

The use of *Verticillium dahliae* and *Diplodia scrobiculata* to induce resistance in *Pinus halepensis* against *Diplodia pinea* infection

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Abstract The pathogenic fungi *Verticillium alboatrum* and *Diplodia scrobiculata* were assayed for biological control of *Diplodia pinea* on Aleppo pine (*Pinus halepensis*) in Catalonia (north-eastern Spain). Young shoots were pre-treated with inoculations of either *V. dahliae* or *D. scrobiculata*, by placing colonized agar plugs on wounds made by removing a single needle fascicle. An inoculation with *D. pinea* was performed 15 days later. Two months after the shoot inoculations, the canker length on the stems was measured and the percentage of shoot dieback calculated. *Verticillium dahliae* and *D. scrobiculata* were found to significantly reduce the canker length of *D. pinea* ($P < 0.05$) when compared with positive controls. *Diplodia pinea* was slightly more sensitive to *V. dahliae* than to *D. scrobiculata*, but no significant differences ($P > 0.05$) were observed in the mean canker length between the two treatments. Trees pre-inoculated with *V. dahliae* resulted in 31.12% shoot dieback, while those pre-inoculated with *D. scrobiculata* resulted in 32.18% shoot dieback, compared with positive controls (42.85%).

Keywords Aleppo pine · Fungi · Induced resistance · Tip blight

Introduction

Diplodia pinea (syn. *Sphaeropsis sapinea* the causal agent of *Sphaeropsis* tip blight, is one of the most significant pathogens of pine in Catalonia (Nadal et al. 2005). Although most *Pinus* spp. are susceptible, the Aleppo pine (*Pinus halepensis*), one of the most common species of *Pinus* in this country, is particularly vulnerable. The parasite can produce a broad range of disease symptoms: dead top, twig blight, seedling damping-off and collar rot, root disease, stem canker and sapstain (Palmer and Nichols 1985; Sinclair et al. 1987). Although tip blight primarily kills the shoot tips, repeated infections over several years can result in the death of the entire tree.

Two morphotypes (A and B) of *S. sapinea* have traditionally been described, based on spore morphology, cultural characteristics (Wang et al. 1986; Palmer et al. 1987), fatty acids in cell walls (Moret et al. 1995) and molecular studies (PCR, RAPD and ISSR markers) (de Wet et al. 2000; Burgess and Wingfield 2001; Zhou et al. 2001; Flowers et al. 2003). de Wet et al. (2003) and Smith and Stanoz (2006) have recently recognized *D. pinea* as the currently accepted name for morphotype A, while morphotype B has been recognized as a distinct species under the name

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Diplodia scrobiculata (J. de Wet, B. Slippers and M.J. Wingfield). Significant variability has been reported in the pathogenicity of *D. pinea* and *D. scrobiculata*. *Diplodia pinea* generally causes greater incidence and severity of symptoms than *D. scrobiculata*, shows more rapid growth on potato dextrose agar (PDA), and is pathogenic to both wounded and unwounded hosts; while *D. scrobiculata* displays slower growth and requires a wound for infection. (Palmer et al. 1987; Blodgett and Stanosz 1999; Blodgett and Bonello 2003).

Verticillium dahliae is a vascular wilt pathogen which causes disease in many woody and herbaceous plants. This fungus usually infects plants through roots, or through wounds caused naturally by root growth or soil organisms (Engelbrecht and Gleason 2006). Studies indicate that plants inoculated with pathogenic organisms develop resistance to subsequent infections by different pathogens. Jacobs and Guries (2003) showed that mechanical inoculations with *V. dahliae* minimized the incidence and severity of *Ophiostoma novo-ulmi* in elms, and Bonello et al. (2001) demonstrated that repeated inoculations of *P. radiata* with *Fusarium circinatum* (the causal agent of pitch canker in pines) induced a significant reduction in lesion length. This defence mechanism includes a number of physical changes, such as cell wall lignification, or the induction of systemic acquired resistance (SAR) (Schneider et al. 1996; Sticher et al. 1997). SAR is a plant immunity system that confers protection against a broad spectrum of pathogens including fungi, bacteria, viruses and nematodes; it is associated with an accumulation of pathogenesis-related (PR) proteins, which contribute to resistance (van Loon 1997; Durrant and Dong 2004; van Loon et al. 2006). This resistance can be expressed locally, at the site of the pathogen attack, or systemically, in uninfected parts of the plant.

