INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

Detection and identification of *Phytophthora* sp. associated with ink disease of chestnut (*Castanea sativa*) from natural soils

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ABSTRACT

One of the biggest threats for the chestnut tree in Portugal and in Europe is the ink disease. The disease is caused by two *Phytophthora* species (*Phytophthora cinnamomi* and *Phytophthora cambivora).* These root parasites have biological and epidemiological characteristics that provide them with rapid adaptation to the environmental conditions and have a high capacity for survival. With this work we intend to develop molecular methods for the detection and identification of *Phytophthora* in chestnut soils, providing greater sensitivity of detection and greater specificity of identification. In this study, two methods were compared for the detection of *Phytophthora* from soils infected with chestnut ink disease (natural soil from a stand with diseased chestnut trees and potting soil from greenhouses). In the classical method (baiting method), for the detection of *Phytophthora*, leaf discs of chestnut (C*astanea sativa*) were used and subsequent identification of isolates. For molecular methods, soil DNA was extracted from the DNeasy® PowerSoil® Pro Kit (Qiagen) and was amplified from the ITS region of ribosomal DNA (rDNA) using the universal primers ITS4 and ITS6. It was possible to obtain 67% of positive detection from natural soils by baiting method and 75% of positive detection from soil of pots from greenhouse. *P. cinnamomi* was the species identified from the baiting method and the nucleotide identity of the ITS region of the three identified isolates ranged between 98 and 100%. Trap tissue assays are very useful when the aim is to obtain isolates of different *Phytophthora* species and DNA-PCR assays are valuable and basic requirements for integrated management programs to evaluate the control measures applied to minimize impacts and control the disease.

Keywords: ink disease, chestnut tree, *Phytophthora cinnamomi*, molecular biology.

RESUMO

Uma das maiores ameaças para o castanheiro em Portugal e na Europa é a doença da tinta. A doença é causada por duas espécies de *Phytophthora* (*Phytophthora cinnamomi* e *Phytophthora cambivora*). Estes parasitas radiculares têm características biológicas e epidemiológicas que lhes proporcionam uma rápida adaptação às condições ambientais e têm uma elevada capacidade de sobrevivência. Com este trabalho pretendemos desenvolver métodos moleculares para a deteção e identificação de *Phytophthora* em solos naturais, proporcionando uma maior sensibilidade de deteção e uma maior especificidade de identificação. Neste estudo, foram comparados dois métodos para a deteção de *Phytophthora* em solos infetados com a doença da tinta do castanheiro (solo natural de um povoamento com castanheiros doentes e solo de vasos de estufas). No método clássico (tecido de armadilha), para a deteção de *Phytophthora*, foram utilizados discos de folhas de castanheiro (*Castanea sativa*) e subsequente identificação dos isolados. Para métodos moleculares, o ADN do solo foi extraído do DNeasy® PowerSoil® Pro Kit (Qiagen) e foi amplificado da região ITS do ADN ribossómico (rDNA) utilizando os primers universais ITS4 e ITS6. Foi possível obter 67% de deteção positiva de solos naturais através do método de tecido armadilha e 75% de deteção positiva de solo de vasos de estufa. *P. cinnamomi* foi a espécie identificada a partir do método de tecido armadilha e a identidade nucleotídica da região ITS dos três isolados identificados variou entre 98 e 100%. Os ensaios de tecido de armadilha são muito úteis quando o objetivo é obter isolados de diferentes espécies de *Phytophthora* e os ensaios de DNA-PCR são requisitos valiosos e básicos para programas de gestão integrada para avaliar as medidas de controlo aplicadas para minimizar os impactos e controlar a doença

Palavras-chave: doença da tinta, castanheiro, *Phytophthora cinnamomi*, biologia molecular.

