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Histology of *Austrocedrus chilensis* roots during infection by *Phytophthora austrocedri*†

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Abstract: *Phytophthora austrocedri* was identified as the primary pathogen causing the "Mal del ciprés" disease in Patagonia, which affects the endemic conifer *Austrocedrus chilensis*. Trees present root necrosis that may extend to the stem. This study aimed to describe the histological alterations occurring during *P. austrocedri* infection of roots of *A. chilensis* seedlings. Plants were inoculated at the roots and histological studies were performed four weeks post-inoculation. Safranin-fast green, phloroglucinol-HCl, toluidine blue, lugol and diaminobenzidine stains were used to describe and compare anatomo-histological features observed in roots of non-inoculated versus inoculated seedlings. In healthy tissues, the presence of Phi thickenings in cortical cells is reported for the first time for *A. chilensis*. In inoculated roots, it was observed necrosis of the epidermis and of the cortical parenchyma, and alterations in parenchymal cells (loss of turgor and content, without starch, presence of phenolic compounds). Lignin content remained unaffected by the presence of *P. austrocedri*. The area occupied by Phi thickenings was smaller in *P. austrocedri*-colonized tissues, and these structures showed an accumulation of polyphenols, absent in healthy tissue. Parenchymal cells, tracheids, and rays, showed production of hydrogen peroxide. Results evidenced that *A. chilensis* triggers mechanisms to restrict the infection, but *P. austrocedri* manages to evade them.

Keywords: Patagonian cypress, root rot, *Phytophthora* diseases

1. Introduction

"Cypress" [*Austrocedrus chilensis* (D.Don) Pic. Ser. et Bizzarri] is a native conifer of Subantartic forests of Argentina and Chile, in the Cupressaceae family (subfamily Callitroideae tribu Libocedreae). Cypress represents a keystone tree species in most of the austral montane regions of Argentina and Chile covering a today a total estimated area of 185,000 ha in South America, grows between the 32°39′ and 43°35 parallels and between altitudes of 250 to 2,200 m., between Neuquén and Chubut provinces on the Argentinean side [1]. It is especially important from the economical point of view due to the quality of its wood which is very appreciated for building proposes, as well as for furniture manufacture. It also has a high ecological value since this species can form forests in the ecotonal region entering the steppe [2]. However, the ecological as well as the socio-economic importance is being affected, mostly because of a disease which affects this species since many years ago. The disease, which can be observed in many sites along *A. chilensis* forests, has been named as "Mal del ciprés", and the species *Phythopthora austrocedri* has been identified as being the primary cause [3-5].

Trees present withering foliage and defoliation. The main sign caused by *P. austrocedri* in naturally infected trees is a necrotic lesion extending from roots up to the collar that may reach up to 1 meter high above the ground, and may lead to the death of the trees. Necrosis affects the cambium, the phloem, and the sapwood [4, 6-7]. Anatomo-histological changes and alterations in conductive tissues as well as in supporting tissues of *A. chilensis* roots infected with *P. austrocedri* have not been previously studied. These aspects are crucial to understand the histopathology of the disease.

2. Materials and Methods

2.1. Plant material

Twenty *A. chilensis* plants (one-year-old) grown in a greenhouse under fertigation were used for the trials. Plants were produced in the greenhouses of the Esquel Institute of Biotechnology (UNPSJB). All plants originated from seeds collected from *A. chilensis* forests at the southern region of the distribution of the species [8]. One month before the initiation of the assays, randomly selected plants were transferred to a controlled environmental chamber under 16-h light/8-h dark at 17–19°C.

2.2. Inoculations

Ten seedlings were removed from the substrate and the root system was washed in sterile water. Inoculations were made by placing 5-mm-diameter clarified vegetable juice agar plugs containing actively growing *P. austrocedri* mycelium in contact with roots, at distances of 0.5-1 cm from their tips. Each inoculation was covered with sterilized, moist muslin cloth, wrapped with aluminum foil, and sealed with adhesive tape to prevent desiccation [9]. Ten plants were used as non-inoculated controls. Four weeks after the inoculations, plants were evaluated as described below.

2.3. Histochemical Analysis

Freehand sections were made with the help of a stereoscopic microscope, in the area of the inoculation point and in the zone of the advance of the necrotic lesion. Observations were made using a Leica DM500 optical microscope. Photomicrographs were obtained using a Canon EOS Rebel T3i digital camera.

