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RESEARCH PAPERS

Control of *Diplodia pinea* and *D. scrobiculata* in *Pinus halepensis* by 5-chloro-salicylic acid

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Summary. *Diplodia pinea* (syn. *Sphaeropsis sapinea*) and *D. scrobiculata* are destructive pathogens of conifer species in many parts of the world. The sensitivity of these fungi to externally applied 5-chloro-salicylic acid on *Pinus halepensis* was studied. Trees treated with 2 mM 5-chloro-salicylic acid were more resistant to the fungi than untreated trees. After 15 days of treatment shoot dieback affected 30% of trees inoculated with *D. pinea*, compared to 60% of untreated trees. *D. scrobiculata* caused shoot dieback in 30% of untreated trees but only in 20% of trees pretreated with 5-chloro-salicylic acid. The controls never developed tip blight. The direct effect of 5-chloro-salicylic acid on the mycelial growth of *D. pinea* and *D. scrobiculata* was tested *in vitro* using PDA amended with 5-chloro-salicylic acid at five concentrations (0.2, 1, 2, 2.5, and 3.0 mM). The radial growth of colonies was measured after 48 and 72 h of incubation at 24°C. After 48 h, 5-chloro-salicylic acid significantly inhibited mycelial growth of *D. pinea* at 3mM, although there was no longer any significant difference in growth rates after 72 h of incubation. *D. scrobiculata* was slightly more sensitive to 5-chloro-salicylic acid than *D. pinea*. After 48 h, significant differences were observed in the mean colony diameter of *D. scrobiculata* when directly exposed to 5-chloro-salicylic acid at dilutions from 0 to 3 mM. After 72 h, however, mycelial growth was reduced significantly only at the highest concentrations (2; 2.5 and 3 mM) (*P*-value <0.05).

Key words: Aleppo pine, tip blight.

Introduction

Diplodia pinea (Desmaz.) J. Kick f. (syn. *Sphaeropsis sapinea* [Fr.: Fr.] Dyko and Sutton), the causal agent of *Sphaeropsis* tip blight and stem canker, is one of the most important pathogens of pine in Catalonia (north-eastern Spain) (Nadal *et al*., 2005). Although most *Pinus* spp. are susceptible, *Pinus halepensis* Mill. (Aleppo, Jerusalem or Mediterranean pine), one of the most common species of *Pinus* in this country, is particularly vulnerable. The most obvious symptoms are necrotic and stunted shoot tips and needles. Small drops of resin often form on the needles, while fruiting structures (pycnidia) are formed at the base of the needles, on the stems, and the cones. In addition, *D. pinea* causes seedling damping off and collar rot, root disease, stem canker, and blue stain (Millikan and Anderson, 1957; Wingfield and Knox-Davies, 1980; Sinclair *et al.,* 1987; Stanosz *et al*., 1999).

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Many of the symptoms become apparent when wounds or other stress factors occur (Bega *et al*., 1978; Swart and Wingfield, 1991).

Although tip blight primarily kills the shoot tips, repeated infections over several years can result in the death of the entire tree. The pathogen also occurs in the stems and branches of asymptomatic pines, and in the tissues of apparently healthy pine trees (Stanosz *et al*., 1997, 2005; Flowers *et al*., 2001, 2003). These latent infections however only result in disease symptoms if the host tree becomes stressed (Blodgett *et al*., 2003).

Two morphotypes (A and B) of *S. sapinea* have been described based on spore morphology, culture characteristics (Wang *et al*., 1986; Palmer *et al*., 1987), differences in fatty acid cell walls (Moret *et al*., 1995) and molecular studies (PCR, RAPD and ISSR markers) (De Wet *et al*., 2000; Burgess *et al*., 2001; Zhou *et al*., 2001). De Wet *et al*., (2000, 2002) using RAPD markers and morphological characters, has shown the existence of a third morphotype (morphotype C). Morphotype A exhibits a fluffy white to gray-green mycelium, smooth conidia, and grows faster than morphotype B. Morphotype B has dark gray mycelium closely appressed to the agar, and pitted conidial walls. Morphotype C has conidial characteristics similar to those of morphotype A, but is more virulent than A and B. In Catalonia, all three morphotypes have been identified by Moret *et al*. (1995) based on differences in the fatty acid walls. There has been considerable controversy over the correct name for this morphotype. *D. pinea* is the currently accepted name for morphotype A, and morphotype B has been recognized as a distinct species under the name *D. scrobiculata* J. de Wet, Slipers and M.J. Wingfield (De Wet *et al*., 2003; Smith *et al*., 2006).

