Environmentally friendly methods for controlling pine pitch canker

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Fusarium circinatum is a highly virulent invasive pathogen that causes the disease commonly known as pine pitch canker (PPC). On mature trees, the most common symptoms of PPC include resin bleeding, resinous cankers, wilting of needles and dieback. In nurseries the main symptoms are yellowish needles, and wilting of foliage and shoots. PPC is considered one of the most important diseases of conifers globally and at least 60 species of *Pinus* along with *Pseudotsuga menziesii* are known to be susceptible. Quarantine regulations are crucial to minimize the risk of new introductions into disease-free countries. However, if these measures fail, implementation of environmentally friendly control methods is currently encouraged in an integrated management approach that minimizes the use of chemicals in forests. This review therefore summarizes current knowledge of biological control using endophytic fungi, bacteria and viruses, and other environmentally friendly methods to control PPC, including thermotherapy, natural products with fungicidal effects, promoters of innate host resistance mechanisms and breeding for resistance. Key research gaps to be addressed for improvement of effective management of this disease are discussed.

Keywords: endophytes, *Fusarium circinatum*, genetic resistance, natural compounds, thermotherapy, viruses

Introduction

Forests are highly important ecosystems, covering approximately one-third of the world's land area, providing significant sources of commercial products and ecosystem services, and exerting a powerful influence on the global carbon cycle. Conifers, mainly comprising

Pinus spp., account for 112.95 million ha of the European forested area (de Rigo *et al.*, 2016). The genus *Pinus* is amongst the most ecologically and economically significant trees globally, which, in addition to numerous biological roles in forest ecosystems, represents a major source of timber, pulp and paper, edible seeds, charcoal and resin (Richardson & Rundel, 2000).

　Forest pathogens, especially invasive alien species, introduced into countries as a result of globalization of

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trade and free market practices, not only result in serious environmental and social impacts, but also important economic losses (Holmes *et al.*, 2009; Vettraino *et al.*, 2015; Wingfield *et al.*, 2015). Phytosanitary regulations often fail to prevent biological invasions (Liebhold *et al.*, 2012; Eschen *et al.*, 2015; Klapwijk *et al.*, 2016) and, as a result, the recorded number of invasive and damaging forest pathogens has increased exponentially in the recent past (Stenlid *et al.*, 2011; Santini *et al.*, 2013). New approaches to pest and disease management are urgently required to preserve the multiple services provided by forests in the face of increasing risks imposed by globalization and climate change (Boyd *et al.*, 2013; Wingfield *et al.*, 2015; Oliva *et al.*, 2016).

Fusarium circinatum (teleomorph = *Gibberella circinata*) is a highly virulent invasive pathogen that causes the disease commonly known as pine pitch canker (PPC). The disease is considered as one of the most important of conifers globally (Wingfield *et al.*, 2008), and at least 60 species of *Pinus* along with *Pseudotsuga menziesii* are known to be susceptible to PPC (Dwinell, 1999; Bezos *et al.*, 2017). Spores and mycelial fragments produced by *F. circinatum* in infected trees can be disseminated by wind, rain, insects, and via humans through management and recreational activities. Insects and other agents can create wounds that provide entry points for the pathogen. Infection success appears to be associated with locations and seasons where atmospheric moisture is readily available and temperatures are relatively warm. Seeds can become infected and if these infected seeds are collected and planted in nurseries, the resulting seedlings will be infected. Additionally, once the fungus has established in a nursery, previously healthy seedlings can become infected. When these infected seedlings are planted in the forest, the disease will establish in the plantations, thereby closing the cycle.

On forest or plantation trees, the most common symptoms of PPC include resin bleeding, resinous cankers on the main stems, terminal or large lateral branches, wilting of needles and dieback. Roots, shoots, female flowers and mature cones can also be affected. Cankers on the main stems are lethal when the stems are girdled or as a result of loss in structural integrity and stem breakage at the site of canker formation (Hepting & Roth, 1946; Barrows-Broaddus & Dwinell, 1985; Barrows-Broaddus, 1990; Pintos *et al.*, 2008). In nurseries the main symptoms are yellowish green needles, foliar and shoot wilting, foliar discolouration (Viljoen *et al.*, 1994) and sometimes resin at the neck section of cuttings and mother plants (R. Ahumada, Bioforest S.A., Concepción, Chile, personal communication). The fungus also causes dieback and mortality in hedge and cutting production, as well as damping-off in seedlings, with mortalities of up to 100% (Martínez-Álvarez *et al.*, 2014a). The occurrence of these symptoms depends on many factors, including the host species, and the biotic and abiotic conditions of the host (Wingfield *et al.*, 2008) and mother tree (Vivas *et al.*, 2013). However, some infected trees in affected forests and nurseries can remain symptomless

for long periods of time (Storer *et al.*, 1998; Mitchell *et al.*, 2004; Kim *et al.*, 2008; Vivas *et al.*, 2012b; Elvira-Recuenco *et al.*, 2015; Swett *et al.*, 2016).

Fusarium circinatum infection of pines was first detected in 1945 in the southeastern United States (Hepting & Roth, 1946), but is now reported from Haiti (Hepting & Roth, 1953), Japan (Kobayashi & Muramoto, 1989), South Africa (Viljoen *et al.*, 1994), Mexico (Guerra-Santos, 1998), South Korea (Lee *et al.*, 2000), Chile (Wingfield *et al.*, 2002), Uruguay (Alonso & Bettucci, 2009), Colombia (Steenkamp *et al.*, 2012) and Brazil (Pfenning *et al.*, 2014). Spain was the first European country where the disease was officially detected in 2003 (Landeras *et al.*, 2005), and subsequently the disease has become established in Portugal (Bragança *et al.*, 2009). *Fusarium circinatum* was also reported in France (EPPO, 2006) and Italy (Carlucci *et al.*, 2007), although PPC disease in these countries has been officially eradicated.

Fusarium circinatum is included in the EPPO A2 list, which includes species present in the EPPO region but not widely distributed, and recommends their regulation as quarantine pathogens. Consequently, the presence of *F. circinatum* in a European country entails several restrictions (Vettraino *et al.*, 2018). The European Union established emergency measures to prevent its introduction into, and spread within, the Community in 2007 (Commission Decision 2007/433/EC). In Spain, for example, legislation includes bans on planting susceptible species like *Pinus* spp. and *P. menziesii* in affected areas, the movement of plant material and wood from infected to uninfected areas (Spanish Royal Decree 637/2006 and 65/2010), and disease monitoring and control in the infested areas or facilities (in nurseries) over at least 2 years.

The European Food Safety Authority (EFSA) has established that over 10 million hectares of pine forests in Europe are at risk of *F. circinatum* infection (EFSA, 2010). This estimation was based on current host distribution ranges and the climatic conditions that are likely to be conducive for disease. The current priority in Europe is avoiding the dispersal of *F. circinatum* into disease-free countries; therefore, efforts have been largely devoted to controlling the main pathways of introduction. These include the movement of plants for planting and especially seed lots (EFSA, 2010). Recently, Martín-García *et al.* (2017) reported the susceptibility of *Picea abies* seedlings, the main forest tree in central to northern and eastern Europe, to *F. circinatum*. Moreover, the inoculation of *P. abies* and *Larix decidua* seedlings from the Czech Republic with *F. circinatum* resulted in infected but symptomless plants, representing a new risk of introducing the pathogen from other species in the Pinaceae (Martín-García *et al.*, 2018).

Several studies have tested the efficacy of fungicides to control *F. circinatum* growth *in vitro* (Allen *et al.*, 2004; Mitchell *et al.*, 2004; Carey *et al.*, 2005; Landeras *et al.*, 2006; Ramón-Albalat *et al.*, 2010; Iturritxa *et al.*, 2011, 2013b; Berbegal *et al.*, 2015; Mullett *et al.*, 2017), and

using *in vivo* assays for seeds (Muñoz López *et al.*, 2009; Berbegal *et al.*, 2015) and woody material (Serrano *et al.*, 2015). Although some chemicals were promising, their use in forests is highly restricted in Europe (Directive 2009/128/EC), while more environmentally friendly management approaches are encouraged. In this regard, it is proposed that other environmentally friendly methods, including biological control, are implemented in an integrated management approach towards PPC control, including methods such as thermotherapy, natural products with fungicidal effects, promoters of innate host resistance mechanisms and breeding for resistance.

