

The Decline of *Austrocedrus* Forests in Patagonia (Mal del Ciprés): Another *Phytophthora*-caused Forest Disease¹

Alina G. Greslebin² and Everett M. Hansen³

Abstract

Austrocedrus chilensis, an indigenous Cupressaceae of the Patagonian Andes forests, is suffering a disease that has been called “Mal del Ciprés” (MDC). This disease was first reported more than 50 years ago but, in spite of many studies, its causes remained unclear until recently. The disease begins in the root system, the distribution and pattern of spread of mortality in a stand is consistent with a soil-borne pathogen, and it is associated with seasonally poorly drained soils. Symptoms include defoliation, basal resinous exudates and red-brown necrotic lesions in the inner bark extending up the bole from killed roots. Brown cubic rots in roots and sapwood caused by wood-decomposer fungi are frequently- but not always- associated with dead or dying trees. These characteristics have led several workers to suggest that a *Phytophthora* species might be the causal agent of the disease. Several attempts to find a *Phytophthora* species responsible for the disease have been made. Five species were isolated from soil and/or associated streams: *P. syringae*, *P. cambivora*, *P. gonapodyides* and the undescribed taxa “Pgchlamydo” and “P. taxon raspberry” and another two species -*P. pseudotsugae* and *P. cactorum*- were reported from soil and/or fine roots in a previous study. None of them showed a clear relationship with the disease. Isolations from the margins of the necrotic lesions in the inner bark using *Phytophthora*-selective media initially failed, but an ELISA test on necrotic phloem tissues was positive for *Phytophthora*, and subsequent DNA extraction from necrotic bark and amplification of ITS DNA using *Phytophthora*-specific primers was successful. Thus encouraged, isolation attempts were renewed and were finally successful. The isolated species was an undescribed taxon of *Phytophthora* that was formally named *Phytophthora austrocedrae*. It is homothallic with amphigynous antheridia and semi-papillate sporangia, very slow growing with a maximum radial growth rate ranging from 1.0–1.8 mm/day in V8A at optimal temperature (17.5C). ITS rDNA sequence places it near *P. syringae* in phylogenetic clade 8 of the genus. It was isolated from symptomatic trees in all localities affected by MDC throughout the range of the disease, showing that the pathogen is widely distributed. Pathogenicity tests fulfilled Koch’s postulates demonstrating it is the primary cause of the disease. This work presents the results of pathogenicity tests and their implications on the aetiology of the disease. A discussion of subjects that should be addressed in future work is also presented.

Introduction

Austrocedrus chilensis (D. Don.) Pic. Serm. & Bizarri (Ciprés de la cordillera) is an endemic tree of the Cupressaceae of southern Argentina and Chile. Among the few

¹ A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

² Área de Protección Forestal, Centro de Investigación y Extensión Forestal Andino Patagónico (CIEFAP), CC 14, 9200, Esquel, Chubut, Argentina.

³ Department of Botany and Plant Pathology, Oregon State University, Cordley Hall 2082, Corvallis, OR 97331-2902, USA.

Corresponding author: agreslebin@ciefap.org.ar.

conifers inhabiting southern Argentina it has the largest distribution, covering ca. 140,000 ha. (Bran and others 2002). It is a very valuable species because of its ecological role, the high quality of its wood and its scenic appeal. Throughout its range, *A. chilensis* suffers a lethal disease known as “Mal del Ciprés” (MDC). This disease was first detected 60 years ago and, since then, the affected area has been constantly increasing. Several studies on biotic and abiotic factors related to the disease have been done but the origin and causes of MDC remained unclear until recently. For a detailed description of the disease and background see Greslebin and others (2005).

