of a Petri dish and bacterial inocula were streaked at a distance of 3 cm from the fungal plugs. Bacterial

antifungal activity was assessed by comparing the areas of mycelial growth inhibition with those on control plates where fungal pathogens alone had been inoculated.

Table 2. Endophytic bacteria able to biologically control *B. cinerea*.

Microorganism*	Plant Species	Reference
Actinobacteria *Bacilli* *Alfaproteobacteria* *Betaproteobacteria* *Gammaproteobacteria*	*Rubus fruticosus*	Contreras et al. 2016
Bacillus amyloliquefaciens ssp. *plantarum*	*Hedera hélix*	Soares et al. 2015
B. amyloliquefaciens	*Capsicum annuum*	Mari et al. 1996
B. cereus	*Arabidopsis thaliana*	Hong et al. 2015
B. mojavensis *B. halotolerans* *B. subtilis* *B. amyloliquefaciens*	*Lycopersicon esculentum*	Kefi et al. 2015
Bacillus sp. CHM1	*Oryza sativa*	Wang et al. 2009a
B. subtilis	*Opuntia ficus-indica*	Boubakri and Schmitt 2015
B. subtilis	*Triticum* sp.	Liu et al. 2010
B. subtilis *B. pumilus*	*Vitis vinifera*	Zhang et al. 2017
B. subtilis	*Lycopersicon esculentum* Mill.	Wang et al. 2009b
B. velezensis ZSY-1	*Catalpa ovata*	Gao et al. 2017
Brevibacillus brevis	*Lycopersicon esculentum*	Yang et al. 2011
Burkholderia cepacia Cs5	*Prunus dulcis*	Kilani-feki and Jaoua 2011
B. phytofirmans PsJN	-	Miotto-Vilanova et al. 2016
Lysisnibacillus sp. 3Y25 *Pantoea* sp. 15T13	*Vitis vinifera* cv. Corvina	Andreolli et al. 2015
Micromonospora	*Medicago sativa*	Martinez-Hidalgo et al. 2015
Pantoea agglomerans PTA-AF1 *Pseudomonas fluorescens* PTA-CT2	*V. vinífera* L., cv Chardonnay	Trotel-Aziz et al. 2008
Phyllobacterium sp.	*Epimedium brevicornu* Maxim	He et al. 2009
Pseudomonas sp. strain PsJN	*Allium cepa*	Barka et al. 2002
P. stutzeri (E25) *Stenotrophomonas maltophilia* (CR71)	*Physalis ixocarpa*	Rojas-Solís et al. 2018

* Microorganisms listed in alphabetical order

The strain *Lysisnibacillus* sp. 3Y25 was the one exhibiting the largest inhibition zone (approximately 17 mm). The strains identified as *Microbacterium* sp. 15Y9, *Pantoea* sp. 15T13, *Pseudoxanthomonas* sp. 15R38 and *Rhizobium* sp. 15R41 which were isolated from 15-year-old plants, also exhibited an inhibitory effect on *B. cinerea*. *Pantoea* sp. 15T13 showed the largest inhibition area (approximately 7 mm) (Andreolli et al. 2015). The studies conducted by Kilani-feki and Jaoua (2011) showed that the sterile cell-free culture supernatant of the endophytic strain *Burkholderia cepacia* Cs5 was active against *B. cinerea* at concentrations 0.9 % and 1.25 % in bioassays on solid and liquid media respectively. Microscopy revealed morphological changes to the hyphae of *B. cinerea* grown on the sterile cell-free culture supernatant, which were completely empty with a larger diameter and rather more branched. Grape vines inoculated with *B. cepacia* Cs5 and exposed to *B. cinerea* spores remained viable, vigorous and had enhanced root development.

Barka et al. (2002) assessed the ability of the *Pseudomonas* sp. PsJN strain which had been isolated from the surface of sterilized *Allium cepa* roots, to promote growth in *Vitis vinifera* L.cv. Chardonnay and act as a biocontrol agent against *B. cinerea*. Grape vines that were treated with bacteria and subsequently inoculated with *B. cinerea* remained healthy after 7 days, with only small areas of necrosis on some leaf surfaces. Simultaneous inoculation with bacteria and fungus did not stop fungal growth. However, inhibition was observed when *B. cinerea* was inoculated two days after the *Pseudomonas* sp. This could be because the bacteria need a sufficient population density to control the fungus or because the bacteria did not have enough time to biosynthesize the compounds with anti-fungal activity. Microscopic analysis of the mycelium of the fungus co-cultivated with *Pseudomonas* sp. showed changes in the structures of the hyphae with coagulation of the cytoplasm, vesicles in the cell walls and lack of organelles.

Miotto-Vilanova et al. (2016) evaluated the capacity of the bacteria *Burkholderia phytofirmans* PsJN to confer resistance to *Vitis vinifera* L.cv. Chardonnay against *B. cinerea*. The leaves of plants which had been inoculated with *B. phytofirmans* PsJN and were then infected with drops of the 630 strain of *B. cinerea*, exhibited a significant reduction in necrosis (approximately 50%) after 72 hours of inoculation. Plants infected with bacteria and subsequently infected with a *B. cinerea* spore suspension, exhibited significantly reduced symptoms of the disease. *B. phytofirmans* PsJN was observed on the surface of the leaves surrounding the fungal mycelium, showing that this bacterium is able to colonize the plant through the stomata of the leaves and form a biofilm around *B. cinerea*. A spore germination bioassay in the presence of *B. phytofirmans* PsJN showed a 32%, 62% and 88% inhibition of the germ tube growth after the addition of 10^2, 10^4, and 10^6 CFU/mL of bacteria, respectively.

