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Alteration of physiological parameters of *Austrocedrus chilensis* by the pathogen *Phytophthora austrocedrae*

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The effect of the pathogen *Phytophthora austrocedrae* on tree physiology of *Austrocedrus chilensis* in Patagonia was studied in a 4-week study. In the first week, stem-inoculated saplings showed a significant decrease in photosynthesis (A) without alteration of stomatal conductance (g_s) or stem-specific hydraulic conductivity (Ks). From the second week on, progressive decreases in A, g_s and Ks were observed, concomitantly with development of significant stem lesions. Water use efficiency (WUE) increased in the second week and declined progressively from the third week. Hyphae and resinous materials were observed in tracheids and rays below lesions. Necrosis of parenchyma ray cells and blockage of tracheids torus were observed. Healthy xylem showed no resinous materials or tracheid blockage, but abundant starch in rays, which was absent in altered xylem. The culture filtrate (CF) of the pathogen was shown to induce changes in extracellular pH and conductivity, and increased necrosis in tissues of leaves and stem challenged with CF *in vitro*. Similar results were obtained in leaf tissues of the inoculated saplings *in vivo*. CF injection into xylem of saplings induced a decline in A and disturbance of leaf tissue integrity, without altering g_s, WUE or Ks. The decrease of A correlated with changes in tissue integrity. A possible mechanism of *A. chilensis* decline induced by *P. austrocedrae* is discussed.

Keywords: cypress root rot, effectors, hydraulic conductivity, mal del ciprés, photosynthesis, xylem colonization

Introduction

Austrocedrus chilensis (*ciprés de la cordillera*) is a tree in the Cupressaceae, endemic to southern Argentina and Chile. It is the most widely distributed species among the few conifers inhabiting southern Argentina. It is found across 140 000 ha (Bran *et al.*, 2002) in a wide variety of ecological niches (Veblen *et al.*, 1995) and in different soil types (La Manna, 2005). It grows between 36°30′ and 43°35′S on the eastern slopes of the Andes, and between 32°39′ and 44°S on the western slopes (Veblen *et al.*, 1995). In Argentina it grows in a 60- to 80-km-wide strip along the Andean foothills, across a broad moisture gradient (170 cm per year in the west to 50 cm per year in the east). In the west, *A. chilensis* can be found either in mixed stands with *Nothofagus* spp. or in pure *Austrocedrus* stands in drier sites. In the north, it can be found mixed with *Araucaria araucana*. It also grows in open, xeric forests or in isolated clumps at the limit of the Andean forest and the Patagonian steppe, acting as a barrier against desert advance. *Austrocedrus chilensis* is valued not only because of its ecological function but also because of the quality of its wood and its aesthetic importance.

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High levels of mortality of *A. chilensis* trees were reported in 1948 on Isla Victoria (Nahuel Huapi National Park) in Patagonia, Argentina. Since then, mortality has been reported in many places within the native range of *A. chilensis*. The disease has been named *mal del ciprés* (MDC, cypress sickness) (Havrylenko *et al.*, 1989). Major symptoms of MDC include chlorosis and foliage withering (Havrylenko *et al.*, 1989). Trees may die rapidly, in which case foliage changes from chlorotic to red, or slowly, with chlorosis followed by progressive defoliation leading to tree death after several years (Filip & Rosso, 1999). The disease originates in the root system (Havrylenko *et al.*, 1989). Other characteristics of MDC are that the disease is associated with poorly drained and high-moisture soils (Baccalá *et al.*, 1998; Filip & Rosso, 1999; La Manna & Rajchenberg, 2004), and that tree mortality presents an aggregated pattern (Rosso *et al.*, 1994). In accordance with the major features of MDC, a novel pythiaceous species was found to be the primary pathogen to *A. chilensis* causing the disease. The pathogen was named *Phytophthora austrocedrae* (Greslebin *et al.*, 2007). This pathogen produces necrotic lesions that affect the entire thickness of the phloem, evidenced by discoloration of the tissue, and also induces superficial staining of the sapwood (Greslebin *et al.*, 2007; Greslebin & Hansen, 2010).

In a few locations, mortality of *A. chilensis* attributed to MDC could not be explained by *P. austrocedrae*

infection because of the absence of the necrotic lesions. In these cases crown dieback was present but roots were unaffected, and they were associated with deep and well-drained soils. These facts indicate that other, undescribed agents could affect *A. chilensis* (Greslebin & Hansen, 2010). The work reported in the present paper was restricted to study of the disease caused by the pathogen *P. austrocedrae*.

The mechanistic basis for *A. chilensis* root rot caused by *P. austrocedrae* remains unknown. As the major symptom of the disease is the presence of necrotic lesions in the phloem, it has been speculated that trees are killed by extensive death of bark and cambium tissues and by the disruption of phloem transport. However, *P. austrocedrae* has been isolated from superficially discoloured sapwood, indicating that the pathogen colonizes at least the outer layers of xylem. Microscopic examination of these tissues showed coloured resinous contents in the cells, especially in xylem ray parenchyma, indicating a possible reaction of the tree (Greslebin & Hansen, 2010). It has been reported that other *Phytophthora* species are also capable of infecting the sapwood of different trees. *Phytophthora ramorum* colonizes the xylem of *Notholithocarpus densiflorus* trees and *Rhododendron macrophyllum* twigs (Parke *et al.*, 2007; Collins *et al.*, 2009). *Phytophthora lateralis* infects xylem of *Chamaecyparis lawsoniana* trees (Oh & Hansen, 2007), and xylem of *Quercus* and *Fagus* is colonized by *P. ramorum*, *P. kernoviae*, *P. cambivora*, *P. citricola* and other species (Brown & Brasier, 2007).

It has been observed that the infection of compatible plants with *Phytophthora* spp. usually leads to an alteration of physiological parameters such as photosynthesis, stomatal conductance and hydraulic capacity (Dawson & Weste, 1982; Crombie & Tippett, 1990; Schnabel *et al.*, 1998; Maurel *et al.*, 2001; Robin *et al.*, 2001; Fleischmann *et al.*, 2005; Parke *et al.*, 2007; Clemenz *et al.*, 2008; Collins *et al.*, 2009). Decline in photosynthetic rate accompanied reduced water supply caused by root or xylem necrosis induced by the pathogen, although in some cases the magnitude of the reduction in photosynthesis did not correspond to the magnitude of changes in hydraulic conductivity (Dawson & Weste, 1982; Maurel *et al.*, 2001). Similarly, Manter *et al.* (2007) detected declines in photosynthesis, in response to inoculation of stems of *Rhododendron* plants with *P. ramorum*, prior to any visible damage to tissues or any measurable decrease in hydraulic conductivity that could have been caused by necrosis of tissues. The response of tissues distant from the inoculation point suggested the presence of toxins or translocated molecules acting as elicitors of host defence.

