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Research article

Phosphite shifts physiological and hormonal profile of Monterey pine and delays *Fusarium circinatum* progression

Andreia Cerqueira [a], Artur Alves [a], Helder Berenguer [a], Barbara Correia [a], Aurelio Gómez-Cadenas [b], Julio Javier Diez [c, d], Pedro Monteiro [a], Glória Pinto [a, *]

[a] Department of Biology, CESAM (Centre for Environmental and Marine Studies), University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[b] Universitat Jaume I, Departamento de Ciencias Agrarias y del Medio Natural, 12071 Castelló de la Plana, Spain
[c] Sustainable Forest Management Research Institute, University of Valladolid - INIA, Avenida de Madrid 44, Palencia, Spain
[d] Department of Plant Production and Forest Resources, University of Valladolid, Avenida de Madrid 44, Palencia, Spain

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ABSTRACT

Fusarium circinatum is the causal agent of pitch canker disease affecting *Pinus* spp. and *Pseudotsuga menziesii* worldwide. Under strict quarantine measures, alternative approaches for disease control are necessary. Phosphite (Phi) salts are known for their fungicidal activity and as plant resistance elicitors; however, its potential is yet to be acknowledged in the *Pinus-F. circinatum* model. The main aim of this study was to assess whether the application of a Phi-based commercial formulation would delay the progression of the pitch canker on *Pinus radiata* plants, and on the in vitro fungal growth. In vitro assays were performed using different Phi concentrations (1% and 4%) and a non-treated control (0%), and repeated in vivo using inoculated and non-inoculated plants. Plant physiological parameters and hormonal content were evaluated. Phi was effective at inhibiting in vitro mycelial growth in a dose dependent manner. Regardless of fungal inoculation, Phi application induced positive effects on plant performance, despite phytotoxic effects found at 4%. *Fusarium circinatum* infection led to a reduction in gas exchange and chlorophyll fluorescence (Fv/Fm and φPSII), while proline and hormone (JA, ABA and SA) levels increased. Phi was effective in delaying disease symptom development in a dose dependent manner, concurrent with in vitro observations: gas exchange and chlorophyll fluorescence (Fv/Fm) were unaffected; proline, MDA and ABA decreased; electrolyte leakage and total soluble sugars increased. This suggests a direct (pathogen growth inhibition) and indirect (host defense priming) action of Phi, showing that Phi represents a potential strategy to control *F. circinatum* infection.

1. Introduction

Forests play vital roles at natural and anthropic levels in Europe and represent 33% of the total land area (215 million ha). Conifers, mainly composed by *Pinus* spp., account for 112.95 million ha of the European forested area (de Rigo et al., 2016). *Pinus* is one of the most ecologically and economically significant tree genera in the world, and, in addition to numerous roles in ecosystems, pines represent an important source of timber, pulp and paper, seeds, charcoal, resin, and construction materials (Richardson and Rundel, 2000).

Phytopathogenic organisms pose a serious threat to the conservation and productivity of both native forests and plantations. The necrotrophic fungus *Fusarium circinatum* Nirenberg & O'Donnell (= *Gibberella circinata*) is one of the most important pathogens affecting *Pinus* spp. and *Pseudotsuga menziesii* (Mirb.) Franco worldwide, and the causal agent of pitch canker disease. Pine species present a differential resistance to *F. circinatum*, and one of the most economically relevant pine species in the world, Monterey pine (*Pinus radiata* D. Don), appears to be the most susceptible species (Wingfield et al., 2008). Since it was first described in 1946 in the United States, the pathogen has been recorded in numerous countries worldwide (Ganley et al., 2009), and affects all stages of tree development, provoking large resinous cankers that impregnate tissues and hamper the movement of water and nutrients (Martín-Rodrigues et al., 2013). In mature trees, typical disease

* Corresponding author.
E-mail address: gpinto@ua.pt (G. Pinto).

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symptoms include wilting and chlorosis of the needles, causing canopy dieback and tree mortality. In nurseries, *F. circinatum* is responsible for contamination of seeds and damping-off of seedlings (Gordon et al., 2015).

There is a growing body of evidence showing that successful pathogens have the ability to subvert plant defense mechanisms, either by avoiding their recognition or by reprogramming host metabolism, leading to changes in host growth and development (Dalio et al., 2014). On the other hand, plants have the ability to respond to infection through several mechanisms, which includes induction of defense as well as changes in primary metabolism, with further implications in growth and plant performance. Altered metabolism involves changes in the photosynthetic performance, carbohydrate and nitrogen metabolism, as well as changes in the oxidative stress dynamics (Berger et al., 2007; Bolton, 2009). Other important signaling players involved in the induction of defense include jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) (Verma et al., 2016).

To date, there are no suitable approaches available to control the pine pitch canker disease, being associated with elevated economic losses to the forestry sector. Strict quarantine measures have been applied to prevent *F. circinatum* dissemination, and management programs include appropriate nursery and silvicultural management, importation bans and genetic selection of resistance species (Wingfield et al., 2008). The induction of plant resistance is a potential alternative approach that has been widely explored in agriculturally important crop plants to manage plant health. Induced resistance (IR) represents a physiological state of enhanced defensive capacity stimulated by biotic or abiotic elicitors, whereby the plant's innate defenses are enhanced against subsequent challenges. This capacity for augmented defense is called priming, and offers a wide spectrum of protection against biotic and abiotic stresses (Machinandiarena et al., 2012).

