Forest Pathology WILEY-BLACKWELL

For. Path. doi: 10.1111/efp.12216

Action of fosetyl-al and metalaxyl against *Phytophthora austrocedri*

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Summary

Fosetyl-Al and metalaxyl, the most commonly used systemic fungicides against *Phytophthora*, were evaluated for their efficacy to control *Phytophthora austrocedri*, the pathogen that causes a serious disease at the *Austrocedrus chilensis* forests in Patagonia. The effect of the chemicals on pathogen development *in vitro* and *in planta* was analysed. Both chemicals were shown to protect plants from the pathogen. *In vitro* assays showed that asexual reproduction was sensitive to both chemicals. However, mycelial growth and sexual reproduction, which were clearly sensitive to metalaxyl, were sensitive only to high concentrations of fosetyl-Al. Fosetyl-Al and metalaxyl had almost the same efficacy when applied preventively by soil drench to seedlings. This difference between *in vitro* and *in planta* results can be attributed to the dual action of fosetyl-Al, not only inhibiting the pathogen but also stimulating host defence. In adult trees, preventive and curative treatments were tested, but only the fosetyl-Al preventive treatment was effective in the assayed conditions. Interestingly, seedlings pretreated with both fungicides were less susceptible to the effectors secreted by the pathogen. Our results indicate that fosetyl-Al and metalaxyl provide some resistance to the plant besides the fungistatic direct action on the pathogen. Further studies to elucidate a possible resistance-inducing activity of these chemicals and the mechanisms involved are underway.

1 Introduction

Phytophthora austrocedri has been found to be a primary pathogen causing '*Austrocedrus* root disease (ARD)', a lethal disease of *A. chilensis* trees that has also been named as 'Mal del Ciprés' (cypress sickness). It is a soilborne pathogen that causes large necrotic lesions in the cambium and phloem of roots and stem base, necrosis of parenchyma rays and tracheids blockage, and progressive decrease in net photosynthesis, stomatal conductance and stem-specific hydraulic conductivity (Greslebin and Hansen 2010; Vélez et al. 2012). It has also been demonstrated that the symptoms of *Phytophthora* infection can be observed well away from the site of infection due to the presence of *Phytophthora*-derived effectors that cause alteration of leaf tissue integrity, necrosis and net photosynthesis decrease in infected plants, prior to significant changes in other parameters and when necrosis of vascular tissues is still incipient (Vélez et al. 2012).

Austrocedrus chilensis is a key species of Patagonian Andes forests with high economical, ecological and cultural relevance. It is the most widely distributed species among the few conifers inhabiting southern Argentina, covering 140 000 ha (Bran et al. 2002) in a wide range of environments (Veblen et al. 1995; La Manna 2005). Cypress mortality has great impacts on the local economy but also on ecosystem functions and processes because, as a foundation species (Ellison et al. 2005), *A. chilensis* defines forests structure, microclimate and forest dynamics.

The disease was firstly detected in the 1940s in an island of Nahuel Huapi National Park (Neuquén province, Argentina). Today, it is widely distributed throughout the cypress range and causes serious damage, especially in those areas where site conditions favour pathogen development (La Manna and Matteucci 2012; La Manna et al. 2012).

The study of the genetic diversity of *P. austrocedri* populations in Patagonia showed high levels of within-species genetic similarity and no evidence of partitioning of genetic diversity among the distribution range, suggesting that the pathogen represents a single population that has been introduced into Argentina (Vélez et al. 2014).

Phytophthora austrocedri was first described causing '*Austrocedrus* root disease', and it had not been reported from other hosts or from other sites outside Patagonia until recently, when it was reported causing root disease and mortality on *Chamaecyparis nootkatensis and C. lawsoniana* in Scotland (EPPO Reporting Service N° 6, 2011, Path News issue 23: autumn 2011. www.forestry.gov.uk) and, afterwards, causing a damaging disease in rare native *Juniperus communis* bushes in England and Scotland (Path News issues 27 and 28: autumn 2013 and spring 2014. www.forestry.gov.uk), and also affecting *J. horizontalis* (Path News issue 27: autumn 2013. www.forestry.gov.uk). It was also recorded in Germany on *Juniperus horizontalis* (Werres et al. 2014). In all these cases, it was assumed that the pathogen was introduced into these areas.

No measures to control *P. austrocedri* in Patagonia have been implemented yet. Preventive actions to avoid pathogen spread were not applied previously because the pathogen remained unknown until 2006 (Greslebin et al. 2007). Assuming that the first report of the disease coincides with the introduction of the pathogen, it has been spreading for more than 60 years without any control measures being implemented.

Even tardily, a desirable management goal is to stop the spread of the pathogen and the disease it causes. However, this strategy would be effective only to protect disease-free sites, whilst the vast area of forest affected by the pathogen requires other actions.

Received: 29.12.2014; accepted: 19.6.2015; editor: W. Osswald

Phytophthora diseases in agricultural crops, natural ecosystems and commercial nurseries are frequently managed through chemical treatments. The effects of fungicides has been the subject of a large number of studies, and key issues, as the mode of action, dosage, phytotoxicity, time and mode of application, as well as development of pathogen resistance, are widely discussed in the literature (see Erwin and Ribeiro 1996 for literature review). The complexity of natural ecosystems adds additional difficulties to chemical control but, despite this, there are some successful experiences of fungicide application in natural vegetation to control *Phytophthora* diseases (Hill et al. 1995; Aberton et al. 1999; Fernández-Escobar et al. 1999; Hardy et al. 2001; Shearer et al. 2004; Jung et al. 2009).

Systemic fungicides, such as phosphite (phosphonate), and phenylamide compounds are widely used to control *Phytophthora* infection in crops and nurseries. Metalaxyl is a phenylamide-based systemic fungicide with specific activity against oomycetes, widely used to prevent and to control diseases caused by *Phytophthora* and *Pythium* in many different crops (Cohen and Coffey 1986). Phosphite, also called phosphonate, refers to the salts of phosphonic acid (H_3PO_3). The anionic form of phosphonic acid (HPO_3^{2-}) controls many plant diseases caused by *Phytophthora*, even at low concentrations. Systemic fungicides based on $HPO_3^{2-}$are called 'phosphites' and the most commonly used are fosetyl-Al and potassium phosphite, which are the salts of phosphonic acid with aluminium and potassium, respectively. Phosphite exhibits a complex mode of action, acting directly on the pathogen and indirectly by stimulating host-defence responses (Guest and Grant 1991). Plants pretreated with this compound accumulate phytoalexins faster (Raynal et al. 1980; Guest 1984). The antifungal effect of fosetyl-AI is decreased by glyphosate and α-aminooxyacetic (AOA), metabolic inhibitors of phytoalexin synthesis (Bompeix et al. 1981). Oxidative burst, rapid (hypersensitive) cell death and accumulation of phenolic compounds have been demonstrated as phosphonate-elicited defence responses in *Arabidopsis thaliana* infected by *P. palmivora* (Daniel and Guest 2006; Eshraghi et al. 2011, 2014a,b). On the basis of these studies, it has been hypothesized that fosetyl-Al has an indirect action inducing hypersensitive and/or systemic acquired disease resistance (SAR) responses, but other studies, showing that oomycete strains selected for insensitivity to fosetyl-AI *in vitro* are no longer controlled by the compound *in planta*, contradict this hypothesis (Fenn and Coffey 1985; Dolan and Coffey 1988). In the same way, selection for decreased sensitivity of *P. cinnamomi* to phosphite *in planta* has been demonstrated in sites with prolonged use of phosphite (Dobrowolski et al. 2008). Similar results have been obtained in studies on metalaxyl action in the plant, and it has been proposed that metalaxyl treatments may result in the stimulation of host-defence mechanisms in infected plants by the release of elicitors of phytoalexin biosynthesis (Cahill and Ward 1989).