In the studies conducted by Zhang et al. (2017) two bacterial strains were isolated from grapevine leaves and identified as *Bacillus subtilis* and *B. pumilus*. These bacteria had an inhibitory effect against *B. cinerea* of between 71% and 80% in *in vitro* bioassays. Bacterial biocontrol capacity against *B. cinerea* in tomato during the postharvest stage was also evaluated using 175 endophytic bacteria isolated from the subepidermis of various horticultural sources (cucumber, eggplant, pepper, tomato, zucchini, apricot, peach and plum) (Mari et al. 1996). Of the 175 strains tested, 7% (thirteen) were active against the phytopathogen and reduced the percentage of infected fruit by more than 50% after 7 days of storage at 20°C. In order to evaluate antagonistic activity against *B. cinerea*, bacterial suspensions were introduced into wounded tomato fruits at a depth of 3 mm. A conidial suspension of gray mold was then introduced into the wound of these same fruits and rot incidence (%) was recorded. The strain which was identified as *Bacillus amyloliquefaciens*, and had been isolated from internal pepper

tissue, was able to reduce the incidence of the disease by 90%. The bacterial extract was completely ineffective thus indicating a direct competition of the bacteria with *B. cinerea*.

In studies on *Lycopersicon esculentum* Mill., *Speranskiae tuberculatae* and *Dictamnus dasycarpus* Turcz, Wang et al. (2009b) isolated three bacterial strains which were identified as EB-15, EB-28 and EB-122, and exhibited 70%, 71% and 69% inhibition against *B. cinerea in vitro* respectively. In the *in vivo* bioassay, strain EB-28 which was identified as *B. subtillis* and isolated from *L. esculentum* Mill., reduced infection by *B. cinerea* by 45% in *Cucumber* cotyledons and by 52% in tomato leaves. The endophytic bacteria of *L. esculentum* identified as *Brevibacillus brevis* and isolated by Yang et al. (2011), exhibited a 78% inhibition index against *B. cinerea* and the fermentation filtrate achieved 100% inhibition. Endophytic bacteria isolated from the stems of *Oryza sativa* were identified as *Bacillus* sp. CHM1. The culture filtrate, the sterile filtrate and the supernatant of the culture medium of CHM1 showed an antifungal index against *B. cinerea* of approximately 61%, 31% and 73% respectively (Wang et al. 2009a). In the *in vitro* bioassay, the strain identified as *Phyllobacterium* sp. presented inhibition of 22 mm and 12 mm respectively when bacterial inoculum and the cell-freeculture supernatant were used. This bacteria was isolated by He et al. (2009) from root tissue of *Epimedium brevicornu* Maxim.

Boubakri and Schmitt (2015) isolated two strains of *B. subtilis* identified as EBS1 and EBS2 from *Opuntia ficus-indica* roots. In the antagonism bioassay against *B. cinerea*, the control showed growth of 40 mm after 5 days of incubation whereas those faced with strains EBS1 and EBS2 showed growth of 9 mm and 10 mm, respectively. The application of cell-free filtrates of both *B. subtilis* strains presented growth of 9 mm and 16 mm, for EBS1 and EBS2, respectively. This indicates that extracellular metabolites secreted by the bacteria are involved in the inhibition of *B. cinerea*. By

removing apoplastic fluid from *Arabidopsis thaliana*, Hong et al. (2015) isolated a leaf-inhabiting endophytic bacteria identified as *Bacillus cereus*. Tomato plants were sprayed with a suspension of *B. cereus* and at 27 days after inoculation, the tomato leaves were infected with a conidial suspension of *B. cinerea*. The tomato leaves inoculated with *B. cereus* had smaller lesion areas compared to the control, indicating that this strain could be effective in biocontrol applications in agricultural biotechnology.

Martinez-Hidalgo et al. (2015) evaluated the biocontrol capacity of *Micromonospora* isolated from *Medicago sativa* nodules. Ten of the 13 strains which were evaluated, were able to inhibit *B. cinerea*. Two *Micromonospora* strains were tested for their efficiency in increasing the resistance of tomato to grey mould. Plants treated with the bacterial strains presented lesions of approximately 13 to 14 mm, while control lesions were over 16 mm. 102 endophytic bacteria belonging to the *Actinobacteria*, *Bacilli*, *Alfaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* classes were isolated from the roots of *Rubus fruticosus*, and 3.9% of the isolates were successful at inhibiting over 50% of *B. cinerea* (Contreras et al. 2016).

4. Biocontrol of *B. cinerea* by endophytic fungi

Although there are few reports of endophytic fungi capable of protecting their host by inducing a systemic response, they are a rich source of bioactive metabolites and extracellular enzymes that play a fundamental role in the biocontrol of pathogens (Suryanarayanan et al. 2009; Fouda et al. 2015; Hardoim et al. 2015). Table 3 summarizes the species of endophytic fungi with biocontrol capacity against *B. cinerea* and the plant species from which they were isolated, revealing the wide diversity of endophytic fungi that can be used for the biocontrol of grey mould.

1 **Table 3**. Endophytic fungi with biocontrol capacity against *B. cinerea*.

Microorganism*	**Plant species**	**Reference**
Aspergillus clavatonanicus	*Taxus mairei*	Zhang et al. 2008
A. fumigatus LN-4	*Melia azeda*	Li et al. 2012
Aureobasidium pullulans	*Prunus avium*	Schena et al. 2003
Alternaria sp. *Botryosphaeria ribis* *Phoma medicaginis* *Bionectria ochroleuca* *Aureobasidium pullulans* *Chaetomium spirochaete*	*Vitis vinifera* L.	Cosoveanu et al.2014
Chaetomium globosum	*Houttuynia cordata* Thunb	Pan et al.2016
Cryptosporiopsis sp. *Phialocephala spharoides* B.J. Wilson	*Picea abies*	Terhonen et al. 2016
Daldinia cf. concentrica	*Olea europaea* L.	Liarzi et al. 2016
Drechslera biseptata *Tricladium splendens* *Leptosphaeria* sp. *Entrophospora* sp. *Pyrenochaeta lycopersici*	*Aralia elata* *Aralia continentalis*	Narayan et al. 2007
Fusarium oxysporum CanR-46	*Brassica napus*	Zhang et al. 2014
Hypoxylon sp.	*Persea indica*	Tomsheck et al. 2010
Microsphaeropsis solivácea *Penicillium janczewskii*	*Araucaria araucana* *Austrocedrus chilensis* *Fitzroya cupressoides* *Pilgerodendron saligna* *P. nubigena* *P. uviferum* *Prumnopitys andina* *Saxegothaea conspicua*	Hormazabal and Piontelli 2014
Nigrospora oryzae 2693 *N. oryzae* 2778 *Trichoderma asperellum* 2739 *Penicillium commune* 2748 *Fusarium proliferatum* 2751 *Chaetomium globosum* 2773	*Espeletia grandiflora* *E. corymbosa*	Miles et al. 2012
Nigrospora sp.	*Moringa oleífera*	Zhao et al. 2012
Paecilomyces lilacinus	*Cannabis sativa* L.	Kusari et al. 2013
Penicillium sp.	*Artemisia absinthium*	Noumeur et al. 2016
Phoma terrestris	*Panax ginseng*	Park et al. 2015
Phomopsis sp. By254	*Gossypium hirsutum*	Fu et al. 2011
Ramularia pratensis *Phoma aliena* *Fusarium acuminatum*	*Vitis riparia*	Kernaghan et al. 2017