The symptomatology of the disease was traditionally described as a chlorosis, withering and subsequent progressive defoliation of the crown (Varsavsky and others 1975, Havrylenko and others 1989, Rajchenberg and Cwielong 1993), and a decline of radial growth of the tree (Filip & Rosso 1999). The disease originates in the root system, where the death of the tissue precedes defoliation of the crown (Havrylenko and others 1989). Death of the roots is followed, many times, by the development of brown rots in the sapwood (Varsavsky and others 1975, Havrylenko and others 1989, Rajchenberg and Cwielong 1993, Barroetaveña & Rajchenberg 1996). Resin exudation has been pointed out either as related (Varsavsky and others 1975) or as unrelated (Havrylenko 1989) to the disease. The death of the tree may occur slowly, as a progressive defoliation, or rapidly, in which case foliage changes from chlorotic to red with little or no defoliation (Filip & Rosso 1999, Greslebin & Hansen 2006). Affected stands are usually associated with poorly drained sites (La Manna & Rajchenberg 2004a, b). The origin of the disease in the roots and its association with poorly drained sites suggested the action of a Pythiaceus organism (Pythiaceae, Oomycota). Several species of *Phytophthora* were isolated from streams and soils in affected stands (Greslebin and others 2005), but attempts to isolate from necrotic tissues were negative up to recently when a new *Phytophthora* species was detected on them (Greslebin and others 2007).

The new species, *Phytophthora austrocedrae*, was the first organism consistently associated with cypress mortality suggesting it might be the primary cause of MDC. To confirm this hypothesis and fulfill Koch's postulates, pathogenicity of *P. austrocedrae* on *Austrocedrus chilensis* was evaluated. *P. syringae*, the principal species recovered from soil near symptomatic trees, was also tested. This work presents the results of pathogenicity tests and their implications on the aetiology of the disease.

Materials and Methods

Isolation

Isolates of *P. austrocedrae* (Table 1) used for pathogenicity tests were obtained from the advancing necrotic zone of phloem lesions of symptomatic trees studied in previous work (Greslebin and others 2007). The isolate of *P. syringae* (Table 1) was recovered from soil in an *Austrocedrus* stand exhibiting symptoms of MDC. Isolates were identified by their morphological features and ITS sequences (Greslebin and others 2007).

Koch's Postulates

Pathogenicity tests were performed:

- a) In the field through stem and root inoculation of adult trees; and
- b) In the laboratory through stem inoculation of five-year-old seedlings; and
- c) Through soil infestation.

Field root and stem inoculation

Field inoculations were performed in an *A. chilensis* stand located at INTA (National Institute of Farming Research) Trevelin, where *P. austrocedrae* had been previously recorded. Ten trees showing no symptoms were selected for stem inoculation. Inoculations were made in 2 different seasons: summer (January 2005) and fall (May 2005). Five trees were inoculated (stem and roots) in each season. Results were combined for analysis.

Root inoculation

Up to four superficial main roots of each tree were excavated and four treatments [T0: control; T1: *P. austrocedrae* (strain CIEFAP Py 190); T2: *P. austrocedrae* (strain ATCC MYA-4074); T3: *P. syringae* (strain CIEFAP Py 5)] (Table 1) were randomly assigned. Two inoculations per root were made in those trees with less than 4 main roots. Cores (7 mm diam) of bark were aseptically removed using a borer. V8-agar discs 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 cheese-cloth was placed over each inoculation point, covered with aluminum foil, and sealed with adhesive tape. Controls received uninfested V8-agar discs. After inoculation roots were covered again with the removed soil.

Stem inoculation

Each tree received 4 inoculations corresponding to the following treatments: T0: control; T1: *P. austrocedrae* (strain CIEFAP Py 190); T2: *P. austrocedrae* (strain ATCC MYA-4074); T3: *P. syringae* (strain CIEFAP Py 5) (Table 1). Inoculations were made on the sides of the tree facing each cardinal direction following the same procedure as in roots. Treatments were randomly assigned to each side of the tree. After 4 months 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 to detect *Phytophthora* 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 between mean lesion size of treatments. Significant differences between mean lesion size of each treatment and control were tested through a Mann-Whitney test. Homogeneity of variances was tested using the Levene test.

Stem inoculation of seedlings

Thirty 5-year-old seedlings were selected for stem inoculation. Two strains of *P. austrocedrae* were tested (Table 1): CIEFAP Py 190 (T1) and CIEFAP Py 232 (T2). Treatments were randomly assigned to each seedling (ten seedlings for each treatment and ten controls). Cores (5 mm diam) of bark were aseptically removed using a borer. T-agar discs 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 cheese-cloth was placed over each inoculation, covered with aluminum foil and

sealed with adhesive tape. Controls received uninfested T-agar discs. Lesion length and width in phloem were recorded after 2 months. Re-isolation was attempted from the edges of the lesions, and ELISA immunoassays to detect *Phytophthora* were performed on necrotic tissues associated with each treatment and controls. A Kruskal-Wallis Nonparametric Analysis of Variance was applied in order to detect significant differences between mean lesion sizes of treatments. Significant differences between mean lesion size of each treatment and control were tested through a Mann-Whitney test. Homogeneity of variances was tested using the Levene test.