Induced disease resistance to pathogens has been demonstrated in many angiosperms (Ryals et al. 1996), although information on conifers remains scarce. To protect themselves against insects and pathogens, conifers have developed constitutive and inducible defenses such as resin production, synthesis of new phenolics, traumatic resin duct formation, and initiation of a wound periderm (Campbell and Ellis 1992; Krokene et al. 1999; Franceschi et al. 2000; Luchi et al. 2005).

No available fungicides provide a reliable level of control of *D. pinea*. One approach to the control of *Sphaeropsis* tip blight is presented in this paper, which evaluates the ability of isolates of *D. scrobiculata* and *V. dahliae* to protect the Aleppo pine against *D. pinea*.

Materials and methods

Fungus material

Diplodia pinea and *D. scrobiculata* were obtained by direct isolation from *P. halepensis* (Girona, Catalonia); *V. dahliae* was isolated from tomato plants in the laboratory of Plant Pathology (Barcelona). Isolates were maintained on PDA, (Sigma, Aldrich, St. Louis, USA) 39 g l⁻¹, in tubes at 4°C and stored until use. They were recovered by plating a small piece of mycelium on PDA and incubating it at 24°C for 6 days in the dark.

Experimental design

Experiments were carried out in a greenhouse during the summer of 2006 to examine the pathogenicity of *D. pinea* in *P. halepensis* after inoculation with *V. dahliae* or *D. scrobiculata*. A total of 85 2 year-old seedlings ranging from 50 to 60 cm in height and 10 to 12 mm in diameter were used. They were transplanted into 6.5 l plastic pots containing a peat–vermiculture–perlite mixture (2:1:1 v/v), and fertilized with osmocote® 14–14–14 (Scott OM España, Spain). The 85 seedlings were divided into 2 groups. The first group of 50 plants was used to compare the aggressiveness of *D. pinea*, *D. scrobiculata* and *V. dahliae* in *P. halepensis*: 15 of these seedlings were inoculated with *D. pinea*, 15 with *D. scrobiculata*, 15 with *V. dahliae* and 5 with sterile PDA (negative controls). The second group of 35 pines was used to test whether *V. dahliae* or *D. scrobiculata* could induce resistance in *P. halepensis* to *D. pinea*: 15 of these were inoculated with *V. dahliae*, 15 with *D. scrobiculata* and 5 with sterile PDA (positive controls). After 15 days, all seedlings of this second group received inoculum of *D. pinea*. Trees were irrigated daily to field capacity throughout the experimental period (May–June–July).

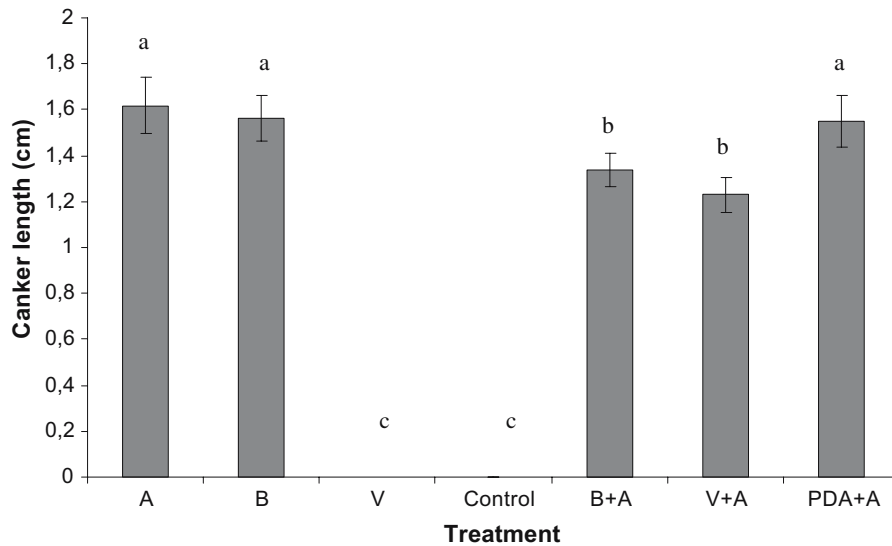


Fig. 1 Canker length on *Pinus halepensis* 2 months after inoculation with *Diplodia pinea* (A), *D. scrobiculata* (B), *Verticillium dahliae* (V) and pre-inoculated with *V. dahliae* (V+A) or with *D. scrobiculata* (B+A) 15 days before inoculation with *D. pinea*. Negative controls were inoculated with

sterile PDA (C) and positive controls were pre-inoculated with sterile PDA 15 days before inoculation with *D. pinea* (PDA+A). Bars with different letters were significantly different by the LSD test ($P=0.05$) following ANOVA