It is well documented that localized infections by some pathogens cause the host to develop resistance to various other pathogens as well, including fungi, bacteria and viruses. This resistance can be expressed locally, at the site of pathogen attack, or systemically, in uninfected parts of the plant. This defense mechanism includes a number of physical changes, such as cell wall lignification, or the induction of systemic acquired resistance (SAR) (Kessman *et al*., 1994; Schneider *et al*., 1996; Sticher *et al*., 1997).

Salicylic acid (SA) plays an important role in the signal transduction pathway leading to SAR (Ross, 1961; Hunt and Ryals, 1996; Murphy *et al*., 1999). Systemic acquired resistance to pathogens has been demonstrated in many angiosperm plant species (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 (Franceschi *et al*., 1998, 2000; Christiansen *et al*., 1999; Raffa, 1991; Luchi *et al*., 2004). Resistance mechanisms have also been induced in suspension cultures of various pines (Hotter, 1997; Lesney, 1989; Campbell and Ellis, 1992).

Other studies reported that defense mechanisms in plants can also be induced by treatment with a chemical agent. The exogenous application of SA and similar agents induces resistance to pathogens in some plants. Spletzer and Enyedi (2000) demonstrated that SA applied directly to the root system of tomato activated a form of systemic resistance against *Alternaria solani*. They found a significant increase in the level of free SA after pathogen infection of SA treated tomato plants.

An increase in phenylalanine ammonia-lyase activity in *P. radiata* seedlings following foliar application of 5-chloro-salicylic acid has been associated with the induction of lignification and other host resistance mechanisms to protect against *S. sapinea* (Reglinski *et al*., 1998). Murphy *et al*. (2000) reported that treatment of tobacco plants with SA reduced their susceptibility to *Botrytis cinerea*. They also stated that SA-responsive gene products inhibited *B. cinerea in vitro,* and restricted the growth of this fungus in SA-treated plants.

The objectives of this paper were: (i) to test *in vitro* the direct effect of 5-chloro-salicylic acid (5CSA) on the radial growth of *D. pinea* and *D. scrobiculata*, and (ii) to assess the effectiveness *in vivo* of 5CSA on the control of *D. pinea* and *D. scrobiculata* in *P. halepensis*.

Materials and methods

Fungal material

Diplodia pinea and *D. scrobiculata* were obtained by direct isolation from *P. halepensis* tissues. *D. pinea* (isol 290305-1), isolated from the needles (Tarragona, Catalonia), and *D. scrobicu-*

lata (isol 070305-21), isolated from the cones (Girona, Catalonia), were maintained in potato dextrose agar (PDA, [Sigma, Aldrich, St. Louis, USA] 39 g l^{-1}) in tubes at 4ºC and stored until use (Fig. 1). Isolates were recovered by plating a small piece of mycelium on PDA and incubating it at 24ºC for 6 days in the dark.

Plant material

The study was carried out on 50 4-year-old *P. halepensis* seedlings ranging from 60 to 75 cm in height and 12 to 15 mm in diameter and grown in 6.5 l plastic pots with a peat-vermiculite-perlite mixture (2:1:1 v:v). The seedlings were maintained in a greenhouse and fertilized with osmocote 14-14-14.

Effect of 5CSA on the radial growth of *D. pinea* and *D. scrobiculata*

The direct effect of 5CSA (Sigma-Aldrich, St. Louis, MO, USA) on the mycelial growth of both species was tested *in vitro* using PDA amended with 5CSA. For each species, five concentrations of 5CSA (0.2, 1, 2, 2.5, and 3.0 mM) were tested. The 5CSA was incorporated in the PDA medium at 60ºC and poured into 90 mm Petri dishes to yield a total volume of 15 ml $dish^{-1}$. A 5-mm-diameter disc from an actively growing PDA culture was placed fungus-side down in the centre of each Petri dish. One control dish (PDA without 5CSA) was used for each morphotype. A total of 30 Petri dishes were prepared for each dilution and incubated at 24ºC. Colony diameters were measured 48 and 72 h after treatment, and colony growth on PDA amended with 5CSA was compared with growth on PDA without 5CSA.

