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Biocontrol of cypress canker by the phenazine producer *Pseudomonas chlororaphis* subsp. *aureofaciens* strain M71

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ABSTRACT

Pseudomonas chlororaphis subsp. *aureofaciens* strain M71 was tested for its efficacy in controlling *L. Seiridium cardinale*, the fungus responsible for bark canker of common cypress (*Cupressus sempervirens*). The bacterium was able to completely inhibit the mycelial growth and conidium germination of the fungus *in vitro* and prevented canker induction in field trials. Strain M71 produced two phenazine compounds, phenazine-1-carboxyilic acid and 2-hydroxyphenazine. They were extracted from the bacterial culture, purified, identified and tested for their activity against *S. cardinale* and three other fungi responsible for cypress canker, viz. *Diplodia cupressi*, *Seiridium cupressi* and *Seiridium unicorne*. Phenazine-1-carboxylic acid was the sole compound active against the four fungi. The application *in vivo* of this phenazine molecule against *S. cardinale* reduced canker size indicating that the compound is directly involved in the control of the fungal pathogen by *P. chlororaphis* subsp. *aureofaciens* strain M71. Furthermore, the antagonist showed an interesting capacity for epiphytic fitness since it was able to establish itself on the crown of cypress plants and survive on it for more than three months.

1. Introduction

Common cypress (*Cupressus sempervirens* L.) plays an important ecological and ornamental role in the Mediterranean area. Since ancient times this species has been used as a multipurpose tree and currently it is appreciated for the quality of its timber, for its value as an ornamental plant, for its tolerance to drought and poor soils and for its usefulness in agriculture (Graniti, 1998). Cypress has suffered from heavy epidemics of *Seiridium cardinale* (Wagener) Sutton and Gibson bark canker, that has developed into a true pandemic since 1928 (Graniti, 1998). In Italy and in the Mediterranean region, major epidemics spread during the last four decades and the disease incidence was reported to be as high as 90% in Peloponnese and 80% in Tuscany in 1980, resulting in severe damage and economic losses (Panconesi, 1990; Xenopoulos, 1991). The necrotic action of *S. cardinale* has been associated with the activity of cell wall degrading enzymes and with the killing of living bark cells due to the production of non-host specific toxic compounds such as four seiridins (Evidente et al., 1986; Sparapano et al., 1986; Evidente and Sparapano, 1994) and three seiricardines (Ballio et al., 1993; Evidente et al., 1993). Other canker-causing fungi such as *Diplodia cupressi* (sp. nov. Phillips and Alves), *Seiridium cupressi* (Guba) Boesewinkel anamorph of *Lepteutypa cupressi* (Nattrass et al.) Swart and *Seiridium unicorne* (Cooke and Ellis) Sutton were also reported to cause serious damage in many cypress plantations in various temperate regions throughout the world (Panconesi, 1990; Graniti, 1998). Control of *S. cardinale* canker of cypress has been based on sanitation, aimed at reducing the inoculum potential, on breeding for resistance (Danti et al., 2006) and on chemical prevention in nurseries and young ornamental plantations. Experiments conducted between 1970 and 1980 demonstrated that benzimidazole compounds and other fungicides prevented the onset of new infections in nurseries (McCain, 1984; Panconesi and Raddi, 1986). However, an adequate level of disease control on adult plants has never been achieved, so heavy losses due to *S. cardinale* attacks still occur on several Cupressaceae plants in the Mediterranean basin (Tsopelas et al., 2008; Danti et al., 2009). Current European Community control measures are directed towards a reduction of the use of chemicals in agriculture, including several products applied for the control of cypress canker (i.e. many benzimidazole formulations).

Biological control using antagonistic microorganisms may be a safer alternative to fungicides for management of cypress canker disease. To our knowledge, only one research study dealing with biological control of *S. cardinale* canker by a *Trichoderma viride* Pers. strain has been published so far (Magro et al., 1984). Several species of the genus *Pseudomonas* possess traits that make them useful for biological control, including the ability to colonize plant

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surfaces, produce antibiotics and adapt to environmental stresses (Weller, 2007).

In the present study a bacterial strain belonging to *Pseudomonas chlororaphis* (Guignard and Sauvageau, 1894, Bergey et al. 1930) species has been evaluated for: (i) antagonistic activity against *S. cardinale*, (ii) ability to colonize the cypress phylloplane and (iii) inhibition of canker induction on cypress plants. The main secondary metabolites produced by *P. chlororaphis* strain M71, were isolated, identified and assayed for their activity against different cypress pathogenic fungi.

2. Materials and methods

2.1. Antagonist isolation and identification

Strain M71 was isolated on nutrient agar amended with 0.25% of glucose (NAG) and 100 ppm cycloheximide from the rhizosphere of a tomato plant grown in a farm located near Salerno (Italy). The bacterium was previously identified to the species level by Biolog GN system (Hayward, California, USA) and phylogenetic analysis of *recA* (Puopolo et al., 2011). In order to determine the subspecies of *P. chlororaphis* strain M71 (Peix et al., 2007), tests for L-arabinose and 5-ketogluconate assimilation were performed on mineral salts (MS) medium (Ayers et al., 1919) at a final carbon source concentration of 0.1% (w/v). Strain M71 was grown on NAG plates for 24 h at 27 °C. Bacterial culture was used to prepare a 1×10^7 cfu/ml suspension in saline buffer (NaCl 0.8% w/v). Aliquots of 100 μl of this suspension were added to 5 ml of MS medium supplemented with the single carbon sources and to MS (control). For each treatment three tubes were inoculated. Suspensions were incubated at 27 °C for one week. The variation of the MS medium colour to yellow indicated the occurred assimilation of the specific carbon source. Non-fluorescent pigment production assay was performed by growing the strain on King's B (KB) agar medium for 48 h at 27 °C. Strain M71 has been deposited in the collection of phytopathogenic and antagonistic bacteria of the Istituto per la Protezione delle Piante-CNR, (IPP-CNR) Florence, Italy.