Soil infestation

Inocula consisted of agar discs with sporangia. Agar discs were cut from the edges of 15-day- old *P. austrocedrae* cultures (T1: strain CIEFAP Py 190, T2: strain CIEFAP Py 232) (Table 1) and placed in T-broth for 4 days. Then, agar blocks were rinsed 10 times in distilled water to remove nutrients and placed in soil extract for 4 days. Pots with a 1:1 mix of tindalized soil and sterilized volcanic sand were used for planting the seedlings. Twenty pots were assigned to each treatment and the control. Two seedlings were planted in each pot and one agar block with sporangia was placed in the same hole where each seedling was planted. Pots were flooded for 48 hours every 14 days. Controls received uninfested T-agar discs in the same hole where each seedling was planted. Mortality of seedlings was recorded weekly.

Table 1ð Isolates used for pathogenicity tests

Strain number	Species	Origin
ATCC MYA-4074 CIEFAP 203	<i>P. austrocedrae</i>	Argentina, Chubut, Los Alerces National Park, Braese stream, isolated from necrotic inner bark of <i>A. chilensis</i> , Oct. 2005
CIEFAP 190	<i>P. austrocedrae</i>	Argentina, Chubut, Futaleufú, Río Grande Valley, "La 106" Ranch, isolated from necrotic inner bark of <i>A. chilensis</i> , Set. 2005
CIEFAP 232	<i>P. austrocedrae</i>	Argentina, Chubut, Los Alerces National Park, near to Los Pumas stream, isolated from necrotic inner bark of <i>A. chilensis</i> , Oct. 2005
CIEFAP 5	<i>P. syringae</i>	Argentina, Chubut, Futaleufú, Río Grande Valley, "Los cerezos" Ranch, isolated from soil of <i>A. chilensis</i> stand. June 2001

Results

Pathogenicity of Phytophthora austrocedrae on Austrocedrus chilensis

Root and stem inoculations of adult trees

Since Levene test showed unequal variances (P-value < 0.001) for lesion size in both roots and stems, non parametric analyses were applied. All roots inoculated with *Phytophthora austrocedrae* developed brown, necrotic lesions that affected the phloem reaching and staining the sapwood (fig 1). Lesions developed after *P. austrocedrae* inoculations (both T1 and T2) were significantly longer and wider (one-

sided P-value < 0.0001) than those in the Controls (fig. 2). Mean lesion length and width of *P. syringae* lesions did not differ from those of the controls (fig. 1). Stem inoculations with *P. austrocedrae* developed lesions similar to those observed in root inoculations. Two exceptions were found: one tree developed small and superficial lesions and another did not develop lesions in the stem but it did in the roots. Lesions developed after *P. austrocedrae* inoculations (both T1 and T2) were significantly longer (one-sided P-value < 0.0001) and wider (T1: one-sided P-value < 0.0001, T2: one-sided P-value = 0.003) than those in Control inoculations (fig. 2). Mean lesion length and width of *P. syringae* inoculations did not differ from Control lesions (fig. 2).

Mean lesion length and width of T1 treatments were greater than mean lesion length and width of T2 treatments in both root and stem inoculation but these differences were not significant except for mean lesion width of stem inoculations (one-sided P-value = 0.02).



Figure 1δ Lesion caused by *Phytophthora austrocedrae* on a root on an adult tree of *Austrocedrus chilensis*. Left: bark removed showing necrotic phloem. Right: phloem removed showing sapwood superficially affected.