Experimental design

The 50 pines were divided into two equal groups: 25 trees were used to compare the aggressiveness of *D. pinea* and *D. scrobiculata*: 10 of these trees were inoculated with *D. pinea*, 10 with *D. scrobiculata* and 5 were the controls. Trees were watered daily to field capacity throughout the experimental period (June-July-August) as the air temperature inside the greenhouse during the day rose to around 35ºC. The second group of 25 pines was used to assess the susceptibility of *P. halepensis* to *D. pinea* and *D. scrobiculata* after the trees had been treated with 5CSA: 10 of these trees were inoculated with *D. pinea*, 10 with *D. scrobiculata* and 5 trees were the controls. The trees were watered as the trees of group 1, but 15 days before wound inoculation they were watered with 2 mM 5CSA every day.

Plant inoculation

Trees were inoculated on 16 June 2005. Prior to inoculation, the bark surface was disinfected in 95% ethanol for 10 s. A superficial wound 15 mm long was made in the bark tissues with a sterile scalpel approximately 15 cm below a shoot apex. A 5-mm diameter plug was removed from the margin of an actively growing culture and placed mycelium-side down on the wound. The inoculated area was sealed with parafilm to prevent desiccation. Control trees were treated in the same way but with plugs of sterile PDA. Tip-blight severity was evaluated after two months (on 16 August 2005) as the percentage of shoot dieback. At the end of the experiment, isolations were made from all the trees at the point of inoculation by plating wood pieces that had been disinfected with ethanol 70º, in PDA, and incubating them at 24ºC for fungal identification.

Statistics

Differences between concentrations of 5CSA on the radial growth of the fungus were evaluated by analysis of variance (ANOVA) for each time at which measurements were taken (48 h and 72 h of incubation). Fisher's least significance difference (LSD) test at P=0.05 was used to compare the radial growth means. The effectiveness of 5CSA *in vivo* was evaluated as percent deadtop incidence.

Results

Effect of 5CSA on the radial growth of *D. pinea* and *D. scrobiculata*

Strain 5CSA did not inhibit mycelial growth of *D. pinea* and inhibited that of *D. scrobiculata* only slightly. After incubating for 48 h at 24ºC, the mean colony diameter of *D. pinea* was not significantly different when directly exposed to 5CSA concentrations from 0 to 2.5 mM, but did differ significantly when exposed to 5CSA at 3 mM (P<0.001). In addition, after 72 h, *D. pinea* had completely overgrown all Petri dishes at all 5CSA concentrations and in the controls (P=0.4385).

With *D. scrobiculata*, mycelial growth was sig-

nificantly affected by the three highest 5CSA concentrations (2; 2.5 and 3 mM) (P<0.001) after 48 h. After 72 h, mycelial growth of this fungus was still reduced significantly (P<0.001) by 5CSA at these concentrations (Table 1). At both inoculation times, *D. scrobiculata* was significantly more sensitive to 5CSA than *D. pinea* (P<0.05).

Effectiveness of 5CSA against *D. pinea* and *D. scrobiculata* in *P. halepensis*

The effectiveness of 5CSA against *D. pinea* and *D. scrobiculata* affected the susceptibility of *P. halepensis* to these fungi. Non-pretreated trees inoculated with *D. pinea* developed tip blight symptoms 1 month after inoculation 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 stromata (Fig. 2). A gradual yellowing and wilting of the needles led to the death of the entire inoculated shoot (Fig. 3).

Pretreatment with 5CSA reduced the size of the lesions and made the symptoms less severe. Only 30% of the trees treated with 2 mM 5CSA developed shoot dieback when infected with *D. pinea*, compared with 60% of non-treated trees.

In untreated seedlings infected with *D. scrobiculata*, the cankers had the appearance of discoloured areas 1 month after inoculation, but necrosis of the bark became apparent later than it did in untreated seedlings infected with *D. pinea*. By the end of the experimental period, 30% of the infected seedlings without 5CSA developed deadtop, compared with 20% of seedlings that had received 5CSA.

Control seedling inoculated with sterile PDA plugs did not develop deadtop. 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 fungus was isolated from any of the control seedlings.