Rhizopus oryzae	*Radula marginata*	Kusari et al. 2014
Saccharomycopsis fibuligera	*Psidium guajava* L.	Abdel-rahim and Abo-elyousr 2017
Xylaria sp.	*Abies holophylla*	Park et al. 2005

*Microorganisms listed in alphabetical order

Miles et al. (2012) studied the diversity and biocontrol potential of endophytic fungi isolated from the leaves of *Espeletia grandiflora* and *E. corymbosa*. In examining the production of secondary metabolites on a solid medium, the fungi identified as *Nigrospora oryzae* 2693, *Trichoderma asperellum* 2739, *Penicillium commune* 2748, *Fusarium proliferatum* 2751, *Chaetomium globosum* 2773 and *N. oryzae* 2778, showed an inhibition index against *B. cinerea* of 17%, 58%, 27%, 69%, 56% and 57% respectively. In the antagonistic activity tests with crude extracts, the fungi *Aureobasidium pullulans* 2679, *Beauveria bassiana* 2749, *Scopulariopsis brevicaulis* 2758, *Epicoccum nigrum* 2759 and *E. nigrum* 2764 showed an inhibition index against *B. cinerea* of 65%, 68%, 65%, 66% and 68% respectively.

Volatile organic compounds (VOCs) are low molecular weight compounds that can vaporize at normal atmospheric temperatures and pressure (Hung et al. 2015; Toffano et al. 2017). Over 300 distinct VOCs have been identified from fungi. Chemically they occur as mixtures of simple hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives (Morath et al. 2012; Hung et al. 2015). VOCs generally have a low water solubility and often have a distinctive odor (Hung et al. 2015). Fungal VOCs are interesting for agricultural research because of their potential as biological control agents (Morath et al. 2012; Schalchli et al. 2016). In the study carried out by Zhang et al. (2014), the endophytic fungus *Fusarium oxysporum* CanR-46 isolated from *Brassica napus*, produced VOCs with a strong inhibitory effect against *B. cinerea*. In the "double-dishes" test consisting of two potato dextrose agar

dish containing an agar plug with mycelia of *B. cinerea* and agar plug with mycelia of *F. oxysporum* to , which were immediately sealed with a piece of parafilm, the VOCs of *F. oxysporum* CanR-46 had an inhibition index against *B. cinerea* of 91%. Tomatoes treated with *F. oxysporum* CanR-46 and *F. oxysporum* CanR-46 plus *B. cinerea* remained healthy or showed very few signs of soft rot after 8 days of incubation at 20ºC, while tomatoes inoculated with only *B. cinerea* showed both soft rot and mold symptoms. In the studies carried out by Narayan et al. (2007), the endophytic fungi identified as *Drechslerabi septata*, *Tricladium splendens*, *Leptosphaeria* sp., *Entrophospora* sp. and *Pyrenochaeta lycopersici* were isolated from roots of *Aralia elata* and *A. continentalis*. These fungi showed antifungal activity against *B. cinerea*. The fungi *Entrophospora* sp. and *Pyrenochaeta lycopersici* were the most active, with inhibition zones of >10 mm between *B. cinerea* and the endophytes.

The post-harvest rot of sweet cherries and table grapes was examined by Schena et al. (2003) who studied the biocontrol capacity of different strains of *Aureobasidium pullulans* which had been isolated from *Prunus avium* tissue. In sweet cherries, the isolate identified as 547 was the most effective against *B. cinerea* reducing the damage of gray mold by 90% on single-wounded berries. In post-harvest tests with cherries, this same isolate reduced the number of rotten berries by between 58% -80%. Rot reduction in grapes was between 59% -64%.

Hormazabal and Piontelli (2009) conducted studies on endophytic communities of eight gymnosperm species: *Araucaria araucana*, *Austrocedrus chilensis*, *Fitzroya cupressoides*, *Pilgerodendro nuviferum*, *P. nubigena*, *P. saligna*, *Prumnopitys andina* and *Saxegothaea conspicua*. The fungi which were identified as *Microsphaeropsis olivácea* and *Penicillium janczewskii*, were isolated from these plants and their ethyl acetate extracts exhibited antifungal activity against *B. cinerea* with minimal inhibitory

concentration (MIC) values (μg/mL) of 250 and 500, respectively. The ethyl acetate extract from the endophytic fungus *Chaetomium globosum* isolated by Pan et al. (2016) from *Houttuynia cordata* Thunb, exhibited a 100% inhibition index against *B. cinerea*. Kusari et al. (2013) isolated an endophytic fungus which was identified as *Paecilomyces lilacinus* from apical buds of *Cannabis sativa* L. This had a 100% inhibition index against *B. cinerea* in antagonism assays. Kusari et al. (2014) also isolated the fungus *Rhizopus oryzae* from *Radula marginata* which again showed a 100% inhibition index against *B. cinerea*.