Plant inoculation

Seedlings from the first group were inoculated approximately 20 cm below the shoot apex (May 17). Before inoculation the bark surface was disinfected in 10% sodium hypochlorite (NaOCl) for 10 s and rinsed in sterile distilled water (SDW). A superficial wound 15 mm long was made in the bark tissues with a sterile scalpel, and a small disc removed from the edge of an actively growing culture was placed mycelium-side down on the wound. The inoculated area was sealed with parafilm to prevent desiccation. Controls were inoculated with sterile PDA. The seedlings from the second group were treated in the same way with either *V. dahliae* or *D. scrobiculata* isolates 25 cm below the shoot apex (May 3). The subsequent inoculation with *D. pinea* was made 5 cm above the pre-treatment inoculation (May 17). On 17 July 2006, 2 months after the shoot inoculations, the canker length on the stems was measured and the percentage of shoot dieback calculated. At the end of the experiment, isolations were made from all the trees at the point of inoculation by plating small pieces of tissue from the side of the canker in PDA and incubating for fungal identification.

Statistics

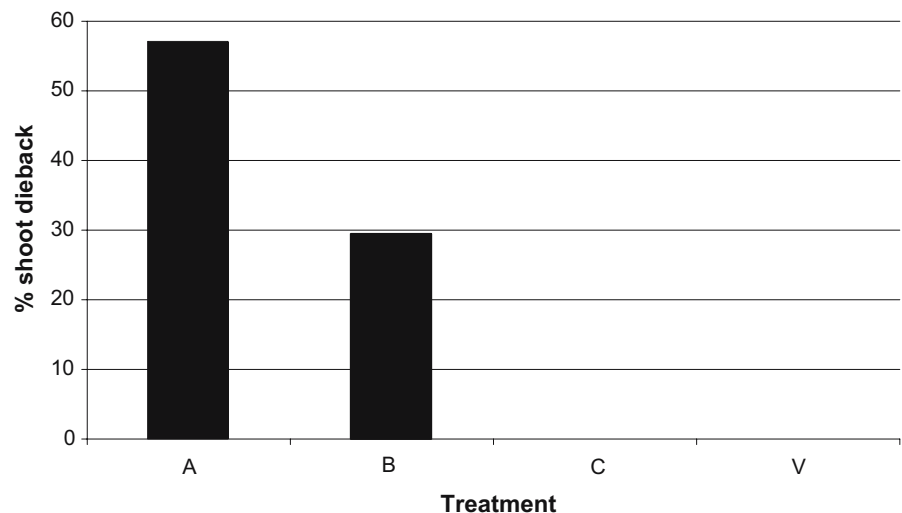
Differences in mean canker length between the two groups of seedlings were calculated, and the results were analysed using analysis of variance (ANOVA) and Fisher's least significance difference (LSD) at $P=0.05$. Incidence of disease was evaluated as percentage of shoot dieback.

Results

After 2 months canker lengths on the two groups of seedlings were measured. Cankers on shoots inoculated with *D. pinea* were not significantly longer ($P>0.05$) than those on shoots inoculated with *D. scrobiculata*. Seedlings inoculated with *V. dahliae* or sterile PDA (negative control) appeared capable of completely healing the wound (Fig. 1).

Pre-inoculations with *D. scrobiculata* or *V. dahliae* significantly reduced canker length ($P>0.05$) of *D. pinea* compared with the seedlings inoculated with sterile PDA (positive control). *Diplodia pinea* was slightly more sensitive to *V. dahliae* than to *D. scrobiculata*: no significant differences ($P>0.05$)

Fig. 2 Percentage of shoot dieback on *Pinus halepensis* 2 months after inoculation with *Diplodia pinea* (A), *D. scrobiculata* (B), *Verticillium dahliae* (V). Controls were inoculated with sterile PDA (C)



were detected in the mean canker length between the two treatments (Fig. 1).

