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Pathogenicity of *Phytophthora austrocedrae* on *Austrocedrus chilensis* and its relation with *mal del ciprés* in Patagonia

A. G. Greslebin[a]* and E. M. Hansen[b]

[a]*Protección Forestal, Centro de Investigación y Extensión Forestal Andino Patagónico (CIEFAP), CC 14, 9200, Esquel, Chubut, Argentina; and* [b]*Department of Botany and Plant Pathology, Oregon State University, Cordley Hall 2082, Corvallis, OR 97331-2902, USA*

Field observations, isolations and pathogenicity tests were performed on *Austrocedrus chilensis* (Cupressaceae) trees to determine the pathogenicity of *Phytophthora austrocedrae* and its role in the aetiology of the cypress disease *mal del ciprés* (MDC) in Argentina. It was found that *P. austrocedrae* is a primary pathogen of *A. chilensis*. It was isolated from large necrotic lesions in the inner bark, and superficially in the sapwood, at the root collar and stem, in most of the MDC-affected stands surveyed along the range of *A. chilensis* in Argentina. The main symptom in naturally infected trees was a necrotic lesion extending from killed roots up to 1 m up the tree bole. Seedlings, saplings and adult trees were all susceptible to inoculation with *P. austrocedrae*. Under favourable experimental conditions (flooding), inoculated seedlings suffered massive mortality in less than a month. The importance of diseases caused by *Phytophthora* spp. in South American forests is discussed.

Keywords: cypress, forest pathology, root rot, soilborne pathogens

Introduction

Austrocedrus chilensis (*ciprés de la cordillera*) is an endemic tree in the Cupressaceae, found in southern Argentina and Chile. It is the most widely distributed 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 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 (Dimitri, 1972; Dezzotti & Sancholuz, 1991), across a broad moisture gradient (170 cm year^{-1} in the west to 50 cm year^{-1} in the east). In the west, *A. chilensis* can be found either in mixed stands with *Nothofagus* spp. or in pure *Austrocedrus* stands on dryer 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 because of the quality of its wood and its scenic importance.

High levels of mortality of *A. chilensis* trees were reported in 1948 in Isla Victoria (Nahuel Huapi National Park) in Patagonia, Argentina. In 1953, a similar case was reported in a stand located near a forest nursery in Epuyen, about 150 km away. Since then, mortality has been reported in many places along the range of *A. chilensis* and it has been assumed that every case has the same cause (Havrylenko *et al.*, 1989), a disease named *mal del ciprés* (MDC, cypress sickness). The first above-ground symptoms of MDC in individual trees are chlorosis and withering of the foliage (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 (Varsavsky *et al.*, 1975; Havrylenko *et al.*, 1989). Root death is frequently followed by the development of brown rots in the sapwood. These decay columns are mainly caused by basidiomycetes, *Postia dissecta* and *Coniophora arida* (Barroetaveña & Rajchenberg, 1996), common saprotrophic wood-decay fungi that apparently act as opportunistic pathogens (Rajchenberg & Cwielong, 1993).

MDC is clearly associated with site conditions, particularly high soil moisture and poor drainage (Baccalá *et al.*, 1998; Filip & Rosso, 1999; La Manna & Rajchenberg, 2004a,b) at both microsite and landscape scales (La Manna *et al.*, 2008). Tree death tends to occur in clusters in stands, at least when incidence is low (Rosso *et al.*, 1989, 1994).

Several causes have been proposed for MDC: (i) a root-rot pathogen (brown-rotting fungi) (Varsavsky *et al.*,

*E-mail: agreslebin@ciefap.org.ar

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1975; Deschamps & Vizcarra Sánchez, 1989); (ii) a pathogen in the rhizosphere, probably a pythiaceous oomycete (Havrylenko *et al.*, 1989; Rajchenberg *et al.*, 1998; Greslebin *et al.*, 2005, 2007); (iii) a forest decline (Calí, 1996; Filip & Rosso, 1999; La Manna & Rajchenberg, 2004a,b); and (iv) inappropriate soils for growth of *A. chilensis* forests (Colmet Dâage, 1992).

Since symptoms begin in the roots, the disease is associated with poorly drained sites, and tree mortality is aggregated, attention has been recently focused on the possible action of a pythiaceous pathogen. Several species of *Phytophthora* have been isolated from streams and soils in affected stands (Greslebin *et al.*, 2005), but only one, *Phytophthora austrocedrae*, has been isolated from necrotic tissues of roots and the root collar (Greslebin *et al.*, 2007).

On account of its potential participation in the aetiology of MDC, the pathogenicity of *P. austrocedrae* on *A. chilensis* was assessed, as well as its presence in affected forests throughout the range of cypress in Argentina. This work presents a description of the symptomatology associated with *P. austrocedrae* infection, the known distribution of the pathogen in *A. chilensis* forests and the results of pathogenicity tests. The role of *P. austrocedrae* in the aetiology of MDC is discussed.