This paper reviews and discusses the environmentally friendly control strategies that could potentially be applied to manage PPC. A general background on biological control is provided, followed by how these approaches have been applied against PPC. Other non-chemical methods, such as hot water treatment (HWT) of seed to limit the spread of PPC and the potential of exploiting genetic resistance against *F. circinatum*, are discussed. Finally, potential areas of research are highlighted that would ultimately enable effective management of this important disease.

The use of biological control for combating *F. circinatum*

Biological control has been applied in agriculture since the early twentieth century as part of an integrated strategy to increase crop production while minimizing the environmental contamination caused by fungicides and pesticides (Cook & Baker, 1996). Biological control has gained relevance in the last decades not just because of environmental awareness, but also due to the growing social demand for effective plant disease control using environmentally friendly management strategies as alternatives to xenobiotic pesticides. The term biological control developed from 'any control achieved through a living system with the exception of man' (Cook & Baker, 1996) to 'the use of microbial antagonists to suppress diseases, including natural products extracted or fermented from various sources' (Pal & McSpadden Gardener, 2006). In this paper, biological control is considered as any control measure based on the use of antagonistic living organisms, including endophytic fungi, bacteria and viruses.

Several mechanisms of biological control that reduce the abilities of plant pathogens to cause disease have been identified. Antibiosis is the inhibition or destruction of an organism by metabolites, enzymes or other lytic agents produced by the antagonist (Jamalizadeh *et al.*, 2011). Competition occurs when the antagonist competes for limited space or nutrients more efficiently than the pathogen inside and/or around the host (Sharma *et al.*, 2009; Di Francesco *et al.*, 2016). Parasitism or predation occurs when the antagonist is able to attack the pathogen killing it either directly or indirectly by targeting its propagules (Heydari & Pessarakli, 2010). Induction of plant resistance is another mechanism by which antagonists can minimize disease incidence (Alves-Santos &

Diez, 2011), but this aspect will be treated separately in this review.

As frequently occurs with other disease management methods, biological control measures alone are often not effective enough to give full control of a given disease, especially in forest environments, where biotic and abiotic factors can significantly modulate the efficacy of the antagonists. Biological control methods against PPC, for example, are most efficiently implemented in an integrated management approach, which also includes sanitary and silvicultural measures (Wingfield *et al.*, 2015; Cazorla & Mercado-Blanco, 2016).

Endophytic fungi and antagonism by *Trichoderma* spp.

Microbial diversity, in particular fungal diversity, has been poorly studied in comparison to macroscopic life forms. Of the estimated 5.1 million fungal species on Earth (Blackwell, 2011), only approximately 70 000 fungi have been actually described (Blackwell, 2011). It is likely that endophytic species in complex biomes comprise a large proportion of those undescribed taxa.

Endophytic fungi are able to spend the entire or part of their life cycle within plant tissues, in either the apoplast or symplast, without causing visible symptoms to the host plants (Saikkonen *et al.*, 1998). Many endophytic fungi promote host growth and provide increased tolerance towards biotic and abiotic stresses (Pirttilä & Frank, 2011; Sanz-Ros *et al.*, 2015). There is increasing evidence that the way by which endophytes confer higher tolerance to pathogens is linked to interactions between beneficial microorganisms and plants, which in turn trigger an immune defensive response reaction against pathogens (Zamioudis & Pieterse, 2012).

The potential for endophytes as biological control agents (BCAs) has mainly been tested in agricultural plant pathosystems, but more recent studies have also suggested that endophytes could also be used against forest diseases (Martínez-Álvarez *et al.*, 2012; Blumenstein *et al.*, 2015; Romeralo *et al.*, 2015; Martínez-Álvarez *et al.*, 2016; Terhonen *et al.*, 2016). Many endophytic fungi have coevolved with their hosts (Krings *et al.*, 2012) and are widespread in forests (Saikkonen *et al.*, 1998; Sieber, 2007; Partida-Martínez & Heil, 2011).

Several endophytic fungi have been tested as BCAs against *F. circinatum* (Bezos *et al.*, 2017). *Clonostachys rosea* parasitized hyphae, reducing mycelial growth of *F. circinatum* in *in vitro* assays, and decreasing post-emergence mortality, with an increase in *Pinus radiata* seedling survival from 5% to 69% (Moraga-Suazo *et al.*, 2011). On the other hand, of 154 isolates tested from a broad range of genera (*Diaporthe* sp., *Bionectria* sp., *Phomopsis* sp., *Biscogniauxia* sp., *Truncatella* sp., *Macrophomina* sp., *Nectria* sp., *Alternaria* sp.), 138 showed antagonist effects towards *F. circinatum in vitro* (Martínez-Álvarez *et al.*, 2016; Fig. 1). The same authors also reported reduction of damage caused by *F. circinatum* under field conditions in 2-year-old *P. radiata* seedlings previously inoculated with *Chaetomium aureum*.

Penicillium chrysogenum and *Fusarium lateritium*, endophytic fungi associated with phloem-feeding insects colonizing *P. radiata*, also outcompeted *F. circinatum* (Romón *et al.*, 2008). *Penicillium chrysogenum* is well known for producing large amounts of secondary metabolites with antifungal activity, including hypocrellin B (Meng *et al.*, 2011), and *F. lateritium* produces enniatins, which exhibit strong antifungal activity (Tsantrizos *et al.*, 1993).

Trichoderma species are the best-studied fungal BCAs and several isolates are marketed as commercial biopesticides (Vinale *et al.*, 2008; Hermosa *et al.*, 2012). The wide range of specific biocontrol mechanisms for species in this genus is probably linked to the broad spectrum of pathogens on which the BCA properties of *Trichoderma* species have been demonstrated, which include diseases caused by pathogens in the genera *Fusarium*, *Rhizoctonia*, *Botrytis*, *Sclerotinia*, *Phytophthora* and *Pythium*, amongst many others (Benítez *et al.*, 2004; Harman *et al.*, 2004; Vinale *et al.*, 2008; Aleandri *et al.*, 2015). Although the beneficial role of *Trichoderma* species on plant fitness is well documented, a few reports also describe negative effects on plants (Menzies, 1993; Marín-Guirao *et al.*, 2016).

Several *Trichoderma* species, including *T. harzianum*, *T. viride*, *T. atroviride*, *T. asperellum*, *T. virens* and *T. spirale*, have been tested as BCAs against *F. circinatum* (Iturritxa *et al.*, 2011; Moraga-Suazo *et al.*, 2011; Martínez-Álvarez *et al.*, 2012, 2016; López-López *et al.*, 2016; Martín-García *et al.*, 2017). Evidence for antagonism by *Trichoderma* species towards *F. circinatum* was consistent in *in vitro* experiments. *Trichoderma* species significantly inhibited growth of the pathogen in *in vitro* assays, ranging from 40% to 60% (Moraga-Suazo *et al.*,

Figure 1 Antagonistic effect of *Chaetomium aureum* (right) against *Fusarium circinatum* (left) *in vitro*.

