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First identification of the pathogen causing tumor malformations in holm oak in Spain

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Abstract

Aim of study: In recent years an increase in pests and diseases associated with truffle plantations has been detected in Spain. The appearance of tumor malformations in trunks and branches of *Quercus ilex* L. must be highlighted. These bumps have expanded dramatically since the increase in the number and density of truffle plantations. This pathology is not only found in plantations, but also in forests, and in trees of all ages.

Area of study: the eastern mountains and the truffle plantations of the Iberian Peninsula.

Material and methods: Positive results were obtained by using two types of PCR: Real-Time PCR and nested-PCR. They were carried out with primers that amplified 16S ribosomal gene sequences that are common to all known phytoplasmas.

Main result: The disease manifests itself as an irregular thickening in branches of any age and in the trunk that results in the woody tissue cracking open, forming wounds. The affected branches usually undergo necrosis and in case of affecting the trunk, the tree will eventually die. After an extensive literature review and several failed attempts to isolate fungal and bacterial species from these tumors and wounds, the disease-causing organism has been identified as a *Candidatus* Phytoplasma.

Research highlights: The appearance of this disease may endanger the profitability of an *a priori* profitable crop. Due to the intrinsic characteristics of the organism, and knowing that no phytosanitary treatment is able to control phytoplasmas, future works should be directed towards identifying the transmitter in order to control the disease.

Key words: *Candidatus* Phytoplasma; PCR; *Quercus ilex*; black truffle; *Tuber melanosporum*.

Introduction

The black truffle *Tuber melanosporum* Vittad. domestication and cultivation has meant in Spain an ecological profitable alternative in a low-fertility land that is suffering the effects of depopulation. Truffle culture is offering in these areas a sustainable agronomic option, not only because of its direct benefits, obtained from the sale of fruiting bodies, but also through indirect benefits (Samils *et al.*, 2008).

Truffle plantations high profitability in the region of Aragón (North East Spain), as well as the support of the Public Authorities, are leading Spain to become a worldwide reference in black truffle cultivation.

Black truffle establishes symbiotic relationships with different species of spermatophytes, mainly of the genus *Quercus*. However, the holm oak (*Quercus ilex* L.) is the most used host tree in Spanish truffle plantations. This is due to its ecological plasticity, its small size, its ease of handling, and its high yields in truffle production.

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Abbreviations used: PCR (polymerase chain reaction); bp (base pairs).

As the trend to monoculture grows and plantation densities increase, the quantity of pests associated with truffle plantations keeps rising. In fact, insects that lived in balance in the natural environment have evolved to pests in recent years. This is the case, for example, of *Kermes ilicis* L. and *Kermes vermilio* Planch. in plantations, or *Leiodes cinnamomea* Panzer in fruiting bodies (Barriuso *et al*., 2012).

But in recent years a pathology that worries growers, hunters, and researchers must be highlighted. It consists of tumor malformations that appear in branches and trunks of holm oaks. There is evidence of their presence in natural areas, but their occurrence is very rare. As densification of truffle plantation is increasing, its incidence is growing in both forestry areas and plantations.

The symptoms of this disease are a general plant decay, whose leaves become chlorotic, and a significant leaf loss in the most advanced cases. The emergence of sprouts in the basal part of the trunk is usual. However, the most obvious symptom is the appearance of tumor malformations in branches and trunk, which prevent sap flow. Eventually very affected trees may reach a peak in truffle production which is followed by a generalized weakness and even death in the most severe cases. In addition, they are usually affected by secondary pests.

This symptomatology has been detected in both young seddlings of two or three years of age, and old plants already established. Tumor malformations are observed on branches and young stems.

In preliminary studies which tried to identify that a causative agent of this disease, the possibility that a fungus, bacteria, or virus was the inducer organism which ruled out (unpublished data). The absence of necrosis in the vessels, as well as the characteristics of the symptoms, suggested the possibility of a phytoplasma being involved in the etiology of this disease.

Phytoplasmas are unculturable cell wall-less prokaryotic organisms grouped in the class Mollicutes. They inhabit phloem sieve elements (specialized cells for the translocation of nutrients in plants) in infected plants. Usually, plants infected by the phytoplasmas exhibit symptoms like virescence, phyllody, yellowing, leaf roll, flower sterility, axiliary buds proliferation, resulting in appearance of the "witches broom" as well as the shoot stunting and generalized decay of diseased plants (Bertaccini and Duduk, 2009).