Materials and Methods

Field survey

Road-accessible *A. chilensis* forests in Argentina were visited from the southernmost extreme near Corcovado, Chubut, 43°43′S, to the northern extreme near Villa Pehuenia, Neuquén, 38°54′S. Marginal xeric forests in the north and east of the range were excluded since they did not exhibit an unusually high level of mortality (Filip & Rosso, 1999). Because of the extremely arid conditions, involvement of *Phytophthora* spp. was considered improbable. During the tours, the forests were inspected for dead individuals as well as trees showing possible symptoms of MDC (i.e. foliar colour change, defoliation). In addition, information on affected forests was gathered from forest services and from the Administration of Protected Areas (APN), and all sites with mortality that were indicated by these organizations were visited. All stands showing *A. chilensis* mortality and/or symptoms were surveyed for *P. austrocedrae* and other possible causal agents. Living trees with symptoms were inspected at the root collar for necrotic tissues, and isolations from those tissues were attempted in the field. A minimum of three and a maximum of 12 necrotic lesions were sampled at each site. A sample (including affected inner bark and associated xylem) of each lesion was taken to the laboratory in a portable cooler to re-attempt isolation and to perform ELISA immunoassays. A sample of healthy tissue of one tree per stand was also taken as a negative control for ELISA immunoassays.

Isolation

Isolates were obtained from the margins of necrotic zones, mostly in phloem lesions, but also in xylem, of trees with symptoms. In the field, isolations were attempted by direct plating of necrotic phloem tissue onto one or more of the following selective media: corn meal agar (CMA 17 g L^{-1}, Sigma) amended with: (i) PARNBP (10 mg pimaricin, 200 mg ampicillin, 10 mg rifampicin, 50 mg nystatin, 15 mg benomyl and 50 mg PCNB L^{-1}); (ii) PAR (10 mg pimaricin, 200 mg ampicillin and 10 mg rifampicin L^{-1}); (iii) NAR (25 mg nystatin, 200 mg ampicillin and 10 mg rifampicin L^{-1}); or (iv) BARP (10 mg benomyl, 200 mg ampicillin, 10 mm rifampicin and 50 mg PCNB L^{-1}). Isolation was re-attempted in the laboratory by direct plating of affected phloem and/or xylem on unamended CMA. A minimum of two and a maximum of five plates, each with four or five pieces of necrotic tissues, were made per medium and per lesion. Isolation plates were incubated at 16°C in the dark. The temperature of incubation was selected according previous studies (Greslebin *et al.*, 2007).

Phytophthora austrocedrae isolates were identified by their morphological features (homothallic with amphigynous antheridia, semipapillate sporangia and very slow growth) and ITS sequences.

Isolates of *P. austrocedrae* (Table 1) used in pathogenicity tests were obtained from necrotic tissues, and the isolate of *Phytophthora syringae* (Table 1) was recovered from soil in an *Austrocedrus* stand with symptoms of MDC.

ELISA immunoassays

A portion of each lesion and a portion of healthy tissue from at least one tree of each stand visited were taken to

Table 1 *Phytophthora* isolates used for pathogenicity tests

Isolate	Species	Location	Source	Date
ATCC MYA-4074 CIEFAP 203	*P. austrocedrae*	Argentina, Chubut, Los Alerces National Park, stand near Braese stream	Necrotic inner bark of *Austrocedrus chilensis*	Oct. 2005
CIEFAP 190	*P. austrocedrae*	Argentina, Chubut, Futaleufú, Río Grande Valley, 'La 106' ranch	Necrotic inner bark of *A. chilensis*	Sept. 2005
CIEFAP 232	*P. austrocedrae*	Argentina, Chubut, Los Alerces National Park, near Los Pumas stream	Necrotic inner bark of *A. chilensis*	Oct. 2005
CIEFAP 5	*P. syringae*	Argentina, Chubut, Futaleufú, Río Grande Valley, 'Los cerezos' ranch	Soil of *A. chilensis* stand	June 2001

the laboratory and kept at −20°C for use in ELISA immunoassays (DAS ELISA reagent set for *Phytophthora*, ADGIA Inc.). Assays were performed according to the manufacturer's instructions.

Koch's postulates

In order to fulfil Koch's postulates, the pathogenicity of *P. austrocedrae* on *A. chilensis* was evaluated. *Phytophthora syringae*, the main species recovered from soil near trees with symptoms (Greslebin *et al.*, 2005), was also tested.

Pathogenicity tests were performed in the field through stem and root inoculation of adult trees, and in the laboratory by stem inoculation of 5-year-old saplings, but also by planting seedlings in artificially infested soil.