Noumeur et al. (2016) isolated 12 endophytic fungi from the roots of *Artemisia absinthium* which in the *in vitro* bioassay, had an inhibition index of between 33% and 50% against *B. cienerea.* Two of the isolates, identified as *Penicillium* sp., significantly reduced the incidence and diameter of lesions on white grape berries. Kernaghan et al. (2017) isolated the endophytic fungi *Ramularia pratensis, Phoma aliena* and *Fusarium acuminatum* from *Vitis riparia* which showed an inhibition index >100 against *B. cinerea*. Other fungi from the genus *Hypoxylon*, *Biscogniauxia*, *Peyronellaea* and *Lecythophora* which were also reported in this study showed some inhibitory activity against grey mould.

The studies conducted by Abdel-rahim and Abo-elyousr (2017) evaluated the biocontrol capacity of the yeast *Saccharomycopsis fibuligera* isolated from fruits of *Psidium guajava* L. on *B. cinerea*. These studies showed that *S. fibuligera* was able to inhibit the growth of *B. cinerea* by 48% in the *in vitro* bioassay, with inhibition areas of 27 mm. Moreover, *S. fibuligera* inhibited gray mold rot in guava fruit by 68%. Cosoveanu et al. (2014) isolated the endophytic fungi identified as *Botryosphaeria ribis, Phoma medicaginis, Bionectria ochroleuca, Aureobasidium pullulans, Chaetomium spirochaete* and A*lternaria* sp. from *Vitis vinifera* L. These fungi exhibited antagonistic activity

against *B. cinerea* and the extracts of *C spirochaete* and *B. ochroleuca* were those with the lowest effective concentration EC50 (the concentration which reduced mycelia growth by 50%), of 0.008 mg/mL and 0.09 mg/mL, respectively.

5. Compounds isolated from endophytic microorganisms with bioactivity against *B. cinerea*

Although biocontrol resulting from the synthesis of bioactive molecules has focused more on rhizospheric bacteria, this same mechanism applies to other endophytic microorganisms (Saraf et al. 2014). Many endophytes have the ability to biosynthesize a wide range of bioactive molecules with insecticidal, antibacterial, and antifungal properties (Dutta et al. 2014; Hardoim et al. 2015). The biosynthesis of these compounds can be induced by the presence of a pathogen in the host plant (Combés et al. 2012). Moreover, a single endophytic strain can produce multiple variants of each type of antimicrobial compound that confer a competitive advantage by eliminating other microorganisms (O´Brien 2017).

Lipopeptides are amphiphilic molecules that are synthesized non-ribosomally through multienzyme complexes and consist of a short peptide chain linked to a lipid tail, whose variations in the length and branching of fatty acid chains and the amino acid composition lead to remarkable heterogeneity (Stein 2005; Ongena and Jacques 2008; Farace et al. 2015). Lipopeptides are involved in processes such as plant tissue colonization, activation of the immune system in plants, induction of plant resistance to phytopathogens and direct antagonism against phytopathogens (Ongena and Jacques 2008; Farace et al. 2015). In the study conducted by Kefi et al. (2015) four strains identified as *Bacillus mojavensis, B. halotolerans, B. subtilis* and *B. amyloliquefaciens* that inhibited the growth of *B. cinerea* by 46%, 42%, 27% and 53% respectively, were

isolated from the roots, leaves and stems of *Lycopersicon esculentum*. The capacity of these strains to produce the lipopeptides, surfactin (**1**), fengycin (**2**) and iturin (**3**) was established using liquid chromatography-mass spectrometry. The strain *B. mojavensis* and *B. halotolerans* produced fengycin (**2**) and surfactin (**1**), while *B. subtilis* produced iturin (**3**) and surfactin (**1**). *B. amyloliquefaciens* secreted bacillomycin D (**4**), fengycin (**2**) and surfactin (**1**) (Figure 2). All four strains inhibited the lesions induced by *B. cinerea* in tomato leaves, *B. amyloliquefaciens* being the one which most reduced their severity (to 11%). The highly efficient antagonistic activity of *B. amyloliquefaciens* probably resulted from the synergy between bacillomycin D (**4**), surfactin (**1**) and fengycin (**2**). Soares et al. (2015) isolated the endophytic bacteria identified as *B. amyloliquefacien* sp. *plantarum* from *Hedera hélix*. This bacteria had an inhibition index of 50.0 ± 1.9% against *B. cinerea*. The genes responsible for the biosynthesis of surfactin (**1**), inturin (**3**), bacillomycin D (**4**), and fengycin (**2**) were detected in this strain and are related to the antifungal activity.

