FIRST REPORT OF PHYTOPHTHORA CINNAMOMI CAUSING INK DISEASE ON CASTANEA SATIVA IN GREECE

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SUMMARY

In October 2012 a heterothallic Phytophthora was isolated from soil and roots of European chestnut (Castanea sativa) trees from three orchards in Northern Greece. The pathogen was identified as Phytophthora cinnamomi on the basis of morphology and ITS sequence analysis. A pathogenicity test, following the soil infestation method, was performed and P. cinnamomi was re-isolated confirming Koch's postulates. This is the first report of P. cinnamomi as the cause of ink disease in Greece.

Key words: chestnut, ITS sequence analysis, pathogenicity, nursery stock, invasive plant pathogens

Castanea sativa Mill. is one of the most important forest trees in Greece. It is cultivated in coppice forests with relatively short rotation for the production of highly valued timber and in orchards for the production of nuts on an annual basis.

In October 2012, approximately 20% of eight to ten-year-old chestnut trees in three orchards in the vicinity of Orma (village in the Prefecture of Pella, Central Macedonia, Northern Greece) showed disease symptoms including decline of the crown, dead leaves and burrs which remained attached to the trees, and dark necrosis of the inner bark below the collar with flame-shaped margins. In some cases wilting was followed by a progressive or quick death of the diseased trees.

For pathogen isolation root and soil samples containing the rhizosphere and fine roots of declining C. sativa plants were collected (Table 1). Roots from diseased plants were rinsed in running water overnight, then tissue fragments were excised and plated directly onto PARBHy selective medium (10 mg pimaricin, 250 mg ampicillin, 10 mg rifampicin, 15 mg benomyl, 50 mg/l hymexazol, 20 g agar, 200 ml V8 juice and 800 ml H2O). Each soil sample, containing the fine chestnut roots, consisted of four monoliths of soil (20x30x30 cm) which were collected at the four compass points around the tree at a distance of 50-100 cm from the stem base (Jung et al., 1996). After collection, soil samples were moistened with sterile distilled water and kept at 20°C. About 200 ml of soil were flooded with 500 ml of distilled water in plastic containers (Jung et al., 1996) and subjected to rhododendron leaf baiting (Themann and Werres, 1998). Newly well developed leaves of Rhododendron catawbiense cv. Cunningham’s White were floated over the flooded soil and, after 2 to 8 days, tissue pieces around the developed necrotic spots were plated onto PARBHy medium. Petri dishes with PARBHy medium were then incubated at 20°C in the dark and examined daily under the microscope for developing Phytophthora colonies which were then sub-cultured on V8-juice agar (V8A). Hyphal cultures of the isolates grown for 10 days on V8A in 90 mm Petri dishes at 20°C in darkness were used for species identification. Phytophthora isolates were maintained on V8A at 20°C in darkness and sub-cultured at 4 weeks intervals.

The colonies on V8A were characterized by a coral-loid mycelium and clusters of thin-walled chlamydospores. Sporangia were produced by placing a mycelial disk from a 7-day-old culture grown on V8A in non-sterile soil-extract water. Sporangia usually formed after 3 to 4 days of incubation at 20°C. Soil extracts were prepared according to Chee and Newhook (1965) by mixing 10 g of soil with 100 ml of distilled water, then allowing it to rest for one day to let soil particles settle before filtering the supernatant through a Whatman No. 1 filter paper. Morphology was observed by light microscopy, and the length and width of 100 sporangia were measured for each isolate. Sporangia were ovoid to ellipsoid, persistent, non-papillate, 42 to 75 μm long and 25 to 38 μm wide.

Since the isolates did not produce oogonia in single culture two different mating tests were performed to determine whether the isolates were heterothallic and to which mating type they belonged. In the first test, a V8A plug of each isolate was placed directly on a microscope slide in contact with a V8A plug of an A1 or A2 tester strain from a heterothallic Phytophthora species (Vetraino et al., 2001, 2005). As A1 tester strain a Phytophthora cambivora isolate (21/95-KII) from Chamaecyparis lawsoniana from Italy was used, while four P. cambivora isolates from C. sativa from Greece (SK2, SK3, M3, PYRI; Vetraino et al., 2005) acted as A2 mating tester strains. Slides were incubated...
PCR products were purified with PureLink™ PCR purification kit at 95°C for 30 sec with final extension at 72°C for 10 min. Initial denaturation step was carried out at 95°C for 2 min, followed by 30 cycles of annealing at 55°C for 1 min, and final extension at 72°C for 2 min. 