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1. INTRODUTION

1.1. Species associated with ink disease

Phytophthora cinnamomi and *Phytophthora cambivora*, both members of the *Phytophthora* genus, cause ink disease. The word "*Phytophthora*" comes from Greek and meaning "plant destroyer" (*phyto*-plants, *phthora*-destructor). The *Phytophthor*a genus is found in the kingdom Chromista, the class Oomycetes, the order Peronosporales, and the *Pythiaceae* family. Even though biological and physiological uniqueness has always been attributed to these physiological singularities to this genus in relation to the kingdom where it belonged, these species were for a long time included in the kingdom Fungi (*Myceteae*) because they have characteristics that are very similar to fungi (Erwin and Ribeiro, 1996; Gouveia, 2004). According to Zentmyer (1987), the genus *Phytophthora* has traits that are not found in other fungi:

- Zoospores have two morphologically different flagella;
- During the vegetative phase they are diploid organisms unlike fungi that are haploid;
- Accumulate as reserve substance mycolaminarins (β 1-3-glucans and mannitol) while fungi accumulate mannitol as their main reserve substance;
- They do not synthesize sterols and for this reason are not sensitive to fungicides that interfere with their biosynthesis.

Only the presence of oogonia ornamented with protuberances in *P. cambivora* and the presence of coralloid mycelium on agar media or large, spherical dilations in water in *P. cinnamomi* allow the two species to be distinguished, according to the Stamps *et al*. (1990) key that evaluates characters related to the sporangia, antheridia, oogonia, are created by the coupling of mycelium from the same or different species with opposite sexual compatibility. They have no taxonomic relevance in this circumstance. Although *P. cinnamomi* and *P. cambivora*, species responsible for ink disease caused very similar symptoms in chestnut, Fernandes (1966) considered the first of those species as the preponderant pathogen in the development of ink disease in Portugal.

The mycelium of *P. cinnamomi* consists of tubular hyphae (with an average diameter of 8 µm), (multinucleate aseptates that result from repeated division of the nucleus without

division of the division of the cytoplasm) and of hyaline color (crystalline almost transparent). The hyphae have numerous spherical swellings (a distinguishing characteristic). The growth of the mycelium occurs at the at the end of each hypha and the lateral branches usually present a slight constriction at the base (Erwin and Ribeiro, 1999) (Figure 1).

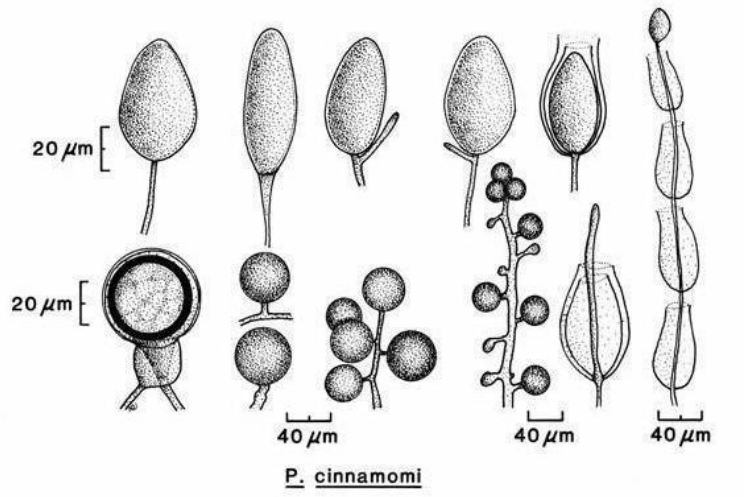

Figure 1 Morphology of *Phytophthora* Cinnamomi

Phytophthora species can have either asexual or sexual reproduction, although asexual reproduction is more common. In Robin (2013) it is stated that in Europe there are no reports of sexual reproduction of *P. cinnamomi*. The sporangia produced by *P. cinnamomi* species are ovoid or ellipsoid, non-papillate with an average size of 75x40 µm (length x width). The zoospores released from the sporangia are reniform (kidney-shaped) with two morphologically distinct flagella, one longer than the other (heterokont), which emerge from the concave part of the zoospore and confer mobility. In culture and in infected tissues, a *P. cinnamomi* species produces a lot of chlamydospores. The chlamydospores are globose, with an average diameter of 41 µm, and the cell wall is thicker than the sporangia's, though it is thinner than that of most *Phytophthora* species. These structures can be seen at the terminals of hyphae or

intercalated between the mycelium and the septum (Erwin & Ribeiro, 1996; Gouveia, 2004).