Successful chemical treatments using phosphonate and/or phenylamide compounds have been reported to control *Phytophthora* species in various forest tree species (Erwin and Ribeiro 1996; Fernández-Escobar et al. 1999; Jackson et al. 2000; Navarro Cerrillo et al. 2004; Heungens et al. 2005; Shearer et al. 2006; Garbelotto et al. 2009; Scott et al. 2013). Based on this background and the lack of information on the effect of systemic fungicides on *P. austrocedri,* the aim of this study was to evaluate the sensitivity of *P. austrocedri* to systemic fungicides and their efficacy to control the development of the pathogen in the host.

2 Materials and methods

2.1 *In vitro* assays

Five *Phytophthora austrocedri* isolates (Table 1), deposited in the culture collection of Centro de Investigación y Extensión Forestal Andino Patagónico (CIEFAP), were obtained from declining *Austrocedrus chilensis* stands during different surveys according to Greslebin et al. (2007). Isolates were maintained on clarified vegetable juice 15% agar supplemented with 30 mg ml^{-1} β-sitosterol at 17°C. Vegetable juice agar (VA) was prepared as follows: 375 ml (520 g) of tomato juice (La Campagnola; ARCOR, Córdoba, Argentina) was buffered with 10 g of calcium carbonate, stirred for at least 30 min, centrifuged for 20 min at 1000 ***g***, and the supernatant was used (200 ml l^{-1}) to prepare the medium.

In vitro evaluation of fungicide activity was based on inhibition of mycelial growth, sporangia and oospore formation. Fungicides used were metalaxyl (M) (Apron Gold®, Syngenta, 35% N-[2,6-dimethylphenyl]-N-[methoxyacetyl]-alanine methyl ester) and fosetyl-Al (F) (Aliete®, Bayer, 80% aluminium tris-o-ethyl-phosphonate).

Table 1. Isolates used in the study: number of isolates at culture collection, geographic origin and GenBank accession number of sequences.

Isolate number	Origin	GenBank accession N°
Phy-195 ATCC N° MYA-4073	'La 106' property, Río Grande Valley, Chubut, Argentina (43°12′55.5′′S, 71°32′50.9′′W)	ITS: DQ995184
Phy-203 ATCC N° MYA-4074	Braese stream, Los Alerces National Park, Argentina (42°46′29.97′′S, 71°32′11.27′′W)	ITS: DQ995184
Phy-233	Río Grande Valley, Los Alerces National Park, Argentina (43°9′55.8′′S, 71°42′18.4′′W)	–
Phy-256	Traful Lake, Neuquén, Argentina (40°39′48.024′′S, 71°22′20.71′′W)	–
Phy-271	Corcovado, Chubut, Argentina (43°34′31.11′′S, 71°41′10.64′′W)	ITS: JX121856; coxI: JX448318

Fosetyl-Al was added to the medium and then autoclaved, whilst metalaxyl was sterilized by filtration with a 0.2-μm filter before its addition to the autoclaved medium at 45°C. Plates containing medium without addition of the fungicides were used as a control.

2.1.1 Effect on hyphal growth

Metalaxyl and fosetyl-Al were incorporated to the VA medium at different concentrations (0.01, 0.1, 1, 10, 100, 1000 μg ml^{-1}). Petri dishes 90 mm in diameter were inoculated with single 5-mm-diameter discs from 20-day-old cultures of *P. austrocedri* and then incubated in the dark at 17°C for 4 weeks. There were 10 plates per treatment, inoculated with five isolates (two replicates per isolate). Two colony diameter measurements were taken weekly on each plate. The mean colony diameter per replicate plate was calculated and used to calculate the mean colony diameter per treatment. Concentrations which were able to reduce vegetative growth by 50 and 90%, EC_{50} and EC_{90} values, were calculated from function plotting relative growth *vs.* concentration. Comparisons among treatments were performed with data taken from the 4th week by the nonparametric Kruskal–Wallis test and the Mann–Whitney *U*-test as *a posteriori* test (SPSS Statistics 17.0, SPSS Inc., Chicago, IL, USA).

2.1.2 Effect on oospore formation

The same plates assayed for linear growth were observed under light microscope at 10× magnification (Axioplan; Zeiss, Oberkochen, Germany) to detect and count the presence of oospores. On each Petri dish, oospores were counted in 4 radius of 15 mm × 1000 μm, covering an area of 60 mm^2. The oospore number per plate was used to calculate the mean oospore number per treatment. Comparisons among treatments were performed by ANOVA (SPSS Statistics 17.0).

2.1.3 Effect on sporangia production

Non-sterile soil extracts were prepared by mixing 50 g (fresh weight) of *A. chilensis* forest soil with 1 l of distilled water which was allowed to stand 48 h and then was filtered through Whatman N° 40 filter paper. Three plugs taken from the margins of colonies on medium amended with 0.01 and 1 μg ml^{-1} metalaxyl, and 1 and 1000 μg ml^{-1} fosetyl-Al were placed in 60-mm-diameter Petri dishes containing soil extract and incubated at 17°C in the darkness for 72 h. Sporangia were detected by observation under a stereoscopic microscope (Wild M 3Z; Leica, Wetzlar, Germany) and quantified in an area of 5 mm^2. Comparisons among treatments were performed by Kruskal–Wallis test and Mann–Whitney *U*-test as *a posteriori* test (SPSS Statistics 17.0).

2.2 In planta assays

2.2.1 Soil application assays (seedlings)

Two-year-old seedlings growing in 0.75-l free-draining pots were obtained from the tree nursery of Patagonia National University (PAIDER Forestal-UNPSJB). All plants originated from seeds of *A. chilensis* forests at the NW of Chubut Province and had been cultivated in the same manner and conditions in a substrate 2 : 1 : 1 soil, volcanic sand and compost. One month before the initiation of the assays, 60 randomly selected plants were transferred to a controlled environment room, under 16-h light/8-h dark at 17–19°C, where the inoculations and fungicide preventive treatments were performed. At the beginning of the assays, seedlings averaged 12 cm high and 2.3 mm in diameter at the root collar. Plants were randomly assigned to one of the six treatments: mock-inoculated control, *P. austrocedri*-inoculated control or one of the four fungicide preventive application treatments (M = 0.5 mg per pot of metalaxyl, 10× M = 5 mg per pot of metalaxyl, F = 2 mg per pot of fosetyl-Al, 10× F = 20 mg per pot of fosetyl-Al). There were 10 replicates of each treatment. Each plant of the fungicide pretreated treatments received the dose twice, 20 days apart each, of the assayed fungicide diluted in 50 ml of distilled water. The lowest concentrations were based on the manufacturer's recommendations and the highest were 10-fold greater than the manufacturer's recommendations. Controls received the same amount of distilled water. Plants were inoculated 30 days after the last chemical application. Inoculations were performed in the stem with a 5-mm-diameter hyphal plug, cut from a 3-week-old *P. austrocedri* culture growing in clarified vegetable juice agar. An incision performed with a sterile scalpel, less than 10 mm long, was made in the bark, and the plug was placed over the incision to favour the pathogen colonization. Each inoculation was covered with sterilized, moist muslin cloth, wrapped with aluminium foil and sealed with adhesive tape. Plants designated as mock-inoculated controls were inoculated with a 5-mm-diameter plug of sterile vegetable juice agar. Four weeks after, the treatments were evaluated as described below.