2011; Martínez-Álvarez *et al.*, 2012) to 100% (Iturritxa *et al.*, 2011). However, the potential antagonistic effects of *Trichoderma* species *in planta* are inconclusive. López-López *et al.* (2016) reported that the presence of *T. asperellum* led to a *c*. 50% reduction in disease incidence on *P. radiata* seedlings in terms of both pre- and post-emergence damping-off. In contrast, Martínez-Álvarez *et al.* (2012) found no antagonism exerted by *T. viride* towards *F. circinatum* in *P. radiata* seedlings. These differences may be due to the timing of the application of *Trichoderma*, because Martínez-Álvarez *et al.* (2012) applied the pathogen and the BCA at the same time. The importance of adding the *Trichoderma* at least 7 days prior to *F. circinatum* inoculation was reported previously (Iturritxa *et al.*, 2011; Moraga-Suazo *et al.*, 2011). In later work, the application of the same *Trichoderma* strain used by Martínez-Álvarez *et al.* (2012) 1 week before *F. circinatum* inoculation presented promising results on Romanian provenances of *Pinus mugo* and *P. abies* (Martín-García *et al.*, 2017).

In the field, strains of *Trichoderma* that were efficient *in vitro* did not exert any protection towards *F. circinatum* in 2-year-old *P. radiata* plants (Martínez-Álvarez *et al.*, 2016), probably because *Trichoderma* was inoculated in the stem instead of the soil. In this regard, the microbiota present in the soil are able to modulate the antagonistic effect exerted by *Trichoderma* (López-López *et al.*, 2016) and the initial positive responses decrease with time (Mitchell *et al.*, 2004). In Chile, several trials have been carried out using over 15 different biocontrol organisms applied to growing substrates during the plant production process (R. Ahumada, Bioforest S.A., Concepción, Chile, personal communication). The most common organisms used were *Trichoderma* species (*T. viride*, *T. harzianum*, *T. longibrachiatum*, *T. polysporum* and *T. atroviride*), giving up to *c*. 50% reductions in mortality (control of *F. circinatum*) during plant production. Further work in the field with *Trichoderma* and other potential BCA species is needed to unravel the effects of the environment and application timing before implementing the use of *Trichoderma* species as BCAs against *F. circinatum* in the production of pine plants.

Little is known regarding the mechanisms of antagonism of *Trichoderma* spp. against *F. circinatum*. Moraga-Suazo *et al.* (2011) demonstrated that the capacity of *Trichoderma* spp. to grow rapidly within plant tissues, provides these species with a competitive advantage for nutrients and space over *F. circinatum*. This mechanism of 'competition' had been already reported with *Trichoderma* spp. against other pathogens, including *Fusarium* species (Benítez *et al.*, 2004; Vinale *et al.*, 2008). However, Moraga-Suazo *et al.* (2011) found no evidence of parasitism in their work, despite the phenomenon being well known with species of *Trichoderma* on other fungi, with effects including hydrolysis of cell walls, hyphal penetration and subsequent dissolution of the host cytoplasm (Howell, 2003; Brotman *et al.*, 2010). Several proteins secreted by *T. atroviride* and *T. harzianum* have been associated with the
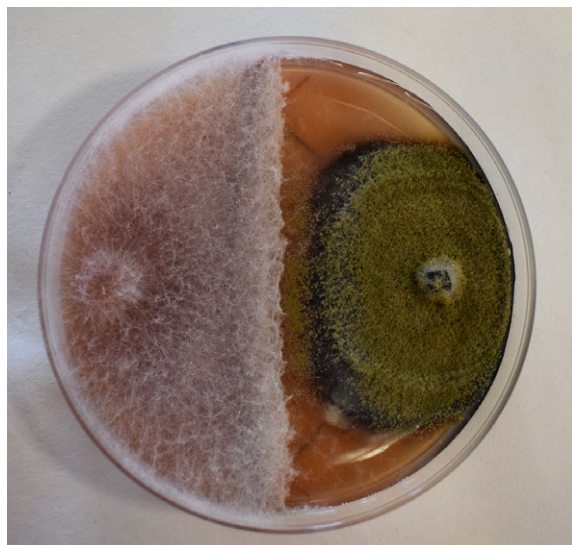
process of mycoparasitism (de Lima *et al.*, 2016, 2017). Similarly, although several studies have reported antibiosis as a mechanism of action of *Trichoderma* spp. to other *Fusarium* species (Calistru *et al.*, 1997; Ferre & Santamarina, 2010), no information is available for *F. circinatum*.

Bacteria

Endophytic bacteria have long been applied in agricultural soils as plant growth-promoters (de Souza *et al.*, 2015) and BCAs of plant diseases (Compant *et al.*, 2005). Although the use of endophytic bacteria to control *Fusarium* species is common in agricultural crops (Singh *et al.*, 1999), the available information on bacteria as BCAs of forest tree diseases is comparatively scarce. *Pseudomonas fluorescens*, *Bacillus simplex* and *Erwinia billingiae* isolated from the endorhizosphere of a symptomless *P. radiata* pine reduced mycelial growth of *F. circinatum in vitro* and lesion lengths on 2-year-old *P. radiata* seedlings inoculated with the pathogen by 17–29% and 22–25%, respectively (Iturritxa *et al.*, 2017). *Bacillus subtilis* and *Burkholderia* sp. inhibited *F. circinatum* growth *in vitro* (Soria *et al.*, 2012). Inhibition of growth of *F. circinatum* by strains of *Micromonospora* isolated from alfalfa root nodules was also reported *in vitro* (Martínez-Hidalgo *et al.*, 2015). *Micromonospora* strains were effective in reducing leaf infection caused by *Botrytis cinerea* on tomato (Martínez-Hidalgo *et al.*, 2015), but have not been tested on pines to date. In Chile, experience with bacteria showed very variable results in the reduction of pine mortality (in the range of 20–60%) caused by *F. circinatum* during plant production in nurseries. Bacteria tested included *B. subtilis*, *Bacillus megaterium* and *Bacillus licheniformis*. However, other species such as *Acidovorax facilis*, *Azotobacter chroococcum*, *Pseudomonas aureofaciens*, *Pseudomonas fluorescens* and *Rhodococcus rhodochrous* showed similar results (R. Ahumada, Bioforest S.A., Concepción, Chile, personal communication). The abilities of bacteria to control pathogens in woody plants (e.g. Ren *et al.*, 2013; Cazorla & Mercado-Blanco, 2016) emphasizes their potential for controlling *F. circinatum*.

Viruses

Mycoviruses are intracellular parasites of fungi, which spread via cytoplasmic contact that results from hyphal fusions. Most mycoviruses are composed of double-stranded RNA, while linear single-stranded RNA and circular DNA genomes are less represented (around 30%; King *et al.*, 2012). Virocontrol (biocontrol with viruses) of fungal plant pathogens was demonstrated when hypoviruses (mycoviruses that reduce their host's ability to cause plant disease) were used to control the chestnut blight fungus *Cryphonectria parasitica* in Europe (Rigling & Prospero, 2018). Although the success of chestnut blight disease virocontrol has been limited (Robin *et al.*, 2000; Milgroom & Cortesi, 2004), it has inspired researchers throughout the world to search for viral

control agents against other fungal diseases, leading to the discovery of many novel virus taxa that mediate hypovirulence in plant pathogenic fungal species (Huang & Ghabrial, 1996; Lakshman *et al.*, 1998; Preisig *et al.*, 2000; Chu *et al.*, 2002; Deng *et al.*, 2003; Kanematsu *et al.*, 2004; Chiba *et al.*, 2009; Yu *et al.*, 2010). Protoplast fusion is a promising methodology that enabled transmission of hypovirulence, caused by viral infection in *Fusarium graminearum*, to other *Fusarium* species (Lee *et al.*, 2011). However, most mycoviruses tested have very little, variable or no effects on their host fungi (e.g. Vainio *et al.*, 2010; Hyder *et al.*, 2013) and may even be mutualists with the hosts (Márquez *et al.*, 2007). Thus, only a tiny fraction of fungal viruses may be considered as potential BCAs.

In order to develop an efficient virocontrol application for fungal pathogens of plants, several steps are required: (i) identify a virus with the potential to reduce the damage caused by the target pathogen; (ii) check the ability of the virus to infect most of the strains of the pathogen; (iii) develop a cost-efficient method to disseminate the virocontrol agent into the pathogen population; and (iv) test if the virocontol agent is able to persist and infect most of the fungal strains in natural conditions.