1.2. Ink disease in Chestnut in Portugal

Over the last centuries there has been a regression of the chestnut tree (*Castanea* spp.) occupation areas in North America and Europe. Associated with this regression is the chestnut ink disease, being considered as one of the main causes of the disappearance of the chestnut tree. In America the disease appeared in 1904 in the East American leading to the death of thousands of chestnut trees. In Europe the beginning of this regression is much earlier than in America, however, since it's the disease expanded very quickly destroying millions of chestnut trees (Cortizo *et al*., 1999).

The chestnut culture is found across Portugal, but it is particularly prominent in Trasos-Montes, Beira-Alta, Alcobaça, Fundão, Monchique, and Portalegre all have some welldefined sites (Praxis, 2000). According to data from the National Institute of Statistics (INE, 2012), there were 34 656 hectares of chestnut tree area in 2012, with 30 586 ha (88%) in the North region (mostly Trás-os-Montes), 3 529 ha in the Center region (10%), 520 ha in Alentejo (1.5%), and the rest distributed throughout the Algarve, Lisbon, and the Islands.

Even though the planted area of chestnut trees (*Castanea sativa* Mill.) in Portugal has increased in recent years, the output of chestnut fruit has not (FAOSTAT, 2020). A variety of phytosanitary issues, the most common of which is ink disease, have been harming the species, causing mature trees to die and producers to regularly replace them with less productive young trees (Martins *et al*., 2007; Arrobas *et al*, 2018; Rodrigues *et al.,* 2019).

This is not, however, a local issue, as statistics from all around the world, including European countries, reveal the same tendency (FAOSTAT, 2020).

The first signs of ink disease were noticed in Portugal about 1838 on the banks of the Lima River, with premature yellowing and leaf fall, as well as the formation of a moist rot in the roots, which eventually led to the tree's death (Fernandes, 1953). Later resulted in the tree's demise (Fernandes, 1953). Infected regions of the plant produce violet or dark blue exudates, which are commonly referred to as ink disease. When

symptoms occur in the tree's crown, it may indicate that the infection has progressed to the point where it is tough to eliminate (Serrazina, 2004; Gomes-Laranjo *et al*., 2009).

According to Gouveia, citing other authors, the effect of ink disease is considered by many authors as the main cause of decline and degradation of the souths, the ink disease has reached epidemic proportions in Portugal today (Gouveia, 1993; Abreu *et al.* 1999).

In the Trás-os-Montes region (northeastern Portugal), the pathogen *P. cinnamomi* is responsible for the ink disease affecting *C. sativa* (Gouveia, 2004)

The chestnut occupation area in Portugal has been suffering a sharp decrease since the onset of this disease. The advance of the disease has been so devastating, that today there are almost no chestnut trees in Minho and the areas of occupation Trás-os-Montes and Beira Alta, regions where the largest chestnut groves still exist, have the largest chestnut stands still exist. (Abreu, 1995).

The ink disease, which kills chestnut trees inexorably, is an endemic disease in all chestnut locations. The disease affinity for chestnut known as the Terra Fria Transmontana has an impact of 15% of the trees in the Trás-os-Montes region (Carvalheira, 1997; Martins, 1997).

According to data from the Food and Agriculture Organization (FAOSTAT, 2021), world chestnut production is estimated at 1.2 million tons for an area of about 360,000 hectares. China is the world's largest producer with an annual volume of 1 million tons, which represents 73% of world production. Europe produces 9% of world production, where Italy leads with 4% (55,000 ton) followed by Portugal with 2% (42,180 ton). Figure 2 shows the evolution of chestnut area and production in Portugal in the last years.