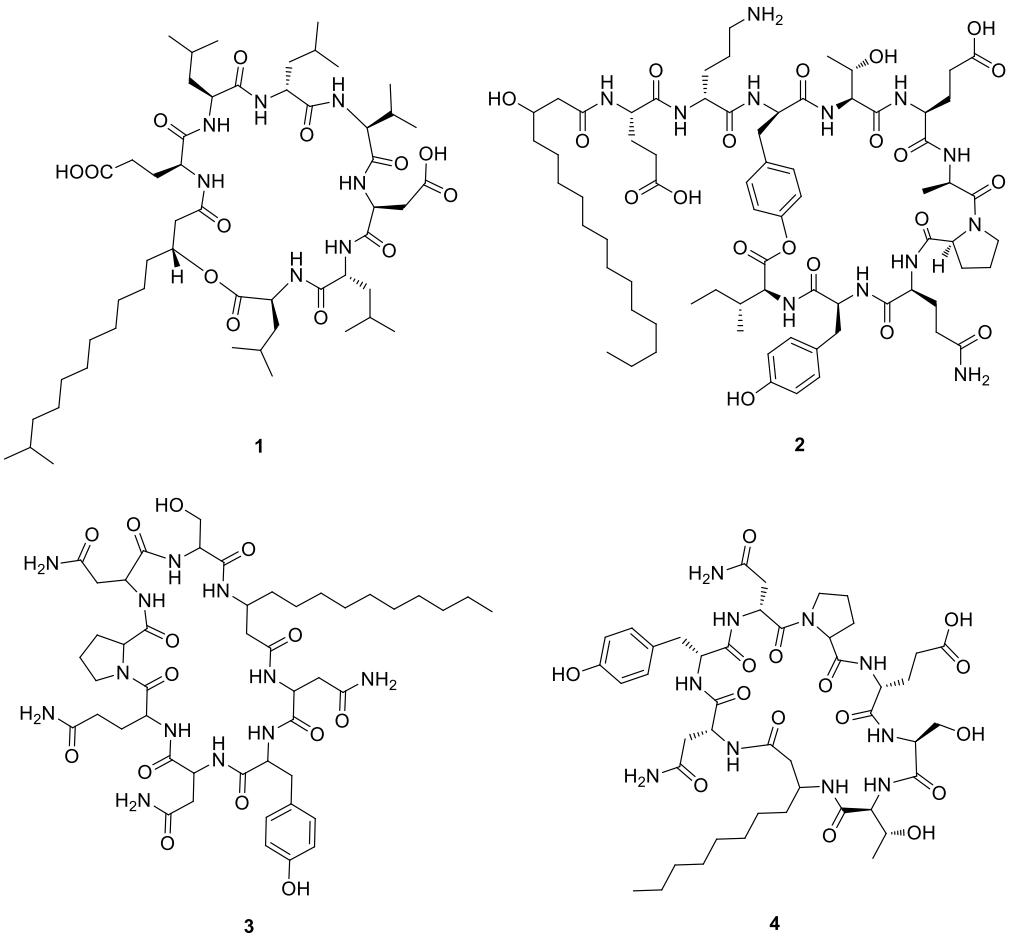

Fig 2 Chemical structure of compound **1-4**.

Liu et al. (2010) isolated and partially characterized the antifungal protein E2 synthesized by *B. subtilis* which had been obtained from the roots of *Triticum* sp. The Oxford cup assay established that the antifungal protein E2 at a concentration of 1.04 μg/mL, produced an inhibition area of 155 mm against *B. cinerea* after 3 days of incubation. Gao et al. (2017) characterized and evaluated the antifungal capacity of the VOCs produced by the endophytic bacteria *Bacillus velezensis* ZSY-1 isolated from leaves of *Catalpa ovata*. The VOCs produced by *B. velezensis* ZSY-1 exhibited significant antifungal activity against *B. cinerea* with an inhibition index of 92%. Twenty nine VOCs were detected in *B. velezensis* ZSY-1, 28 of which were evaluated against *B. cinerea*. Four of these compounds were identified as 2,5-dimethylpyrazine (**5**),

benzothiazole (**6**), 4-chloro-3-methylphenol (**7**), and 2,4-bis (1,1- dimethylethyl) phenol (**8**). Compounds **5**-**7** had an inhibition index of 100% against *B. cinerea*, whilst compound **8** had an index of 91%. However the provenance of some of these compound as natural products is uncertain.

Liarzi et al. (2016) isolated and characterized the endophytic fungus *Daldinia cf. concentrica* from a branch of *Olea europaea* L. and evaluated its ability to produce VOCs. They identified 27 different compounds including alcohols, dienes, ketones, aldehydes, and sesquiterpenes. The VOCs of *D. cf. concentrica* had an inhibition index of 100% against *B. cinerea*, *trans*oct-2-enal (**9**) being the most active compound against this phytopathogen with 100% inhibition of its growth and viability

In the study carried out by Park et al. (2015), an endophytic fungus from *Panax ginseng* was isolated and identified as *Phoma terrestris*. It was found to inhibit the growth of *B. cinerea* by 59% and 31% using disk diffusion and fermentation broth tests respectively. The ethyl acetate extracts of *P. terrestris* had an inhibition index of 89% against *B. cinerea* at a MIC of 100 $\mu g.\mu L^{-1}$, and an inhibition of more than 90% in spore germination at a concentration of 10 $\mu g.\mu L^{-1}$. The major metabolites in the *P. terrestris* extract were identified as *N*-amino-3-hydroxy-6-methoxyphthalimide (**10**) (32% of the total metabolites), 5H-dibenz[B, F]azepine (**11**) (7%), 3-methylthiobenzothiophene (**12**) (4%), 2-phenylindole (**13**) (4%), 5-(methoxycarbonyloxy) pent-3-yn-2-ol (**14)** (4%), and 5-hydroxydodecanoic acid lactone (pentylpyrone) (**15**) (4%).

Fu et al. (2011) studied the antifungal capacity of the endophytic fungus *Phomopsis* sp. By254 which had been isolated from the roots of *Gossypium hirsutum*. Three compounds identified as epoxycytochalasin H (**16**), cytochalasin N (**17**) and cytochalasin H (**18**) were isolated from the organic extract of *Phomopsis* sp. By254 cultured on a solid medium. These compounds were evaluated *in vitro* against *B. cinerea*

and showed an inhibition radius of between 1.0-5.0 mm for each of the compounds and an IC50 (μg/mL) of approximately 6 for epoxycytochalasin H (**16**), cytochalasin N (**17**) and cytochalasin H (**18**). The chemical structure of the compounds of **5-18** are shown in Figure 3.