Field, root and stem inoculations

Field inoculations were performed in an *A. chilensis* stand located at INTA (National Institute of Farming Research) Trevelin, where *P. austrocedrae* and MDC had been previously recorded. Ten trees showing no symptoms were selected for root and stem inoculation. Diameter at breast height (DBH) ranged from 13 to 50 cm, and estimated root diameter at inoculation points varied from 3 to 15 cm. Inoculations were made in summer (January 2005) and autumn (May 2005), and incubated for 4 months. Five trees were inoculated (stem and roots) in each season. Temperature was not monitored during experiments, but they were conducted in summer–autumn and autumn–winter periods to cover the extreme soil and air temperature conditions of these forests. Significant differences in lesion sizes of the experiments of both periods (summer–autumn and autumn–winter) were tested with a *t*-test.

Root inoculations

Where possible, up to four superficial (5–7 cm deep) main roots of each tree were excavated and four treatments were randomly assigned [T0: control; T1: *P. austrocedrae* (isolate CIEFAP Py190); T2: *P. austrocedrae* (isolate ATCC MYA-4074); T3: *P. syringae* (isolate CIEFAP Py5)] (Table 1). Two inoculations per root were made in trees having less than four main roots. Bark cores (7 mm diameter) were aseptically removed using a borer. V8-agar discs taken from the edges of 15-day-old cultures of *P. austrocedrae* and 7-day-old cultures of *P. syringae* 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. Controls received uninfested V8-agar discs. After inoculation, roots were covered again with the removed soil. Assessment was carried out after 4 months: the outer bark was removed to expose the phloem, and the length and width of the lesion (necrotic phloem) was recorded. Re-isolation was attempted from the top and the bottom edges of the lesions and ELISA immunoassays (DAS ELISA reagent set for *Phytophthora*, ADGIA Inc.) were performed on necrotic tissues associated with each treatment, including controls. A Kruskal–Wallis nonparametric analysis of variance was applied in order to detect significant differences in mean lesion size between treatments. Significant differences between mean lesion size of each treatment and control were tested by a Mann–Whitney test. Homogeneity of variances was tested using the Levene test.

Stem inoculations

Inoculations were made at a height of 1·3 m. Each tree received four inoculations: T0: control; T1: *P. austrocedrae* (isolate CIEFAP Py190); T2: *P. austrocedrae* (isolate ATCC MYA-4074); T3: *P. syringae* (isolate CIEFAP Py5) (Table 1). Inoculations were made on four sides of the trees, facing each cardinal direction, following the procedure described above. A single treatment was applied to each hole and treatments were randomly selected for each side of the tree. Trees were assessed in the field after 4 months: the outer bark was removed to expose the phloem, and the length and width of the lesion (necrotic phloem) was recorded. Re-isolation was attempted from the top and bottom edges of the lesions and ELISA immunoassays were performed on necrotic tissues associated with each treatment, including controls. Statistical analyses were carried out on the data as described above for root inoculations.

Stem inoculation of saplings

Thirty 5-year-old *A. chilensis* saplings were inoculated. Plants were raised from seed and were about 35–55 cm tall and 1–1·5 cm in diameter at the ground line at time of inoculation. Two isolates of *P. austrocedrae* were tested (Table 1): CIEFAP Py190 (T1) and CIEFAP Py232 (T2). Treatments were randomly assigned to each sapling (10 saplings per treatment and 10 controls). Bark cores (5 mm diameter) were aseptically removed using a borer. Tomato-juice agar (TA) discs taken from the edges of 15-day-old cultures of *P. austrocedrae* were placed in the holes and covered with the removed bark. A piece of sterilized, moist muslin cloth was placed over each inoculation, covered with aluminium foil and sealed with adhesive tape. Controls received uninfested TA discs. Seedlings were kept at the laboratory with a 12-h photoperiod, at temperatures varying from 17 to 22°C and watered as necessary. Assessment was carried out after 2 months: the outer bark 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 controls. Statistical analysis was as described above for root inoculations.

Soil inoculation of seedlings

Two-month-old seedlings of *A. chilensis* were transplanted into infested soil. Inocula consisted of agar discs with sporangia. Agar discs were cut from the edges of 15-day-old *P. austrocedrae* cultures (T1: isolate CIEFAP Py190, T2: isolate CIEFAP Py232) (Table 1) and placed

in clarified T-broth for 4 days. Clarified T-broth was prepared as clarified V8 juice broth (Erwin & Ribeiro, 1996), but using tomato juice (La Campagnola) instead of V8 juice. The discs were then rinsed 10 times in distilled water to remove nutrients and placed in non-sterile soil extract water for 4 days [50 g soil in 1 L water for 48 h then filtered through filter paper no. 40 (Whatman International Ltd)]. Non-sterile soil extract was chosen because it was very difficult to obtain sporangia using sterile soil extract. Incubation conditions were 16°C in the dark. Seedlings were planted in pots with a 1:1 mix of tyndalized soil and sterilized volcanic sand. Soil was tyndalized three times, heating it with steam to about 90°C for 2 h and re-heating it after 24 and 48 h. Tyndalization was applied instead of sterilization to prevent possible negative effects of high temperatures on soil. Volcanic sand was sterilized at 130°C for 48 h. Twenty pots were assigned to each treatment, including controls. Two seedlings were planted in each pot; one agar block with sporangia was placed in the hole where each seedling was planted. Controls received uninfested TA discs, placed in non-sterile soil extract for 4 days, in the hole where each seedling was planted. Pots were flooded for 48 h every 14 days by blocking drainage holes and adding water to soil saturation. After 48 h, normal drainage was allowed. Mortality of seedlings was recorded weekly. Assessment was carried out after 1 month. Mortality of each treatment was evaluated and isolations from rotten roots of dead seedlings were attempted on selective media (PAR) as previously described.