2.2.2 Photosynthesis (A) and Stomatal conductance (gs)

Stomatal conductance (gs, mol m^{-2} s^{-1}) and net photosynthetic rate (A, CO_2 assimilation, μmol m^{-2} s^{-1}) were measured with a LICOR 6200 portable photosynthesis system (Li-Cor). Duplicate measurements were taken on an area basis in the ten *A. chilensis* plants of each treatment. Briefly, two small branches of each plant were used to perform independent measurements, and each branch was scanned and their area determined. A and gs values were related to the estimated area.

Comparisons among treatments were performed using nonparametric Kruskal–Wallis test and Mann–Whitney *U*-test as *a posteriori* test (SPSS Statistics 17.0).

2.2.3 Hydraulic conductivity

Stem-specific hydraulic conductivity (Ks, kg m^{-1} MPa^{-1} s^{-1}), an estimation of water transport capacity, was measured in five random selected plants of each treatment. Ks was determined on a segment of the stem taken from the collar root to the first branch and including the inoculation site. We followed the procedures outlined in Gyenge et al. (2008). Specific hydraulic conductivity was calculated as follows:

$$\mathrm{Ks} = (QL) \times (\Delta\Psi \mathrm{TAS})^{-1},$$

where Q is water flux (ml s^{-1}), L is length of the segment (cm), $\Delta\Psi$ is the difference of water pressure (MPa) between the segment and a reservoir of water placed 1 m above, and TAS is the transverse area of the segment (cm^2). The nonparametric Kruskal–Wallis test and the Mann–Whitney *U*-test as *a posteriori* test were used to detect statistical differences among treatments (SPSS Statistics 17.0). Bark was removed to expose discoloured inner bark, and the severity of symptoms was assessed by measuring lesion lengths (in millimetres) and perimeters (%) at the inoculation point. Differences in lesion size were analysed by Kruskal–Wallis test and Mann–Whitney *U*-test. Verification of *P. austrocedri* infection within the tissues of the artificially inoculated *A. chilensis* plants was determined by direct plating of pieces of tissue taken from the necrotic margin of the lesions on a *Phytophthora* spp.-selective medium [pimaricin–ampicillin–rifampicin agar, (PAR)]. If isolation results were negative from necrotic tissues, ELISA immunoassays were performed according to the manufacturer's instructions (DAS ELISA reagent set for Phytophthora, ADGIA Inc.) (Greslebin and Hansen 2010).

2.2.4 Evaluation of the efficacy of the fungicides as preventive and curative treatment by trunk injection assays (adult trees)

Twenty-seven healthy trees, 17–37 cm diameter at breast height, were selected from a stand with low incidence of the disease located inside a diseased area in Los Alerces National Park. Selection included a close inspection of each tree to discard the presence of symptoms of disease (defoliation, resin exudates, cankers and necrotic inner bark of root collar). In each tree, four to eight small incisions were made at the root collar to discard the presence of necrotic phloem.

Each tree was randomly assigned to one of three treatments: injection of fosetyl-Al [1 ml of aqueous solution (75 g l^{-1}) per centimetre of stem perimeter at 1.3 m high], injection of metalaxyl [1 ml of aqueous solution (8 g l^{-1}) per centimetre of stem perimeter at 1.3 m high] and injection of sterile distilled water (control). Injections were applied as described by Shearer et al. (2006). First inoculation was made 15 days before fungicide application to evaluate the efficacy of the fungicide as curative treatment. The second inoculation was made 15 days after fungicide application to evaluate the efficacy of the fungicide as preventive treatment.

Each tree was inoculated with *Phytophthora austrocedri* at 1.3 m high, in one side randomly assigned. Bark cores (from the outer bark up to the xylem) of 7 mm diameter were aseptically removed using a borer. Vegetable juice agar discs (7 mm diameter) taken from the edges of 15-day-old cultures of *P. austrocedri* (isolate Phy-233, Table 1) were placed in the holes and covered with the removed bark. A piece of sterilized, moist muslin cloth was placed over each inoculation point, covered with aluminium foil and sealed with adhesive tape. Fifteen days after inoculation, each tree was injected, at 0.4 m high, with one of the three treatments. Ten trees received fosetyl-Al, seven received metalaxyl and ten received sterile distilled water. The dose of fosetyl-Al was calculated according to the one used by Shearer et al. (2006). The proportion between fosetyl-Al and metalaxyl doses used in this assay correspond to the proportion of active ingredient recommended by the manufacture's of these products for the same purpose. As the assay was conducted in a protected natural area, it was not allowed to release these chemicals to the environment; therefore, the fungicides had to be applied as stem injection, but the point of application was below the point of inoculation of the pathogen to allow the upwards translocation to the inoculated tissues. Fifteen days after systemic fungicide injection, the same trees were inoculated with *P. austrocedri* as described above on the opposite side to the previous inoculation.

Assessment was carried out 3 months after inoculation. Outer bark around the inoculation point was removed to expose the phloem, and the length and width of the lesion (necrotic phloem) was recorded. Re-isolation was attempted from the edges of the lesions, and ELISA immunoassays were performed on necrotic tissues associated with each treatment and control. One-way ANOVA was applied to detect significant differences among treatments. Nonparametric ANOVA (Kruskal–Wallis) was performed when the assumptions of normality and homoscedasticity were violated and as *a posteriori* test Mann–Whitney was applied (SPSS Statistics 17.0).