Fusarium circinatum hosts several species and types of mitoviruses (Martínez-Álvarez *et al.*, 2014b; Vainio *et al.*, 2015), but the role the viruses play in pathogen virulence is not clear. While virulence of *F. circinatum* was enhanced on 1-year-old *P. radiata* seedlings (Muñoz-Adalia *et al.*, 2016), mycelial growth of *F. circinatum* colonies and spore germination were significantly reduced by the virus presence under *in vitro* conditions (Flores-Pacheco *et al.*, 2017). Current understanding of the occurrence of hypovirulence caused by viruses in *F. circinatum* remains poor. Indeed, the occurrence of these viruses in *F. circinatum* populations outside Europe has not been investigated. In addition, viruses other than mitoviruses may have remained undetected so far.

Development of a cost-efficient method for disseminating the identified viruses is also complex. The work-intensive manual treatment used in chestnut trees is too expensive for other forest trees that form continuous and large forests. The nature of *F. circinatum* as a stem canker disease with separate infections on each tree also complicates the dissemination of viruses for control purposes. However, the clonal population structure of *F. circinatum* in many areas of introduction (Hammerbacher, 2005; Berbegal *et al.*, 2013) is an advantage for the natural spread of mycoviruses, as spread is often extremely limited between mycelia belonging to different vegetative compatibility groups (e.g. Anagnostakis & Day, 1979).

Other environmentally friendly approaches for controlling *F. circinatum*

Thermotherapy and atmospheric pressure nonthermal plasma treatments

Fusarium circinatum can be transmitted through infested seeds and this pathway is a major source of nursery

infections (Storer *et al.*, 1998; Wingfield *et al.*, 2008). Seeds can carry the pathogen not only externally, where the seed surface carries inoculum, but also internally, where the pathogen is present inside the endosperm (Storer *et al.*, 1998; Wingfield *et al.*, 2008). Nonsuperficial infections can be either active or latent, and the pathogen can be isolated even from symptomless seedlings (Storer *et al.*, 1998; Mitchell *et al.*, 2004; Kim *et al.*, 2008; Vivas *et al.*, 2012b; Elvira-Recuenco *et al.*, 2015). The use of uninfected seeds is the most effective measure to prevent PPC in nurseries and avoid the introduction of *F. circinatum* into areas currently free of the pathogen (Agusti-Brisach *et al.*, 2012; Berbegal *et al.*, 2015). In this regard, early detection at border controls is essential to avoid new introductions and, therefore, proper surveys and diagnostic methodologies (EPPO, 2009) should be implemented. Numerous treatments have been proposed to minimize infection from seeds. Chemical treatments, including fungicides, can effectively reduce surface contamination of pine seeds by *Fusarium* spp. (Dumroese *et al.*, 1988; Runion & Bruck, 1988; Allen *et al.*, 2004). However, few fungicides penetrate the seed coat and reduce internal seed contamination without a significant negative effect on seed germination (Agusti-Brisach *et al.*, 2012).

Hot water treatment, where heat penetrates the seeds, controls nonsuperficial infections (Baker, 1962; Gratwich & Southey, 1986; Grondeau *et al.*, 1994). HWTs have been shown to successfully control a number of seedborne pathogens, including other *Fusarium* species, without a significant effect on seed germination (e.g. du Toit & Hernandez-Perez, 2005; Bennett & Colyer, 2010). Immersion of *Pinus palustris* seeds in 60 °C water for 2 min reduced *F. circinatum* to trace levels, while still producing high numbers of viable seedlings (Jones *et al.*, 2002). A more extensive study by Agusti-Brisach *et al.* (2012), using four Spanish isolates of *F. circinatum* and *P. radiata* seeds, showed significant survival rate differences between mating types and individual isolates of *F. circinatum* to HWT, both in terms of maximum temperature and duration of the heat treatment. Spanish MAT-2 isolates were more sensitive to HWT temperatures than Spanish MAT-1 isolates (Agusti-Brisach *et al.*, 2012). Temperatures above 50 °C were lethal to mycelium when exposed for 30 min. *Pinus radiata* seed germination was reduced with increasing HWT temperatures and duration but this reduction remained below 30% for temperatures below 53 °C. Therefore Agusti-Brisach *et al.* (2012) concluded that a HWT at 51–52 °C for 30 min can be used to substantially reduce *F. circinatum* contamination of *P. radiata* seeds. Similar results were obtained for naturally infected *P. radiata* seedlots tested in the work (Agusti-Brisach *et al.*, 2012).

Fusarium circinatum was totally eliminated from artificially inoculated *P. radiata* seed coat, embryo and gametophyte after a thermotherapy treatment at 55 °C for 8 h or longer, with no decrease in seed germination (Iturritxa *et al.*, 2011). *Fusarium circinatum* infection of artificially inoculated *P. radiata* seeds was negligible after HWT at 52 °C for 30 min (Berbegal *et al.*, 2015), but the authors pointed out that germination could be reduced as a result of thermotherapy treatment. The differences observed in seed germination rates reported in the publications may be due to the application of an initial water soaking (24 h) prior to HWT. In fact, reduction of seed germination took place when water soaking was applied before HWT (Agusti-Brisach *et al.*, 2012; Berbegal *et al.*, 2015), in contrast with experiments in which no initial water soaking was carried out (Dumroese *et al.*, 1988; Iturritxa *et al.*, 2011). This initial water soaking (priming) is a commonly used method that triggers seed germination processes by partial hydration of internal seed tissues, stimulating the activity of starch-degrading enzymes and making sugars available for embryo growth (Ashraf & Foolad, 2005; Farooq *et al.*, 2006). So, the activation of these enzymes may make embryos susceptible to heat when HWT is applied after the initial soaking in water. However, further studies are required to confirm these assumptions.

Hot water treatment is probably the most useful and economic method to reduce *F. circinatum* infection in seeds, although temperatures to be used vary between species and provenances and on occasion, complete elimination of the pathogen may be not possible. This point is especially relevant for quarantine pathogens, such as *F. circinatum*, in which even very low rates of infestation are sufficient to allow introduction of the pathogen to disease-free areas. Dielectric heating treatments, and microwave and radio frequency technologies, used successfully to eradicate other *Fusarium* species in agricultural seeds could, therefore, also be promising techniques to control *F. circinatum* (Leal *et al.*, 2010).

Wood material can be an important source of inoculum of *F. circinatum*, with trade in wood products potentially contributing to long-distance dispersal of the pathogen (EFSA, 2010). *Fusarium circinatum* showed reduced survival on wood blocks exposed to 56 °C for 30 min or longer, but models predicting negligible survival, 1% or 0.1%, would only be achieved with exposure for 30 min to a minimum temperature of 61.7 or 68.9 °C, respectively (Ramsfield *et al.*, 2010).

Nonthermal plasma (NTP) technology is an emerging field that uses active particles, radicals, electrons, metastabiles, ions and radiation in various applications (Moreau *et al.*, 2008), including surface sterilization. Atmospheric pressure NTP can be used for the sterilization of living and nonliving materials (Scholtz *et al.*, 2015) and may inactivate bacteria (Ziuzina *et al.*, 2014), fungi (Dasan *et al.*, 2016) and viruses (Wang *et al.*, 2016). The idea of using plasma to sterilize the surface of seeds has been tested recently: air gas or sulphur hexafluoride gas was used for decontamination of *Aspergillus parasiticus*, which produces aflatoxins on the surface of hazelnuts (*Corylus* sp.), peanuts (*Arachis hypogaea*) and pistachio (*Pistacia vera*) (Basaran *et al.*, 2008). Seeds of several crop species were also decontaminated, inactivating *Fusarium* spp., *Trichothecium roseum*, *Aspergillus*

spp. and *Penicillium*, with success depending on the seed surface, plasma gas type, treatment duration and the microbial population density (Selcuk *et al.*, 2008; Zahoranová *et al.*, 2016). Seed contamination was reduced below 1%, without lowering seed quality below the commercial threshold of 85% germination (Selcuk *et al.*, 2008). Zahoranová *et al.* (2016) suggested that NTP technology is useful in surface sterilization of *Fusarium* spp. as a result of fungal cell membrane rupture (Stepczyńska, 2016). Testing this process on *P. radiata* seeds infected with *F. circinatum* was performed with NTP (air gas) for 0, 5, 10, 60, 180 and 300 s; infected seeds treated for 60 s or more were free of viable *F. circinatum* inoculum (Šerá *et al.*, 2019). However, germination of seeds was affected, even with short exposure times, but research is in progress to improve this innovative environmentally friendly method of control.