Results

Field survey

Mortality of *A. chilensis* was located in some stands in all parts of the range. Mortality or crown symptoms suggestive of MDC were found in 47 stands. Two patterns of disease development on affected trees were recorded:

1 Necrotic lesions in the inner bark of roots and root collar, extending up the bole (Fig. 1). Trees at 43 out of the 47 (91·5%) visited sites showed this pattern of root necrosis.
2 Symptoms first present in the crown as dieback, with only healthy tissues at the root collar. Trees at 4 out of 47 (8·5%) visited sites showed this pattern of symptoms.

The study was focused on the first type of disease pattern, since the symptoms were consistent with a disease caused by *Phytophthora*. It was the most frequently found expression of MDC, and *P. austrocedrae* was isolated from the necrotic inner bark of roots and root collar.

In the 47 stands with symptoms 316 lesions (227 of them active and 89 inactive) on 234 trees were sampled. Affected tissues of roots and root collar appeared as necrotic lesions extending upward from dead roots to more than 1 m up the tree stem. No outer bark symptoms were associated with necrotic lesions, but resin exudates, when present, usually indicated the presence of a necrotic lesion. The necrosis affected the entire thickness of the cambium and phloem and also the superficial sapwood. Lesions appeared as active or inactive. When active,

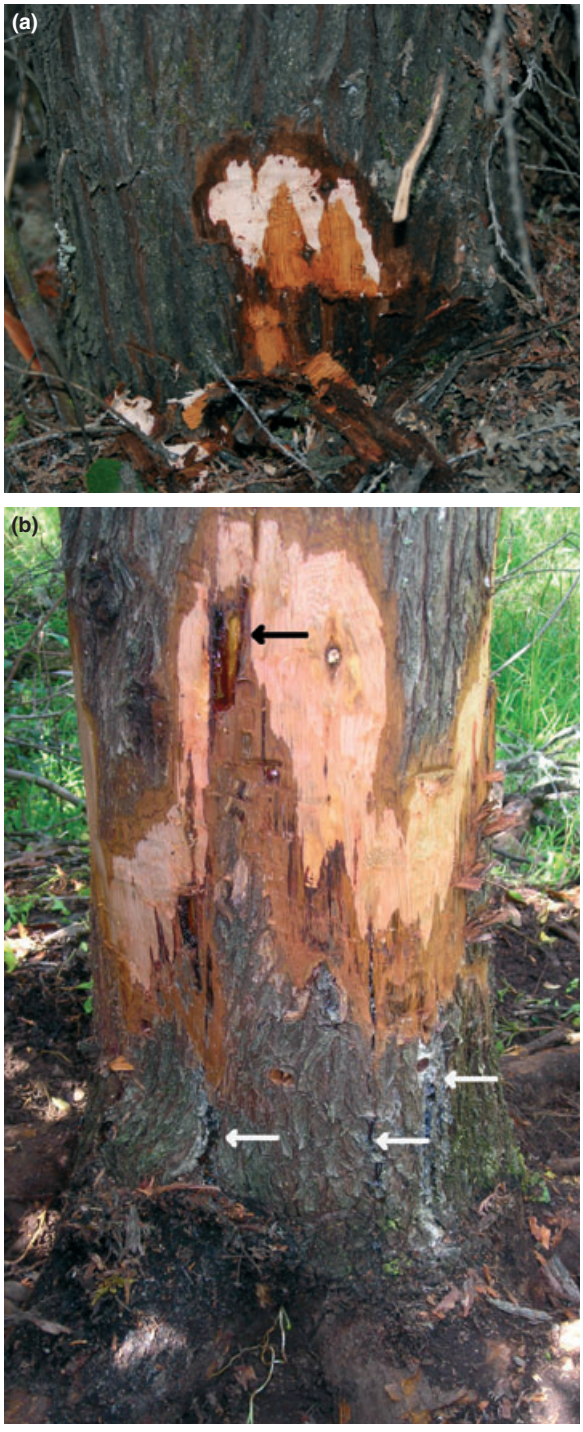

Figure 1 Necrotic lesions of *Phytophthora austrocedrae* in the inner bark of an *Austrocedrus chilensis* tree. (a) Active lesion, bright chestnut brown in colour and moist. (b) Inactive lesion, dark brown and dry; a resin pocket can be observed in the advancing zone (black arrow), as well as resination in the outer bark (white arrows).

below the outer bark lesions were bright chestnut brown in colour, moist and flexible (Fig. 1a). When inactive, they were dark brown, dry and hard, and it was very difficult to distinguish them from the outer bark (Fig. 1b). In the xylem, active lesions appeared brown instead of the normal brilliant white colour. Microscopic examination of affected xylem showed coloured resinous contents in the cells, especially in xylem ray parenchyma. The brown zone in the xylem associated with the lesions in the phloem seemed to be a reaction zone of the tree, but *P. austrocedrae* was isolated from the wood, indicating at least superficial penetration.