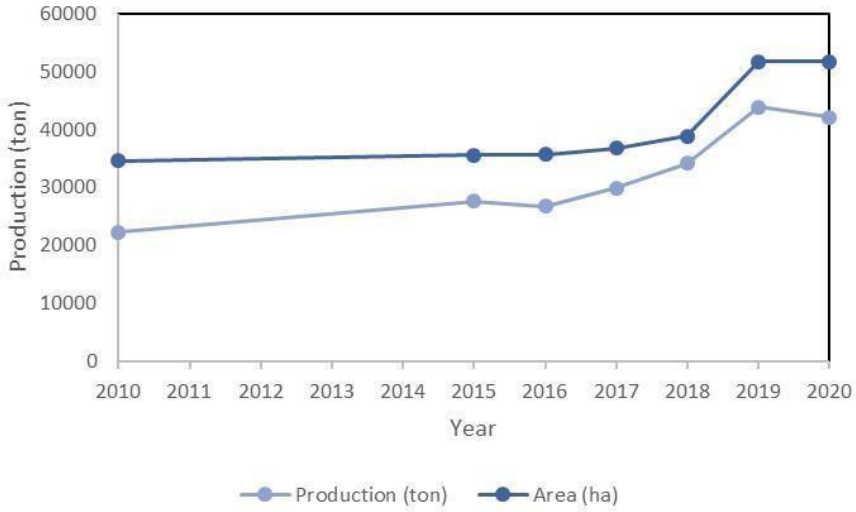

Figure 2 Production and area of chestnut in Portugal. (FOASTAT 2021).

1.3. Disease incidence

Ink disease causes chestnut trees to decline and die, and once introduced into the soil, the parasites multiply quickly and spread to larger areas. The production of parasite-free chestnut propagation material and planting in soils where the parasites are absent are thus essential requirements for the success of new chestnut plantations. To ensure the quality of propagation material and to determine the health status of soils, sensitive and quick detection methods are required (Gouveia *et al*., 2009). However, because to *P. cinnamomi's* high capacity for survival, developing tactics to control this oomycete is extremely difficult (Hwang and Ko, 1978).

The roots of the plants release exudates that attract the zoospores, which accumulate on new or damaged roots, thus initiating the infection process. infection process begins. Roots with lesions are more susceptible to infection because of a greater release of exudates and consequently a greater attraction of zoospores. This attraction through the release chemical substances into the rhizosphere (vitamins, amino acids, enzymes, organic acids, etc.) is called chemotaxis (Gouveia, 1993; Erwin and Ribeiro, 1996). Some authors, cited in Gouveia (1993), argue that the movement of zoospores is induced by electrical charges of the particles and this phenomenon is called electrotaxis.

The zoospores have the characteristic that they adhere easily to a solid surface, which in the case of roots, leaves the zoospores in a prime position to infect root tissues. Germination (cysts) occurs through the emission of one or more germ tubes that penetrate the epidermis. Once inside the plant the germ tube grows, and the mycelium

develops between and within the cells. Between and inside the cells (inter- and intra-cellular) feeding on the nutrients present in the cell contents. Normally the infection starts in the thinner (young) undignified roots, spreading to the lignified roots, collar root and later to the basal trunk of the tree (Gouveia, 1993).

Root exudates affect zoospore motility as well as chlamydospore germination, which is one of *P. cinnamomi*'s key survival structures. Germination can also take place through the emission of one or more germ tubes, followed by the formation of mycelium, and is dependent on the medium's nutritional parameters (quantity of root exudates) (Mircetich and Zentmyer, 1970, cited in Gouveia, 1993).