For prokaryotes of the class Mollicutes, which include phytoplasmas, vector insects are needed for transmission, spread and multiplication (Arismendi *et al.,* 2010*)*. In addition, these insects must feed on plant phloem. Phytoplasmal diseases are spread primarily by sap-sucking insect vectors (order Hemiptera; suborder Homoptera) belonging to the families Cicadellidae, Fulgoridae (Auchenorryncha) and Psyllidae (Sternorryncha) (Nielson, 1979; Batlle *et al.,* 2008; Carraro *et al*., 1998; Tedeschi *et al*., 2003). Other insects (even Heteroptera) are suspected to be possible transmitters of phytoplasma diseases (Mathen *et al.* 1990; Hiruki, 1999).

The identification of a phytoplasma as the causal agent, serves as the ground work for future and necessary studies on this disease epidemiology including prevalence, severity and incidence (Agrios, 2011). To achieve this goal, an effective strategy for combating the disease throught the vector should be proposed.

Material and methods

Study area

There is evidence of the presence of this symptomatology in several Spanish regions, mainly in Aragón, Catalonia, Comunidad Valenciana, Castilla la Mancha and Castilla y León. It is in the foothills of the Sistema Ibérico mountain range (provinces of Castellón and Teruel) where there is evidence of a higher incidence of this disease. For this reason, samples were obtained in different areas from the province of Teruel, in the region of Gúdar-Javalambre.

Teruel is the southernmost of the three provinces of the Autonomous Community of Aragón. The region of Gúdar-Javalambre is in the south-east of the province. It meets the optimum conditions for the development of the black truffle, as it features a xeric Mediterranean climate with a continental trend, characterized by low seasonal rainfall (about 450-550 mm per year) and marked contrasts in temperature. Currently, over 6.000 ha are used for this agroforestry crop, and the actual trend is a positive growth for the next years (Samils *et al.,* 2003).

Plant material

Different samples to be used as contaminated plant material were collected from symptomatic *Q. ilex* trees from May 2011 until May 2013. Samples were taken

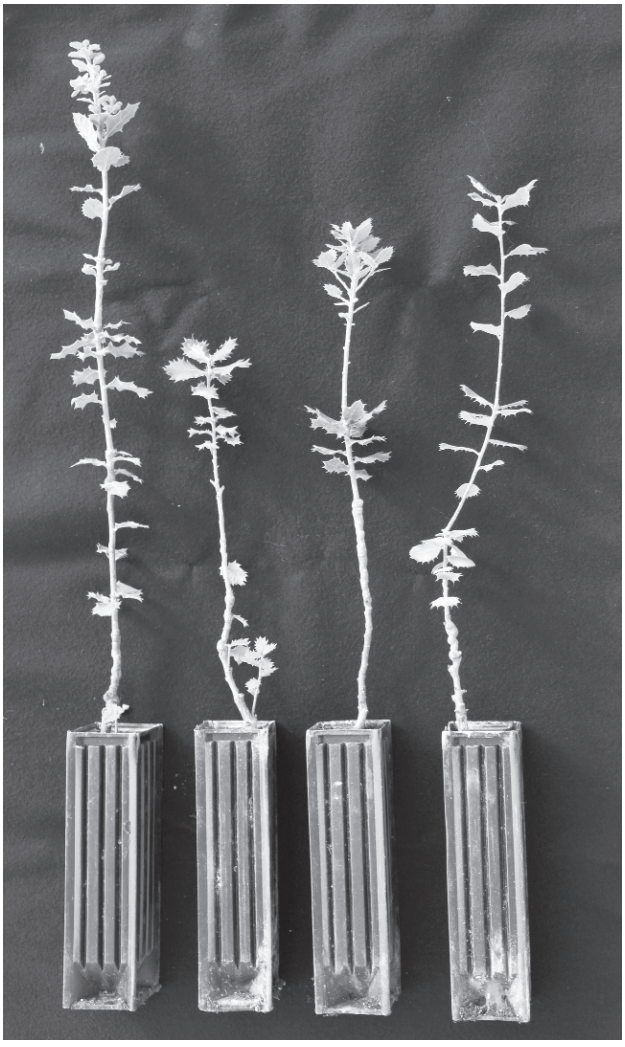

Figure 1. Five-year-old plants grown in nursery. It is possible to observe tumors developing in their stems and an abnormal development of internodes and branches as well.

from nursery plants and from trees already established in plantations.