Inactive lesions were frequently found in trees with symptoms. Most isolations were made from active lesions, whilst samples were taken from both active as well as inactive lesions to perform ELISA immunoassays in the laboratory.

Isolation

Isolations from the first 62 trees sampled were attempted on all four *Phytophthora*-selective media (PARNBP, PAR, NAR and BARP) and also on unamended CMA. Since PAR was slightly more effective than the others, from tree 63 onwards, only PAR and unamended CMA were used. Since these two media gave similar results, from tree 88 onwards only PAR was used. The results of isolation and ELISA tests are shown in Table 2. *Phytophthora austrocedrae* was isolated from affected phloem and also from affected xylem. ELISA immunoassays were positive in most of the necrotic lesions whether active or inactive. Isolated mitosporic fungi were mostly *Penicillium* spp., *Cladosporium* spp., *Papulospora* spp. and other unidentified hyphomycetes, most of them dematiaceous. Other organisms recovered, very infrequently, from active lesions were basidiomycetes (i.e. *Serpula himantioides* and *Postia dissecta*) and Mucorales. Isolations from inactive lesions of 14 trees on selective media were also attempted, mostly resulting in no microbial growth, or saprotrophic hyphomycetes. *Phytophthora austrocedrae* was never recovered from inactive lesions. Other organisms recovered were bacteria (7%), *Mortierella* spp. (5%) and *Coniophora arida* (1%).

ELISA immunoassays

ELISA immunoassays for *Phytophthora* were successful in detecting *Phytophthora* in active as well as inactive necrotic lesions. The rate of detection was much higher for active lesions than for inactive ones. Since *P. austrocedrae* is the only *Phytophthora* species that has been isolated from necrotic lesions of *A. chilensis*, positive ELISA tests on *A. chilensis* necrotic phloem were assumed to be positive detections of *P. austrocedrae*.

Detection of *Phytophthora austrocedrae* in *Austrocedrus chilensis* forests of Argentina

Phytophthora austrocedrae was isolated and/or detected by ELISA immunoassays from 273 out of 316 necrotic lesions in 220 out of 234 *Austrocedrus* trees with symptoms inspected in 43 out of 47 sites visited throughout the tree's range in Argentina (Fig. 2).

The species was isolated from 24 out of the 43 visited sites exhibiting root symptoms, whilst at all 43 sites, detection by ELISA immunoassay of the necrotic lesions at the root collar was positive. ELISA immunoassays performed on healthy tissues were always negative. Difficulties of isolation could be related to the sensitivity of the species to temperatures over 20°C. The highest isolation rates were achieved in autumn and spring, whilst in summer no isolates were obtained, even though isolation was attempted from lesions that looked very active. The use of a portable cooler improved isolation rate, but it was not a total solution to the problem.

Four stands exhibited crown decline without root lesions. These stands were located in Los Alerces National Park, (42°50′75·3″S, 71°30′43·5″W), on the road to Los Alerces National Park near the Percey river (42°58′58·3″S, 71°29′37·4″W), at Estancia Santa Teresita, Corcovado, Chubut (43°32′15″S, 71°33′00″W) and Cerro Llao-Llao, Nahuel Huapi National Park (41°02′21·8″S, 71°33′01·77″W). In these cases roots were unaffected (even in trees with dead crowns) and necrotic lesions typical of *P. austrocedrae* were not present at the base of the trees. In addition, site conditions at these stands appeared to be less favourable for *Phytophthora* development (i.e. well-drained soils, steep slopes). In these stands the cause of mortality remains unknown.