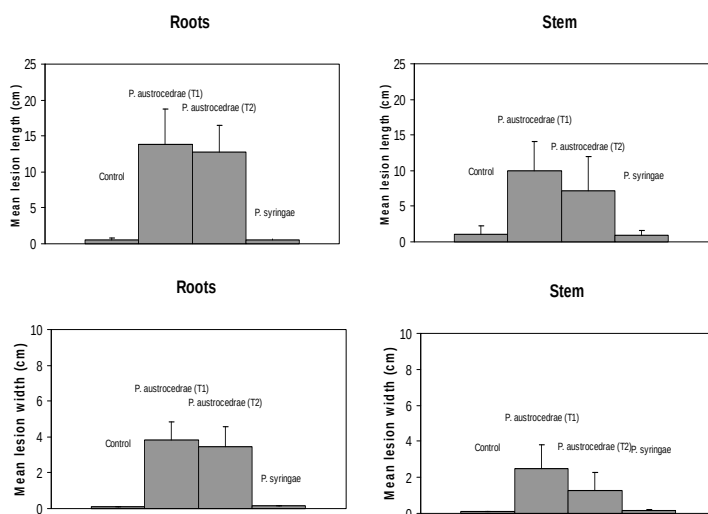


Figure 2—Mean length and width of necrosis (cm) produced by *P. austrocedrae* and *P. syringae* on roots and stems of *A. chilensis* adult trees.

Trees showed no external foliar symptoms at the end of the study. A few trees did exhibit resin exudation from the inoculation point.

Phytophthora austrocedrae was re-isolated from 78 percent of the lesions and ELISA tests were positive from 100 percent of them. *Phytophthora syringae* was not reisolated from inoculation points, and ELISA tests were negative for both *P. syringae* and control treatments.

Stem inoculation on five year old seedlings

After two months all seedlings inoculated with *Phytophthora austrocedrae* developed brown, necrotic lesions that affected the phloem, reaching and staining the sapwood. A few seedlings showed external symptoms (i.e. dark red foliage) (fig. 3) but most showed no symptoms even though they developed lesions similar to those of symptomatic seedlings. Since Levene test showed unequal variances (P -value < 0.002) non parametric analyses were applied. Lesions of both treatments were significantly longer (one-sided P -value < 0.0001) than those in Controls (Fig. 4). *Phytophthora austrocedrae* was successfully re-isolated twice (from one plant of each treatment) the other re-isolation attempts failed, being over-grown by Deuteromycetes. ELISA tests were positive for 100 percent of the lesions and negative for the Controls. Mean lesion length of T2 was greater than T1 one (fig. 4) but the difference was not significant (one-side P -value = 0.11)



Figure 3d Five year old seedlings inoculated with *Phytophthora austrocedrae* after two month of inoculation. A) Symptomatic seedling and it respective lesion. B) Asymptomatic seedling and it respective lesion.

Five year old seedlings

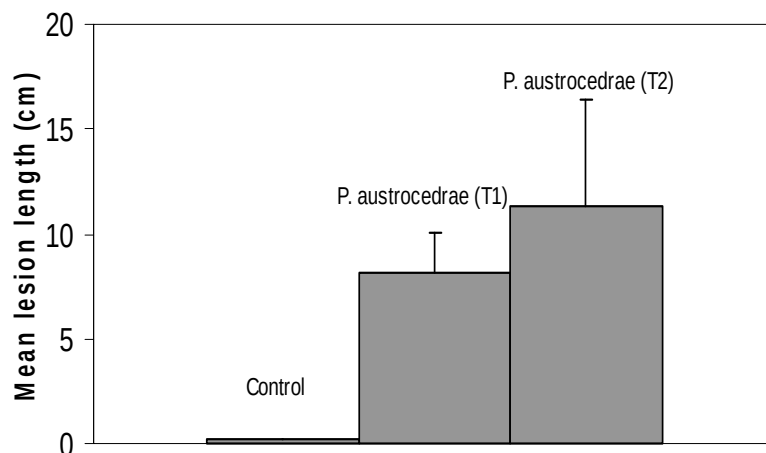


Figure 4d Mean length of necrosis (cm) produced by *P. austrocedrae* on stems of *A. chilensis* seedlings.

Soil Infestation

In *P. austrocedrae* treatments (T1 and T2) 95 percent of the seedlings were dead after the second flooding (20 days of treatment). Mortality was mostly observed after each flooding (about 40 percent of the seedlings after the first flooding and about 55 percent after the second one). Roots of dead seedlings were brown or were severely rotted. Only two control seedlings were dead at the end of the study (one month) and they died the first week of treatment.