To study the sexual behavior, mycelial DNA of six isolates was extracted using the DNeasy plant mini kit (Qiagen, USA) according to the manufacturer's instructions. Template DNA (8 μl), 0.5 μl each of reverse primer ITS4 and forward primer ITS6a, 5 μl of a commercial red reaction buffer, containing MgCl₂ and dNTPs (Bioline, USA) one unit of Taq polymerase and bi-distilled H₂O were added to each reaction tube to make a final volume of 25 μl. PCR was performed with a thermocycler (Mastercycler, Eppendorf, USA) with the following amplification conditions (Cooke and Duncan, 1997): initial denaturation step at 95°C for 2 min, 30 cycles of annealing at 55°C for 30 sec, extension at 72°C for 1 min and denaturation at 95°C for 30 sec with final extension at 72°C for 10 min. PCR products were purified with PureLink™ PCR purification kit (Invitrogen, USA) and custom sequenced (ChromasLite, Technelysium, Australia), visually aligned and deposited in GenBank under the accession Nos. KF559324 to KF559329. A BLAST comparison with sequenc- ers retrieved from GenBank revealed 100% homology with those of various P. cinnamomi isolates (e.g. with sequence GU799635 of the P. cinnamomi strain CMW33386 isolated from Quercus cerris in South Africa) thus confirming the morphological identification.

Pathogenicity of these six P. cinnamomi isolates was tested by soil infestation tests (Jung et al., 1996) in a growth chamber at 22°C using 15 two-year-old potted seedlings per isolate, and 15 non-inoculated plants as controls. P. cinnamomi inoculum was prepared by growing the above six cultures for 4 weeks at 20°C on sterilized millet seeds moistened with V8 broth (200 ml V8 juice, 3 g CaCO₃ and 800 ml H₂O₂). Each seedling was inoculated with 30 ml/l of the inoculum, while in control plants sterilized non-inoculated millet seeds were added to the potting mixture. All plants were flooded for 24 h at two weeks intervals. Five weeks post inoculation, all inoculated seedlings showed wilting, collar and root rot symptoms, while control plants remained symptomless. P. cinnamomi was re-isolated from artificially inoculated plants, fulfilling Koch’s postulates.

P. cinnamomi has been reported as the causal agent of ink disease of C. sativa from many countries of the world (Farr and Rossman, 2013). In Greece, ink disease of chestnut trees has previously been associated with P. cambivora (Chitzanidis and Kouyecas, 1970) and P. cryptogea (Perlerou et al., 2010). To our knowledge, this is the first report of P. cinnamomi causing ink disease on chestnut in Greece.

P. cinnamomi and P. cambivora are considered as the two species most commonly associated with ink disease in Europe and most aggressive to chestnut (Vannini and Vettraino, 2001; Vettraino et al., 2005). In Greece, P. cambivora is the prevailing species in chestnut orchards and natural stands (Vannini and Vettraino, 2001). Nonetheless, as P. cinnamomi is more aggressive than P. cambivora to European chestnut (Vettraino et al., 2001), it constitutes a potential major threat to C. sativa in Greece. In fact, in the present case, circumstantial evidence of aggressiveness was provided by the severity of the disease, as the trees were killed within one growing season, without showing typical symptoms like small-sized foliage, thin crown and attached burrs from the previous season. Furthermore, as P. cinnamomi has a wide range of hosts, it could be a potential threat to horticultural, ornamental and forestry plant species with economic and aesthetic importance.

In a previous Europe-wide study P. cinnamomi was not detected in declining chestnut stands in Greece (Vettraino et al., 2005). Most likely, P. cinnamomi was introduced more recently in the three orchards object of this study, subsequently in the surrounding area, through transport of infected nursery stock, confirming the importance of the nursery pathway in the spread of aggressive invasive plant pathogens (Jung and Blaschke, 2004; Jung et al., 2009).

### Table 1.

<table>
<thead>
<tr>
<th>Orchard</th>
<th>Number of samples</th>
<th>Number of positive isolations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soil</td>
<td>Roots</td>
</tr>
<tr>
<td>Orchard 1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Orchard 2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Orchard 3</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Total number</td>
<td>14</td>
<td>9</td>
</tr>
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</table>
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