Pathogenicity of *Phytophthora austrocedrae* on *Austrocedrus chilensis*

Root and stem inoculations of adult trees

Nineteen inoculations of *P. austrocedrae* (10 of isolate CIEFAP Py190 (T1) and nine of isolate ATCC

Table 2 Results of isolations and ELISA tests from lesions on 234 *Austrocedrus chilensis* trees sampled in Argentina

No. lesions sampled	Lesion state	ELISA-positive (%)	Isolation			
			No. lesions	Media	*Phytophthora* (%)	Negative (no fungal growth) or other organisms isolated (%)
227	Active	95	219 (active)	Selective media	23	Negative: 38% Mitosporic fungi: 37% Others: 2%
			88 (active)	Unamended-CMA	14	Negative: 46% Mitosporic fungi: 40% Others: 0%
89	Inactive	66	14 (inactive)	Selective media	0	Negative: 50% Mitosporic fungi: 37% Bacteria: 7% Others: 6%

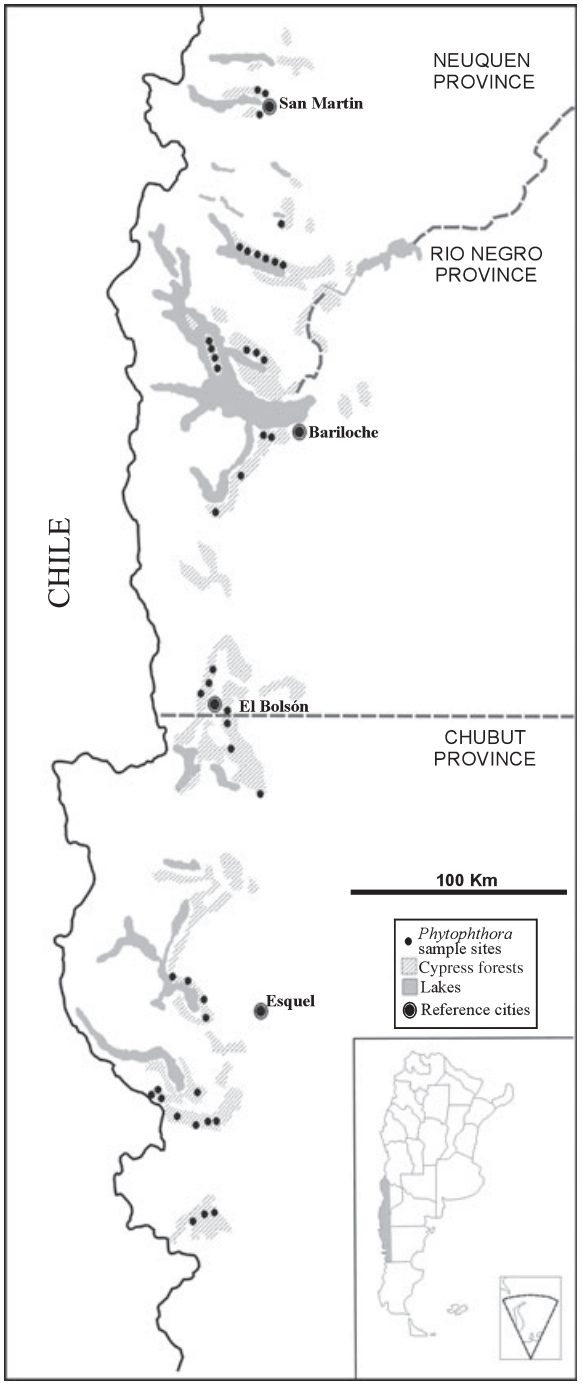

Figure 2 *Phytophthora austrocedrae* distribution in *Austrocedrus chilensis* forests of Patagonia.

MYA4074) were made on roots, and 20 inoculations (10 of isolate CIEFAP Py190 (T1) and 10 of isolate ATCC MYA4074) were made on stems.

All inoculations of *P. austrocedrae* on roots developed into brown, necrotic lesions in the phloem, reaching and staining the sapwood, and so did 18 out of 20 stem inoculations. There was only one inoculated tree where no lesions developed from the stem inoculations, presumably as a result of inoculum failure, but lesions developed from root inoculations of the same tree.

Since the Levene test showed unequal variances in lesion size ($P < 0{\cdot}001$) in both roots and stems, nonparametric analyses were applied. Lesions that developed in the roots after *P. austrocedrae* inoculations (both T1 and T2) were significantly longer and wider (one-sided $P < 0{\cdot}0001$) than those seen in the negative controls in both January and May inoculations (Table 3). Mean length and width of the 10 inoculations of *P. syringae* did not differ from those of the negative controls (Table 3). Lesion lengths of January root inoculations (summer–autumn period) were significantly longer (one-sided $P < 0{\cdot}0001$) than those of May inoculations (autumn–winter period) for both *P. austrocedrae* treatments (T1 and T2).

Lesions that developed in the stems after *P. austrocedrae* inoculations (both T1 and T2) were significantly longer (one-sided $P < 0{\cdot}0001$) and wider (T1: one-sided $P < 0{\cdot}0001$, T2: one-sided $P = 0{\cdot}003$) than those observed in Control inoculations (Table 3). Mean lesion length and width of the 10 stem inoculations of *P. syringae* did not differ from those in the negative controls (Table 3). Lesion length of January stem inoculations (summer–autumn period) did not differ significantly from those of May inoculations (autumn–winter period) for both *P. austrocedrae* treatments (one-sided $P = 0{\cdot}43$ and $0{\cdot}16$ for T1 and T2, respectively).