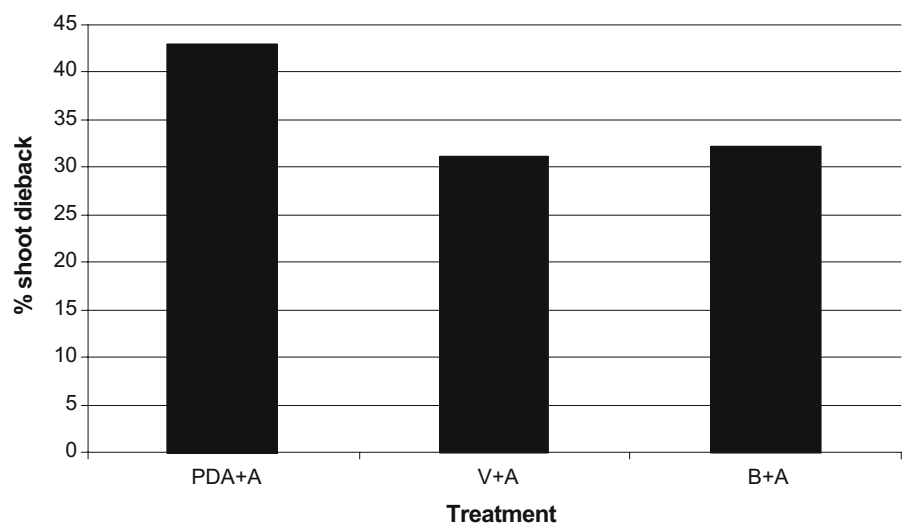
Diplodia pinea was more aggressive on *P. halepensis* than *D. scrobiculata*. Trees inoculated with *D. pinea* developed tip blight symptoms in the form of localized cankers around the inoculation site. The bark on the canker was split and slightly swollen, and pycnidia were produced in dark-brown erumpent stomata. A gradual yellowing and wilting of the needles led to the death of the entire inoculated shoot. Cankers on seedlings infected with *D. scrobiculata* had the appearance of discoloured areas and symptoms were less severe; needles appeared slightly chlorotic compared with the necrotic needles of trees inoculated with *D. pinea*. No necrotic needles were observed either on trees inoculat-

ed with *V. dahliae*, or on the negative controls. The incidence of disease was greater among seedlings inoculated with *D. pinea* than among seedlings inoculated with *D. scrobiculata*. After 2 months shoot dieback affected 57.14% of trees inoculated with *D. pinea*, compared with 29.54% of trees inoculated with *D. scrobiculata*. Seedlings inoculated with *V. dahliae* or sterile PDA did not develop deadtop (Fig. 2).

Trees pre-inoculated with *V. dahliae* showed shoot dieback at 31.12%, while those pre-inoculated with *D. scrobiculata* had shoot dieback of 32.18% relative to positive controls (42.85 %) (Fig. 3).

The occurrence of *D. pinea* and *D. scrobiculata* in the inoculated seedlings was confirmed by positive re-isolations at the end of the experimental period. No

Fig. 3 Percentage of shoot dieback on *Pinus halepensis* pre-inoculated with *Verticillium dahliae* (V+A), or *Diplodia scrobiculata* (B+A) 15 days before inoculation with *D. pinea*. Positive controls were pre-inoculated with sterile PDA (PDA+A)



fungus was isolated from any of *V. dahliae* or negative control seedlings.

Discussion

The results of our experiment indicate a significant decrease in the mean canker length of *D. pinea* on pines previously inoculated with the pathogenic organisms *D. scrobiculata* or *V. dahliae*. Our results are in accordance with the observations of Gordon et al. (1998) and Storer et al. (1999) who found that repeated inoculations with *Fusarium subglutinans* f. sp. *pini* and *F. circinatum* (the causal agents of pitch canker in Monterey pine (*P. radiata*)) produced a significant decrease in the lesion size. These authors reported systemic induced resistance (SIR), the first report in conifers. The present study shows that similar responses can be activated in *P. halepensis*. Both *D. scrobiculata* and *V. dahliae* were effective inducers of resistance; there was no significant difference in the reduction of *D. pinea* canker length between the two treatments.