Phosphites ($H_2PO_3^-$; Phi), a group of alkali metal salts of phosphorus acid, are used as fertilizers, have fungicidal activity and potential for activating plant resistance (Dalio et al., 2014) functioning as elicitors. Despite its mode of action being complex and not fully understood, it most certainly includes mycelial growth inhibition and host defense stimulation (e.g. production of reactive oxygen species [ROS]) (Liu et al., 2016). Phosphites are known for reducing infection by oomycetes, bacterial and fungal pathogens (Guest and Grant, 1991; Percival and Banks, 2015). Although Phi application has been studied in *P. radiata* for *Phytophthora cinnamoni* control (Ali et al., 2000) with low phytotoxicity (Scott et al., 2016), no reports where Phi was used in the control of *F. circinatum* disease could be found.

In this study, we investigated the mode of action of potassium phosphite (Phi) on plant physiological performance and hormonal content of pre-treated plants having *Pinus radiata-Fusarium circinatum* as model. We hypothesized that Phi foliar application would have a protective role and alter the pitch canker disease progression. The effects of Phi in the in vitro pathogen growth were also evaluated. With this study, we expect to contribute to the improvement of the current *F. circinatum* control strategies as well as to fulfil some knowledge gaps related to the interaction between fungus, elicitor, and the plant physiological performance in forest species.

2. Materials and methods

2.1. In vitro assay

An isolate of *Fusarium circinatum* (FcCa6) was obtained from the collection of the Forest Entomology and Pathology Lab at the University of Valladolid. The isolate was grown on half strength potato dextrose agar (PDA; Merck, Darmstadt, Germany) medium supplemented with Phi, prepared from a potassium phosphite commercial formulation (Trafos Sinergy, Nutrisapec, Portugal), nutrient enriched, at 1% and 4% in distilled water (v/v; pH 5.6). A control, not-treated with potassium phosphite (Phi 0%), was also prepared. Uniform pathogen-colonized PDA plugs (2.5 mm^2) were inoculated at the center of a Petri dish filled with fresh medium, followed by incubation at 20 ± 2 °C. Five replicates per treatment were used. Measurements of the mycelial radial growth were performed daily until the control reached the edge of the Petri dish.

2.2. In vivo assay

2.2.1. Plant material

Six-month old *Pinus radiata* (25 ± 1 cm height) plants were obtained from Cultiflor Nursery (Pombal, Portugal). The plants were placed in 1 l plastic pots filled with 3:2 (w/w) peat:perlite and kept in a climate chamber (Fitoclima D1200, Aralab, Portugal) under 16 h light/8 h darkness at 25 °C/15 °C (day/night), and 60% relative humidity. Photosynthetic photon flux density (PPFD) during the day was 500 μmol m^2 s^{-1}. The plants were regularly well-watered and fertilized (Frutifol, Nufarm, Portugal) every week.

2.2.2. Experimental design

Two Phi concentrations ($Phi_{1\%}$ and $Phi_{4\%}$, pH 5.6) were prepared from a commercial formulation of Phi (Trafos Sinergy, Nutrisapec, Portugal). A control, not-treated with Phi commercial formulation (C, pH 5.6), was also prepared. Plants were acclimatized for two months and three groups of 30 *P. radiata* plants each were treated as follows: plants were foliarly sprayed with $Phi_{0\%}$, $Phi_{1\%}$ and $Phi_{4\%}$ until run-off, using a hand-held sprayer 17 days prior to inoculation, to allow the Phi absorption and uptake before plant inoculation.

For inoculations, the *F. circinatum* isolate was grown on half strength PDA medium at 20 ± 2 °C for 7 days. Inoculation was performed as described by Cinelli et al. (2015). The stems were wounded with a sterile scalpel and an agar plug colonized with *F. circinatum* mycelium was placed mycelial surface down onto the wound and sealed with Parafilm®. Sterile PDA plugs were used as a negative control. The above pre-treated plants (including control) were then divided into two groups of non-inoculated and *F. circinatum* inoculated plants resulting in a total of six treatments: pre-treated with 0% Phi (C); inoculated plants pre-treated with 0% Phi (F); pre-treated with 1% Phi ($Phi_{1\%}$); inoculated plants pre-treated with 1% Phi (F + $Phi_{1\%}$); pre-treated with 4% Phi ($Phi_{4\%}$); and inoculated pre-treated with 4% Phi (F + $Phi_{4\%}$). The experiment was conducted under the environmental conditions as described for the acclimatization period. Plants were watered every other day and randomly moved every two days.

2.2.3. Sample collection

Each treatment (inoculated and respective controls) was sampled once when 50% of the inoculated plants within each treatment displayed the initial typical disease symptoms (tip dieback). After in vivo morpho-physiological related parameters measurement, such as disease symptoms, growth, water potential, gas exchange, chlorophyll fluorescence and electrolyte leakage, needles were collected, frozen in liquid nitrogen, and stored at −80 °C for biochemical analysis (pigments, proline, total soluble sugar and lipid peroxidation quantification) and hormone quantification. Controls and inoculated symptomatic plants were measured. Also, to confirm Koch's postulates, stem portions (above the inoculation point) were placed in PDA medium and incubated at 20 ± 2 °C for 7 days.