Numerous effectors have been found to be secreted and translocated by different *Phytophthora* spp. (Kamoun, 2006; Hardham & Cahill, 2010). These molecules are involved in colonization and pathogenicity, altering host tissue structure and function, including interference in plant defence pathways. Mechanisms of action include facilitation of infection processes (toxins or virulence factors) or limitation of the pathogen infection (elicitins or avirulence factors). Depending on the extent of host response and temporal differences in the process, the same effector could be a toxin for a certain host or an avirulence molecule for another host (Desender *et al.*, 2007).

The present work aimed to characterize the impact of *P. austrocedrae* on the physiological status of the host by monitoring changes through time in net photosynthesis, stomatal conductance and hydraulic conductivity of *A. chilensis* inoculated with *P. austrocedrae*. Tissue integrity in leaves distant from the inoculation site was also evaluated. The presence of effectors in the culture filtrate of the pathogen was tested and the effect of the culture filtrate on tissue integrity and physiology was studied. Colonization of xylem by the pathogen was also investigated. A possible mechanism of *A. chilensis* decline induced by *P. austrocedrae* is discussed.

Materials and methods

Plant material

Two-year-old saplings used in inoculation assays were purchased from the tree nursery of Patagonia National University (PAIDER Forestal-UNPSJB). All plants originated from seeds from *A. chilensis* forests in the northwest of Chubut province, and had been cultivated in the same manner. One month before the initiation of the assays, randomly selected plants were transferred to a controlled-environment chamber under 16 h light/8 h dark at 17–19°C, where the inoculations were performed. Another set of randomly chosen saplings of *A. chilensis* (30 individuals) was used to study the effect of *P. austrocedrae* culture filtrate (CF) injection on tree physiology. At the beginning of the assays, saplings averaged 12 cm high and 2·3 mm in diameter at the root collar. Six-month-old seedlings used for *in vitro* experiments to test for toxin activity in the CF of *P. austrocedrae* were obtained from seeds. Seeds were collected from healthy *A. chilensis* trees at 16 de Octubre Valley (Chubut province), surface-sterilized and cultivated in the controlled-environment chamber, using fertigation in 0·3-L pots with a sterilized inert substrate.

Stomatal conductance (g_s), photosynthesis (A) and water use efficiency (WUE)

To study the physiological response of *A. chilensis* to *P. austrocedrae*, 40 seedlings (2 years old) were inoculated in the stem with a 5-mm-diameter hyphal plug cut from a 3-week-old *P. austrocedrae* culture (isolate no. Oo-306, CIEFAP culture collection) growing in clarified vegetable juice agar (Erwin & Ribeiro, 1996). An incision, < 10 mm long, was made in the bark and the plug was placed over the incision to favour pathogen colonization. Each inoculation point was covered with sterilized, moist muslin cloth, wrapped with aluminum foil, and sealed with adhesive tape. Plants designated as controls (40) were inoculated with a 5-mm-diameter plug of sterile vegetable juice agar (Greslebin & Hansen, 2010).

Stomatal conductance (g_s, mol m^{-2} s^{-1}) and net photosynthetic rate (A, CO_2 assimilation, μmol m^{-2} s^{-1}) were monitored weekly, starting 1–4 weeks after inoculation, with a LICOR 6200 portable photosynthesis system (LI-COR). Duplicate measurements were made on an area basis in 10 *A. chilensis* plants per time and treatment. At the same times, photosynthetic photon flux density (PPFD, μmol m^{-2} s^{-1}) was measured with the radiation sensor provided with the same equipment. Values of net photosynthesis and stomatal conductance were recorded at PPFD > 300 μmol m^{-2} s^{-1}. Under these values of PPFD, stomatal conductance reaches the maximum level and photosynthesis has adequate measurable values for the species (Gyenge, 2005; Gyenge *et al.*, 2007). WUE was estimated as the ratio between A and g_s when PPFD was higher than 500 μmol m^{-2} s^{-1} (light saturation). Under these conditions photosynthesis and stomatal conductance reach the highest values for this species; also, photosynthesis and stomatal conductance have a linear relationship (A is proportional to g_s) in normal, well-watered *A. chilensis* plants (Gyenge *et al.*, 2007). Comparisons between inoculated and control plants for each period of time were performed using *t*-tests (SPSS Statistics 17·0).

Hydraulic conductivity

Stem-specific hydraulic conductivity (Ks, kg m^{-1} MPa^{-1} s^{-1}), an estimation of water transport capacity, was measured for each control and inoculated plant used for stomatal conductance and net photosynthetic rate. Ks was determined on a segment of the stem taken from the collar root to the first branch and including the inoculation site, 7–28 days after inoculation. For a more detailed evaluation of Ks decrease, an additional measurement was made on 10 extra plants on the ninth and 11th days after inoculation. The procedures outlined in Gyenge *et al.* (2008) were followed. Specific hydraulic conductivity was calculated as:

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

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). A *t*-test was used to detect statistical differences between controls and inoculated plants at each time point (SPSS Statistics 17·0).

The same saplings sampled for Ks were used to assess the severity of the infection by measuring lesion lengths (in millimetres) and perimeters (%) at the inoculation point. Lesions were evaluated by recording discoloured phloem tissue after removal of bark tissue. Severity was determined by calculating the proportion of stem affected by the lesion (percentage of the length × percentage of the perimeter). Verification of *P. austrocedrae* infection within the tissues of the artificially inoculated *A. chilensis* plants was determined by direct plating on a *Phytophthora* spp.-selective medium (pimaricin–ampicillin–rifampicin agar (PAR)) (Greslebin & Hansen, 2010). On each sample date, pieces from phloem tissues with (lesion margin) and without symptoms were plated on PAR medium, incubated in the dark at 16–19°C, and examined daily for 4 weeks for *P. austrocedrae* emergence. If isolation results were negative from necrotic tissues, then ELISA immunoassays, according to the manufacturer's instructions (DAS ELISA reagent set for *Phytophthora*; Agdia Inc.), were performed (Greslebin & Hansen, 2010).

Microscopy

Stems of inoculated seedlings with lesions caused by *P. austrocedrae* were microscopically studied in order to assess the effects of the pathogen on the xylem. The portions of affected stems were sliced into transverse, tangential and radial sections of 15 μm using a microtome (Leica Hn 40). Before slicing, stems were placed in saturated phloxine water solution for at least 2 h, to highlight the presence of hyphae. Slices were mounted in distilled water and observed and photographed under a light microscope (Axioplan Universal; Zeiss).

Test for effector activity in *P. austrocedrae* culture filtrate (bioassay)

Whole leaves and stem portions of non-inoculated 6-month-old *A. chilensis* seedlings were used to study the effect of the CF of *P. austrocedrae* on membrane permeability.