Isolations from rotted roots of dead seedlings were overgrown by *Mortierella* spp. and *P. austrocedrae* could not be recovered, but ELISA tests were positive. ELISA tests from the two dead seedlings found in the Controls were negative.

Discussion

The experimental evidence confirms that *Phytophthora austrocedrae* is the causal agent of MDC. The pathogenicity tests indicate that *P. austrocedrae* is an aggressive pathogen of *Austrocedrus chilensis*. Adult trees and seedlings were susceptible to infection and lesions developed quickly (up to 4.7 cm month⁻¹ in adult trees and up to 11.5 cm month⁻¹ in seedlings). A root pathogen has been frequently suggested as cause of MDC (Havrylenko and others 1989, Rosso and others 1994, Rajchenberg and others 1998, Filip & Rosso 1999), but a specific organism had not been implicated. *P. syringae* has been isolated from soil and streams in diseased stands, but never from trees (Greslebin and others 2005), and was not pathogenic in stem or root inoculations in these tests.

The apparent aggressiveness of *P. austrocedrae* was unexpected. MDC has traditionally been described as a slow decline, with progressive defoliation (Rajchenberg and others 1998), where death of individual trees may take several

decades (Calí 1996, Filip & Rosso 1999). It is not unusual to find apparently healthy trees very close to dead or dying ones. This also disagrees with an aggressive pathogen. The questions are: why do some trees die quickly while others die decades after they begin to decline, and why do some trees remain unaffected even when they are next to dying trees? We have observed that some lesions in the stem become inactive and trees begin to wall off old lesions with callus tissues (fig. 5). Whether the lesions become inactive because of tree defenses or because of ambient conditions unfavorable for the pathogen is an issue that remains unclear and should be considered in future research. *P. austrocedrae* growth in vitro is affected by high temperatures (Greslebin and others 2007), showing no growth at 25 C. Maximum air temperature in summer in areas where *Austrocedrus* grows can reach 35 C (exceptionally more). High summer temperatures could be responsible for the inactivation of the lesions on some trees. The effect of ambient temperatures on lesion development as well as the variation of *A. chilensis*' resistance/susceptibility to *P. austrocedrae* should be evaluated in future work.



Figure 5—Callus tissues walling off an old, inactive lesion.

MCD symptomatology is consistent with a *Phytophthora* disease but similar crown symptoms may result from other causes. We propose that the disease name Mal del Ciprés be reserved for the disease of *Austrocedrus* caused by *Phytophthora austrocedrae* characterized by root infection and necrotic lesions extending up the bole. This pathogen has now been isolated from dying *A. chilensis* trees in Isla Victoria (Nahuel Huapi National Park) the place where MDC was first reported, and in most of the other areas throughout the tree's range that have been affected by MDC (Greslebin and others 2007).

Another very important issue that should be resolved is the geographic origin of *P. austrocedrae*. Is it a native species or an introduced pathogen? The area where the disease was first observed (Isla Victoria) was planted early in the 20th century with exotic tree species from many places in the world and a nursery was built to provide seedlings to many other places in Patagonia. *P. austrocedrae* could be an exotic pathogen that was introduced in Isla Victoria and spread from this point. On the other hand, today it is a widely distributed species present throughout the cypress

distributional range, and its closest genetic relative is *Phytophthora syringae*, another species that is also present in *A. chilensis* forests. Perhaps it is a native species that acts as a pathogen under favorable site conditions including poor soil drainage (La Manna & Rajchenberg 2004a,b) or has increased its activity because of the climate change. To elucidate the origin of *P. austrocedrae* as well as *P. syringae* will be the objective of our future work.

Acknowledgments

We want to thank Dr. Charles G. Shaw III and Dr. Paul Hennon, USDA Forest Service, Alaska Region, Forest Health Protection and ICSU-TWAS-UNESCO-UNU/IAS Visiting Scientist Programme for making possible this collaborative work by supporting AGG and EMH visits to OSU and to CIEFAP respectively. Dr. Carlos Buduba, Instituto Nacional de Tecnología Agropecuaria (National Institute of Farming Technology) INTA of Argentina, is warmly acknowledged for kindly permits the realization of pathogenicity tests in Cypress stands of INTA Trevelin. This work was supported by Agencia Nacional de Promoción Científica y Técnica (ANPCyT, FONCyT) of Argentina (PICT 1-13305) and National Research Council (CONICET) of Argentina (PIP 5660). AGG is researcher for CONICET.

