Systemic Immunity against Soil Borne *Phytophthora* and Control of Ink Disease of Chestnut by Foliar Spray of Potassium Phosphonate

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Abstract

Chestnut ink disease represents a great risk for chestnut (*Castanea sativa* Mill.) in all regions of chestnut production of Europe and North America. Induced by soil borne oomycetes, *Phytophthora cinnamomi* and *P. cambivora*, both parasites infect the root system and cause root and collar rot resulting in dieback, decline and finally, the death of infected trees. There are no single control measures for root rot pathogens and control with systemic and selective oomycetes biochemical substances can be important as part of the integrated disease management of these soil borne parasites. In this work we studied the effect of potassium phosphonate to protect roots from *P. cinnamomi*. Chestnut seedlings were planted in potting mix previously inoculated with *P. cinnamomi* (Pr 120). One group of five pots, with three seedlings each were submitted to potassium phosphonate foliar spraying (3 ml L^{-1}Atlante®) and another group of five pots were water foliar sprayed. Seedlings had been grown in a nursery for 120 days. At the end of the experiment, visual symptoms of the crown and root system were assessed. Parameters related with roots: root length, root rot length, root number, root rot number, crown diameter, height and biomass were evaluated. Statistic significant differences between treatments were obtained in all root studied variables. Potassium phosphonate by foliar spraying prevented *P. cinnamomi* infections of roots of chestnut and can be a key factor in the management of ink disease of chestnut.

INTRODUCTION

Chestnut ink disease represents a great and continuing threat to chestnut (*Castanea sativa* Mill.) in all regions of chestnut production. Induced by soil borne oomycetes, *Phytophthora cinnamomi* and *P. cambivora* (Tucker, 1931; Pimentel, 1947; Urquijo, 1947; Crandall, 1950; Grente, 1961), both parasites infect the root system and cause root and crown rot. Symptoms can be apoplectic and the tree rapidly dies or results in dieback, long-lasting decline and in the end, death of the infected trees occurs. Dead roots reduce water and mineral uptake which stresses the tree over several years before symptoms of dieback and rearing of the crown are visible above ground. Symptoms of the disease may be non-specific and diverse which hamper diagnoses and management. Despite the presence of the pathogens and their devastating epidemics in Europe since the second half of the 19th century (Zentmyer, 1980) and even though many and varied approaches to control the disease were studied, there are no simple solutions for these root rot pathogens. Chemical control with systemic and selective oomycetes products can be an important factor and part of the integrated management of soil borne *Phytophthora*. Phosphonates are salts or esters of phosphonic acid (Guest and Grant, 1991) but in recent literature, when these products were applied for protection of plant diseases the term phosphite is also frequent. Daniel et al. (2005) and Daniel and Guest (2006) stated that following phosphonate treatment challenged cells in normally susceptible plant species undergo rapid cytological changes that include nuclear migration and hypersensitive cell death, activate defence-related biosynthetic pathways and accumulate higher levels of phytoalexins and deposit of physical barriers around challenged cells. Activities of

enzymes involved in defence mechanisms are also elevated in normally susceptible *Eucalyptus marginata* seedlings treated with phosphonate that includes phenylalanine ammonia lyase (PAL), 4- coumarate coenzyme-A ligase (4-CL), cinnamyl alcohol dehydrogenase (CAD), and the synthesis of phenolic compounds (Jakson et al., 2000).

Phosphonates are officially approved to control Sudden Oak Death associated with *P. ramorum* (Garbelotto and Douglas, 2009) in California and "Jarrah" dieback associated to *P. cinnamomi* (Jakson et al., 2000) in Australia and some reported experiments reveal phosphonate efficacy in many others *Phytophthora* host interactions (Wilkinson et al., 2001). A range of responses to phosphonate application are also reported depending on the plant species and time of application (Wilkinson et al., 2001; Navarro et al., 2006). The present work provides data on biological activity of potassium phosphanate ($H_2PO_3^-$), the anionic form of phosphonic acid, against *P. cinnamomi* in chestnut seedlings and its efficacy in controlling ink disease by preventive foliar application. To take into account some potentially negative effects of phosphonate, direct toxicity against beneficial soil micro-organisms were also evaluated in vitro to provide useful information to aid in the implementation of a viable management treatment for ink disease of chestnut.