To obtain the CF, three isolates of *P. austrocedrae* (Oo-273, Oo-290 and Oo-298, deposited in the CIEFAP culture collection) were grown separately in vegetable juice broth (Erwin & Ribeiro, 1996) for approximately 3·5 months in the dark at 16–19°C. Ten 25 mL tubes containing 10 mL liquid medium and one 5-mm-diameter hyphal plug were prepared per isolate. After the incubation, the cultures were filtered through a sterile 0·2 μm membrane and the filtrates were collected. Equal parts of CF from each isolate were then combined. Five plants were used in the assay; control and CF-treated samples were obtained from each plant. The assay was applied separately to leaves and stems of each plant; 100 mg leaves and 25 mg stems of each plant were placed in sterile tubes containing 3 mL CF. The same amount of leaves and stems of each plant were placed into sterile tubes containing 3 mL sterile liquid medium alone as controls. The procedures followed were as described by Ezeibekwe *et al.* (2009) and Bailey *et al.* (1990), with minor modifications. 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, with 16 h light/8 h dark, then put for 30 min on a shaker (Meridiens Lab) set at 150 strokes per min. After resting the tubes for 5 h, conductance and pH of the solution were determined with a conductivity meter (model #198020-00; Cole Parmer Instrumental Company) and pH meter (model pH 530; Wissenschaftlich Technische Werkstätten, WTW) (final conductivity and pH). The conductance (in μS) and pH of

the zero time point were subtracted from the values of the control and the CF treatment (final), as well as the initial levels of conductivity and pH, to determine the increase in electrolyte leakage and change in pH induced by the CF. All tests were conducted in two replications.

Comparisons between CF-treated and control samples were performed using *t*-tests (SPSS Statistics 17·0).

The possible toxic effects of substances secreted by the pathogen *in vivo* were tested. Leaves from the same *A. chilensis* saplings inoculated with *P. austrocedrae* or controls from the physiological function assay were put into 3 mL sterile distilled water and processed as described above at every time point. To detect statistical differences between controls and inoculated samples, a *t*-test was applied at every time point (SPSS Statistics 17·0).

Lactophenol trypan blue staining

To monitor plant cell death, 10 CF-treated leaves and 10 control leaves from each plant of the bioassay were cleared in methanol for at least 24 h and boiled for 3 min in lactophenol trypan blue stain (10 mL water, 10 mL lactic acid, 10 mL glycerol, 10 g phenol, 10 mg 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 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 between CF-treated and control samples were performed using *t*-tests (SPSS Statistics 17·0).

Similarly, to detect toxic effects *in vivo*, leaves from *A. chilensis* saplings inoculated with *P. austrocedrae* and from controls were processed as described above at every time point. Statistical differences between controls and inoculated plants at every time point were detected using *t*-tests (SPSS Statistics 17·0).

Phytophothora austrocedrae CF effect on physiological parameters, membrane function and cell viability

To study the direct effect of the CF on physiological status, 100 or 300 μL CF (obtained as previously described) were injected into the xylem of 2-year-old *A. chilensis* saplings. Controls were injected with sterile culture medium. Ten individuals per treatment were used. Sterile 1-mL syringes were used and the CF was taken up by the xylem without any external pressure. After complete absorption of the CF, saplings were kept in the controlled-environment chamber for 6 weeks and then measurements of stomatal conductance (g_s) and net photosynthetic rate (A) on an area basis were taken. WUE was also estimated and specific hydraulic conductivity (Ks) determined as described above.

ANOVA was used to compare mean rates of A, g_s, WUE and Ks. Post hoc Tukey or Games–Howell tests were used for multiple comparisons of groups according to homogeneity of variance based on the Levene test (SPSS Statistics 17·0).

In the same injected saplings membrane integrity was examined by monitoring the changes in conductivity and pH. Similar masses of leaves of each injected sapling were placed into tubes containing 3 mL sterile distilled water and conductivity and pH were recorded (initial), then samples were put for 30 min on a shaker set at 150 strokes per min. Five hours later, conductance and pH of the solution were determined (final). The conductance (in μS) and the pH of the distilled water and of the initial values were subtracted from those of the control and the CF treatment (final).

Necrosis was also monitored by lactophenol trypan blue staining of the leaves as previously described. Multiple group comparisons were conducted by ANOVA and post hoc Tukey or Games–Howell tests as described above (SPSS Statistics 17·0).

Results

Physiological parameters in *P. austrocedrae*-inoculated plants

Stem inoculations of *P. austrocedrae* were successful in 87% of the cases judged by combined reisolation and ELISA methods. *Phytophthora austrocedrae* was isolated only from tissues showing symptoms in inoculated plants. Infection success was similar to that previously reported (Greslebin & Hansen, 2010). Lesions reached an average length of 50·8 ± 14·4 mm, affecting 100% of the stem circumference at the inoculation point at the end of the 4-week experiment. Net photosynthesis (A) was the first parameter affected by the inoculation. By the first week it was reduced by ~29% (Fig. 1a; $P < 0{\cdot}05$ vs. control, Student's *t*-test). The declines continued in the following weeks, reaching ~83% reduction in the fourth week (Fig. 1a; $P < 0{\cdot}01$ vs. control, Student's *t*-test).

Stomatal conductance (g_s) decreased by ~38% relative to control values in the second week (Fig. 1b; $P < 0{\cdot}01$ vs. control, Student's *t*-test). Values for the third and fourth weeks were on average below 0·03, indicating that stomata were closed (Fig. 1b).

No alteration in WUE was observed during the first week; but, in the second week WUE increased by ~78% compared to the control value (Fig. 1c; $P < 0{\cdot}05$ vs. control, Student's *t*-test). From the third week WUE diminished, reaching ~45% of the control level in the fourth week (Fig. 1c; $P < 0{\cdot}05$ vs. control, Student's *t*-test).

Physiological changes appeared prior to the development of visible symptoms in the leaves, which were evident as foliage wilt after the third week.

The capacity of the inoculated stem to supply water to host leaves was also tested by monitoring stem-specific hydraulic conductivity (Ks). Ks was not altered in the first week, but was markedly reduced, by ~70%, in the second and third weeks, and by ~78% in the last week (Fig. 2a; $P < 0{\cdot}0001$ vs. control, Student's *t*-test). Because of the