Young plants were kept for five years in 450cm^3 forestry containers. They showed obvious symptoms of the presence of the disease (Fig. 1) from the second year.

Samples from adult plants (Fig. 2) were taken from two 15-year-old orchards, located in the Gúdar-Javalambre district (Teruel). These trees, which were already producing black truffles, had tumor malformations in branches and trunk, and showed a general decay.

The plant material was cut with pruning shears properly disinfected with alcohol to avoid possible contaminations between samples. Once obtained, they were stored at 4°C until processed.

Figure 2. Tumor malformation in an adult tree branch from a truffle plantation. Absence of necrosis in vessels is also observed.

Two-year-old *Quercus ilex trees* were used as negative control samples. The seeds came from certified provenances and they were grown in nursery to avoid any possible contamination. The substrate used had a composition of 80% solarized soil coming from an agricultural plot, 18% Humin Substrat® and 2% perlite. The substrate was enriched with slow release fertilizer (10-1-6) at a ratio of 3 kg/m^3.

Positive controls were obtained from apple-trees and pear-trees infected with *Candidatus* Phytoplasma mali, the causative agent of apple proliferation, and *Candidatus* Phytoplasma pyri, which is responsible for pear decline.

Nested PCR and real-time PCR

For PCR, total DNA was isolated from 1g of fresh plant material (leaf midribs, buds and stems) following the phytoplasma-enrichment procedure of Ahrens and Seemüller (1992) and stored at –20°C until processing. Samples from both affected and symptomless plants were analysed for phytoplasma infection by two PCR methods: Nested PCR and real-time PCR.

Nested PCR was used for specific detection of the phytoplasma following the method of Garcia-Chapa *et al.* (2003). The universal first pair for phytoplasma detection, P1/P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996), located at the 16S rDNA and 23S rDNA gene respectively, was used in the first step to amplify a fragment of about 1800 base pairs (bp) in length. The

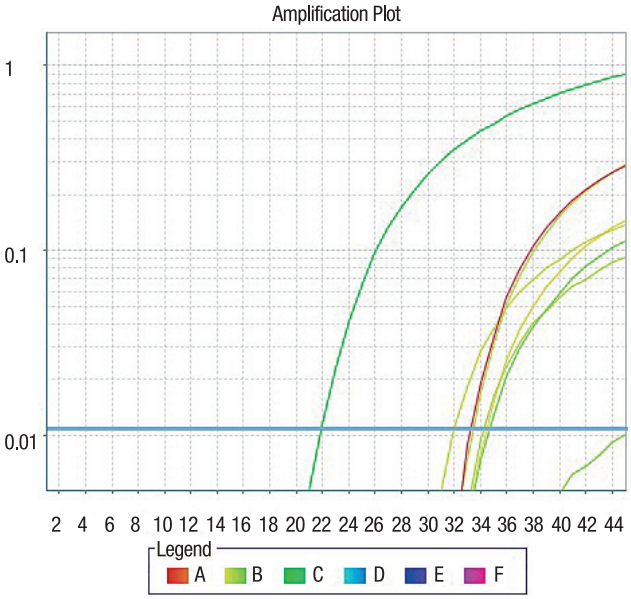

Figure 3. Results for Real Time PCR. A strong positive is shown (positive control), whose signal is produced after 22 cycles. Five weak positives are also shown, which signal is produced after 32 cycles. The five weak positive are from leaf midribs, buds and stems of different trees. A: buds; B: leaf midribs, buds and stems; C: positive control.

second step was performed with the universal primers R16F2/R16R2 (Gundersen and Lee, 1996). The first amplification was carried out in a total volume of 20 µL containing 5-10 ng of DNA and the following mixture: 0.250 µM of each primer, 250 µM dNTPs, 1 unit 100 μL^{-1} Taq DNA polymerase (Promega) and 1x Taq buffer. Two microlitres of a 1:50 dilution of the first amplification product were used for the second step. The second amplification mixture contained the same components, but a different specific primer concentration (0.375 µM each). Amplification products were electrophoresed in a 1.5% D-1 agarose gel (Pronadisa, Madrid, Spain) according to standard procedures. DNA was stained with ethidium bromide and exposed to ultraviolet light.