Literature Cited

- Barroetaveña, C.; Rajchenberg, M. 1996.** Hongos Aphylllophorales (Basidiomycetes) que causan pudriciones en *Austrocedrus chilensis* en pie. *Bol Soc Argen Bot* 31: 201-216.
- Bran, D.; Pérez, A.; Barrios, D.; Pastorino, M. & Ayesa, J. 2002.** Eco-región valdiviana: distribución actual de los bosques de “ciprés de la cordillera” (*Austrocedrus chilensis*) - Escala 1:250.000. INTA, APN, FVSA. Informe preliminar, Bariloche, Argentina. 12 p.
- Calí, S.G. 1996.** *Austrocedrus chilensis*: estudio de los anillos de crecimiento y su relación con la dinámica del “Mal del Ciprés” en el P. N. Nahuel Huapi, Argentina. Tesis de Licenciatura en Cs. Biológicas, Universidad Nacional del Comahue, Bariloche. 143 p.
- Filip, G.M.; Rosso, P.H. 1999.** Cypress mortality (mal del ciprés) in the Patagonian Andes: comparison with similar forest diseases and declines in North America. *European Journal of Forest Pathology* 29: 89-96.
- Greslebin, A.G.; Hansen, E.M. 2006.** Novedades sobre el “mal del ciprés”. *Patagonia Forestal* 12(1): 11-14.
- Greslebin, A.G.; Hansen, E.M.; Winton, L. Rajchenberg, M. 2005.** *Phytophthora* species from declining *Austrocedrus chilensis* forests in Patagonia, Argentina. *Mycologia* 97: 218-228.
- Greslebin, A.G.; Hansen, E.M.; Sutton, W. 2007.** *Phytophthora austrocedrae* sp. nov., a new species associated with *Austrocedrus chilensis* mortality in Patagonia (Argentina). *Mycological Research* 11(3): 308-316.
- Havrylenko, M.; Rosso, P.H.; Fontela, S.B. 1989.** *Austrocedrus chilensis*. Contribución al estudio de su mortalidad en Argentina. *Bosque* 10: 29-36.
- La Manna, L.; Rajchenberg, M. 2004a. Soil properties and *Austrocedrus chilensis* forest decline in Central Patagonia, Argentina. *Plant Soil* 263: 29-41.

La Manna, L.; Rajchenberg, M. 2004b. The decline of *Austrocedrus chilensis* forests in Patagonia, Argentina: soil features as predisposing factors. *Forest Ecol Manag* 190: 345-357.

Rajchenberg, M.; Cwielong, P.P. 1993. El Mal del Ciprés (*A. chilensis*): su relación con las pudriciones radiculares y el sitio. *Actas del Congreso Forestal Argentino y Latinoamericano*, Paraná, Entre Ríos.

Rajchenberg, M.; Barroetaveña, C.; Cwielong, P.P.; Rossini, M.; Cabral, D.; Sívori, A. 1998. Preliminary Survey of Fungi Associated with the decline of Ciprés in Patagonia. In Delatour C, Guillaumin JJ, Lung-Escarmant B, Marçais B (eds.). *Proceedings of the 9th International Conference on Root and Butt-Rots* (Carcans, France), 1-7 Sept., 1997. INRA, Paris. p 235--244.

Rosso, P.H.; Baccalá, N.; Havrylenko, M. Fontenla, S. 1994. Spatial pattern of *Austrocedrus chilensis* wilting and the scope of autocorrelation analysis in natural forests. *Forest Ecol Manag* 67: 273—279.

Varsavsky, E., Bettucci, L.; Rodríguez García, D.; Gómez, C. 1975. Observaciones preliminares sobre la mortalidad del ciprés (*Austrocedrus chilensis*) en los bosques patagónicos. *Fundación Patagonia* N° 19. Bariloche, Argentina. 11 p.