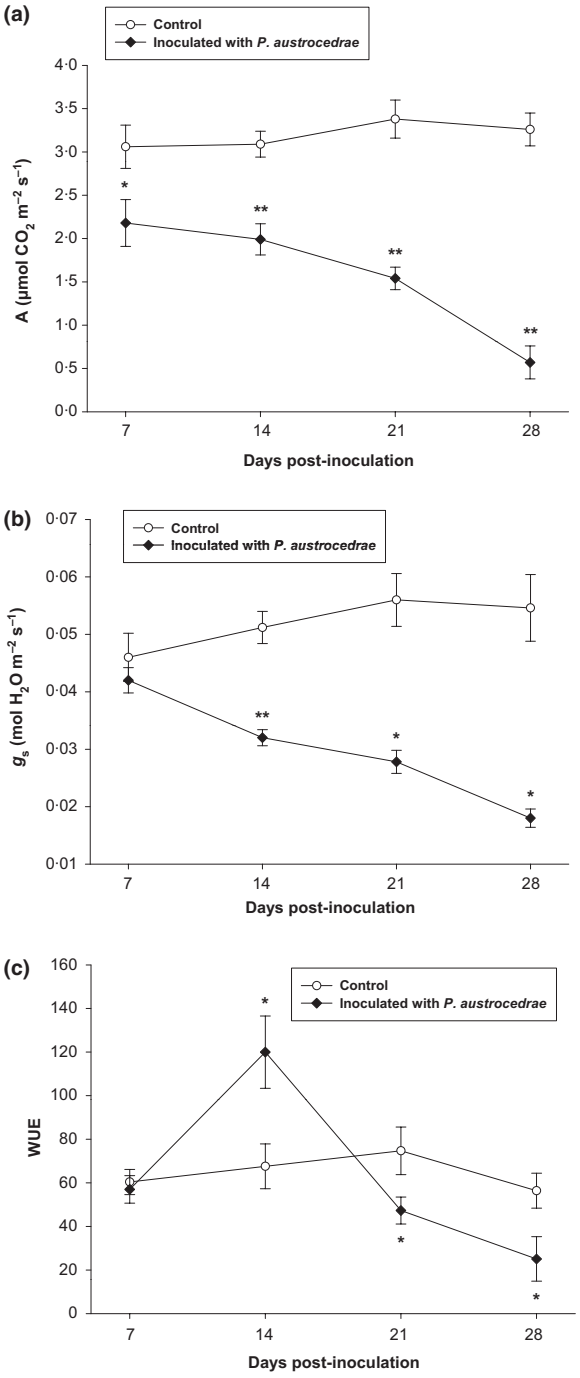

Figure 1 (a) Weekly measurements of net photosynthetic rate (A); (b) stomatal conductance (g_s); and (c) water use efficiency (WUE) in 2-year-old *Austrocedrus chilensis* saplings after mock inoculation (controls, white symbols) or inoculation with *Phytophthora austrocedrae* (black symbols). Each point represents the mean ± standard error of 10 plants, and in each plant the measurements were made in duplicate. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ vs. control, Student's *t*-test.

abrupt decline in the second week, Ks was monitored between the first and the second weeks. It was found that between the eighth and ninth days after inoculation, Ks

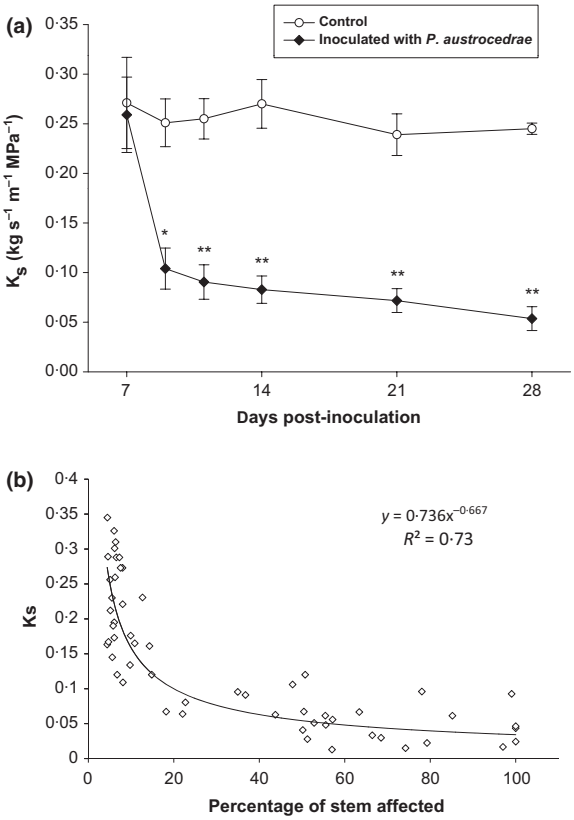

Figure 2 (a) Time-course study of stem-specific hydraulic conductivity (Ks) in 2-year-old *Austrocedrus chilensis* saplings after mock inoculation (controls, white symbols) or inoculation with *Phytophthora austrocedrae* (black symbols). Ks was determined on a segment of the stem taken from the collar root to the first branch as described in Materials and methods. Each value represents the mean ± standard error of 10 plants. $^*P < 0{\cdot}001$, $^{**}P < 0{\cdot}0001$ vs. control, Student's *t*-test. (b) Relationship between Ks and the percentage of the stem affected by the lesion. Each point represents one sapling.

began to decrease, falling to ~41% of the control value on the ninth day (Fig. 2a; $P < 0{\cdot}01$ vs. control, Student's *t*-test).

The Ks of stem segments was negatively and significantly correlated with the percentage of stem affected by the lesion ($R = -0{\cdot}77$, $P < 0{\cdot}01$) (Fig. 2b).

Phytophthora austrocedrae colonization of *A. chilensis* xylem

Microscopic examination of the xylem located below the necrotic lesion in the phloem revealed the presence of hyphae as well as resinous materials of unknown nature (Fig. 3a–d). In tangential and radial sections hyphae were observed in tracheids and rays, passing from rays to tracheids through the cross-field pitting, and from one tracheid to another through the pits. Crossing hyphae filled the torus of the pit completely and consequently blocked the pit (Fig. 3a). A resinous, light brown material

