First Report of Phytophthora Cinnamomi Associated with Decline of the Cypress Plants (Cupressus Sempervirens) in Morocco’s Nurseries

Jihane Touati, Mohamed Chliyeh, Abdelaziz El Asri, Fatima Ait Aguil, Karima Selmaoui, Amina Ouazzani Touhami, Rachid Benkirane and Allal Douira

Laboratoire de Botanique et de Protection des Plantes, Université Ibn Tofail, Faculté des Sciences, B.P. 133, Kénitra, Maroc (Morocco).

ABSTRACT

A survey was done to the cypress plants nurseries in Sidi Yahya. Presence of leaf chlorosis, defoliation and decline in some cypress plants was remarked with the percentage of 1%. Phytophthora cinnamomi was isolated from the stem of cypress plants with the percentage of 44%. Cypress plants were inoculated with P. cinnamomi using two inoculation technics. The first inoculation technic was to dip the cypress roots in the inoculum solution and the second one was to insert a disk of Phytophthora agar culture in the vascular cambium. Chlorosis and root rot symptoms were observed after six weeks of inoculations using a technic1. Phytophthora cinnamomi reduced the root fresh weight of the cypress plant (24.1 g) relative to the control (35.5 g). By contrast, using technic 2: P. cinnamomi showed no effect on the root fresh weight of the inoculated plants (36.5 g) after six weeks of inoculation. Koch’s postulate was verified by reisolating P. cinnamomi from roots and stem of the inoculated cypress plants.

INTRODUCTION

The genus Cupressus (Cupressaceae) consists of twelve species spread across North America, the Mediterranean Basin, and sub-tropical Asia at high altitudes (Rawat et al., 2010). C. sempervirens is a conifer tree about 20-30 m height with a straight trunk. The bark is thin, smooth and gray for quite a long time, later it becomes gray-brown and longitudinally furrowed (Valgimigli, 2005). Shoots grow radiating in all directions; they are about 1 mm in diameter, round or quadrangular. Leaves are scale-like, decussate, small, ovate, and obtuse with a dark green color and a dorsal gland in the shape of longitudinal furrow (Valgimigli, 2005). Flowers appear early in spring. Cones are 2-3 cm long and pendulous, they have short stalks and look glossy, brown to gray; the shape is from globose to elliptic. Seeds are brown, flattened, and minute, without resin blisters and narrowly winged (Valgimigli, 2005). Three species reported as part of North African flora, for convenience called Cupressus sempervirens are often confused, being closely related and similar in external appearance (Greuter et al., 1984). Cypress is typically used for timber due to its high quality wood or as a windbreak (Pedron et al., 2008). These aggregate species include a Moroccan endemic species C. atlantica Gaussean, an Algerian endemic species C. dupreziana, A. camus, and C. sempervirens L., with the latter species in Tunisia comprising three varieties, pyramidalis, horizontalis, and numidica, differing in the direction of the branches (Neffati et al., 1999).

Phyto-preparation obtained from the core and young branches of C. sempervirens were reported to have antiseptic, aromatherapeutic, astringent, balsamic and anti-inflammatory activities (Taha et al., 2007). Cypress is also described to exert antispasmodic, astringent, antiseptic, deodorant, and diuretic effects, to promote venous circulation to the kidneys and bladder area, and finally to improve bladder tone and as a coadjuvant in therapy of urinary incontinence and enuresis (Keller, 1991; Tisserand et al., 1995). In Morocco, Cypress plays an important social role. The wood of the species is used by the local population for construction and for home uses, also as a fuel for cooking and heating, and, during the last decades, for the production of souvenirs for tourists (Sękiewicz et al., 2014). The young twigs with leaves are fed to sheep and goats. The permanent exploitation of Cypress wood and the overgrazing of the terrains are recognized causes for the decrease in the number of individuals and lack of successful regeneration (F.A.O., 1976; Griffith, 1998; El Wahidi, 2004; Farjon, 2005; Ouahmane et al., 2006).

Cypress is attacked by different fungal species: Seiridium cardinale, agent of the dangerous cypress canker (Pedron et al., 2008), Fusarium compactum causing mortality of Italian cypress (Cupressus sempervirens) and Phytophthora lateralis causing the root disease (Torgeson et al., 1954). Seed cones of C. sempervirens are intensively colonized by an aggressive and specific pathogen (the canker fungus Seiridium cardinale, Coelomycetes), associated with seed insect vectors Megastigma wachti (Hymenoptera Torymidae) and Orsillus maculatus (Heteroptera Lygaenidae) (Zocca et al., 2008). In contrast, we observed lower tree damage in the expansion
Several species of fungi have been associated with diseases of natural and cultivated varieties of *C. sempervirens* in Turkey. Among these fungi, *Seiridium cardinale* (Wag.), the causal agent of the canker disease which had caused heavy damage in forests, nurseries and ornamental plantations, especially in the Mediterranean countries, has taken the most attention (Graniti, 1986, 1998). On the other hand, many pathogens, such as *Diplodia pinea* f.sp. *cupressi*, *Phomopsis occulta* (Sacc.) Trav., *Pestalotiopsis funerea* (Desm.) Steyaert, *Fusarium* sp. Link, *Cytospora* sp. and *Lasidiodelphidia theobromae* (Pat.) Griffon & Maubl., (syn: *Botryodiplodia theobromae* (Pat.)) have also been found to be the causal agents of the cankers on cypress (Solel et al., 1987; Bruck et al., 1990; Madar et al., 1991, 1996; Linde et al., 1997; Ducey et al., 1999; Gonthier and Nicolotti, 2002; Bajo et al., 2008).