For the visualization of structures in the non-inoculated healthy plants, it was used the safranin-fast green staining following Ma et al. (1992) [10] with modifications; or no staining and direct mounting in water of the sections. Lugol was used to contrast lignin (yellow) to non-structural carbohydrates, mainly starch (bluish to black) [11]. Toluidine blue was used to contrast lignin and polyphenols (turquoise to green), carbohydrates (lilac color) and oils (no color) [11]. Lignin detection was carried out according to the methodology described by Mauch-Mani and Slusarenko (1996) [12]. Stem sections were placed in a solution of 1% phloroglucinol in 70% ethanol for 5 min. They were then placed on slides and a drop of concentrated HCl was added and incubated for 5 min. The excess of acid was removed, and sections were soaked in water. Sections were inspected within the following 20 min under an optical microscope. The presence of lignin is visualized as a reddish to purple stain. Hydrogen peroxide (H_2O_2) was detected using a 3,3 diaminobenzidine (DAB) stain, according to the method of Thordal-Christensen et al. (1997) [13]. Roots sections were transferred into a solution of 1 mg/ml DAB (pH 3.5) and incubated in the dark at room temperature for 3 h. Hydrogen peroxide was visualized as a dark reddish to brown stain.

3. Results and Discussion

The histological features of non-inoculated healthy root tissues of *A. chilensis* were similar to other species in the Cupressaceae (Figure 1). However, it was evidenced the presence of Phi thickenings as remarkable structures since they are first reported for the species (Figure 1B-C). These structures are lignified thickenings found in the radial and tangential walls of the root cortical cells in some gymnosperms that may play a mechanical supporting role [14-15]. However, some studies

have shown that Phi thickenings can be altered by changes in environmental conditions as an adaptative response [16-17].

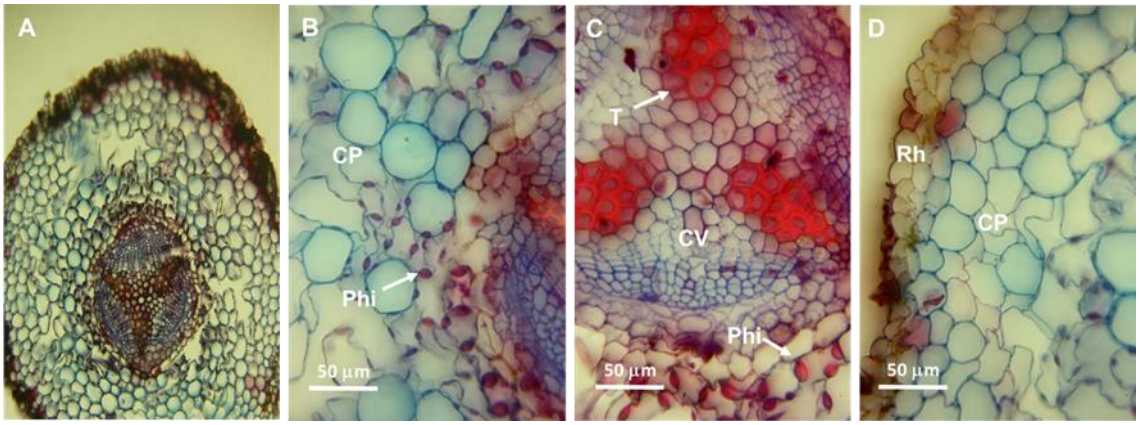

Figure 1. Micrographs of sections of non-inoculated root tissue of *A. chilensis* seedlings (safranin-fast green staining). A. general view (100X). B. cortical parenchyma (CP) and Phi thickenings (400X). C. Vascular cylinder (CV), tracheids (T) and Phi thickenings (400X). D. Rhizodermis (Rh) and cortical parenchyma (CP) (400X).

Austrocedrus chilensis seedlings inoculated with *P. austrocedri* showed symptoms (withering foliage and chlorosis) from the third week of the trial. Root tissues from inoculated plants showed the presence of hyphae and oospores (sexual reproduction structures) which confirmed the successful colonization of the pathogen in root tissues and the efficacy of the inoculation method (Figure 2A-B). A disorganization of the cortical parenchyma with collapse of cells (loss of turgor and content) and reduced numbers of Phi thickenings was observed (Figure 2C). Vascular cylinder presented necrosis of the phloem (Figure 2B, D). Necrosis was also observed in the rhizodermis and in the cortical parenchyma (Figure 2B, D).