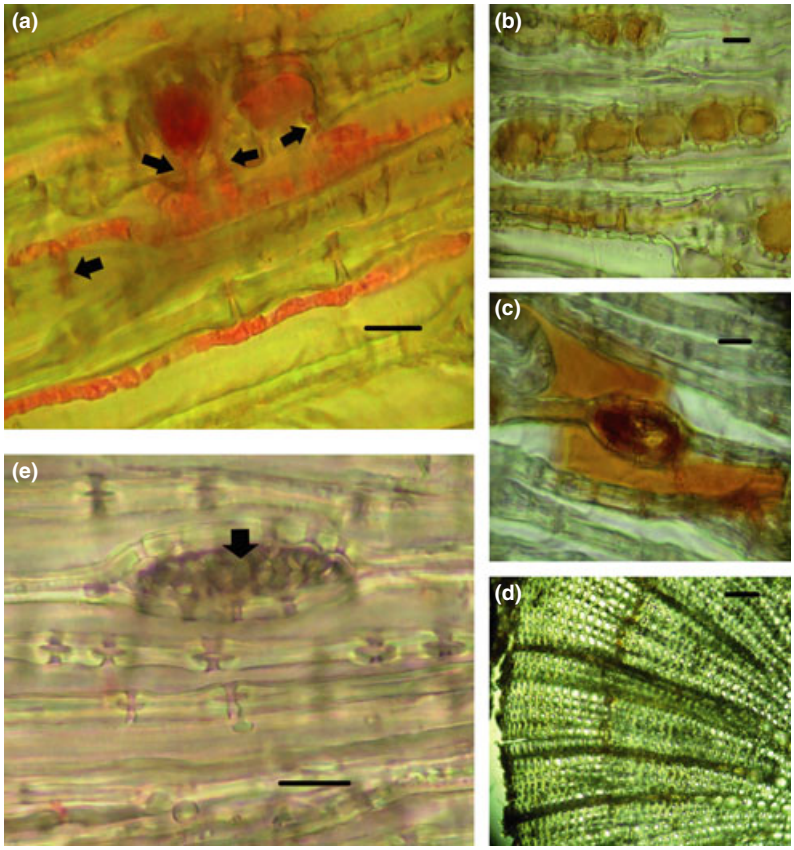

Figure 3 Micrographs of longitudinal and transverse sections of stems of *Austrocedrus chilensis* saplings inoculated with *Phytophthora austrocedrae* (a–d) and mock inoculated (e). (a) Tangential section showing hyphae (pink colour as result of phloxine) in tracheids and rays. Arrows indicate bordered pits crossed and blocked by hyphae. (b) Tangential section showing rays filled with resinous, light brown substance of unknown nature. (c) Tangential section showing a ray filled with the light brown substance also present in adjacent tracheids, probably spread from ray parenchyma. (d) Transverse section showing necrotic rays. (e) Tangential section denoting unaltered xylem with pits free of any material and abundant starch content (arrow) in the ray. Bars in (a), (b), (c) and (e) = 15 μm. Bar in (d) = 50 μm.

of unknown nature was also observed in rays that seemed to block them. The same material was also seen in adjacent tracheids, to which it probably spread from ray parenchyma (Fig. 3b,c). Necrosis of xylem ray parenchyma and presence of resinous materials was observed in transverse section (Fig. 3d). The necrosis of the rays was deeper in big lesions than in small ones. Healthy xylem showed abundant starch, especially in rays, that was absent in affected areas (Fig. 3e). The torus of pits of healthy xylem was free from any material (Fig. 3e).

Toxic effect of *P. austrocedrae* CF

The possibility that *P. austrocedrae* could secrete toxins or substances that in turn affect tissue integrity and function of the host was tested by exposing leaves and stem tissue to the CF of the pathogen. CF induced an increase in electrolytes liberated by the tissues, evidenced by an increase in conductivity of the bathing solution (Fig. 4a; $P < 0{\cdot}001$ and $P < 0{\cdot}0001$ vs. control, Student's *t*-test). Similarly, an alteration of H^+ concentration regulation was evident by the decrease in external pH (Fig. 4b; $P < 0{\cdot}0001$ vs. control, Student's *t*-test). CF treatment produced an increase in cell necrosis in the leaves (Fig. 4c; $P < 0{\cdot}0001$ vs. control, Student's *t*-test). Necrosis was detected mainly in parenchyma cells and, to a lesser extent, in mesophyll cells (data not shown).

The possible distant toxic effect was also studied in stem-inoculated plants. Conductivity, H^+ concentration and necrotic cells in leaves from stem-inoculated plants showed a progressive increase, with massive liberation of electrolytes (Fig. 5a; $P < 0{\cdot}01$ vs. control, Student's *t*-test) and H^+ at the end of the study (Fig. 5b; $P < 0{\cdot}0001$ vs. control, Student's *t*-test), and more extensive necrotic areas reaching values of ~80% in the fourth week (Fig. 5c; $P < 0{\cdot}0001$ vs. control, Student's *t*-test).

Effect of *P. austrocedrae* CF on physiological status and tissue integrity

The direct effect of the CF of the pathogen on physiological parameters was studied by injecting sterile CF into the xylem of 2-year-old saplings. Six weeks after the injection A, g_s and Ks were monitored and WUE estimated. As

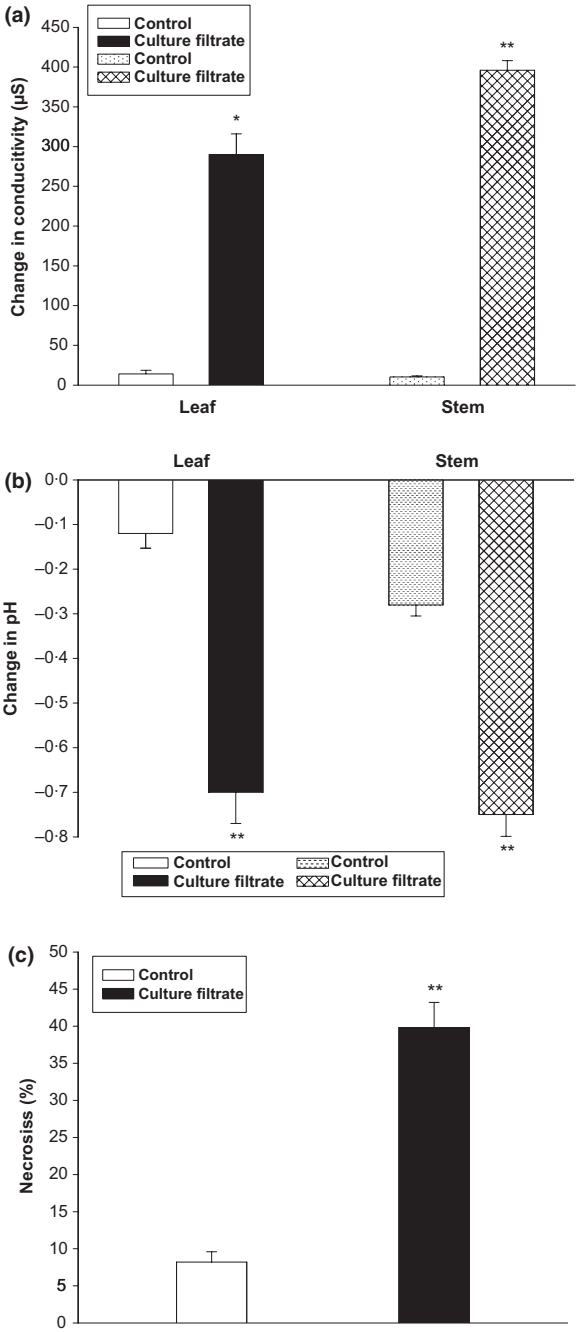

Figure 4 (a) Extracellular change in electrical conductivity; (b) extracellular change in pH; and (c) necrosis of leaves and stems of *Austrocedrus chilensis* exposed to culture filtrate (CF) of *Phytophthora austrocedrae* (black and crosshatched bars, for leaf and stem, respectively) or to sterile liquid medium (controls, white and dotted bars, for leaf and stem, respectively), as described in Materials and methods. Each bar represents the mean ± standard error of five plants. Measurements of electrical conductivity and pH were performed in duplicate. Necrosis is expressed as percentage on an area basis; 10 leaves per seedling were analysed. *$P < 0{\cdot}001$, **$P < 0{\cdot}0001$ vs. control, Student's *t*-test.