METHODS

Nursery Plant Experiments

Experiments where performed at an IPB/ESA nursery and carried out on pot mix previously inoculated with *P. cinnamomi* (Pr 120). Chestnut seedlings similar in size and vigour were selected, rinsed free of original substrate and immediately planted in the uniformly *P. cinnamomi* infested potting mix. Seedlings were sprayed with distilled water or potassium phosphonate (Atlante® - 30% P_2O_5, 20% K_2O). The seedlings were sprayed to run-off using a sprayer. Atlante® was applied as a formulated product at the recommended field dosage application (3 ml L^{-1}). The foliage was allowed to dry naturally for 12h before the watering programme was re-established. Pots were also flooded weekly. Fifteen seedlings were used per treatment with three seedlings per pot of 15 L capacity arranged randomly. After 120 days, chestnut seedlings were removed and the roots rinsed free of soil particles. Root rot length, total root length, root weight, visual above-ground symptoms and biomass of root and stem were evaluated. Additionally, segments from necrotic roots and healthy ones and also their respective potting mix were plated on PVPH medium for re-isolation of the inoculated *P. cinnamomi*. Statistical analysis was conducted using the student t-test for means comparison on untransformed data to analyse the differences between phosphonate and water application. The significance level of the test (p-value) was set at 0,05. The assumptions on normality and heterocedasticity of residual values were checked by Shapiro-Wilk test and Levene, respectively (software SPSS 16.0®).

In Vitro Phosphonate Toxicity

Tests in vitro were performed to provide some information related to phosphonate toxicity to *P. cinnamomi* and *P. cambivora* isolates, *Cryphonectria parasitica*, a virulent fungus that causes chestnut blight, and *Pisolithus tintorious*, a basidyomicete and mycorrhizal chestnut root fungus. Five-mm plugs of each isolate from an active culture grown on PDA (Difco) were transferred to three replicates PDA plates containing 0, 5, 20, 50 µg ml^{-1} of potassium phosphonate, then the plates were incubated at 22-24ºC for 5 days. Colony diameters were measured and the average colony area was determined for each concentration and expressed as the percentage of the no-phosphonate control. Percentage values were plotted as probit versus $\log_{10}$ of the phosphonate concentration and analyzed by linear regression. The regression equation was used to estimate the EC_{50} (concentration inhibiting mycelia growth by 50%) for each isolate.

RESULTS

Nursery Plant Experiment

At the end of the experiment differences in general conditions and above ground symptoms of chestnut seedlings were obvious. All chestnut seedlings that grew in *P. cinnamomi* infested potting mix and treated with phosphonate were apparently healthy whereas the water application seedlings manifested visible and characteristic ink disease symptoms. When chestnut seedlings were removed from the pots, differences in root rot symptoms and root development were also easily observable and marked.

Statistical analyses by the t-test and means comparison revealed significant differences between treated and untreated chestnut seedlings for all root studied parameters, namely, root length, healthy root length, root rot length, root number, root rot number, root weight and biomass of roots (Table 1).

In Vitro Phosphite Toxicity

EC_{50} values for *P. cinnamomi* were generally near or below 1 μg ml^{-1} but the isolate Pr 125 has shown a higher value which revealed the existence of reduced sensitivity in some *P. cinnamomi* isolates. EC_{50} values for *P. cambivora* averaged 16,2 μg ml^{-1} ranged from 9,92 to 22,44 μg ml^{-1} (Table 2). The basidiomycete *Pysolithus tintorius*, a mycorrhizal fungus of chestnut seedlings, evidenced an intermediate value. Higher values of EC_{50} were obtained for the tested ascomycete *Cryphonectria parasitica* virulent strain associated with chestnut blight.