2.2.5 P. austrocedri *toxic effect in leaf tissue of preventive fungicide treated seedlings*

As we previously detected toxic effects in leaves of plants inoculated with the pathogen (Vélez et al. 2012), we monitored tissue integrity under the different treatments. Leaves from five randomly selected *A. chilensis* seedlings coming from each of previously mentioned treatments (F, M, 10× F, 10× M, inoculated control and mock-inoculated control) applied to the soil around seedlings were processed at the end of the assay for the detection of changes in electric

conductivity and pH, and the presence of necrosis. Assays were performed as described in Bailey et al. (1990) and Ezeibekwe et al. (2009) with minor modifications. Briefly, 100 mg of leaves of each plant was put in separate tubes containing 3 ml of sterile distilled water. Conductivity (conductivity meter Cole Parmer Instrumental Company) and pH (pH meter Wissenschaftlich Technische Werkstätten, WTW) were measured in each tube before (zero time point) and immediately after the addition of the leaves (initial level). Then, the tubes were shaken (Meridiens Lab, Buenos Aires, Argentina) at 150 strokes per min for 30 min. After resting the tubes for 5 h, at room temperature and day light, conductivity and pH of the solution were determined (final conductivity and pH). The conductance in μS and the pH of the initial level were subtracted from the values of final level to determine the possible electrolyte leakage and change in pH induced by the presence of effectors derived from the pathogen. Kruskal–Wallis test and Mann–Whitney *U*-test were used to detect statistical differences among treatments for electrical conductivity changes, and ANOVA and Tukey test as *a posteriori* test to assess differences among treatments for pH changes (SPSS Statistics 17.0). To monitor plant cell death, 10 leaves from each plant were cleared in methanol for 24 h and boiled for 3 min in lactophenol trypan blue stain (10 ml of water, 10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, 10 mg of trypan blue). After the leaves had cooled to room temperature for 1 h, the stain was replaced with 1 g ml^{-1} chloral hydrate (Takemoto et al. 2005). Stained leaves were decolorized with methanol overnight and viewed under the light microscope to assess the extent of cell death. Results were expressed as percentage of necrosis on an area basis. Comparisons among treatments were performed by Kruskal–Wallis test and Mann–Whitney *U*-test (SPSS Statistics 17.0).

2.2.6 Effect of the fungicide preventive treatment on the response of tissues to the effectors present in P. austrocedri *culture filtrate*

To test possible effect of the systemic fungicides in the response/sensitivity of plant tissues to toxic effectors (or to test a possible role of the fungicides in ameliorating the response of the plant to the toxic effect of the pathogen), stem portions and leaves from seedlings treated with the chemicals (F and M) and untreated (control) were exposed to the culture filtrate (CF) of the pathogen and their integrity parameters were compared.

Six-month-old *A. chilensis* seedlings were randomly assigned to one of the following treatments: pretreatment with fosetyl-Al at 0.8 mg per 0.3-l pot, the dose recommended by the manufacturer (F); pretreatment with metalaxyl at 0.2 mg per 0.3-l pot, the dose recommended by the manufacturer (M); and pretreatment with sterile distilled water (control). There were 10 plants (replicates) per treatment. Whole leaves and stem portions (1 cm long) of these plants were used to study the effect of the culture filtrate (CF) of *P. austrocedri* on tissue preservation after fungicide treatment. For description of methodology of CF obtaining, see Vélez et al. (2012). Isolates of *P. austrocedri* were grown in VA broth (without agar) for 3.5 months in the dark at 16–19°C. After incubation, the liquid cultures were filtered through a sterile 0.2-μm membrane and the filtrates (CF) collected. The assay was applied separately to leaves and stems of each plant. A mass of 100 mg of leaves and 25 mg of stems of each plant were placed in sterile tubes containing 3 ml of CF. The same amount of leaves and stems of each plant were placed into sterile tubes containing only 3 ml of sterile liquid medium as controls. Conductivity and pH were measured in each tube before (zero time point) and immediately after the addition of the leaves and stems (initial level). Tubes were incubated for 72 h at 16–19°C, 16-h light/8-h dark and then, tubes were put for 30 min on a shaker (Meridiens Lab) set at 150 strokes per min and processed for determination of electrical conductivity, pH and necrosis as described above. Comparisons among treatments in changes in conductivity and necrosis were performed using Kruskal–Wallis test and Mann–Whitney *U*-test. Differences among treatment in changes in pH were tested by ANOVA and Tukey test (SPSS Statistics 17.0).

3 Results

3.1 *In vitro* assays

3.1.1 Effect on hyphal growth

Metalaxyl was effective in reducing hyphal growth in all isolates, and there were no differences among them. There were significant (Kruskal–Wallis $\chi^2 = 200.60$ $p < 0.001$; *post hoc* Mann–Whitney *U*-test $p < 0.001$) differences in colony diameter between metalaxyl treated and untreated controls at all concentrations tested except 0.01 μg $ml^{-1}$with an ED_{50} of 263 μg ml^{-1} and an ED_{90} of 922 μg ml^{-1}. In contrast, fosetyl-Al reduced hyphal growth only at the highest concentration assayed (1000 μg ml^{-1}) (*post hoc* Mann–Whitney *U*-test $p < 0.001$), meanwhile at all other concentrations, colony diameters did not differ significantly from untreated controls (Fig. 1). As there were no differences among isolates, Figure 1 shows the combined results of the five isolates.

3.1.2 Effect on oospore formation

Metalaxyl totally inhibited oospore formation at all concentrations assayed, whilst fosetyl-Al did only at the three highest concentrations (10, 100 and 1000 μg ml^{-1}) tested. Oospores formed in the three lowest concentrations of fosetyl-Al tested and the number did not differ significantly (one-way ANOVA $F3,36 = 2.327$; $p = 0.09$) from the number observed in untreated controls (Fig. 2). Results of the five isolates tested were combined for analysis.

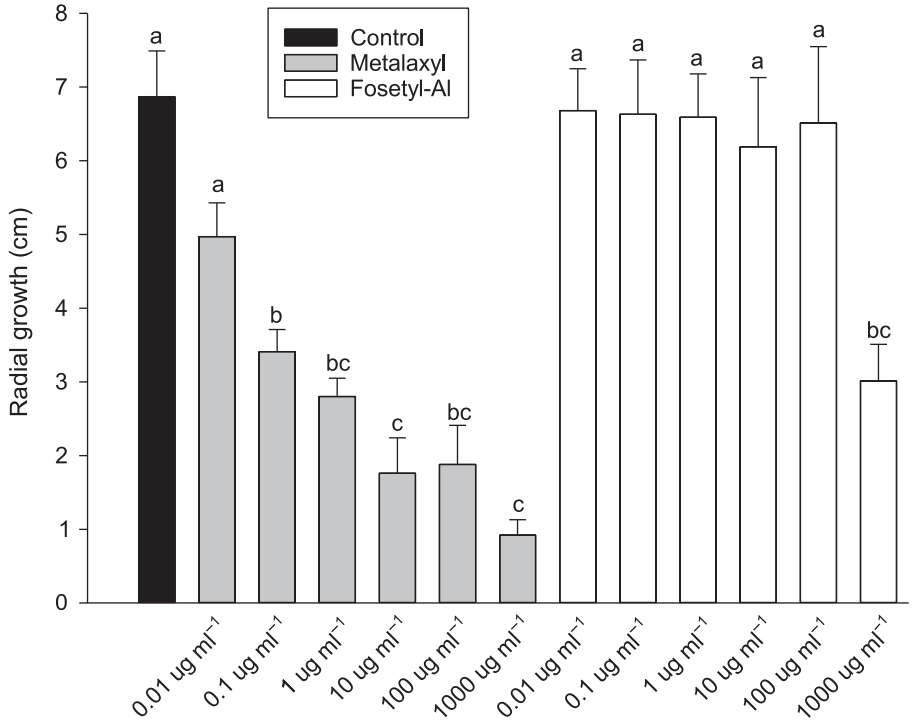

Fig. 1. Radial growth (cm) of *Phytophthora austrocedri* growing on VA vegetable juice media (Black bar) at different concentrations (μg ml^{-1}) of metalaxyl (grey bars) and fosetyl-Al (white bars). Data are plotted as average ± SD. Same letter indicates non-significant ($p > 0.05$) differences, Kruskal–Wallis analysis and Mann–Whitney *post hoc* test.