The use of natural products

The use of natural compounds for disease mitigation and control of *F. circinatum* is receiving increasing attention and several studies have been carried out to test the effect of plants, other organisms or their derived metabolites on *F. circinatum*.

Isothiocyanates (ITCs), released during the hydrolysis of glucosinolates (GSL) in cruciferous plants by the endogenous enzyme myrosinase (Kirkegaard *et al.*, 2000), can suppress fungi, oomycetes and bacteria. Pure ITCs have been shown to be effective in the suppression of mycelial growth and conidial germination of *Fusarium oxysporum*; conidia were the most sensitive fungal structures to the treatment (Ramos García *et al.*, 2012). Similarly, Smolinska *et al.* (2003) reported that treatment with propenyl and ethyl isothiocyanates inhibited mycelial growth, and completely suppressed conidial and chlamydospore germination of all *F. oxysporum* isolates tested. Other isothiocyanates, including benzyl and phenethyl, also displayed a fungitoxic action against *F. oxysporum* conidia and chlamydospores. Based on these results, Smolinska *et al.* (2003) suggested that pathogenic *F. oxysporum* isolates infesting nursery soils could be suppressed by glucosinolates from plant species such as *Brassica carinata*, *Brassica nigra* and *Brassica juncea*. Reduction in pathogen populations resulting from a green-manure crop are probably achievable because chlamydospores are sensitive to ITCs. In this sense, experiments with commercial *B. carinata* pellets (Bio-Fence; Triumph) have recently been carried out showing fungicidal effects on *F. circinatum* at low ED_{50} in *in vitro* tests, and an increase in seed germination in laboratory experiments with artificially inoculated seed batches of *P. radiata* (Morales-Rodríguez *et al.*, 2018). In contrast, *Fusarium* populations of some pathogenic species (e.g. *F. oxysporum*, *F. solani* and *F. avenaceum*, among others) increased dramatically after incorporation of the *Brassica* crop into soil (James *et al.*, 2004).

Plant essential oils comprise a rich source of bioactive molecules (Christaki *et al.*, 2012), and may also provide alternatives to the synthetic fungicides currently used for control of plant pathogenic fungi. A large number of essential oils have been reported to have antifungal activities (Papaefthimiou *et al.*, 2014). The essential oils of *Leptospermum petersonii*, for example, showed antifungal activity against *F. circinatum*, inhibiting mycelial growth *in vitro* (Lee *et al.*, 2008). Essential oils of manuka (*Leptospermum scoparium*) and patchouli (*Pogostemon patchouli*) also showed moderate activity against *F. circinatum* (Lee *et al.*, 2009). Iturritxa *et al.* (2011) screened several essential oils against growth of *F. circinatum* and found that the most effective oils were those obtained from oregano, Japanese mint and cinnamon, all being 100% inhibitory at the lowest dose tested (10% v/v essential oil dilution). Cinnamon, fennel and clove oils also reduced or inhibited (partially or completely) the *in vitro* growth of *F. circinatum*, showing both fungicidal and fungistatic effects (Iturritxa *et al.*, 2017). Antagonistic effects were also demonstrated in inoculated seeds of *P. radiata* using thyme oil (Iturritxa *et al.*, 2011) and in 2-year-old *P. radiata* seedlings using cinnamon, clove or a mixture of the two with promising results on the control of *F. circinatum*, although phytotoxic effects were noted on 1-year-old plant tissues (Iturritxa *et al.*, 2017).

Germination of *P. radiata* seed was inhibited following treatment with essential oils of thyme, tea tree, oregano, niaouli, cinnamon and red thyme. Phytotoxicity seems to be age-dependent, because after application of cinnamon or clove oils to 1-year-old seedlings, epinasty or curling and twisting of needles and stems followed by necrosis occurred, but the effect was not observed in 2-year-old plants (Iturritxa *et al.*, 2011, 2017).

Another natural compound widely used in plant protection for its antiseptic properties is propolis (Özcan *et al.*, 2004), which effectively inhibited mycelial growth (68%) of *F. circinatum* (Silva-Castro *et al.*, 2018b). Moreover, coating of propolis on seeds enhanced survival by approx. 45% of *P. sylvestris* seedlings grown in *F. circinatum*-infested soil (Silva-Castro *et al.*, 2018a). The resin of *P. radiata* and specifically five monoterpene components of this resin (limonene, α-pinene, β-pinene, camphene and myrcene) inhibited mycelial growth, or affected survival and germination of *F. circinatum* spores (Slinski *et al.*, 2015). Application of gorse compost (*Ulex europaeus*) obtained from forest cleaning green wastes also decreased the incidence of *F. circinatum* disease when used as a growth medium for *P. radiata* seedlings, but the mechanism involved in this effect requires investigation (López-López *et al.*, 2016).

Inducers of resistance

The induction of plant resistance is a potential alternative approach to chemical control that has been widely explored in agriculturally important crops to manage plant health (Walters *et al.*, 2013). Induced resistance (IR) represents a physiological state of enhanced plant

defensive capacity stimulated by biotic or abiotic elicitors, whereby innate defences are enhanced against subsequent challenges (Eyles *et al.*, 2010). This capacity for augmented defence offers a wide spectrum of protection against biotic and abiotic stresses. Stimuli from pathogens, beneficial microbes, chemicals and abiotic cues can trigger the establishment of induced responses in pines that enhance their protection against *F. circinatum* (Fig. 2, Table 1). If defence mechanisms are triggered by a stimulus prior to infection by a plant pathogen, disease incidence and severity may be reduced.

Induction of resistance has been observed in young plantations and natural forests of *P. radiata* and is known to be a key process in the interaction between pines and *F. circinatum* (Gordon *et al.*, 2011; Reynolds *et al.*, 2016). Repeated infection of the same tree resulted in progressively shorter lesions (Table 1), implying an effect of prior infection on susceptibility. This observation probably explains disease remission in long-term monitoring plots (Gordon *et al.*, 2001), in which numerous trees that were initially severely diseased were free of symptoms and infections 3 years later. Similarly, exposure of stems and roots of *P. radiata* seedlings to *F. circinatum* enhanced resistance to shoot infections by the same fungus and promoted survival following subsequent stem inoculations (Bonello *et al.*, 2001; Gordon *et al.*, 2011; Swett & Gordon, 2017). Elevated host resistance occurred both when seedlings were grown in infested sand and when exposure to inoculum resulted from emergence through infested leaf litter (Swett & Gordon, 2017). If IR is operating in nature, susceptibility of *P. radiata* stands should decline over time following initial exposure to the pathogen.

It is not yet known if this phenomenon can be exploited on a practical level, but induction treatments on pines susceptible to PPC disease could be performed in both the nursery and in the field (Table 1). A further question is how long the phenomenon persists, an important consideration in long-lived tree species.