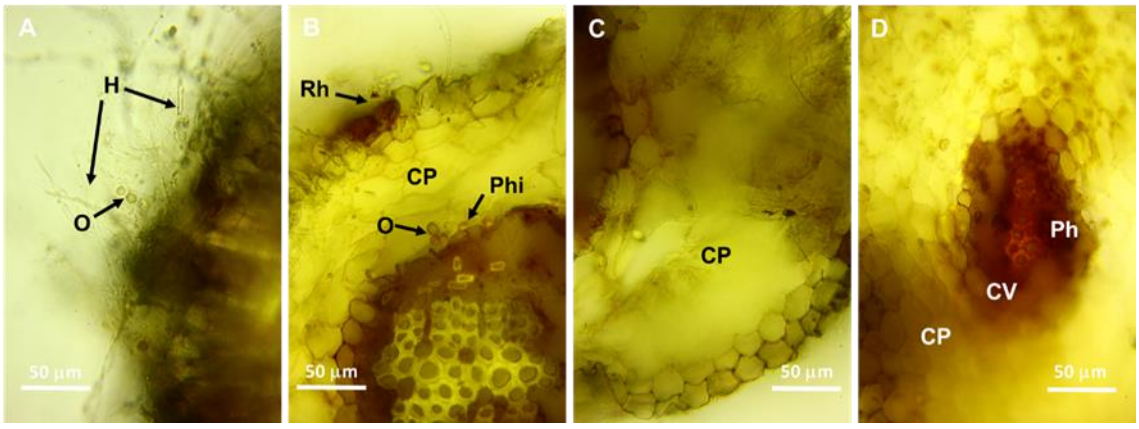

Figure 2. Micrographs of sections of root tissue of *A. chilensis* seedlings inoculated with *P. austrocedri* (400X). A. Hyphae (H) and oospores (O) in the surface of root tissue. B. oospores (O) and Phi thickenings in cortical parenchyma (CP), necrosis in parenchyma and rhizodermis (Rh). C. disintegrated cortical parenchyma (CP) tissue. D. Necrosis of phloem (Ph) and necrosis in cortical parenchyma (CP). Vascular cylinder (CV).

In Figure 3A it can be observed that in non-inoculated healthy root tissue sections stained with toluidine blue parenchymal cells were regularly arranged and isodiametric, and presented a lilac coloration indicating the presence of carbohydrates. Phi thickenings showed a blue color denoting the presence of lignin, in agreement with what was previously described regarding Phi thickenings as lignified structures.

In the inoculated roots, two zones could be differentiated, the inoculation point and the zone of the advance of the necrotic lesion towards both sides of the inoculation point. In the inoculation zone,

a turquoise coloration of the parenchymal cells was observed, indicating the accumulation of polyphenols (Figure 3B). Also, the presence of hyphae of the pathogen growing intracellularly was often observed in parenchyma (Figure 2B). In the advance zone little carbohydrate content was observed in cells of the parenchyma, the tissue was disintegrated and presented many hyphae of the pathogen growing intracellularly in the vascular cylinder (Figure 3C). Phi thickenings were stained turquoise, showing the presence of phenolic compounds, absent in healthy root tissue (Figure 3C).

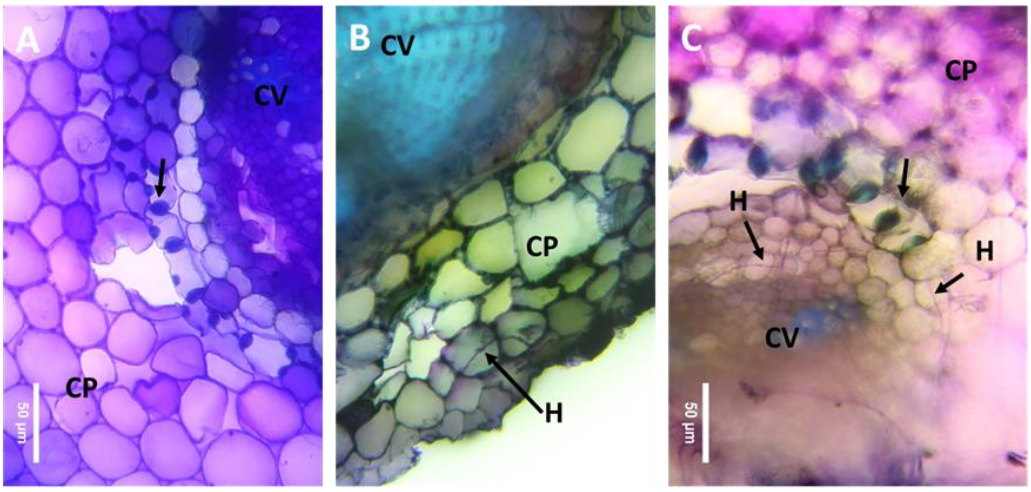

Figure 3. Sections of root tissue of *A. chilensis* seedlings inoculated with *P. austrocedri* stained with toluidine blue (400X). A. Non-inoculated healthy tissue. B. Inoculation zone. C. Advance zone. Vascular cylinder (CV), cortical parenchyma (CP), hyphae of the pathogen (H), arrows indicate Phi thickenings.