SIR has been shown to occur in many plant species in recent years. Helton and Brown (1971) reported SIR in *Prunus domestica* infected with *Cytospora cincta*, and some studies of biological control of Dutch elm disease caused by *Ophiostoma novo-ulmi* provide evidence of SIR to bacteria and fungi (Scheffer 1989; Shi and Brasier 1986). Voeten (2003) reported that *V. alboatrum* induces an autoimmune response in the elm tree: after inoculation, the natural defence mechanisms of the elm are maintained at a higher level. However, this treatment can only be used preventively on healthy trees, since it has no curative properties. In conifers, Christiansen et al. (1999) and Krokene et al. (1999) demonstrated localized induction of resistance in Norway spruce against the bark beetle-associated blue-stain fungus *Ceratocystis polonica*. They reported that there was no pathogen-specific effect on the induction of disease resistance. The induced response may vary between experiments. Fungi that destroy much phloem and cambium or big mechanical wounds that destroy more tissues will induce a stronger response than inoculations with non-pathogenic or less pathogenic fungi or pre-treatment with small or few wounds. The mass-inoculation dosage, the tree resistance and the pathogenicity of the fungal isolate are

other factors that affect the level of disease resistance. This differs from Scots pine, where Krokene et al. (2000) found that pre-treatment with *Leptographium wingfieldii* and *Ophiostoma canum* or even mechanical wounding were equally effective in inducing resistance.

SIR in Aleppo pine appears to be non-specific to the fungal species used in the pre-treatment, since both pathogens *V. dahliae* and *D. scrobiculata* inoculated in this experiment were found to enhance almost the same resistance to *D. pinea*.

Luchi et al. (2005) reported systemic induction of traumatic resin ducts (TRD) in *Pinus nigra* (Austrian pine) when trees were inoculated with *D. scrobiculata*, but no TRD appeared in seedlings inoculated with *D. pinea*. In addition, *D. pinea* invaded the host xylem a few days after inoculation, whereas *D. scrobiculata* was never detected in the host xylem. Their studies reported that wounding and microbial invasion induce systemic resin flow and systemic induction of TRD formation, which are correlated with the SIR phenotype. Blotgett et al. (2007) observed induced lignification as a mechanism involved in the systemic defence responses of Austrian pine; Wang et al. (2006) identified proteins in response to induction with *D. scrobiculata* and *D. pinea*; and Bonello and Blotgett (2004) found several metabolites (such as stilbenes) correlated with resistance.

SIR similar to that observed in Austrian pine and other conifer species such as Norway spruce (*Picea abies*) (Christiansen et al. 1999; Krokene et al. 1999) and Scots pine (*Pinus sylvestris*) (Krokene et al. 2000) appears to be present in Aleppo pine (*Pinus halepensis*).

Blotgett et al. (2003) suggested that responses to canker pathogens are mediated by endogenous signals in which salicylic acid (SA) and analogues (5-chloro-salicylic acid) and jasmonic acid play a signalling role. Expression of SIR is correlated with the accumulation of PR proteins and the induction of secondary metabolic responses (Durrant and Dong 2004). Studies reveal that resistance responses can be induced by the exogenous application of SA and similar agents. Reglinski et al. (1998) and Moret and Muñoz (2007) reported induction of resistance to *D. pinea* in *P. radiata* and *P. halepensis* following applications of 5-chloro-salicylic acid. An increase in phenylalanine ammonia-lyase activity in *P. radiata* seedlings has been associated with the induction of

lignification and other host resistance mechanisms to protect against *D. pinea*.

The aggressiveness differential between two closely related fungal pathogens, *D. pinea*, an aggressive pathogen that causes significant damage to pines, and *D. scrobiculata*, a non-aggressive pathogen, has proved a useful tool for testing the hypothesis that SIR is a phenomenon associated with conifer defence. Because no correlation between alterations in secondary metabolism and expression of SIR has been established, further investigations will contribute to the understanding of the effects of SIR in conifer species. The studies described in this paper provide new information on the ability of the pathogenic fungi *D. scrobiculata* and *V. dahliae* to exhibit biocontrol potential for the reduction of tip blight in the Aleppo pine in Catalonia.

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