Examination of resistance inducers on a range of plant species indicate that some compounds, such as chitosan (Reglinski *et al.*, 2004; Fitza *et al.*, 2013) or phosphites (Cerqueira *et al.*, 2017), may be effective in enhancing resistance to *F. circinatum*, whereas others, such as DL-3-amino-*n*-butanoic (DL-β-aminobutyric) acid (BABA), benzo-[1,2,3]-thiadiazole-7-carbothionic acid-*S*-methyl ester (Bion; BTH), chitin, Kannar, Messenger, methyl jasmonate and salicylic acid were ineffective (Vivas & Solla, 2012; Vivas *et al.*, 2012a; Fitza *et al.*, 2013). Phosphite treatments on *P. radiata* seedlings delayed disease progression in a dose-dependent manner, in part through effects on the physiological status of the plants, including hormonal signalling (Cerqueira *et al.*, 2017). Prophylactic chitosan application reduced disease incidence in *P. radiata* seedlings subsequently inoculated with *F. circinatum* at low inoculum levels (100 spores per wound). Moreover, chitosan coating also enhanced the survival of *P. radiata* and *P. sylvestris* seedlings grown in *F. circinatum* -infested soil (Silva-Castro *et al.*, 2018a). Systemic induced resistance to *F. circinatum* in seedlings of *P. radiata* treated with chitosan lasted *c.* 42 days (Reglinski *et al.*, 2004) and 6 weeks in *Pinus patula* (Fitza *et al.*, 2013). The effects of chitosan and phosphite may result from IR and/or a fungistatic effect. This conclusion is consistent with the findings of Silva-Castro *et al.* (2018b) and Cerqueira *et al.* (2017) who demonstrated that chitosan oligomers and phosphite salts were able to inhibit mycelial growth of *F. circinatum in vitro*. As noted above, pine rhizobacterial isolates of *E. billingiae* and *P. fluorescens* protected young *P. radiata* seedlings
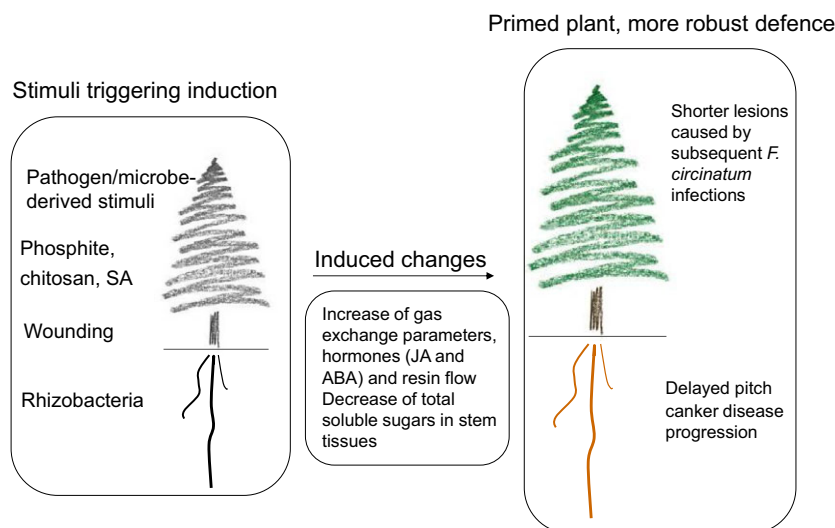

Figure 2 Various biotic and abiotic stressors (left) can trigger an increased defensive capacity of pines to *Fusarium circinatum* (right). Natural infections and artificial inoculations with *F. circinatum*, chemical inducers and mechanical wounding are able to induce local and systemic changes in different parts of the plant.

Table 1 Priming stimuli and changes induced in seedlings and adult pine trees in nurseries and forests, which successfully enhanced resistance to *Fusarium circinatum*.

Environment	Stimuli	Plant material	Enhanced responses	Long-lasting responses	References
Nursery	Phosphite (1%)	0.5-year-old *Pinus radiata* seedlings	Increased plant growth and delayed symptom development	ND	Cerqueira *et al.* (2017)
	Chitosan (0.1 mg mL^{-1})	1-year-old *P. radiata* seedlings	Reduction of disease incidence, lesion length and dead tops	ND	Reglinski *et al.* (2004)
	Chitosan (1 mg mL^{-1})	0.3-year-old *P. patula* seedlings	Delayed symptom development and reduction of lesion size	ND	Fitza *et al.* (2013)
	Pseudomonas fluorescens and *Erwinia billingiae*	2-year-old *P. radiata* seedlings	Reduction of lesion size[a]	ND	Iturritxa *et al.* (2017)
	Clonostachys rosea	*P. radiata* seeds during germination	Reduction of plant mortality		Moraga-Suazo *et al.* (2011)
	Substrate infected with high inoculum density of *F. circinatum*	*P. radiata* seeds during germination	Increased survival and reduction of lesion size	ND	Swett & Gordon (2017)
Field	Repeated inoculations of *F. circinatum* over time	4-year-old *P. radiata* trees in the field	—	64% reduction of lesion length of tree over a period of 2 years	Bonello *et al.* (2001)
	Prior exposure to *F. circinatum*	Plantations of 4-year-old *P. radiata* seedlings	Reduction of lesion length and no reduction of tree growth	ND	Reynolds *et al.* (2016)
	Natural infections of *F. circinatum*	Areas of adult *P. radiata* trees long exposed to the pitch canker	Reduction of lesion length in old versus new areas, and 89% of trees in remission	Elevated resistance in trees with a longer period of exposure to *F. circinatum*	Gordon *et al.* (2011)
	Wounding	2-year-old *P. rigida* and *P. densiflora* seedlings/ increased resin flow but similar lignin content	Delay in disease severity	ND	Kim *et al.* (2010)

ND, not determined.
[a]Probably as a consequence of both antagonism and induced resistance.

against *F. circinatum* (Iturritxa *et al.*, 2017), probably as a consequence of a combined effect of mycelial growth inhibition and induced resistance.

Transgenerational induction of defences is also a putative way to increase the resistance of seedlings. The progeny of mother plants subjected to a biotic challenge may be better defended than seedlings derived from healthy plants (Agrawal, 2002). Although the mechanisms of this nongenetic inheritance, probably of epigenetic nature, are still not well known, it is widely accepted that this is a taxonomically widespread phenomenon (Holeski *et al.*, 2012) that may also operate in pines (Vivas *et al.*, 2015). Moreover, the abiotic environment in which seeds are produced may also affect progeny performance (Cendán *et al.*, 2013) including resistance to biotic threats (Vivas *et al.*, 2013). Seedlings derived from seeds developed under stressful conditions were more susceptible to *F. circinatum* than seedlings obtained from the same genotypes growing under favourable conditions (Vivas *et al.*, 2013). Carbohydrate changes and antioxidant activity during infection were implicated in this transgenerational tree–pathogen interaction (Vivas *et al.*, 2014a,b). This research suggests that seedlings would be more resistant towards *F. circinatum* if cones were collected from vigorous trees.

Mechanical wounding of mature trees is also a well-known elicitor of induced defence responses in pines (e.g. Lombardero *et al.*, 2000). Two-year-old pine seedlings became less vulnerable to subsequent inoculations with *F. circinatum* after being wounded (Kim *et al.*, 2010). However, in forests affected by *F. circinatum*, wounding trees does not seem a good strategy to enhance resistance, as wounds facilitate pathogen access (Gordon *et al.*, 2015).

Although the occurrence of IR in the *F. circinatum–P. radiata* pathosystem has been well documented, the mechanisms behind IR, the compounds involved in the priming phase, and their efficacy over time are still under investigation. An additional practical issue of IR in pines relates to the identification and selection of genotypes able to effectively enhance their resistance after induction (Gordon & Reynolds, 2017). The absence of a negative impact on growth rate associated with IR (Reynolds *et al.*, 2016) suggests that IR may have utility as a tool for the management of PPC in plantations.