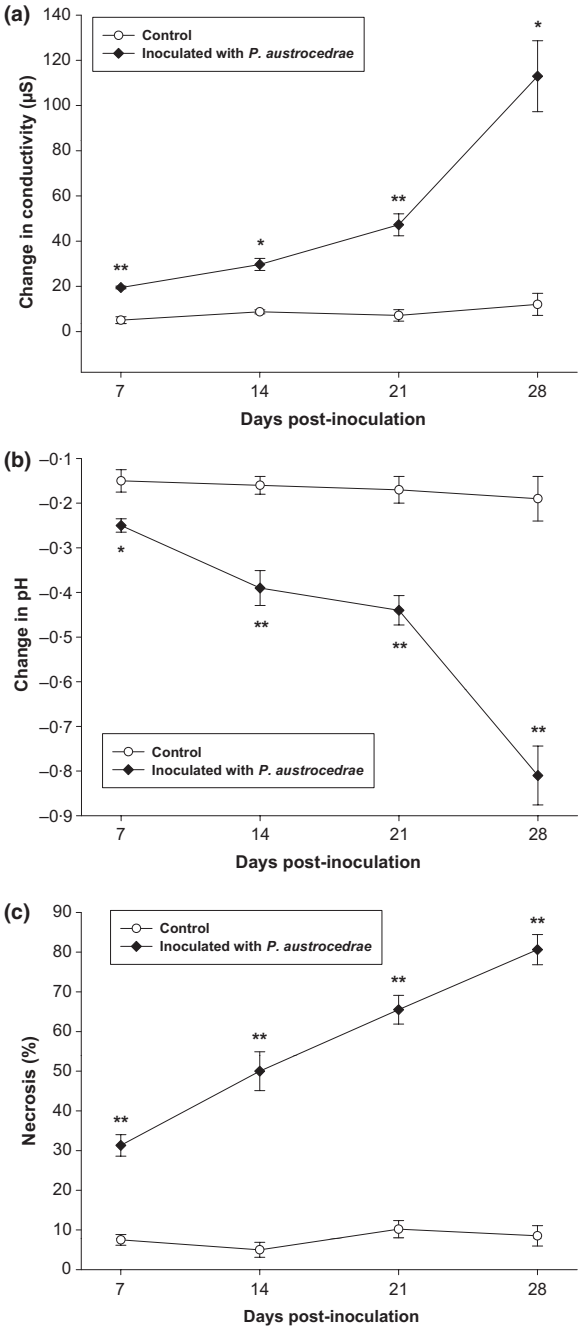

Figure 5 (a) Change in extracellular electrical conductivity; (b) change in extracellular pH; and (c) necrosis in leaves of *Austrocedrus chilensis* saplings inoculated with *Phytophthora austrocedrae* (black symbols) or controls (mock inoculated, white symbols), as described in Materials and methods. Each value represents the mean ± standard error of 10 plants. Measurements of electrical conductivity and pH were performed in duplicate. Necrosis is expressed as percentage on an area basis; 10 leaves per seedling were analysed. *$P < 0{\cdot}01$, **$P < 0{\cdot}0001$ vs. control, Student's *t*-test.

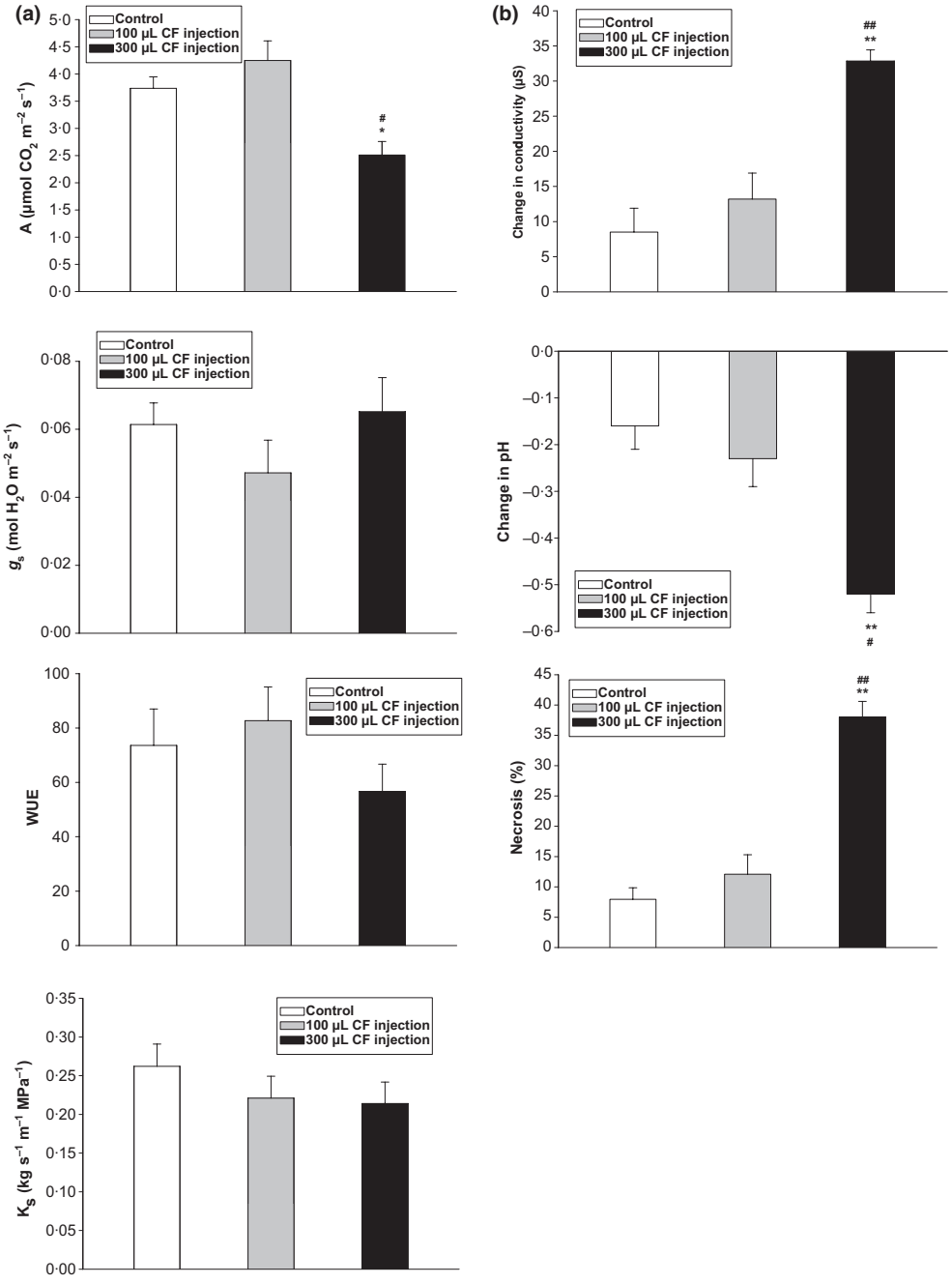

Figure 6 (a) Net photosynthetic rate (A, upper panel), stomatal conductance (g_s, middle panel), water use efficiency (WUE, middle panel), and stem-specific hydraulic conductivity (Ks, lower panel) of 2-year-old *Austrocedrus chilensis* saplings injected with 100 μL (grey bars) or 300 μL (black bars) culture filtrate (CF) of *Phytophthora austrocedrae*, or 300 μL sterile liquid medium (white bars, controls). Each bar represents the mean ± standard error of 10 plants. A and g_s measurements were performed in duplicate. (b) Detection of extracellular change in electrical conductivity (upper panel), extracellular change in pH (middle panel) and necrosis (lower panel), in leaves of the same saplings analysed in (a). Measurements of electrical conductivity and pH were performed in duplicate. Necrosis is expressed as percentage on an area basis, and 10 leaves per sapling were analysed. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}001$ vs. control; $^{\#}P < 0{\cdot}01$, $^{\#\#}P < 0{\cdot}001$ vs. 100 μL CF injection. For statistical analysis of g_s, change in electrical conductivity and in pH, the Games–Howell post hoc test was applied, the rest of the parameters were analysed by the Tukey post hoc test.