In Morocco, fungal diseases of *Cupressus sempervirens* are little studied. *Phytophthora* sp. have never been reported on this forest species, however the typical symptoms of these pathogens are often observed on the same plant nurseries. The objective of this study was to isolate the responsible pathogen of the decline symptoms on the cypress trees, to demonstrate its pathogenicity and to fulfill the Koch’s postulate.

**MATERIALS AND METHODS**

In 2013, a survey was done to the cypress plants nurseries in Sidi Yahia (north west of Morocco), presence of leaf chlorosis, defoliation and decline in some cypress plants was remarked (Fig. 1) with the percentage of 1 %. Isolation test was realized on one hundred root and stems segments of the cypress plants showing decline, segments were washed with water, disinfected with alcohol for five minutes, put on sterile distilled water and then dried with sterile filter paper. Then they were put on PSA agar plates (Potato Sucrose Agar: 200 g potato, 15 g sucrose, 20 g Agar-Agar, and 1,000 ml distilled water) and incubated on darkness at 28 °C. The developing colonies were then observed for the species determination. Isolation percentage (Pi %) was obtained by applying the following formula:

\[
\text{Pi} = \text{N}_{sX} / \text{N}_{T} \times 100
\]

*Pi*: Number of segments containing the fungal species *X*  
*N*: Total number of used segments.

**RESULTS AND DISCUSSION**

Pathogenicity test of *Phytophthora cinnamomi* was realized by inoculating cypress plants with the zoospores solution. Zoospores of *P. cinnamomi* were produced by growing cultures on oatmeal agar at 28 °C in the dark for 14-21 days. The mycelium was transferred to a sterile Petri dish, covered with sterile distilled water (SDW) and incubated overnight at 28 °C, under lights. The mycelia plates were chilled for 5 min at -20 °C to induce zoospores release. The concentration of the inoculum was adjusted at 10⁷ zoospores/ml by SDW.

**Inoculation Test**

The Koch’s postulate was verified by inoculating cypress plants using two inoculation technics. Technic 1. Four cypress plants were inoculated and two others were used as a control. Plants were inoculated according to the method described by Olbracht et al. (2006). The roots of investigated plants were washed under running water to discard soil remnants, trimmed to 2/3 of their length, and subsequently dipped in the prepared inoculum during 6 hours.

Technic 2. A small disk of bark extending down to the cambium is removed from the trunk and a disk of agar culture of *Phytophthora* is inserted in its place. The area is then bound with waterproof wrapping material (Klotz et al., 1930; Rossett et al., 1947; Broadbent et al., 1971).

**Measured parameters**

Root fresh weight and the leaves number showing chlorosis were two parameters used to estimate the severity of *Phytophthora cinnamomi*.

**Pathogen reisolation**

After the appearance of symptoms, twenty five roots and stems segments were taken from inoculated plants and controls, washed with water, disinfected with alcohol for five minutes, rinsed with SDW and then dried with sterile filter paper. After, they were plated on PSA culture and incubated on the obscurity at 24°C.

Analysis of the variance and of the mean comparisons using the LSD test (p = 5%) were performed using the software STATISTICA program.

**RESULTS AND DISCUSSION**

The isolated *Phytophthora* species was identified as *Phytophthora cinnamomi* basing on the morphological and cultural characters especially on sexual and asexual reproduction forms (Gallegly and Hong, 2008; Ristaino, 2012). This fungus was isolated from the stem of cypress plants with the percentage of 44% (Fig. 2). There was wide variation in sporangium shape and size both within and among isolate populations. In general, the shape of sporangia was predominantly globose (Fig 3A) to ovoid (Fig 3B). The mean lengths of sporangia ranged from 50 to 75 µm and the mean breadths ranged from 20 to 43 µm. *P. cinnamomi* produced all the sexual reproductive organs; oogonia (Figure 3C) and Antheridia (Figure 3D).