Real-time PCR amplifications were carried out with the method of Christensen *et al.* (2004), using TaqMan probe.

Results

Positive results have been obtained using both types of PCR, Nested-PCR and Real-Time PCR performed with primers that amplify 16S ribosomal gene sequences common to all known phytoplasmas. PCR products were not detected in the negative controls.

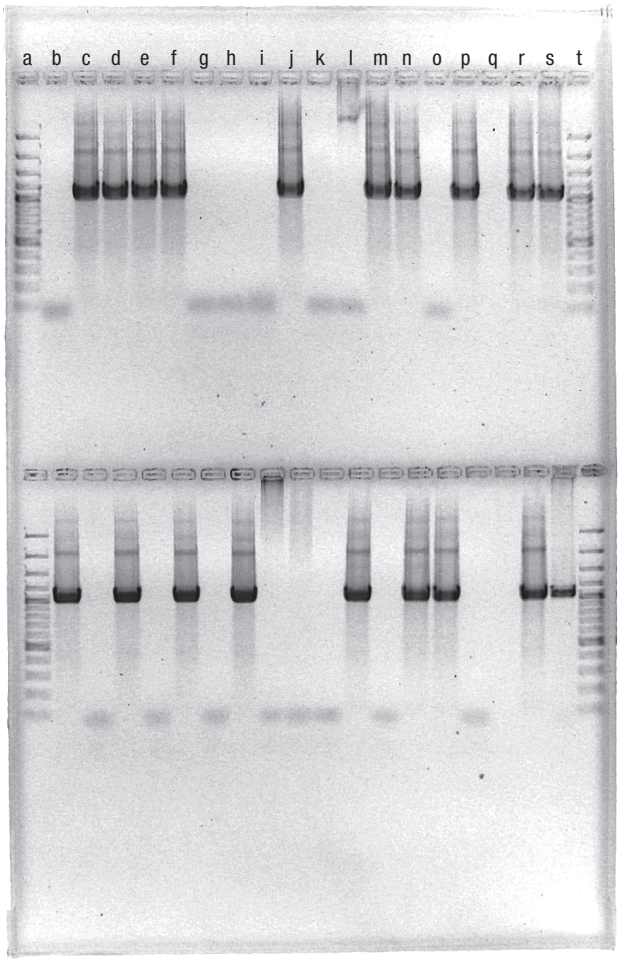

Figure 4. Agarose gel electrophoresis (1.5%) of nested PCR products obtained from DNA extracts of symptomatic *Quercus ilex* trees. Nested PCR with universal primer pair R16F2/ R16R2 after PCR by universal primer pair P1/P7. All the positives (1200 bp) are from leaf midribs, petioles, buds, shoots and phloem of different infected trees. Samples: Leader (a, t); Petiole (b, f, g, h); apical buds (c) phloem (d, e, i, j, k); midribs (l); shoots (m, n, o, p); positive control (r, s); negative control (q)

Real-time usually showed weak positive results (Fig. 3). Of the 18 samples analyzed, 10 were obtained from nursery plants and eight from plantations. All of them showed positive results. The positive control sample gave signal at 22 cycles, while samples obtained from holm oaks started to give signal at 32 cycles. The dissociation curve analysis shows just one peak indicating the presence of a single amplification product. However, the repetition of positives (although weak) in samples obtained from symptomatic trees and

the negative results in all the negative controls was indicative of the relationship of a phytoplasma in the development of tumors.

To confirm this hypothesis Nested-PCR was used. The expected band of 1.200 bp was found, with no PCR products in negative controls (Fig. 4). A total of 26 samples were analyzed, of which 18 were obtained from nursery plants and eight from plantations. All of them showed positive results. The positive control showed a band with the same molecular weight. Results in this case were also positive, confirming the relationship between a phytoplasma and malformations.

Cloned fragments from a sample taken in autumn 2012, which had a very high signal of R16SF2n/R2 amplicons were sequenced (GenBank accession number: KJ415258). Comparison by BLAST analysis with sequences of other phytoplasmas in the GenBank database showed a high similarity to phytoplasmas belonging to 16SrX group.