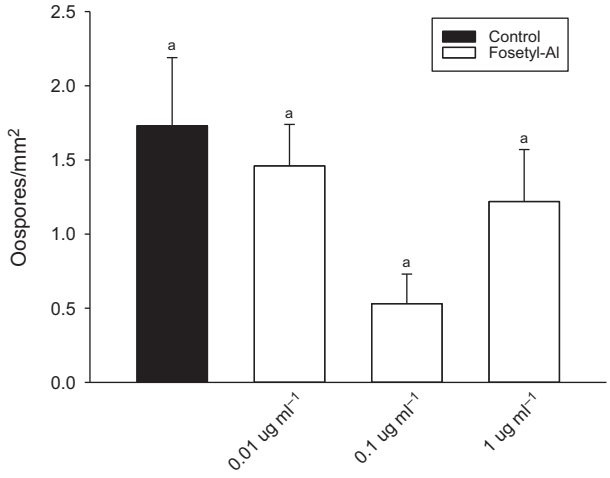

Fig. 2. Number of oospores formed by *Phytophthora austrocedri* growing on VA vegetable juice media (Black bar) at different concentrations (μg ml^{-1}) of fosetyl-Al (white bars). Data are plotted as average number of oospores ± SD. Same letter indicates non-significant ($p > 0.05$) differences, one-way ANOVA.

3.1.3 Effect on sporangia formation

Metalaxyl reduced sporangia formation at both concentrations assayed (0.01 and 1 μg ml^{-1}), and the highest one almost inhibited sporangia development. Fosetyl-Al reduced sporangia formation at the highest (1000 μg ml^{-1}) concentration assayed (Kruskal–Wallis $\chi^2 = 34.529$, $p < 0.001$; *post hoc* Mann–Whitney *U*-test $p < 0.01$ and $p < 0.001$) (Fig. 3). Results of the five isolates tested were combined for analysis.

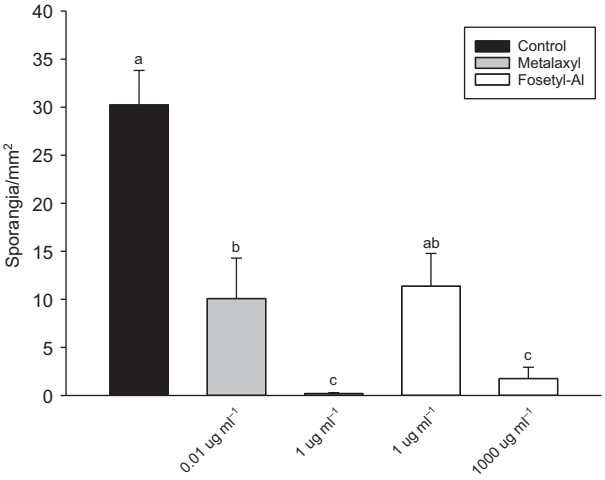

Fig. 3. Number of sporangia formed in soil extract by isolates of *Phytophthora austrocedri* growing on VA vegetable juice media (Black bar) at different concentrations (μg ml^{-1}) of metalaxyl (grey bars) and fosetyl-Al (white bars). Data are plotted as average ± SD. Same letter indicates non-significant ($p > 0.05$) differences, Kruskal–Wallis analysis and Mann–Whitney *post hoc* test.

3.2 In planta assays

3.2.1 Soil application assays

Four weeks after inoculation, inoculated non-treated plants were symptomatic (20%) or dead (80%), and mock-inoculated controls were alive with green and healthy foliage, showing no symptoms of disease. Plants treated preventively with fosetyl-Al and metalaxyl at the concentration recommended by the manufacturer (treatments F and M) were still alive but showing symptoms of the disease (foliage wilt). All plants treated preventively with fosetyl-Al or metalaxyl at the 10-fold higher dose (10XF and 10XM) remained alive and asymptomatic.

Photosynthetic rate (A) of plants preventively treated with fosetyl-Al at the concentration recommended by the manufacturer did not differ from inoculated controls and was significantly lower than the one of mock-inoculated plants (Kruskal–Wallis $\chi^2 = 46.988$; $p < 0.001$; *post hoc* Mann–Whitney *U*-test $p < 0.001$). Metalaxyl pretreated plants showed A values between inoculated and mock-inoculated controls. On the other hand, plants preventively treated with fosetyl-Al or metalaxyl with the 10-fold higher dose concentration than recommended by the manufacturer did not differ from the mock-inoculated control plants (Fig. 4a).

Stomatal conductance (gs) plants pretreated with both chemicals did not differ statistically (Kruskal–Wallis $\chi^2 = 28.97$; $p > 0.05$) from mock-inoculated controls (Fig. 4b).

Hydraulic conductivity of plants preventively treated with metalaxyl at both concentration and fosetyl-Al at 10× concentration recommended by the manufacturer did not differ from mock, and all pretreated plants showed a significantly (Kruskal–Wallis $\chi^2 = 35.75$; $p < 0.001$; Mann–Whitney *U*-test $p < 0.01$) higher Ks than inoculated control plants (Fig. 4c).

As expected by the results obtained in the other parameters, lesion length of all plants preventively treated with fungicides at 10× recommended concentration did not differ (Kruskal–Wallis $\chi^2 = 57.70$, Mann–Whitney *U*-test $p > 0.05$) significantly from the mock-inoculated control. In contrast, lesions of plants preventively treated with fosetyl-Al or metalaxyl at concentrations recommended by the manufacturer's developed bigger lesions (Mann–Whitney *U*-test $p < 0.0001$) and did not differ from the inoculated controls (Fig. 4d).

3.2.2 Evaluation of the efficacy of the fungicides as preventive and curative treatment by trunk injection assays (adult trees)

All inoculated trees developed lesions. In the preventive treatment assay, lesion area showed significant (Kruskal–Wallis, $\chi^2 = 14.09$, $p < 0.001$) differences between treatments. Trees preventively injected with fosetyl-Al developed lesions significantly (Mann–Whitney *U*-test $p < 0.001$) smaller than trees preventively injected with metalaxyl and inoculated controls (Fig. 5a). Lesion area of trees preventively injected with metalaxyl did not differ (Mann–Whitney *U*-test $p > 0.05$) from lesion area of inoculated untreated control trees. In the curative treatment assay, there were no significant (One-way ANOVA, $F = 1.89$, $p > 0.05$) differences between treatments (Fig. 5b). Re-isolation from lesions was mostly negative, but the presence of *Phytophthora* in the margin of the lesions by ELISA test was positive in 100% of cases.