shown in Fig. 6a (upper panel), net photosynthetic rate decreased by ~33% with the injection of 300 μL CF, but was not altered by 100 μL CF ($P < 0{\cdot}05$ vs. control, Tukey post hoc test). Stomatal conductance and stem-specific hydraulic conductivity were unaffected at both doses (Fig. 6a, middle and lower panels, respectively).

Despite the reduction in A, WUE showed no significant modification (Fig. 6a, middle panel).

A CF-induced loss of membrane integrity in leaves was revealed by an increase in conductivity and pH change (Fig. 6b, upper and middle panel, $P < 0{\cdot}001$ vs. control, Games–Howell post hoc test). The extension of the damage induced by the CF was visualized as an increase in necrotic area in the leaves (Fig. 6b, lower panel, $P < 0{\cdot}001$ vs. control, Tukey post hoc test), which involved the death of parenchyma and mesophyll cells (data not shown).

The decline in net photosynthesis was strongly and positively correlated to the increase in electrical conductivity (Fig. 7a; $R = 0{\cdot}951$, $P < 0{\cdot}001$), the decrease in pH (Fig. 7b; $R = 0{\cdot}965$, $P < 0{\cdot}0001$) and the increase in necrosis (Fig. 7c; $R = 0{\cdot}984$, $P < 0{\cdot}0001$).

Discussion

Inoculation of *A. chilensis* with *P. austrocedrae* led to progressive, significant declines in net photosynthesis, stomatal conductance and stem-specific hydraulic conductivity. Values of these functional parameters in control saplings were found to be in the ranges previously reported for the species (Gyenge *et al.*, 2005, 2007, 2008). The strong impact on plant physiology observed from the second week on could be mainly explained by the extensive death of bark and cambium tissues and the disruption of phloem transport, as well as by the blockage of xylem transport by hyphal colonization and resinous plugs in tracheids and rays and death of xylem ray parenchyma, leading to loss of water supply capacity. The resinous material that plugs rays seems to be very similar to that observed in xylem rays of *P. austrocedrae* lesions of naturally infected trees (Greslebin & Hansen, 2010) and could be related to the resin exudates that are observed on the outside of affected trees associated with lesions in the field. Apparently, the exudate is produced by xylem ray parenchyma as a defence against the pathogen, and might contribute to the decrease of water transport. Moreover, a positive correlation was observed between the severity of stem infection of saplings and the decrease of stem-specific hydraulic conductivity. However, an involvement of effectors secreted by *P. austrocedrae* was also indicated by the progressive alteration of tissue integrity of leaves distant from the lesions that was observed throughout the study. Thus, the negative impact of effectors on tissue may contribute to the observed physiological dysfunction. Associations of alterations in physiology with root or shoot necrosis have been observed in other *Phytophthora* spp. in a variety of hosts (Dawson & Weste, 1982; Crombie & Tippett, 1990; Robin *et al.*, 2001; Fleischmann *et al.*, 2005; Manter *et al.*, 2007; Clemenz *et al.*, 2008). Although the alteration of phloem transport has been hypothesized as being the main cause of host decline, there is increasing evidence for the importance of xylem colonization in the aetio-physiopathogenic action of *Phytophthora* spp. *Phytophthora ramorum* and *P. kernoviae* were found to infect the sapwood of

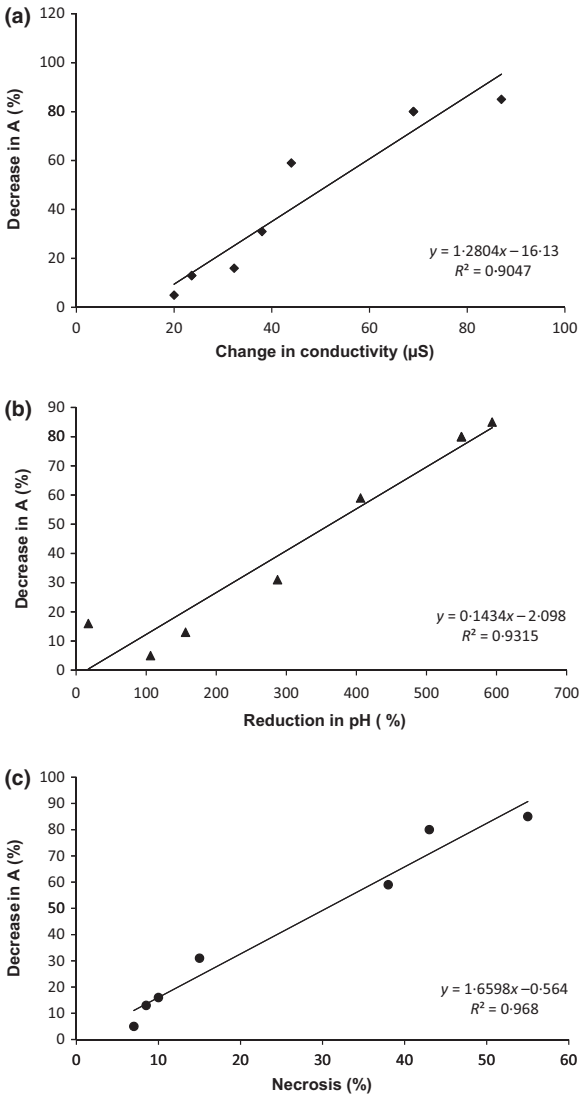

Figure 7 Relationship between the relative decrease in net photosynthetic rate (expressed as percentage of reduction) and (a) extracellular change in conductivity; (b) relative extracellular reduction of pH (expressed as percentage of reduction); and (c) percentage of necrosis, in leaves of *Austrocedrus chilensis* saplings injected with 300 µL culture filtrate (CF) derived from *Phytophthora austrocedrae*. Each point represents one sapling.

Quercus and *Fagus* species leading to, at least, local xylem dysfunction (Brown & Brasier, 2007). Colonization of fibre cells of the xylem was involved in the susceptibility/resistance of Port-Orford-cedar to POC root disease caused by *P. lateralis* (Oh & Hansen, 2007). *Lithocarpus densiflorus* trees with *P. ramorum*-infected xylem exhibited reduced midday sap flux and specific conductivity in the field. The anatomical observations suggested that water transport in the infected trees decreased through obstruction caused by fungal hyphae, chlamydospores and tyloses (Parke *et al.*, 2007; Collins *et al.*, 2009).