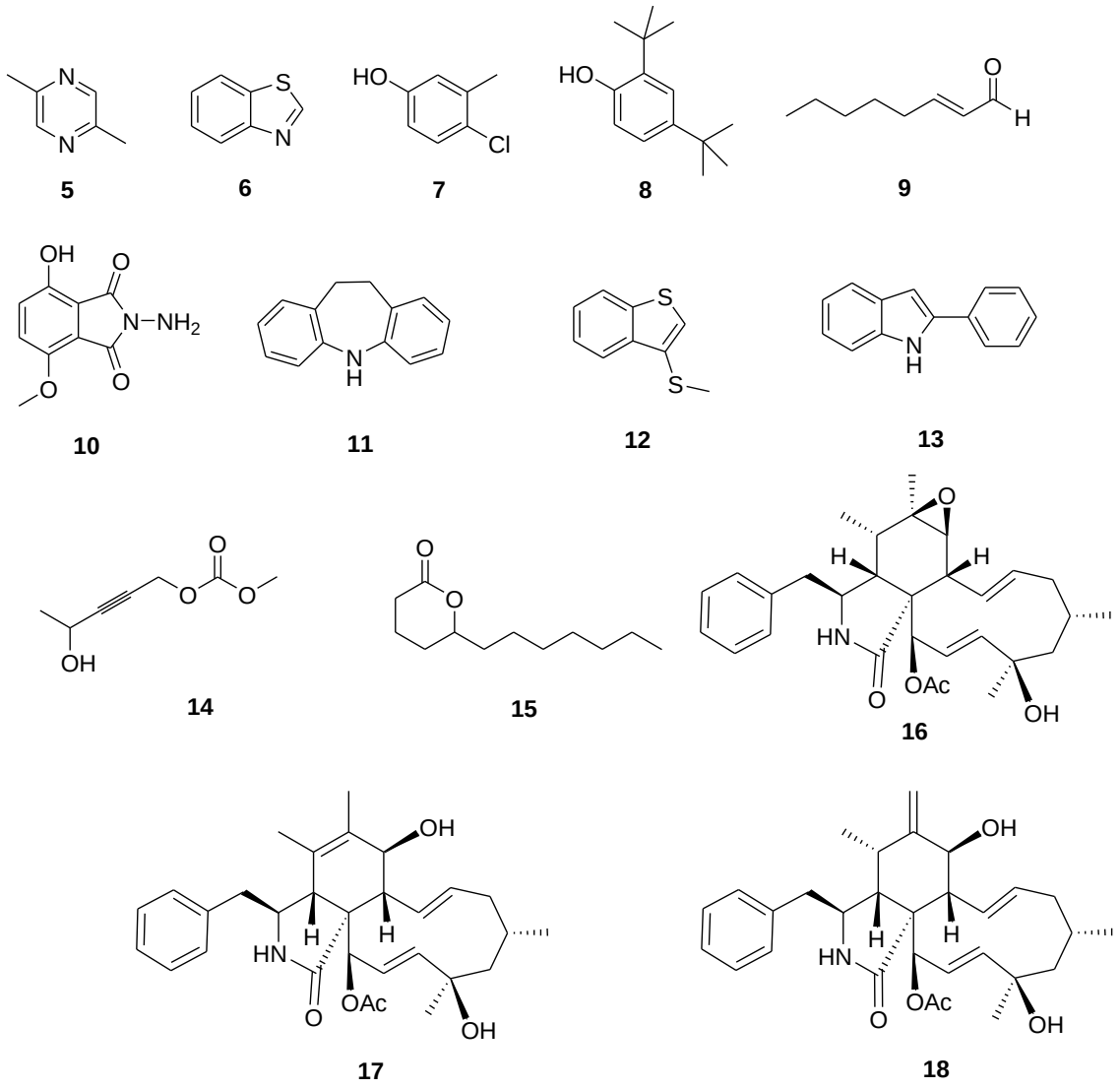

Fig 3 Chemical structure of compounds **5-18**.

Tomsheck et al. (2010) isolated an endophytic fungus from *Persea indica* which was identified as a *Hypoxylon* sp. This fungus produced a wide variety of VOCs including 1,8-cineole (**19**), 1-methyl-1,4-cyclohexadiene (**20**), and a compound which was tentatively identified as *α*-methylene-*α*-fenchocamphorone (**21**). The VOCs produced by *Hypoxylon* sp. had a 100% inhibition index against *B. cinerea*. However, when sub-cultured again, the gray mold remained viable. Zhao et al. (2012) purified four compounds identified as griseofulvin (**22**), dechlorogriseofulvin (**23**), 8-dihydroramulosin (**24**) and mellein (**25**) from a culture of the endophytic fungus *Nigrospora* sp. isolated from roots of *Moringa oleífera*. The four compounds proved active against *B. cinerea* at an IC50 concentration (µg/mL) of 0.20, 40, >100 and 49 for compounds **22-25**, respectively.

Terhonen et al. (2016) isolated two endophytic fungi from *Picea abies* which were identified as *Cryptosporiopsis* sp. and *Phialocephala sphareoides* B.J. Wilson. They inhibited the growth of *B. cinerea* by approximately 50%. The metabolites extracted from *Cryptosporiopsis* sp. also induced apical swelling at the tips of the hyphae and along the mycelium of *B. cinerea.* Zhang et al. (2008) isolated an endophytic fungus identified as *Aspergillus clavatonanicus* from a twig of *Taxus mairei.* They were able to isolate clavatol (**26**) and patulin (**27**), which are two polyketides capable of inhibiting *B. cinerea* with an IC50 mg/mL of 0.058 and 0.021 for **26** and **27**, respectively.

Rojas-Solís et al. (2018) isolated two endophytic bacteria identified as *Pseudomonas stutzeri* (E25) and *Stenotrophomonas maltophilia* (CR71) from *Physalis ixocarpa.* In the VOCs production tests, *P. stutzeri* (E25) and *S. maltophilia* (CR71) reduced the mycelial diameter of *B. cinerea* by more than 40% and 52%, respectively. In the direct co-inoculation assays, *S. maltophilia* (CR71) had an inhibition index of 24% and *P. stutzeri*

(E25) of only 12%. These results show that the antagonistic effect of these two bacterial strains is attributable to the VOCs and not to the production of diffusible compounds. A total of 34 VOCs were produced by the strains, 11 of which were produced by both strains, 7 were exclusive of *P. stutzeri* (E25) and 16 were exclusive of *S. maltophilia*. The VOCs produced in the highest quantity by the two strains were those containing sulfur: S-methylthiobutyrate (**28**), isobutyl isothiocyanate (**29**), 2-methylthioethanol (**30**), and dimethyl disulphide (DMDS) (**31**). Inhibition tests with **31** showed that it was more toxic when it was in direct contact with the phytopathogen and it produced an inhibition effect even at concentrations of 0.1 μM, while as a volatile product showed an inhibition effect at 10 μM. The chemical structure of compounds **19-31** are shown in Figure 4.