3.2.3 P. austrocedri *toxic effect in leaf tissue of preventive fungicide treated seedlings*

Leaves from plants inoculated with the pathogen and pretreated with the fungicides in concentrations suggested by the manufacturer's showed significant (Kruskal–Wallis $\chi^2 = 35.30$, $p < 0.001$) electrolyte leakage, evidenced by the increase in

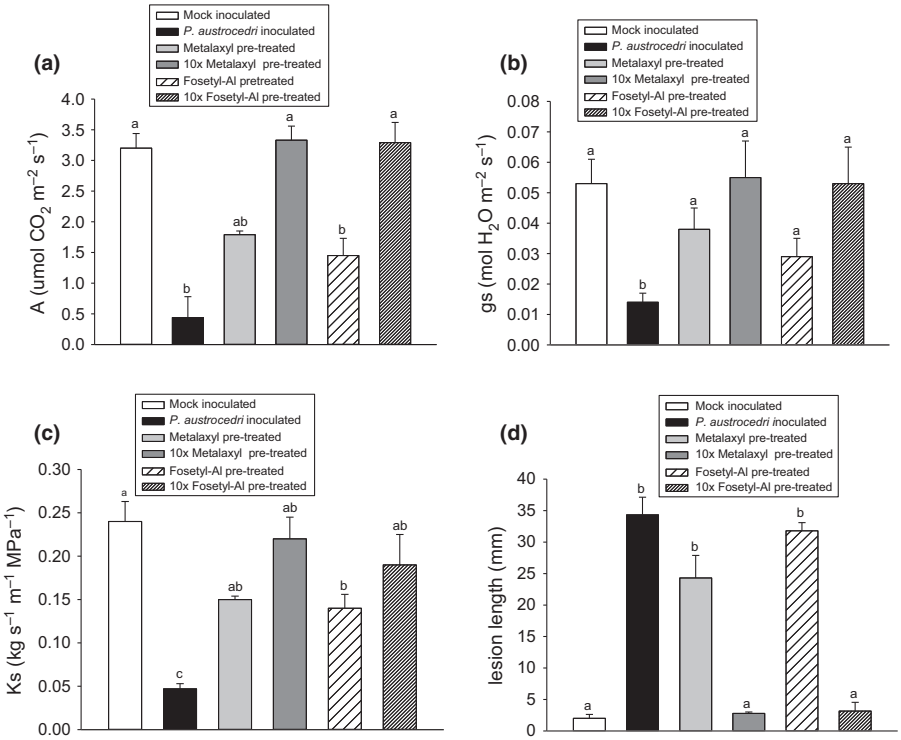

Fig. 4. Physiological parameters and lesion length recorded for 2-year-old *Austrocedrus chilensis* seedlings mock inoculated (striped bar), *Phytophthora austrocedri* inoculated (black bar), *P. austrocedri* inoculated with preventive metalaxyl (grey bars), or fosetyl-Al (dashed bars), applications at recommended or 10× recommended concentration, after 4 weeks of inoculation: (a) net photosynthetic rate (A); (b) stomatal conductance (gs); (c) stem-specific hydraulic conductivity (Ks); (d) lesion length. Data are plotted as average ± SD. Same letter indicates non-significant differences ($p > 0.05$), Kruskal–Wallis analysis and Mann–Whitney *post hoc* test.

extracellular electrical conductivity, with respect to mock-inoculated control plants (Fig. 6a). However, changes were by far smaller (Mann–Whitney *post hoc* $p < 0.01$) than those observed in leaves derived from the inoculated control plants (Fig. 6 a). When plants were pretreated with the fungicides at 10× recommended dose rate, no differences (Mann–Whitney *U*-test $p > 0.05$) in electrical conductivity were observed between leaves of plants treated with the fungicides and the mock-inoculated controls (Fig. 6a).

Necrosis extent was significantly (Kruskal–Wallis $\chi^2 = 36.219$, $p < 0.001$; Mann–Whitney *post hoc* $p < 0.01$) lower in leaves from plants preventively treated than in leaves from the inoculated controls (Fig. 6b). No statistical differences were observed between pretreated plants and mock-inoculated controls except (Mann–Whitney *U*-test $p < 0.01$) in the case of plants pretreated with fosetyl-Al at the lowest dose (Fig. 6b).

When changes in pH were analysed, no differences between leaves from the different chemical preventive treatments and the mock-inoculated control plants were found and showed a significantly (one-way ANOVA $F_{5,24} = 33.917$; $p < 0.001$; *post hoc* Tukey test $p < 0.001$) smaller change in extracellular pH than the inoculated control plants (Fig. 6c).

3.2.4 Effect of the fungicide preventive treatment on the response of tissues to the effectors secreted by the pathogen

Similar results to *in vivo* experiments regarding tissue integrity parameters were obtained. In leaves and stems from plants treated with the fungicides and exposed to CF, an increase in electrolyte leakage was detected, compared to the control not incubated with CF (Kruskal–Wallis $\chi^2 = 25.394$, $p < 0.001$; Mann–Whitney *post hoc* $p < 0.01$; Fig. 7a; Kruskal–Wallis $\chi^2 = 25.677$, $p < 0.001$; Mann–Whitney *post hoc* $p < 0.01$; Fig. 7b). However, the increase of the electrical conductivity was lower than in tissues derived from plants untreated with the chemicals (Mann–Whitney $p < 0.01$, Fig. 7a,b).

Changes in pH in plants treated with fosetyl-Al and incubated with CF were smaller (one-way ANOVA $F_{5,24} = 28.852$, one-way ANOVA $F_{5,24} = 22.670$, *post hoc* Tukey test $p < 0.001$) than in plants exposed to CF without pretreatment (CF incubated controls). However, in plants pretreated with metalaxyl, pH did not differ from CF incubated controls (Fig. 7c,d).

The percentage of necrosis in leaves from plants pretreated with the chemicals and exposed to CF was significantly (Kruskal–Wallis $\chi^2 = 22.715$, Mann–Whitney *U*-test $p < 0.01$) lower than in leaves from plants that were not pretreated with the chemicals, but higher ($p < 0.001$; Mann–Whitney *post hoc* $p < 0.05$ and $p < 0.01$) than in leaves from plants that were only incubated with the sterile medium.

No changes in electrical conductivity, pH or necrosis were observed in tissues derived from plants treated with the products and incubated with the sterile medium (Fig. 7), denoting that the chemicals do not exert a deleterious effect on tissues.