Mean lesion length and width of T1 treatments were larger than mean lesion length and width of T2 treatments both in root and stem inoculations, but the differences were not significant (one-sided $P = 0{\cdot}41$ and $0{\cdot}11$ for root and stem inoculations, respectively).

Trees showed no external foliar symptoms at the end of the study. Most of them exhibited resin exudation from the inoculation point. *Phytophthora austrocedrae* was re-isolated on selective media from the phloem of 78% of lesions. ELISA tests were positive for all the lesions developed from *P. austrocedrae* inoculations. *Phytophthora syringae* could not be re-isolated from inoculation points, and ELISA tests were negative in *P. syringae* and control treatments.

Stem inoculation on 5-year-old saplings

After 2 months, all saplings inoculated with *P. austrocedrae* developed brown necrotic lesions that affected the phloem, reaching and staining the sapwood. A few trees showed external symptoms (i.e. dark-red foliage), but most of them showed no symptoms, even though they developed lesions that were similar to those of plants with symptoms. Since the Levene test showed unequal variances ($P < 0{\cdot}002$), nonparametric analyses were applied. Lesions developed in both treatments were significantly longer (one-sided $P < 0{\cdot}0001$) than those in negative controls (Table 4). *Phytophthora austrocedrae* was successfully re-isolated only twice (from one plant of each treatment). ELISA tests were positive for all the lesions developed from *P. austrocedrae* inoculations and

Table 3 Pathogenicity of *Phytophthora austrocedrae* and *P. syringae* to *Austrocedrus chilensis* roots and stems of adult trees (combined data from January and May inoculations)

Species/Isolate	Inoculation	*n*	Mean lesion length (cm)	Standard deviation	Mann–Whitney test significance
P. austrocedrae/CIEFAP Py 190 (T1)	Roots	10	28·37	6·80	***
	Stem	10	21·53	6·58	***
P. austrocedrae/ATCC MYA4074 (T2)	Roots	9	25·64	7·55	***
	Stem	10	15·27	8·98	**
P. syringae/CIEFAP Py	Roots	10	1·18	0·41	Not significant
	Stem	10	1·58	0·92	Not significant
Controls	Roots	10	1·16	0·60	
	Stem	10	1·07	0·86	

***$P < 0{\cdot}0001$, **$P < 0{\cdot}01$, not significant $P > 0{\cdot}05$.

Table 4 Pathogenicity of two isolates of *Phytophthora austrocedrae* to wound-inoculated *Austrocedrus chilensis* saplings compared to the artificially inoculated control

Species/Isolate	*n*	Mean lesion length (cm)	Standard deviation	Mann–Whitney test significance
P. austrocedrae/CIEFAP Py 190	10	15·18	3·58	***
P. austrocedrae/CIEFAP Py232	10	20·69	7·39	***
Controls	10	0·32	0·04	

***$P < 0{\cdot}0001$.

negative for the control inoculations. Mean lesion length was larger for T2 than T1, but the difference was not significant (one-sided $P = 0{\cdot}11$). The low isolation rate could be caused by desiccation. Since isolation was attempted once all bark was removed and measurements and photographs were taken, and the phloem was very thin, it suffered high desiccation during the process.

Soil inoculation and flooding of seedlings

In *P. austrocedrae* treatments (T1 and T2), 95% of seedlings (76 out of 80 seedlings in 38 out of 40 pots) were dead after the second flooding (20 days of treatment). Roots of dead seedlings were brown or severely rotten. Two control seedlings in one pot were dead at the end of the study (1 month) and they died during the first week of treatment, probably as a result of transplant stress. Isolations from rotten roots of dead seedlings inoculated with *P. austrocedrae* were overgrown by *Mortierella* spp., and *Phytophthora* was not recovered, but ELISA tests were positive. ELISA tests on the two dead seedlings in the control treatment were negative.

Discussion

Field observations and isolations, and pathogenicity tests on saplings and roots and stems of mature trees indicate that *P. austrocedrae* is a primary pathogen to *A. chilensis*. It was isolated from the margins of large necrotic lesions in the inner bark, and superficially in the sapwood, at the root collar and stem. Adult trees as well as saplings and seedlings were susceptible to inoculation and lesions developed quickly (up to 4·7 cm per month in adult trees and up to 11·5 cm per month in small saplings). Under favourable conditions, the pathogen caused massive mortality of seedlings in less than 1 month. Control seedlings receiving the same flooding treatment showed only minor transplant mortality. *Austrocedrus chilensis* is a flood-tolerant species (La Manna, 2005). Isolations were successful only from the margins of actively growing lesions, although ELISA indicated the presence of a *Phytophthora* species in nearly all cases, including fine roots of seedlings, and never from control tissues. *Phytophthora syringae*, the only *Phytophthora* species recovered from soil of stands exhibiting MDC, was not pathogenic in inoculation tests.