Discussion

The establishment of a truffle plantation involves a significant economic investment (Quintana, 2007; Reyna *et al.,* 2002). The initial economic outlay represents an important amount for the truffle grower. The appearance of a disease able to weaken or even kill the trees, as in the case of these tumor malformations, may endanger the profitability of an *a priori* profitable crop.

Furthermore, the main problem of the occurrence of a disease caused by a phytoplasma is the lack of curative measures when it is established in a plantation. Due to the intrinsic characteristics of the organism, no phytosanitary treatment is able to control phytoplasmas, as in the case of viruses. In this sense, once the phytoplasma has been identified as the causative agent of the disease, and knowing that it belongs to the 16SrX group, future works should be directed towards identifying the transmitter in order to control the disease. That is why, nowadays, several studies on transmission mechanisms are being carried out in our facilities (acorns, root anastomosis, grafting, mechanical transmission).

Phytoplasmas belonging to 16SrX group are usually transmitted by Psyllidae. *Cacopsylla pyri* that transmits the Phytoplasma that causes pear decline in Spain (*Candidatus* Phytoplasma pyri) (Garcia-Chapa *et al*., 2005). *Cacopsylla melanoneura* and *Cacopsylla picta* spread the phytoplasma responsible for apple proliferation disease (*Candidatus* Phytoplasma mali) (Laviña *et al*., 2011). And finally, *Cacopsylla pruni* transmits the phytoplasma that causes European stone fruit yellows (*Candidatus* Phytoplasma prunorum) (Laviña *et al.,* 2004).

Psylidae that transmit *Candidatus* Phytoplasma mali and *Candidatus* Phytoplasma prunorum normally hibernate in forests far from the orchards, where they feed on forest trees. However, as soon as the hibernation period is over, they return to their hosts in plantations.

Phytoplasmas belonging to the 16Sr-X group were also detected in *Corylus avellana*, *Fraxinus excelsior*, *Rosa canina*, *Celtis australis*, *Crataegus monogyna*, *Quercus robur, Quercus rubra*, *Carpinus betulus* and *Convolvulus arvensis* (Schneider *et al*., 1997; Seemüller and Schneider, 2004).

Different species of *Quercus* are known to be hosts of phytoplasmas and to develop the disease after infection (Seemüller and Schneider, 2004; Marcone *et al.,* 1999; Berges *et al.,* 2000). Nevertheless, the described symptomatology does not match the one observed in *Q. ilex.* Other similar tumor malformations have been observed in holm oaks, but they were related to insects (mainly of the family Cynipidae) or bacteria (*Agrobacterium tumefaciens*).

Further research is needed on the distribution and incidence of this disease, paying special attention to forest areas. Forests rangers working on the most affected areas (foothills of the Sistema Ibérico mountain range) are alarmed by the increasing incidence of tumors in forests. The high density of holm oak plantations may have augmented vector insect populations, increasing likewise the incidence of tumors in forestry areas.

Most truffle growers believe that the scale insects *K. vermilio* and *K. ilicis* are related to the emergence of this symptomatology. However, due to their low mobility and the short life of males, *Kermes* can´t be considered initially a good candidate to be the vector that spreads the disease, although its is not possible to reject the relationship between these scale insects and the phytoplasmas. Nowadays, several transmission studies are being carried out in our facilities (acorns, root anastomosis, grafting, mechanical transmission.)

Currently little or nothing is known about the etiology of this disease. Based on the experience gained in other studies (Lee *et al.,* 2000), the establishment

of preventive measures to try to avoid the spread of the disease is necessary. The first of these actions could be the use of resistant varieties. However currently this is not a feasible solution due to the difficulty of identifying the resistance genes and to the absence of available plant varieties to work with. The second solution is through the control of vector insects. But it requires knowing the vector transmitting the disease, which is still unknown. The third solution is the eradication of infected plants, with the aim of reducing the amount of inoculum, but this would only be useful if the amount of disease is limited by the available inoculum. Finally the use of healthy seedlings in plantations is necessary, besides being a legal requirement.

Once the causal agent of the disease has been described, researchers, truffle hunters and managers must be alert in the face of the possible occurrence of new outbreaks of this syndrome, not only in agricultural areas, but also in forests. Studies must be continued to learn about the etiology and spread of this disease to avoid that this epiphyte may endanger Mediterranean oaks.

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