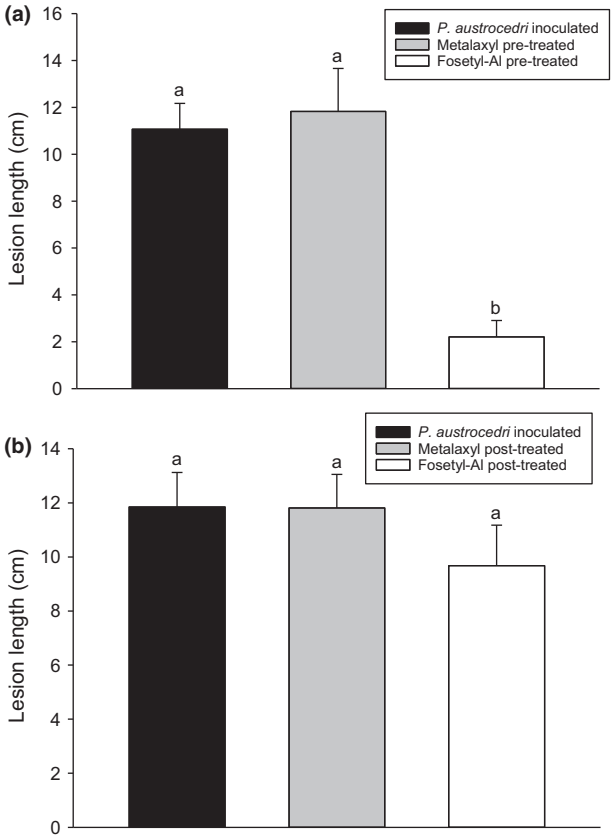

Fig. 5. Effect of preventive and curative application of the metalaxyl and fosetyl-Alon lesion development: lesion length in adult *Austrocedrus chilensis* trees inoculated with *Phytophthora austrocedri* (a) without fungicide treatment (black bar) and after a preventive application of metalaxyl (grey bars) or fosetyl-Al (white bars), and (b) without chemical application (black bar) and before a curative application of metalaxyl (grey bars) or fosetyl-Al (white bars). The data are plotted as average ± SD. Same letter indicates non-significant ($p > 0.05$) differences, Kruskal–Wallis analysis, Mann–Whitney *post hoc* test (a) and one-way ANOVA (b).

4 Discussion

The results of this work show that the tested fungicides are effective in preventing disease caused by *Phytophthora austrocedri* in *Austrocedrus chilensis*.

Even though fosetyl-Al was not effective in reducing mycelial growth *in vitro*, it was clearly effective diminishing lesion progression in seedlings as well as in adult trees infected with the pathogen. This dual behaviour *in vitro*/*in vivo* had already been reported in previous studies (Guest and Grant 1991; Jackson et al. 2000; Garbelotto et al. 2009), and we assumed it was related to the dual action of the fungicide in the plant where it acts directly against the pathogen and indirectly by stimulating the defence mechanisms of the plant. *In vitro* tests showed that metalaxyl is highly effective in reducing, and even inhibiting sexual and asexual reproduction and growth of the pathogen. Meanwhile, fosetyl-Al did not reduce growth of *P. austrocedri in vitro* except at the highest concentration used and showed an inhibitory effect on asexual reproduction in high concentrations.

The effectiveness of fosetyl-Al and metalaxyl was almost the same when applied preventively by soil drench in seedlings, but fosetyl-Al was much more effective than metalaxyl when applied by injection in adult trees. This difference could result from a dose effect or from the application technique, instead of being by an intrinsic difference in the efficacy between both products. Metalaxyl is translocated apoplastically in an upward direction (Staub et al. 1978). It means that this systemic fungicide moves from the point of application upwards. For this reason, it is recommended to apply it as a soil drench because it can move from the roots to stems and leaves. The negative results of this assay, contrasting with the positive results of all the assays where the fungicide was applied as soil drench, allow us to assume that the fungicide was not translocated to the tissues affected by the pathogen, even though it was injected below the point of inoculation. It might be that metalaxyl was transported upwards through the xylem but not translocated to the phloem. This aspect of metalaxyl has been reported in other species (Zaki et al. 1981; Gupta et al. 1985). Fosetyl-Al was shown to be more effective as preventive treatments than as curative treatment in the concentration assayed. This should be taken into account when planning prevention and control management of forests. Also, higher concentrations should be tested to improve curative treatment performance.