The main symptom of *P. austrocedrae* in naturally infected trees was the necrotic lesion extending from killed roots up to 1 m up the tree bole. The necrosis affected the entire thickness of the phloem and the sapwood was superficially stained. Lesions appeared to be active or inactive. When active, they were bright chestnut brown, moist and flexible. When inactive, they were dark brown, dry and hard, and it was very difficult to distinguish them from the outer bark. It was observed that trees were able to begin to wall off old inactive lesions with callus tissues. Defoliation is another symptom of the disease, even though it seems to be an unspecific one. It was associated with the amount of root affected, but it was not totally reliable as an indicator of the percentage of necrotic tissues of main roots and root collar (Floria & Greslebin, 2009). Sometimes, especially in stands where the disease was very active, older foliage inside the crown turned bright yellow and then red by the end of the summer. This symptom was usually associated with the presence of active lesions at the root collar. Resin exudation was often associated with *Phytophthora* lesions. Resin flow usually emerged from a resin pocket in the phloem near the active margin of a necrotic lesion.

The symptoms of MDC, as originally described, agree with those of a *Phytophthora*-caused disease in the following aspects: (i) mortality of the roots, which are often subsequently rotted by secondary invaders (Varsavsky *et al.*, 1975; Havrylenko *et al.*, 1989); (ii) association of the disease with site conditions related to high soil moisture and poor drainage (Baccalá *et al.*, 1998; Filip & Rosso, 1999; La Manna & Rajchenberg, 2004a,b; La Manna *et al.*, 2008); and (iii) aggregated spatial pattern of affected trees, as usually seen in contagious processes produced by root pathogens (Rosso *et al.*, 1989, 1994).

Phytophthora austrocedrae was detected in most of the affected stands surveyed along the cypress range, including those where the disease was first reported (i.e. Isla Victoria, Parque Nacional Nahuel Huapi, and Golondrinas Epuyén). There were four sites showing cypress mortality where *P. austrocedrae* lesions were not detected, but where symptoms (i.e. crown dieback and unaffected root system), as well as the associated site conditions (i.e. deep and well-drained soils), were different from those originally described for MDC (i.e. root necrosis and high soil moisture/poor drainage). This shows that other agents in addition to *P. austrocedrae* may affect *A. chilensis*.

The other hypotheses proposed to explain MDC do not match observations from recent studies. Decayed large roots and wood-rotting basidiomycetes are often present, but they are not consistantly associated with MDC. The associated organisms are saprotrophic or act as opportunistic pathogens when the tree is weakened (Havrylenko *et al.*, 1989; Rajchenberg & Cwielong, 1993; Barroetaveña & Rajchenberg, 1996). In the present study, *Postia dissecta* was found fruiting on necrotic *P. austrocedrae* lesions, and this species, as well as *S. himantioides* and *C. arida,* other wood-rotting fungi, was isolated from the sapwood close to lesions of *P. austrocedrae*. This would indicate that wood-rotting species colonize the roots through the lesions caused by *Phytophthora*. The tree decline hypothesis seems to be inappropriate since mortality affects individuals of all ages, may occur quickly and appears to be associated with a primary pathogen. Finally, the idea that MDC appears when cypress colonizes inappropriate sites seems to be wrong given the fact that the disease is sometimes present even in vigorous forests. However, La Manna *et al.* (2008) showed the soil classes with greatest disease incidence are those with smallest forest area, suggesting that disease-prone sites are those of lower suitability for *A. chilensis* development.

The evidence points to *P. austrocedrae* as the most probable aetiological agent of MDC; however, there are some aspects that need to be elucidated to conclude it is the single primary pathogen causing MDC. More information on the cycle of the pathogen in the tree and how the tree dies is needed, as well as on the geographical origin of *P. austrocedrae*.

The most similar *Phytophthora*-caused forest disease is the root rot of *Chamaecyparis lawsoniana* caused by *Phytophthora lateralis* in the Pacific Northwest USA. Symptoms, affected organs and disease cycle are very similar (Tucker & Milbrath, 1942; Roth *et al.*, 1957; Hansen *et al.*, 2000). Both pathogens are low-temperature species and they show similar aggressiveness in pathogenicity tests (Trione, 1959; Murray & Hansen, 1997). However, *P. lateralis* disease development in the tree seems to be faster than that of *P. austrocedrae*. Infected seedlings are killed within weeks of infection, and large forest trees are usually dead within a year of the first crown symptoms (Hansen *et al.*, 2000).

Phytophthora austrocedrae is the first *Phytophthora* species reported as a pathogen of forest trees in native forests of South America. Recently, Durán *et al.* (2008) reported the new species *Phytophthora pinifolia* as the cause of 'Daño Foliar del Pino' (DFP), a needle disease that affects *Pinus radiata* plantations in Chile. This highlights the need to focus attention on the species of *Phytophthora* that inhabit forests in South America, their ecological roles and their pathogenicity.

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