After six weeks, all the inoculated plants have shown a general leaf chlorosis from the bottom of the branches. Finally, some inoculated plants have been wilted, while, non-inoculated plants didn’t show any wilting symptom (Figure 4A and B). *Phytophthora cinnamomi* had a negative effect on the area, where a non-aggressive fungus (*Pestalotiopsis funerea*, Coelomycetes) was more frequently associated with the same insect vectors (Zocca et al., 2008).
inoculated cypress plants treated with technic 1. Thus, leaves of cypress plants showed chlorosis symptoms after the inoculation with *P. cinnamomi* (Figure 4A and B). Thus, the number of leaves showing chlorosis of the cypress plants treated with technic 1 had arrived to 35 leaves. In the other side, *P. cinnamomi* showed no chlorosis symptoms on the leaves of cypress plants treated with technic 2 (Figure 4B and Table 1). Also, *P. cinnamomi* reduced the root fresh weight of the cypress plants (24.1 g) relative to the control (35.5 g) (Table 1 and Fig. 4A, and B). Using a technic 2, *P. cinnamomi* showed no effect on the root fresh weight of the inoculated plants (36.5 g) relative to the control (36 g) (Table 1).

**Table 1** Root fresh weight and chlorotic leaves number of Cypress plants inoculated with *Phytophthora cinnamomi* using two inoculation technics

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<tr>
<th></th>
<th>Technic 1</th>
<th>Technic 2</th>
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<tr>
<td></td>
<td>Inoculated</td>
<td>Control</td>
</tr>
<tr>
<td>Roots fresh weight (g)</td>
<td>24.1</td>
<td>35.5</td>
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<tr>
<td>Number of leaves showing chlorosis</td>
<td>35°</td>
<td>0°</td>
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The results of the same line followed by different letters differ significantly at 5%.

*Phytophthora cinnamomi* was reisolated from roots and stems of the inoculated cypress plants (Figure 5A and 5B) with the percentage of 80.5 and 43 % respectively, and it was not isolated from the control group from both of roots and stems of cypress plants (Table 2).

**Table 2** Reisolation percentage of *Phytophthora cinnamomi* from roots and stems of inoculated and control cypress plants

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<thead>
<tr>
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<th>Reisolation percentage (%)</th>
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<tr>
<td></td>
<td>Roots</td>
</tr>
<tr>
<td>Inoculated cypress plants</td>
<td>80.5°</td>
</tr>
<tr>
<td>Control</td>
<td>0°</td>
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</tbody>
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The results of the same column followed by different letters differ significantly at 5%.

As the pathogen is able to quickly produce zoospores it may cause severe disease outbreaks even in Mediterranean areas with only short conducive periods (Cahill *et al.*, 2008). One example is in the Western Australian *Eucalyptus marginata* (jarrah) forest, where the spread of the disease increased from approximately 1.5 % of the forest area in 1940 (Dell *et al.*, 2005) to 6 % in 1972 (Podger, 1972), and more recently 14 % (Davison & Shearer, 1989). *P. cinnamomi* has the broadest host range of any *Phytophthora* species. It is especially associated with root diseases of *Eucalyptus*, oaks and chestnuts, pines, and members of the Ericaceae (the heath family,) as well as diverse agricultural crops. Symptoms range from fine root mortality leading to gradual tree decline, to enlarging basal cankers, often with bleeding spots, and tree mortality (Robin *et al.*, 2012).
In Morocco, *Cupressus sempervirens* nurseries are located within the Eucalyptus plantations which are only a reforestation plots that were planted after the cork oak. The growing substrate of the *C. sempervirens* plants is prepared from Eucalyptus litter. The results of this study have shown that 1% of the plants that grow in these nurseries are attacked by *P. cinnamomi*. In general, the plants of these nurseries are cultivated everywhere as alignment plants, windbreak and in public gardens. This circulation of diseased plants should be considered in the management and national programs of reforestation. In this sense, it is also very important to check the presence of *P. cinnamomi* in the cork oak seedlings and Eucalyptus that grow in nurseries of the same region. It is also preferable to investigate the cause of cork oak tree decline in the forest of Mamora. Indeed, this forest species and Eucalyptus trees are known in other countries as a host of *P. cinnamomi* (Dell et al., 2005; Eggers et al., 2012). *P. cinnamomi* is known as a pathogen capable of causing the decline of oak forests in the Iberian Peninsula (Brasier et al., 1993).

In Morocco, *P. cinnamomi* was reported as a responsible agent of avocado trees decline (Vanderweyen, 1977). This tree is cultivated in the region of Chefchaouen and almost all cultivated plots are surrounded by *C. sempervirens*. A prospection will be programmed to confirm or deny the presence of *P. cinnamomi* in the plots surrounded by Cupressus sempervirens and in the areas cultivated by the avocado trees.

**CONCLUSION**

The survey protocol as described above successfully detected *P. cinnamomi* in nurseries stock in Sidi Yahya in the north west of Morocco. The pathogenicity of *P. cinnamomi* on Cypress tree has been confirmed by artificial inoculation under controlled conditions. This fungus had a negative effect on leaves alteration and on the root system.

Because of its contribution to enormous number of plant species decline, the control of the attacked cypress trees should be affected in the nurseries to reduce the risk of the pathogen being dispersed more widely in the Moroccan forests. Plants must be controlled before planting in order to prevent the contamination of the other plant species.

**References**


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