DISCUSSION AND CONCLUSIONS

Phosphite and phosphonate mode of action in plant resistance is still not completely elucidated but appears to be a combination of direct inhibition of pathogen growth and stimulation of plant defence responses, possibly via an increase in the production of pathogen derived elicitors or a decrease in the production of pathogen derived suppressors of the defence response as stated by some authors (Jackson et al., 2000; Guest and Grant, 1991; Hardham, 2005).

In this experiment all parameters related with roots were significantly different in chestnut seedlings sprayed with phosphonate. Healthy status of the root system after four months of phosphonate treatment suggested a defence response in the pre-infection stages similar to those observed in an incompatible interaction.

Potassium phosphonate by foliar application obviously did not remove *P. cinnamomi* from the soil and the pathogen was always in contact with the roots as was confirmed by isolation from the potting mix and root tissues on PVPH medium. Global control strategies of soil borne *Phytophthora* inevitably will have to include other sanitary measures related to the hygiene and sanitation of vegetative material in nurseries to avoid the introduction and spread of pathogens in the field and to minimize environmental determinants.

Higher in vivo activity of potassium phosphonate than observed in vitro makes EC_{50} a weak tool for predicting the final effect of phosphonate on beneficial or pathogenic microbes. Microbe-plant mycorrhizal interaction must be evaluated because plants may become vulnerable by the possible disruption on their symbiotic microbes. Further studies are also needed to determine potential negative effects such as the induced deficiency of phosphorus reported in the literature by some authors (McDonald et al., 2001; Landchoot and Cook, 2005) as well as optimal rates of active ingredient and ideal time of application.

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Tables

Table 1. Statistical summary of root parameters and above ground parameters of chestnut seedlings that grew in potting mix infested with *Phytophthora cinnamomi* with preventive foliar phosphonate or water spraying.

Parameters	Treatment	Mean±std. error
Root length (cm)	Phosphite sprayed	1027,04±120,29 a
	Water sprayed	660,40±85,77 b
Healthy root length (cm)	Phosphite sprayed	991,57±124,06 a
	Water sprayed	31,88±31,88 b
Root rot length (cm)	Phosphite sprayed	31,54±12,71 a
	Water sprayed	628,52±66,79 b
Root number (nº)	Phosphite sprayed	49,57±4,54 a
	Water sprayed	36,62±2,58 b
Root rot number (nº)	Phosphite sprayed	3,07±1,27 a
	Water sprayed	36,23±2,70 b
Root dry weight (g)	Phosphite sprayed	2,52±0,44 a
	Water sprayed	1,05±0,10 b
Stem and foliar dry weight (g)	Phosphite sprayed	4,06±0,49 a
	Water sprayed	4,66±0,45 a
Annual grown (cm)	Phosphite sprayed	9,68±1,11 a
	Water sprayed	11,04±1,35 a
Crown diameter (cm)	Phosphite sprayed	0,63±0,03 a
	Water sprayed	0,53±0,04 b

Means in each parameter followed by the same letter do not differ significantly at P≤0,05 by t-test

Table 2. EC_{50} (µg ml^{-1} of potassium phosphonate - Atlante®) of *Phytophthora cinnamomi*, *P. cambivora*, *Pisolithus tinctorius* and *Cryphonectria parasitica*.

Species	*P. cinnamomi*				*P. cambivora*		*Pisolithus tinctorius*	*Cryphonectria parasitica*
	Pr120	810	804	Pr125	Ar102	Pr135		
EC_{50}*	1,34	0,64	0,97	31,56	9,92	22,44	14,10	44,39

*Concentration of potassium phosphonate (Atlante®) causing 50% of mycelial growth inhibition compared to growth on no amended PDA