An important point regarding the potential use of resistance inducers for protection of forest stands against a quarantine pest such as *F. circinatum* is that resistance inducers influence plant resistance traits but do not effect inoculum eradication. It is important to consider, therefore, whether symptomless infected seedlings with enhanced resistance should be used in plantations. If plants treated with resistance inducers were planted in the field, what implications would this have on the spread of the disease? On sites where the pathogen is present, the introduction of induced plants could be used to enhance the resistance of a forest. However, according to the current legislation of most countries regulating quarantine pathogens, this strategy would not be allowed. Nevertheless, eradication of *F. circinatum*, as established by current regulations, may be rather complicated in most situations. In Spain, for example, removal and burning of several *P. radiata* plantations was performed following national law RD 637/2006, with costs of over €6000 ha^{-1}. As roots, grasses and debris on soil (e.g. needles and wood fragments) are sources of inoculum, it is unlikely that the fungus would be completely eradicated from a forest. In such cases, improving the resistance of the new plantations by the use of resistance inducers will definitely help to minimize the impact of the disease. Long-term disease remission by IR could be a more efficient disease management strategy in the long-term than pathogen eradication.

Exploiting genetic resistance against *F. circinatum*

Evidence is accumulating that the exploitation of genetic resistance is one of the most promising alternatives to manage the PPC disease (Wingfield *et al.*, 2008; Mitchell *et al.*, 2012a; Gordon *et al.*, 2015). Variation in susceptibility occurs at all possible genetic levels, from among-species (e.g. Gordon *et al.*, 1998a; Enebak & Stanosz, 2003; Iturritxa *et al.*, 2012, 2013a; Martínez-Álvarez *et al.*, 2014a) to intraspecific genotypic variation (e.g. Gordon *et al.*, 2006; Matheson *et al.*, 2006b; Hodge & Dvorak, 2007; Mitchell *et al.*, 2012c; Elvira-Recuenco *et al.*, 2014), and the range of variation is extremely large. This huge variation offers a unique environmentally friendly and cost-effective opportunity for disease management. With no other absolute means of controlling *F. circinatum*, selection and/or deployment of resistant material for reforesting high-risk sites is viewed as one of the few operative ways to reduce the impact of

this devastating disease (Mitchell *et al.*, 2012a; Hodge & Dvorak, 2014; Serra-Varela *et al.*, 2017). Several non-exclusive strategies to exploit the genetic variation in resistance within the genus *Pinus* are described below.

Selection of resistant species

The first and easiest solution is to plant alternative resistant species that may substitute the ones commonly used in large-scale forestry (Gordon *et al.*, 1998a; Hodge & Dvorak, 2000; Iturritxa *et al.*, 2013a). This alternative has received particular attention in countries of the southern hemisphere (e.g. South Africa, Chile, Colombia, Uruguay), where no native pines occur, and where the forestry industry mainly relies on pine species that are highly susceptible to *F. circinatum* (e.g. *P. radiata*, *P. patula*, *Pinus elliottii*) (Viljoen *et al.*, 1995; Roux *et al.*, 2007; Porter *et al.*, 2009; Mitchell *et al.*, 2012b, 2013). Independent studies conducted in different countries (South Africa, USA, Spain, South Korea, Central America, Chile, Colombia) have consistently demonstrated that *P. radiata*, *P. patula* and *P. elliottii* are highly susceptible, whereas *Pinus tecunumanii*, *Pinus oocarpa*, *Pinus canariensis*, *Pinus pinea* and *Pinus thunbergii* are highly resistant (Gordon *et al.*, 1998a; Hodge & Dvorak, 2000; Kim *et al.*, 2008; Steenkamp *et al.*, 2012; Martínez-Álvarez *et al.*, 2016).

It is important to note that substituting the main planting species would be complicated when the forestry industry is already well established, centred and fine-tuned around a single or few species. This is a problem, for example, in countries such as South Africa, Chile and New Zealand. The search for alternative species would not only imply the selection of those that are resistant to the pathogen, but also those that are well adapted to local environmental conditions and suited to current industry requirements.

Selection of breeding material from resistant populations

A second strategy to reduce the impact of *F. circinatum* is to select highly resistant populations within species (Gordon *et al.*, 2006; Dvorak *et al.*, 2007; Hodge & Dvorak, 2007). Many pine species, especially those with isolated and fragmented small populations, harbour large intraspecific genetic variation, with high population differentiation in many different life-history traits (Grivet *et al.*, 2013), including resistance to pathogens (e.g. Zas *et al.*, 2015). Specifically, resistance to *F. circinatum* is known to vary among populations in a wide array of pine species, including *Pinus leiophyla* (Dvorak *et al.*, 2007), *P. patula* (Hodge & Dvorak, 2007; Dvorak *et al.*, 2009), *P. tecunumanii* (Hodge & Dvorak, 2007; Dvorak *et al.*, 2009), *P. oocarpa* (Dvorak *et al.*, 2009) and *Pinus pinaster* (Iturritxa *et al.*, 2012; Elvira-Recuenco *et al.*, 2014). For some species, among-population variation has been related to geographical and environmental gradients (Hodge & Dvorak, 2007; Dvorak *et al.*, 2009). For example, populations of *P. tecunumanii* obtained from lower altitudes

consistently showed greater resistance to *F. circinatum* than those from higher altitudes (Dvorak *et al.*, 2009). These environmental gradients may either be the result of contrasting disease pressures under different environmental conditions in species that naturally coevolved with the fungus (e.g. *P. tecunumanii*; Dvorak *et al.*, 2009) or the result of other demographic and adaptive processes that have led to correlated responses in *F. circinatum* resistance traits (Elvira-Recuenco *et al.*, 2014).

Hybridization

Hybridization with resistant species or populations is another alternative that is receiving increasing attention, especially in South Africa, where hybridization between the susceptible *P. patula* and the resistant *P. tecunumanii* has been shown to be highly promising for minimizing the current impact of *F. circinatum* in *P. patula* plantations (Kanzler *et al.*, 2014). Hybridization aims to combine the advantages of both parental species. Although interspecific hybrids were originally developed to improve the adaptation of *P. patula* to the particular environmental conditions in South Africa, it soon became evident that the hybrids were much more tolerant than *P. patula* to *F. circinatum*, especially when highly resistant low elevation populations of *P. tecunumanii* were used for hybridization (Roux *et al.*, 2007; Mitchell *et al.*, 2013; Kanzler *et al.*, 2014). Other hybrids that have shown good results in terms of tolerance to *F. circinatum* are *P. elliottii* × *Pinus caribaea* and *P. patula* × *P. oocarpa* (Roux *et al.*, 2007), although, in general hybridizations between *P. patula* and any other tolerant species improve resistance to *F. circinatum* (Mitchell *et al.*, 2013). Family variation within hybrids was also high, so selection of the parents is critical, while mass clonal propagation of selected hybrid offspring may result in substantial genetic gains (Roux *et al.*, 2007; Mitchell *et al.*, 2013). Vegetative propagation of hybrid material proved relatively easy to apply in some pine species, facilitating the massive deployment of resistant material for reforestation (Ford *et al.*, 2014).

Tolerance of family hybrids between *P. patula* and *P. tecunumanii* seems to be more affected by the specific interaction between the parents (specific hybrid ability, SHA) than to general effects of each parent (general hybrid ability, GHA; Mitchell *et al.*, 2013). For operational deployment of hybrid material, therefore, selection of the best male and female parents should be accompanied by a deep screening of as many parental combinations as possible. This approach is especially required when hybridizing with high-elevation *P. tecunumanii* origins, for which large family and within-family variation has been observed (Mitchell *et al.*, 2013). In contrast, hybrids with low-elevation origins of *P. tecunumanii* showed much less variation (Mitchell *et al.*, 2013; Ford *et al.*, 2014).

Exploiting within-population variation

Taking advantage of the large within-population variation that commonly occurs in most pine species is another alternative for exploiting genetic resistance as a tool for minimizing the impact of *F. circinatum*. There is ample evidence that resistance to *F. circinatum* varies widely within populations, both in species regarded as susceptible (e.g. *P. radiata*, *P. patula*) and in those considered resistant (e.g. *P. tecunumanii*, *Pinus maximinoi*). Variation in resistance appears to be quantitative, and has been attributed to polygenic resistance mechanisms (Gordon *et al.*, 1998b; Kayihan *et al.*, 2005; Quesada *et al.*, 2010), that is, the result of the integrated action of many genes, each contributing small effects (Telford *et al.*, 2015).