Figure 4 shows a reduction in the area occupied by Phi thickenings in the inoculation and in the advance zones, respect to the area occupied in healthy tissue. In the area of inoculation an abnormality was observed in parenchymal cells (Figure 4B), while in the advance zone cells showed a loss of turgor and the rhizodermis presented necrotic areas (Figure 4C). Red color indicates the presence of lignin, as shown by the staining of Phi thickenings. The general lignin content was not affected by the presence of the pathogen, except for the reduction in the number of the lignified Phi thickening structures.

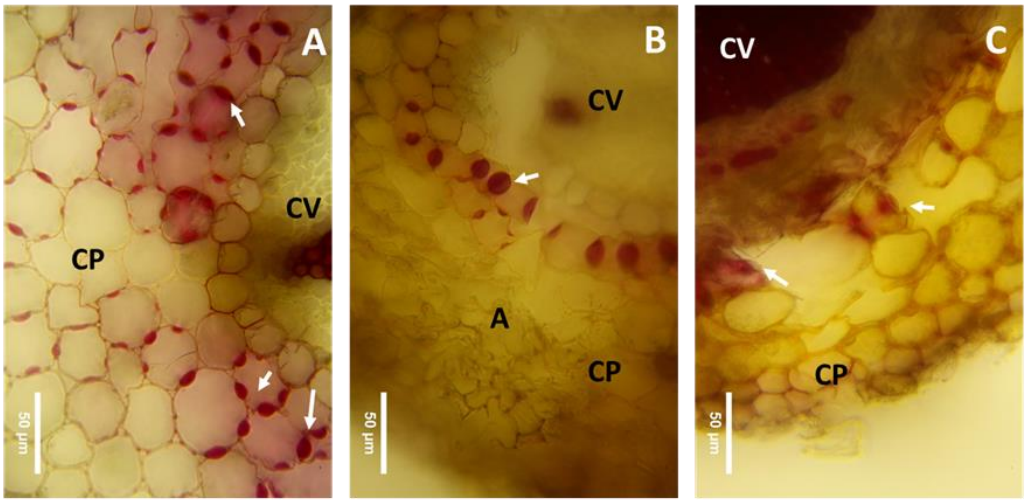

Figure 4. Sections of root tissue of *A. chilensis* seedlings inoculated with *P. austrocedri* stained with phloroglucinol-HCl (400X). A. Non-inoculated healthy tissue. B. Inoculation zone. C. Advance zone. Vascular cylinder (CV), cortical parenchyma (CP), abnormality (A), arrows indicate Phi thickenings.

Figure 5 corresponds to root sections stained with lugol to analyze the presence of starch content in cells. The presence of starch granules could not be observed in any of the sections from inoculated roots. Starch granules were evidenced in a few isolated cells and in some sections of healthy tissue. In accordance with what was observed in the previous figures, a reduction in the area occupied by the Phi thickenings in the inoculated roots was observed. Necrotic areas and presence of hyphae of the pathogen were also observed both in the inoculation zone and in the advance zone.