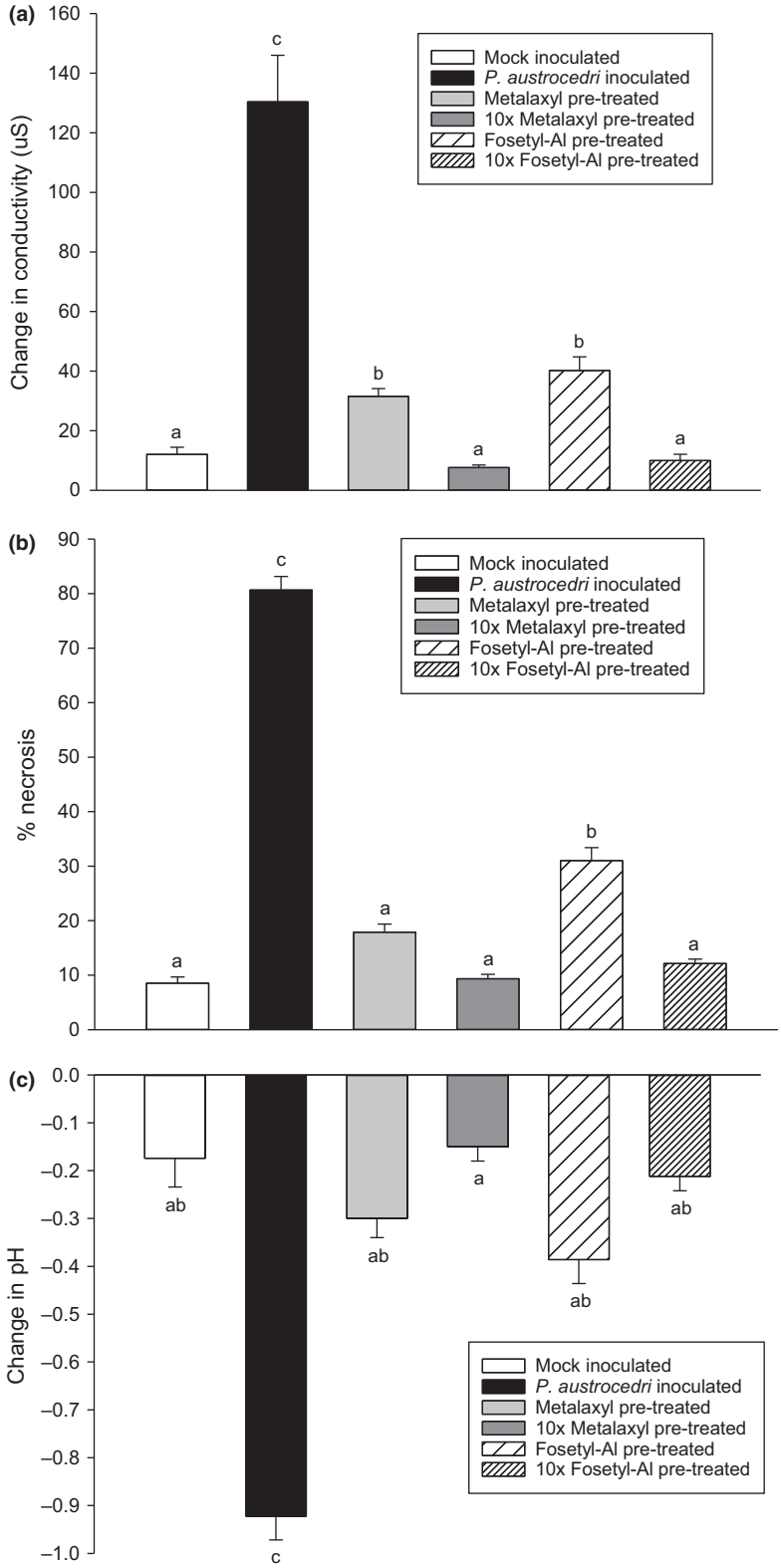

Fig. 6. Detection of extracellular change in electrical conductivity (a); necrosis (b); and extracellular change in pH (c) in leaves of 2-year-old *Austrocedrus chilensis* seedlings mock inoculated (striped bar), *Phytophthora austrocedri* inoculated (black bar), *P. austrocedri* inoculated with preventive metalaxyl (grey bars) or fosetyl-Al (dashed bars) application applications at recommended or 10× recommended concentration. Data are plotted as average ± SD. Same letter indicates non-significant ($p > 0.05$) differences, one-way ANOVA, *post hoc* Tukey test (c), Kruskal–Wallis analysis and Mann–Whitney *post hoc* test (a and b).

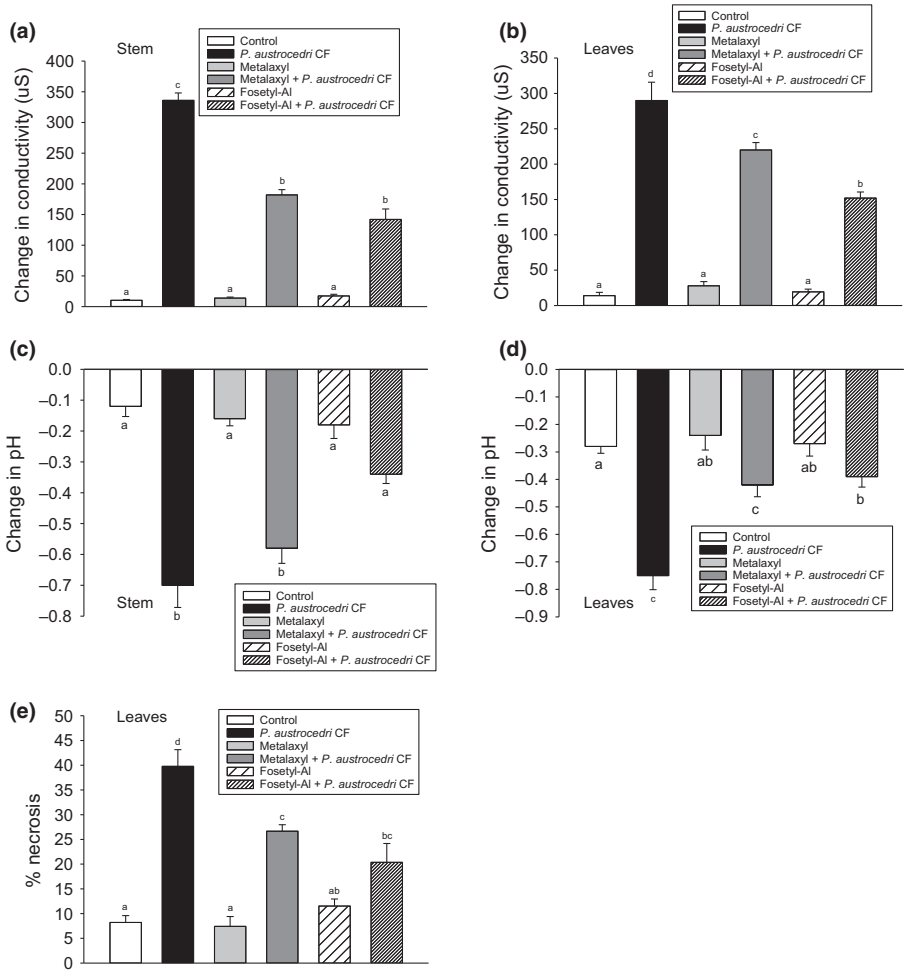

Fig. 7. Detection of extracellular change in electrical conductivity (a–b); pH (c–d); and necrosis (e) of leaves and stems (only leaves in case of necrosis) of 6-month-old *Austrocedrus chilensis* seedlings pretreated with metalaxyl (dark grey bar), with fosetyl-Al (fine striped bar) or non-pretreated (black bar) exposed to the culture filtrate of *Phytophthora austrocedri* (CF), in comparison with leaves and stems of the same plants not exposed to CF [metalaxyl (light grey bar), fosetyl-Al (coarse striped bar) and non-treated (white bar)]. Data are plotted as average ± SD. Same letter indicates non-significant ($p > 0.05$) differences, one-way ANOVA, *post hoc* Tukey test (b), Kruskal–Wallis analysis and Mann–Whitney *post hoc* test (a and c).

According to our results, phosphite has very low phytotoxicity on *A. chilensis*, even at high concentrations. Leaves of seedlings pretreated with fosetyl-Al in 10 times higher concentration than recommended by the manufacturer presented same necrosis than untreated and mock-inoculated controls, indicating that no tissue alteration was produced by the fungicide. This points out that phosphite has great potential to be used in natural ecosystems to control Austrocedrus root disease in Argentina. Of note, it is likely that higher concentrations than those used in the current study can be used to increase the efficiency, persistence and longevity of phosphite and its active effects in the tree. Further work is required to determine the optimal concentration of phosphite that can be applied to trees without causing phytotoxicity.

In accordance, preliminary assays of preventive application of potassium phosphite (Ransa®) in seedlings gave similar results to fosetyl-Al (data not presented), showing it is another chemical effective for preventing the disease.

Special attention is deserved to the results of the assay in which the effect of the fungicides on the response of plant tissues to the effectors produced by the pathogen was tested. These showed that leaves of pretreated plants are more resistant to the toxins/effectors present in the culture filtrate of *P. austrocedri*. These results seem to indicate that fosetyl-Al and metalaxyl provide some resistance to the plant besides their direct fungistatic action on the pathogen. Previous studies on the action of fungicides *in planta* have been conducted through pathogen inoculation. In the present study, as the pathogen was absent and the culture filtrates were obtained in liquid medium free of fungicides, differences cannot be attributed to the inhibitory effect on pathogen growth or metabolism. Therefore, the differences can be attributed exclusively to the plant response. The possible resistance-inducing activity of these chemicals has been previously discussed (Ward 1984; Kessmann et al. 1994; Oostendorp et al. 2001; Walter et al. 2007), but there is not a general agreement and mechanisms are not totally elucidated. According to our results, we can hypothesize that a systemic acquired resistance (SAR) response might be causing the observed differences. Further studies on the possible mechanism involved are underway.

Acknowledgements

Administración de Parques Nacionales, Mr. Santiago Caldiero (Syngenta SRL), Dr. Thomas Kitzberger, Ing. Guillermina Dalla Salda, and Agencia Nacional de Promoción Científica y Técnica of Argentina (ANPCyT, FONCyT, PICT-O 36776), are deeply acknowledged for making possible the realization of this work.

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