Heritability estimates of *F. circinatum* resistance are quite variable depending on the species, the population within the species (e.g. Mitchell *et al.*, 2012a), the material tested (e.g. Matheson *et al.*, 2006a), and the screening procedure (e.g. Aegerter & Gordon, 2006; Table 2). Nevertheless, despite the relatively high variation in heritability estimates, the level of genetic control is commonly sufficiently high to allow improved resistance through selection and breeding using classical procedures (Kayihan *et al.*, 2005; Matheson *et al.*, 2006a; Elvira-Recuenco *et al.*, 2014; Mitchell *et al.*, 2014; Nel *et al.*, 2014). Relatively high coefficients of variation of resistance among genetic entries facilitate large selection differentials that can easily translate into significant genetic gains in resistance.

Gains in resistance can be obtained either by forward recurrent selections, in which the most resistant offspring is selected and bred to obtain new genotypes with increased resistance, or by backward selections, in which the best parents are selected based on the resistance of their progeny, and directly used for producing seeds for reforesting in high-risk sites (Telford *et al.*, 2015). Forward selections are constrained by the long time required for completing the reproductive selection cycle (and the moderate narrow-sense individual heritability estimates; see Table 2), but it can lead to notable gains owing to larger differential selections and the opportunity to capture both additive and non-additive genetic variation. In contrast, backward selections are more rapid and straightforward to apply but genetic gains are typically not as high due to lower differential selections. However, large family heritability estimates (e.g. Nel *et al.*, 2014) favour large genetic gains through family selection. The establishment of seed orchards with selected resistant parents or rogueing current seed orchards to eliminate those parents that led to highly susceptible offspring is, therefore, a very easy, inexpensive, and rapid approach that can lead to substantial improvements of *F. circinatum* resistance in new plantations (Vivas *et al.*, 2012b; Nel *et al.*, 2014). Similarly, in clonal forestry, Bioforest in Chile has defined a selection methodology to rank clones from more susceptible to more tolerant against *F. circinatum* and many clones have been eliminated from the operation due to high susceptibility to *F. circinatum* at the nursery stage (R. Ahumada, Bioforest S.A., Concepción, Chile, personal communication).

The commercial deployment of resistant pine reproductive material through the selection (and breeding) of species, populations, hybrids, families or genotypes is a

Table 2 Narrow-sense heritability estimates of resistance to *Fusarium circinatum* in different *Pinus* species.

Species	Tested material	Age (months)	h_i^2	References
Pinus radiata	More than 500 seedlots, including half-sibs and full-sibs	12	0.34–0.78	Matheson *et al.* (2006a)
P. taeda	Clones of full-sib families from a circular design	12	0.27	Kayihan *et al.* (2005)
P. taeda	498 clones from the whole distribution		0.21–0.38[a]	Quesada *et al.* (2010)
P. pinaster	40 selected half-sib families	6	0.20[b]	Vivas *et al.* (2012b)
P. tecunumanii	28 half-sib families from 8 high-elevation populations	6	0.59	Mitchell *et al.* (2012b)
P. tecunumanii	49 half-sib families from 4 low-elevation populations	6	0.01	Mitchell *et al.* (2012b)
P. maximinoi	105 half-sib families from 13 populations	7	0.01	Mitchell *et al.* (2012b)
P. elliottii	49 half-sib selected families	8	0.22	Mitchell *et al.* (2012b)
P. pseudostrobus	33 half-sib families	7	0.06	Mitchell *et al.* (2012b)
P. patula	13 half-sib families	7	0.06	Mitchell *et al.* (2013)
P. pinaster	165 clones from 47 half-sib families from 10 populations	36	0.45	Elvira-Recuenco *et al.* (2014)
P. patula	78 half-sib selected families	10	0.25	Mitchell *et al.* (2014)
P. patula	63 half-sib selected families	9	0.52	Mitchell *et al.* (2014)
P. patula	65 half-sib families	5	0.27	Nel *et al.* (2014)
P. patula	65 half-sib families	5	0.41	Nel *et al.* (2014)
P. patula	140 half-sib families	6	0.26	Nel *et al.* (2014)
P. patula	18 half-sib families	6	0.30	Nel *et al.* (2014)
P. patula	73 half-sib families	6	0.32	Nel *et al.* (2014)

h_i^2, narrow-sense heritability.
[a]The reported heritability value corresponds to clonal repeatability.
[b]Heritability for symptom scores. In all the remainder cases the reported heritability corresponds to lesion length after inoculation.

realistic and valuable option to improve resistance to *F. circinatum*. The success of the breeding initiatives developed in South Africa has been catalogued as a 'story of major success in plantation forestry' (Hodge & Dvorak, 2014), and is the best demonstration that exploiting genetic resistance is a powerful tool against this disease. But this history of potential success is not free of significant drawbacks that need careful consideration. To properly exploit genetic resistance against *F. circinatum*, special attention should be paid to (i) improving and standardizing the current screening protocols (Oak *et al.*, 1987; Roux *et al.*, 2007); (ii) developing genomic selection protocols (Quesada *et al.*, 2010; Moraga-Suazo *et al.*, 2014; Donoso *et al.*, 2015; Carrasco *et al.*, 2017); (iii) discovery of durable and stable resistance operational across different environmental conditions, *F. circinatum* strains and tree ontogenetic stages; (iv) quantifying the consequences of putative trade-offs between resistance and other life functions (e.g. growth–defence trade-off) (Matheson *et al.*, 2006a; Vivas *et al.*, 2012b); and (v) exploration of other sources of variation with genetic backgrounds, such as inducibility (Agrawal *et al.*, 2002; Moreira *et al.*, 2013), maternal effects (Vivas *et al.*, 2013) or the attraction and susceptibility of trees to vectoring insects (Erbilgin *et al.*, 2005).

Conclusions, future prospects and challenges: identifying gaps of knowledge

It is clear, based on the information reviewed here, that environmentally friendly control methods can be successfully implemented in integrated management of PPC. Plans for this integrated management should differentiate at least three protocols: (i) avoiding new introductions and spread via plant material, (ii) control in the nursery,

and (iii) control in the field. Thermotherapy may be integrated in management plans to avoid spread of the pathogen. Obviously, these techniques should be considered as a valuable complement to rapid detection and efficient monitoring of *F. circinatum*, but not as a substitute. The identification, evaluation and harmonization of known methods (EPPO, 2009), together with the development of rapid and sensitive diagnostic techniques based on new molecular methods (e.g. high-throughput next-generation sequencing methods) or DTBIA (direct tissue blot immunoassay), commonly used for detection of viruses and bacteria, is a priority.

In addition, the use of antagonistic fungi and bacteria as well as natural compounds and inducers of resistance could be implemented in management plans for PPC control in the nursery. However, forest nursery managers should be aware that application of these preparations could minimize seedling mortality and promote vigorous plant growth, but it does not entirely remove the risk of infection. Special attention should be paid to the fact that application might increase numbers of symptomless infected plants. This problem applies in particular to countries where the pathogen is not widely established (e.g. in Europe), in which avoiding spread from nurseries to the field via latent, symptomless infections is crucial.

Breeding for resistance appears to be the best option for the control of PPC disease in the field. Further work is needed to test the susceptibility of alternative species and populations and to obtain resistant material, especially in Europe, where the presence of *F. circinatum* currently is limited to the Iberian Peninsula where a few *Pinus* species are affected. Testing the potential hypovirulence of the *F. circinatum* mycoviruses could be another line of enquiry for controlling PPC in the field. Other gaps in knowledge identified in this review should be

addressed by: (i) defining the temporal and spatial persistence of the various treatments in different environments; (ii) estimating the costs of the application of these environmentally friendly control strategies for PPC disease; and (iii) determining the genetic mechanisms behind the *F. circinatum*–plant interaction, biocontrol and priming strategies, with the aim of improving understanding of the mechanisms of resistance and finally providing evidence for the fine-tuning of potential control methods.

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