2.2. Production, extraction and identification of phenazines

Strain M71 was grown for 1 week at 27 °C in 1 l of Pigment Production Medium (PPMD) (Wood et al., 1997), with continuous shaking (150 rpm). The lyophilized material from the culture filtrate of strain M71 was dissolved in distilled water (200 ml), acidified to pH 2 with concentrated HCl and extracted with benzene (3×200 ml). The organic extracts were combined, dried on Na_2SO_4, and evaporated under reduced pressure. The oily residue (90 mg) was tested for antifungal activity according to the method described below and purified by preparative TLC on silica gel (Merck, Kieselgel 60, F_{254} 0.50 mm, eluent $CHCl_3$: *i*-PrOH 95:5).

Extracted compounds were identified using spectroscopic techniques. The IR spectrum was recorded as a deposited glassy film on a Perkin-Elmer Spectrum One FT-IR Spectrometer and UV spectrum was measured in 0.1 M NaOH on a Perkin–Elmer spectrophotometer; ^1H and ^{13}C NMR spectra were recorded at 600 and 75 MHz, respectively, in $CDCl_3$ on a Bruker spectrometer. The same solvent was used as the internal standard. ESI-MS spectra were recorded on Waters Micromass Q-TOF Micro and Agilent 1100 coupled to JOEL AccuTOF (JMS-T100 LC) instruments.

2.3. In vitro antagonistic activity

Inhibitory activity of strain M71 against *S. cardinale* (ATCC 38654) and other fungal pathogens responsible for cankers on cypress, including *D. cupressi* (IPP-CNR Collection, Italy, n. 110), *S. cupressi* (CBS 122615) and *S. unicorne* (CBS 122613) was assessed using the dual culture method. Fungal strains were grown on Petri dishes containing Potato Dextrose Agar (PDA) at 24 °C for 3–7 days. Five mm diameter plugs from the edge of actively growing colonies were cut and separately placed at the center of PDA plates. At the same time, the bacterial strain was streaked twice at 3 cm from the plug. Three plates were seeded for each treatment, while three plates inoculated with the fungus alone were used as control. Plates were incubated at 25 °C for 7–15 days based on the growth rates of the different fungi. Inhibition activity was determined by the comparison between the average reductions in the diameter size of the fungal colonies in dual culture with the size of the colonies of single fungal culture.

In vitro production of conidia by the four pathogens was obtained by growing the fungi on sterile cypress seeds. Seeds were sterilized by autoclaving (30 min at 120 °C) and put into sterile Petri dishes. Subsequently, 20 ml of water agar was poured in each plate and allowed to dry. Plates were then seeded at the center by placing a plug cut from the edge of actively growing colonies of each of the four phytopathogenic fungal species (*D. cupressi*, *S. cardinale*, *S. cupressi* and *S. unicorne*) and incubated in a growth chamber at 24 °C with a photoperiod of 16 (light)/8 h (dark) for one month. Dark and shiny drops of conidia extruded from the conidiomata that developed on seeds were collected and transferred in malt extract broth. Conidia were counted using a Burker slide under a microscope and suspensions were diluted to a final concentration of 1×10^6 conidia ml^{-1}.

Strain M71 was grown in 5 ml of PPMD broth on a rotary shaker at 100 rpm for 48 h at 25 °C. Cells were separated by centrifugation at 12000 rpm for 10 min, the supernatant was sterilized by filtration (Millipore 0.2 μm) and stored in aliquots of 1 ml in sterile tubes at 4 °C. Five milliliter of the suspensions containing conidia were mixed with five ml of the bacterial filtrate and incubated on a rotary shaker at 25 °C for one month. Controls were done by mixing, at the same ratio indicated above, conidial suspension with sterile nutrient broth and conidial suspension with sterile distilled water. Five tubes for each treatment were prepared. Aliquots of the suspensions were taken at 24 h intervals for the first week and then once a week for the next three weeks and observed under a light microscope to determine the number of germinated conidia. The whole experiment was repeated three times.

Moreover, the purified compounds extracted from the filtrate of the bacterial culture were tested for their activity on germination of conidia of the four fungal species. The surface of PDA plates were spread with 100 μl of conidial suspension (1×10^6 conidia ml^{-1}) and then a sterile disk of filter paper (2 cm diameter) soaked with 30 μl of a 4 mg/ml solution of each compound was placed at the center of the plates. NaOH (0.1 M) solution was used to dissolve both lyophilized compounds. Disks soaked with 30 μl of NaOH (0.1 M) solution were used as control. Formation of an inhibition halo around the filter paper indicated antibiotic activity against the fungi. For each treatment three plates were inoculated. The test was repeated three times. The activity of the compounds was evaluated as the average of the size of the inhibition halo measured for the different replications.

2.4. Epiphytic behavior on cypress plants

A natural derivative of strain M71 resistant to rifampicin was obtained by growing the bacterium on NAG plates amended with increasing concentrations of the antibiotic. The marked strain, named M71 rif^+, was employed to study the epiphytic behavior of the antagonist on cypress. Before its application, the physiological profile of the marked strain was checked with Biolog GN system in order to assess potential metabolic differences with the wild type strain. Moreover, M71 rif^+ *in vitro* antagonistic activity