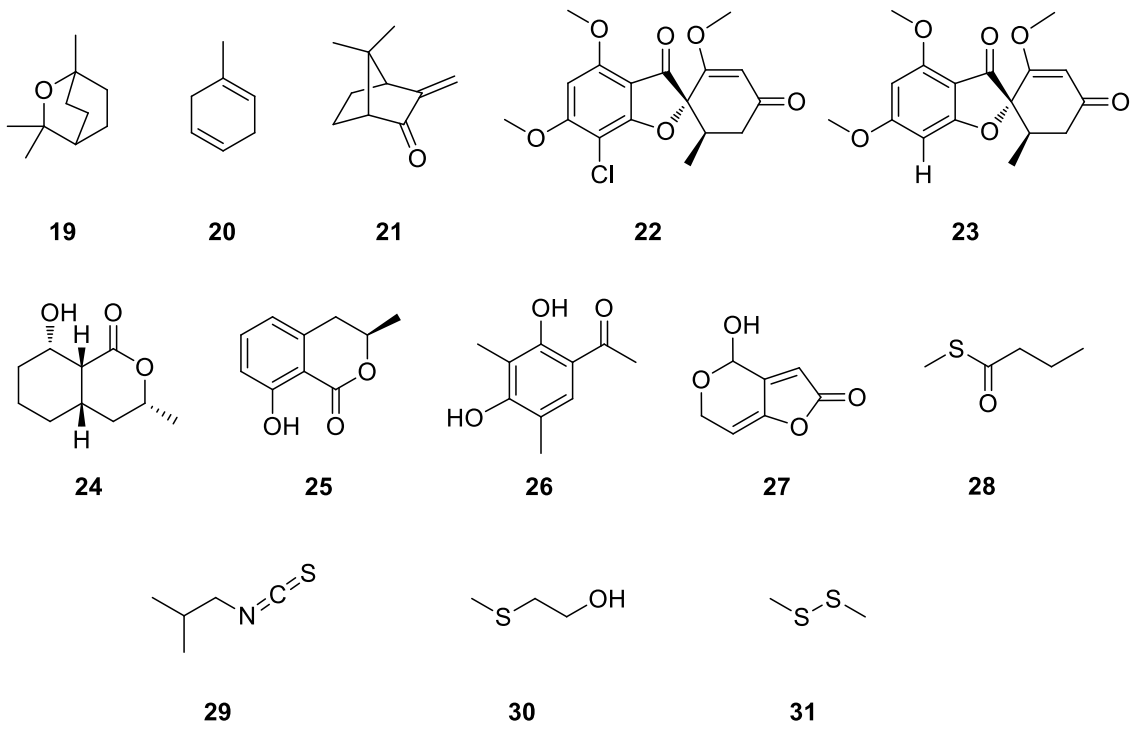

Fig 4 Chemical structure of compounds **19-31**.

Li et al. (2012) studied the metabolites of the endophytic fungus *Aspergillus fumigatus* LN-4 which had been isolated from the stem bark of *Melia azedarach*. Among

the various metabolites of the fungus, the compounds identified as fumitremorgin C (**32**), cyclotryprostatin B (**33**), verruculogen TR-2 (**34**), verruculogen (**35**), 12 *β*-hydroxy-13α-methoxyverruculogen TR-2 (**36**), fumitremorgin B (**37**), fumiquinazolines F (**38**), fumiquinazolines A (**39**), 3-hydroxyfumiquinazoline A (**40**), 4,8-dihydroxy-1-tetralone (**41**) and helvolic acid (**42**) were active against *B. cinerea*. Compounds **36**, **37** and **42** showed the greatest activity with an MIC of 6 µg/mL. Park et al. (2005) evaluated the antifungal capacity of the endophytic fungus *Xylaria* sp. isolated from the inner cortex of *Abies holophylla.* This fungus produced two compounds identified as griseofulvin (**22**) and dechlorogriseofulvin (**23**), which exhibited antifungal activity against *B. cinerea* with an IC50 (µg/mL) of 5 and > 200, respectively. Evaluation of the *in vivo* activity of these two compounds on tomatoes showed that at a dose of 150 µg/mL, griseofulvin has an inhibition index of 60% against *B. cinerea*, while dechlorogriseofulvin at the same concentration had an inhibition index of only 25%. The chemical structure of compounds of **32-42** are shown in Figure 5 and table 4 summarizes the compounds isolated from endophytic microorganisms.