Consistent with these studies, the present work found that *P. austrocedrae* hyphae are abundant in xylem ray parenchyma and in fibre tracheids and it was hypothesized that they might gain access to sapwood through rays from bark tissues. The blockage of the cross-field pitting, pits and tracheid lumen by hyphae, together with the abundant accumulation of resinous material, contribute to stem-specific hydraulic conductivity disruption. However, infection does not appear to spread further in the sapwood than in the bark.

The presence of *P. austrocedrae* in the xylem could have different implications. The obstructions in the water transport system which decrease water supply could lead to rapid death of branches or crown during periods of marked water deficits. Thus, it could contribute to the appearance of rapid tree death seen in some cases (especially in young trees of small diameter) and to the decrease of plant vigour caused by decline of photosynthetic rate. In the same way, Mundo *et al.* (2010) found that, after extreme drought events, the growth of trees showing symptoms of decline was consistently lower than the growth of symptomless trees. This could also be explained by the xylem condition because affected trees suffer a decrease in water transport capacity and, consequently, a marked decrease in the water supply, that leads to a decrease in growth, especially in drought periods. On the other hand, the presence of *Phytophthora* in xylem could also enhance and accelerate the transport and delivery of effectors to active tissues throughout the tree.

The rapid effect on gas exchange prior to any changes in stem hydraulic conductivity suggests a distant effect caused by the presence of an effector secreted by the pathogen. It is well known that *Phytophthora* spp. release a wide range of toxins and elicitors (Tepfer *et al.*, 1998; Costet & Cahill, 1999; Heiser *et al.*, 1999; Kamoun, 2006; Hardham & Cahill, 2010). Many studies reported on the role of these molecules in altering or inducing plant defence mechanisms, and their action on morphological and structural features (Tepfer *et al.*, 1998; Costet *et al.*, 1999; Heiser *et al.*, 1999; Desender *et al.*, 2007). However, little is known about the capacity of these substances to affect different physiological properties. The most studied effectors are the elicitins, low-molecular-weight proteins which induce a hypersensitive reaction (HR), different degrees of necrosis or apoptosis, extracellular changes in pH, ion fluxes and membrane depolarization in different hosts (Pernollet *et al.*, 1993; Churngchow & Rattarasarn, 2000; Vleeshouwers *et al.*, 2000; Brummer *et al.*, 2002; Ivanova & Singh, 2003; Fleischmann *et al.*, 2005; Desender *et al.*, 2007; Manter *et al.*, 2007). *Phytophthora ramorum* elicitins caused a decline in chlorophyll fluorescence (F_v/F_m) in compatible and incompatible species (Manter *et al.*, 2007). Citricolin, an elicitin produced by *P. citricola*, caused a decrease in gas exchange of tobacco leaves but not of beech leaves (Fleischmann *et al.*, 2005). Similarly, cryptogein and parasiticein elicitins did not alter stomatal conductance in chestnut (Maurel *et al.*, 2004). Thus, a variety of responses to elicitins may be observed.

The results obtained here demonstrate the ability of *P. austrocedrae* CF to induce electrolyte leakage, changes in extracellular pH in leaf and stem tissues and necrosis in leaves *in vitro*. Similarly, effects on leaf tissue distant from the necrotic lesion were observed in inoculated plants *in vivo*. These features were observed in previous studies of incompatible as well as of compatible interactions and are associated with HR or HR-like responses, respectively (Pernollet *et al.*, 1993; Vleeshouwers *et al.*, 2000; Desender *et al.*, 2007; Manter *et al.*, 2007). The detrimental effect on net photosynthesis, without the alteration of other physiological functions, of a released *Phytophthora*-derived factor or a host-response, was clearly demonstrated by the fact that the effect of CF injection resembled that observed in the first week after inoculation. The mechanism(s) responsible for this effect have not been explored. However, it is hypothesized here that an HR-like response could be involved, because a strong correlation between the decrease in net photosynthesis and the three parameters of leaf tissue integrity (increase in conductivity, reduction in pH and increase in necrosis) was observed. The necrosis observed in mesophyll cells could explain, at least in part, the decrease in photosynthetic rate. The results obtained here are consistent with the work of Manter *et al.* (2007), who reported a decline in photosynthesis in response to inoculation of stems of *Rhododendron* plants with *P. ramorum*, without any visible tissue damage or measurable decrease in hydraulic conductivity. The effect was also observed in other compatible as well as incompatible hosts challenged with *P. ramorum* elicitins. The decline in photosynthetic rate was associated with an HR-like reaction induced by the elicitins.

The lack of effect in saplings injected with 100 μL *P. austrocedrae* CF denotes a concentration-dependent response. This is a known phenomenon and threshold levels of different *Phytophthora* spp. toxins and elicitors to induce certain effects on hosts have been determined (Costet *et al.*, 1999; Heiser *et al.*, 1999). Whether a toxin or an elicitor is responsible for changes in tissue integrity and photosynthesis remains unknown. Because *P. austrocedrae* is a recently described species, no toxins or other secreted molecules have yet been characterized. Preliminary results show that boiling of CF for 15 min abolishes the negative impact (data not shown), which indicates that proteins may be involved in the observed effect. Assays are currently being performed in the laboratory of the present study to identify proteins present in CF, and determine which of them trigger physiological changes in host tissues.

The WUE increase observed in the second week could be explained mainly by a marked decrease in g_s and a moderate reduction in A value, which imply better use of the resource during water stress. These findings are in agreement with the previous demonstration that the main physiological mechanism that allows *A. chilensis* to survive water deficits is its strong stomatal control (Gyenge *et al.*, 2005, 2007). Later reductions of WUE occurred during a chronic near-blockage

of the stem-specific hydraulic conductivity, which triggered further reduction of g_s and a marked decline in A because of the failure of adequate water supply to maintain this parameter.

To summarize, *P. austrocedrae* inoculation of *A. chilensis* leads to progressive declines in net photosynthesis, stomatal conductance and stem-specific hydraulic conductivity. The mechanism of action of the pathogen may involve a reduction in photosynthesis without significantly altering another physiological parameter, when the necrotic lesion in the stem is incipient. This stage may involve the participation of effectors secreted by *P. austrocedrae* which could trigger an HR-like reaction, leading to a dysfunction in photosynthesis. At a later stage, when the stem is extensively affected, down-regulation of all physiological functions may occur as a result of disruption of phloem transport as well as blockage of xylem transport. In this circumstance, a reduction of photosynthetic products and a drastic decrease in water supply may cause root necrosis and water stress, which may explain, at least in part, the functional decline. *Phytophthora*-derived effectors may also contribute to dysfunction. More studies are necessary to identify the secretome profile of the pathogen and the full mechanism of invasion, colonization and action on tree physiology.

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