Discussion

Previous studies have shown that morphotype A is significantly more aggressive and produces more severe symptoms than does morphotype B (Blotgett and Stanosz, 1997, 1999). In this study the morphotypes differed in the rate at which symptoms developed in the *P. halepensis* seedlings. One month after inoculation, 60% of seedlings inoculated with *D. pinea* had well-developed cankers, and deadtop. In seedling inoculated with *D. scrobiculata,* on the other hand, the progress of the disease was slower. Cankers did not develop until two months after inoculation, and only 30% displayed deadtop. While *D. scrobiculata* is thought to have a much more limited distribution, Blodgett and Stanosz (1997) found that both fungi coexist on *P. banksiana* in north-central USA. Burgess *et al*. (2004) also reported *D. scrobiculata* in association with native *P. radiata* in different parts of North America. Our study suggests that morphotype B or *D. scrobiculata* is widespread in other *Pinus* species since we found it associated with *P. halepensis* in Catalonia.

Table 1. Mycelial growth (mm) of *Diplodia pinea* and *D. scrobiculata* on PDA medium supplemented with 0 (control), 0.2, 1, 2, 2.5 and 3 mM 5-chloro-salicylic acid. Colony diameter was determined 48 h and 72 h after treatment.

Concentration 5CSA (mM)	*D. pinea*[a,b]		*D. scrobiculata*[a,b]	
	48 h	72 h	48 h	72 h
Control	62.2±0.65 a	90±0 a	53.0±1.11 ab	90.0±0 a
0.2	60.6±0.44 a	90±0 a	55.6±1.52 a	77.0±1.76 a
1	61.2±1.29 a	90±0 a	52.8±1.29 ab	76.0±1.17 a
2	60.2±0.54 a	90±0 a	51.0±0.93 bc	68.8±1.63 b
2.5	60.0±0.93 a	90±0 a	48.4±0.57 dc	69.0±1 b
3	56.2±0.89 b	90±0 a	47.8±1.57 d	69.0±1.73 b

[a] Numbers are means ± standard error of 30 replicates.
[b] Means in columns followed by the same letter are not significantly different according to Fisher's least significant difference (P=0.05).

Fig. 1. Symptoms on *Pinus halepensis* inoculated with *Diplodia pinea*. Note the asexual fruiting structures (pycnidia) surrounding the wound on the infected branch.

Fig. 2. Symptoms of shoot blight on *Pinus halepensis* two months after inoculation by *Diplodia pinea*.

Some studies suggest that SA-induced resistance is associated with the systemic expression of a large number of defense genes, most notably those encoding the pathogenesis-related (PR) proteins, which play important roles in the restriction and spread of pathogens (Bowles, 1990; Hunt *et al*., 1996; Van London, 1997).

The present study looked for possible resistance factors in pine seedlings activated by treatment with the chemical activator 5CSA. Several studies have reported that SA pre-treatment enhances subsequent elicitor responses (Kauss *et al*., 1992, 1994; Draper, 1997). Our investigations clearly demonstrate that root feeding of 5CSA, a chemically related derivative of SA, affords effective protection against *D. pinea*.

An important question that remains is whether 5CSA acts as a fungicidal compound *in situ* and whether it is responsible for reducing disease symptoms directly or works indirectly by activating a signal involved in SAR. The present study showed that 5CSA does not directly affect the *in vitro* growth of *D. pinea* and *D. scrobiculata*, since in the *in vitro* test there was no significant decrease in the radial growth of the mycelium of these fungi.

Our findings are consistent with Spletzer and Enyedi (2000), who found that there was a significant decrease in disease incidence when pathogen inoculation was preceded by SA treatment. This suggests that the root system assimilated SA and distributed it throughout the plant, and this activated systemic disease resistance. SA and other, similar agents such as 5CSA, act as an endogenous signal in the development of systemic acquired resistance so that the host plant will either be more

resistant to further infection, or exhibit reduced disease symptoms when it is infected (Durrant and Dong, 2004).

In conclusion, the induction of disease resistance by synthetic chemicals (elicitors) applied to the soil is shown to be another tool for the integrated management of *D. pinea* and *D. scrobiculata* and offers an attractive alternative to traditional fungicides.

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