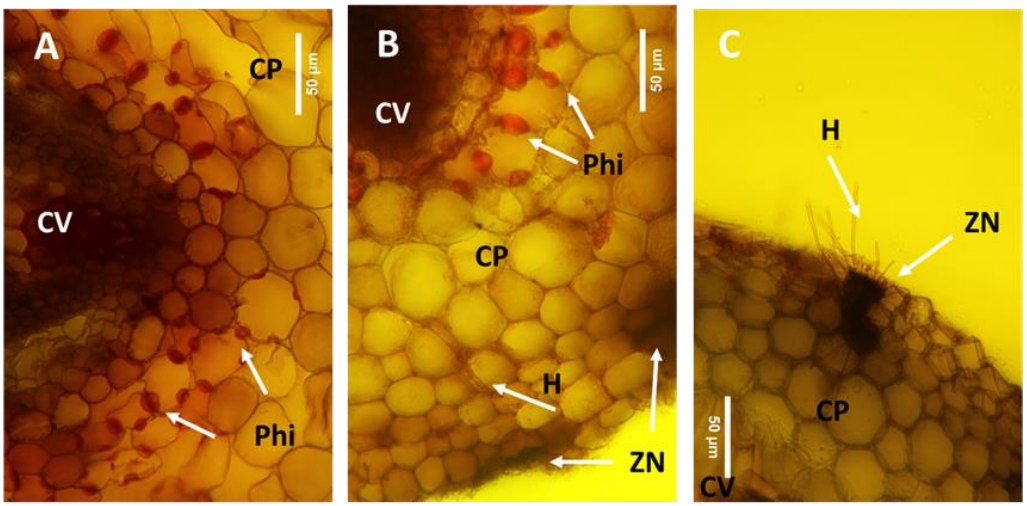

Figure 5. Sections of root tissue of *A. chilensis* seedlings inoculated with *P. austrocedri* stained with lugol (400X). A. Non-inoculated healthy tissue. B. Inoculation zone. C. Advance zone. Vascular cylinder (CV), cortical parenchyma (CP), necrotic zone (ZN), hyphae (H), arrows indicate Phi thickenings.

When sections of inoculated root tissues were stained for the detection of H_2O_2, a discoloration of cells of the cortical parenchyma (Figure 6A), of the cambium and rays (Figure 6B), and also of the first rows of tracheids was observed. The discoloration may indicate the production of H_2O_2. However, discoloration of the cambium and rays may also involve necrosis, as well as the dark color observed in the vascular cylinder, since these features were previously observed and presented similar color to what was observed with DAB staining (dark brown color) (Figure 6A). Cambial cells appeared flattened, with loss of turgor (Figure 6B).

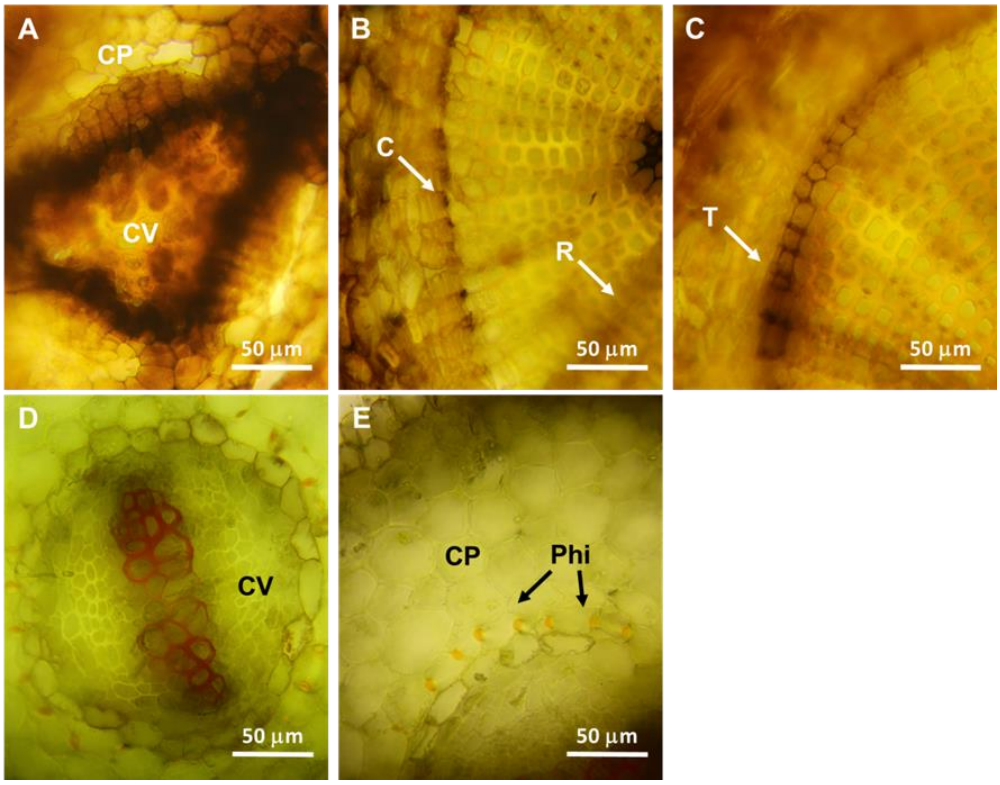

Figure 6. Micrographs of sections of root tissue of *A. chilensis* seedlings inoculated with *P. austrocedri* (A-C) and of non-inoculated (D-E) stained with DAB (400X). Vascular cylinder (CV), cortical parenchyma (CP), cambium (C), ray (R), tracheids (T).

4. Conclusions

This is the first anatomo-histological study done on *A. chilensis* roots. Some alterations occur in a similar way to what was observed at the stems of inoculated seedlings [6] and at the stems of adult trees naturally or artificially infected [18]. Results evidenced that *A. chilensis* triggers mechanisms to restrict and resist the infection, but *P. austrocedri* manages to evade them and finally colonizes and degrades host tissues.

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Conflicts of Interest: The authors declare no conflict of interest.

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