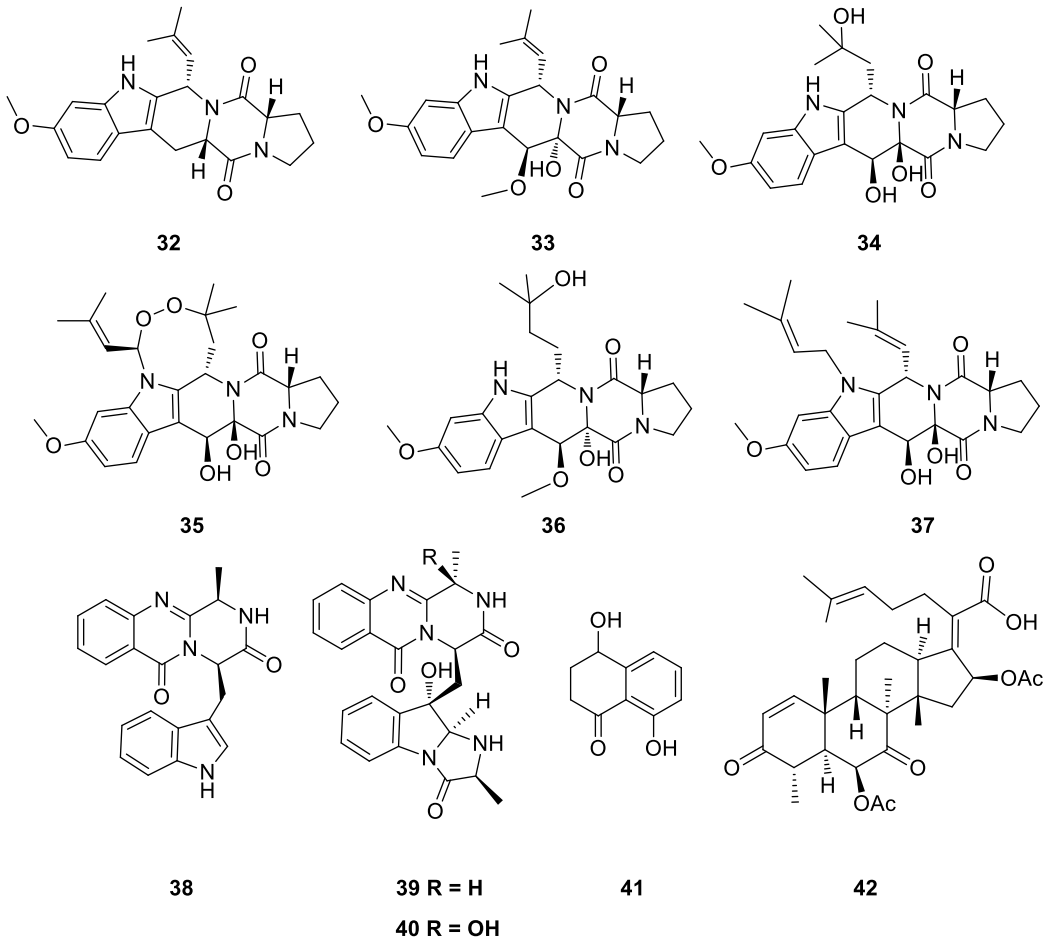

Fig 5 Chemical structure of compound **32-42**

Table 4. Compounds isolated from endophytic microorganisms

Microorganism	Compound	Reference
Bacillus mojavensis *B.halotolerans* *B. subtilis* *B. amyloliquefaciens*	**1-4**	Soares et al. 2015 Kefi et al. 2015
B. subtilis	Protein E2	Liu et al. 2010
B. velezensis ZSY-1	**5-8**	Gao et al. 2017
Daldinia cf. concentrica	**9**	Liarzi et al. 2016
Phoma terrestris	**10-15**	Park et al. 2015
Gossypium hirsutum	**16-18**	Fu et al. 2011
Hypoxylon sp.	**19-21**	Tomsheck et al. 2010
Nigrospora sp.	**22-25**	Zhao et al. 2012
Aspergillus clavatonanicus	**26-27**	Zhang et al. 2008
Pseudomonas stutzeri (E25) *Tenotrophomonas maltophilia* (CR71)	**28-31**	Rojas-Solís et al. 2018
Aspergillus fumigatus LN-4	**32-42**	Li et al. 2012

Conclusions

In this review we have noted that *B. cinerea* is considered to be a high-risk pathogen in terms of its resistance to fungicides. There are now limiting factors in terms of its chemical control. The use of many fungicides may become unsustainable in the context of their effect on human health and the environment. Consequently the search for new environmentally-friendly alternatives for the control of *B. cinerea* which do not have adverse effects, is an important area for study. In this context the study of endophytic micro-organisms that establish a close relationship with their host plant could lead to the discovery of new biological control agents and bioactive molecules of interest.

In order to examine the control of *B. cinerea* by this means, it is worth considering aspects of the interaction between endophytic organisms and nectrotrophic organisms such as *B. cinerea* in the wild. The role of the necrotrophic organism is to facilitate the decay of the plant after senescence and the recycling of its constituents and, in the case of fruit containing the seed, to provide a nutrient base for the seed to germinate. Amongst its other properties, the role of the endophytic organism in this context is to protect the plant against premature attack by a necrotrophic organism prior to senescence. Thus the endophytic organism is playing a regulatory role in the life cycle of the plant. When the plant reaches senescence the conditions within the plant that favour the growth of the endophyte (water, nutrient, nitrogen source) may cease allowing the nectrotrophic organism (e.g. *B. cinerea*) to flourish. Thus for the use of endophytic organisms to protect plants, the conditions that favour their growth and metabolite production particularly within the plant, must be understood and these must be maintained especially as the plant reaches maturity.

The fact that the same plant may host several different endophytic organisms, each with its own special range of anti-microbial metabolites, could be considered as the

natural way of overcoming the development of resistance. If the invasive organism, in this case *B. cinerea* begins to develop resistance to one set of anti-microbial metabolites, there are different metabolites that are also present which have been produced by other endophytic organisms that can combat the resistant strains before they can pass on the resistance to the next generation. This multiplicity of endophytic organisms needs to be considered when they are being evaluated for use as biocontrol agents. It might be wise not to rely on just one organism as a biocontrol agent against phytopathogenic fungi.

In the immediate future there are several questions that must be solved in order to provide a rational basis for the biocontrol of phytopathogenic fungi by endophytes. As previously indicated, endophytic organisms are not 'inert passengers' within their host. There is already evidence for a chemical communication between the endophyte and its host which needs to be explored much more thoroughly particularly in the context of the stressed plant. A well-known strategy for restoring secondary metabolite production by a fungus weakened by repeated sub-culturing, is to grow it on its host plant. There is a question as to whether this chemical communication changes when a plant is infected and produces a phytoalexin. Does the phytoalexin have an effect on the endophytic organisms by, for example, activating the silent or 'orphan' genes to produce 'cryptic' metabolites which might be anti-microbial? It is known that plants when attacked produce volatile organic compounds such as methyl salicylate which convey a warning to healthy plants that an attack by an invasive organism may be imminent. The healthy plants respond by activating their defense mechanisms. Are their endophytic organisms part of this response and do they then start to produce anti-microbial metabolites?

It is important to point out that most of the metabolites listed in this review have been isolated from the endophyte which has been cultured in the absence of its host. Fungal metabolite production is notoriously sensitive to the medium. Consequently

in the future the metabolite production by the endophyte needs to be examined in terms of bipartite host:endophyte and tripartite host:endophyte:pathogen relationships.

Although a number of endophytic bacteria and fungi and their metabolites have exhibited the potential to exert biocontrol against *B. cinerea*, their widespread use requires development. Furthermore of the more than 300,000 species of plants that have been described, the accompanying microbiota of only a handful of these have been studied. This relatively unexplored field is therefore seen as an interesting source of new micro-organisms and of their metabolites particularly in the context or their ecological role and exploitation.

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