Water aids the spread of *P. cinnamomi* by transporting propagules (zoospores, chlamydospores, and oospores) and mycelium fragments on natural drainage of soils and water lines. In the field, it disperses in the shape of oil slicks and in the direction of the land's slope (where the water runs off). In cultural operations carried out in agriculture and forestry, soil movements and the transport of infected also constitute a way of spreading the oomycete, namely using of machinery in cultivation operations, which is likely to spread the inoculum from contaminated soil. Animals can also contribute to the transport of propagules of *P. cinnamomi* (Gouveia, 1993).

The phytopathogen that causes the so-called ink disease is *P. cinnamomi*. The term stems from the fact that affected plants emit a violet or dark blue liquid from the damaged tissues, which is caused by polyphenols released from the cortical, phloem, or cambium cells being oxidized. As previously stated, this oomycete starts the infection process at the root system (Figure 3). The degradation of the cortex causes a blackening with a rotten appearance in the thinner roots. Because of the degeneration of the cortex and cambium, the bigger diameter, and lignified roots, which are later infected, have black patches (Grente, 1961).

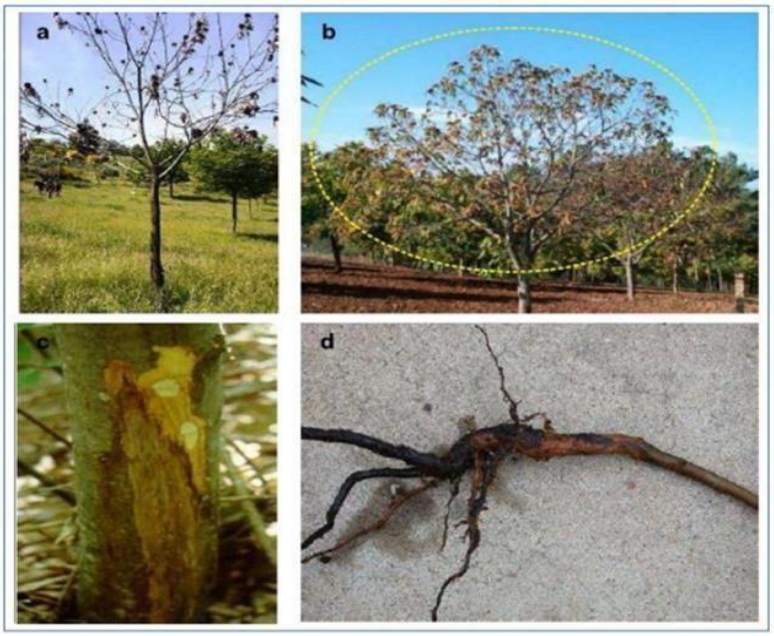

Figure 3 Symptoms caused by *Phytophthora Cinnamomi* in chestnut tree at crown (A) and (B), trunk (C) and root (D) level (Carvalho, 2014).

1.3.1. Control Methods

P. cinnamomi has extraordinary survival strategies that make control of this oomycete extremely difficult (Hwang and Ko, 1978).

In culture and in infected tissues, *P. cinnamomi* species produces a lot of chlamydospores. The pathogen, the host, and the environment are the three most critical factors to consider when developing control measures. Effective disease control is achieved when actions that alter their interaction are taken (Erwin and Ribeiro, 1996; Gouveia, 2004).

The control methods to control ink disease based on:

- **hygiene and sanitary measures:** Sanitary measures consist of preventing the appearance of inoculum in the soil and reducing the amount of inoculum present in the soil by removing infected plant material from the site. Without inoculum there is no disease development, but once in the soil its eradication is not economically viable or even feasible. The prevention of the appearance of inoculum in the soil is therefore of great significance, and even more so when applied in nurseries where sanitary measures besides being adequate should be a requirement. In the nurseries it is fundamental to obtain propagation material

free of *P. cinnamomi* to avoid the transport of inoculum to the field. (Gouveia, 1993; Erwin and Ribeiro, 1996).

- **cultural methods:** In places where stands are irrigated and rainfall is not abundant, irrigation management to avoid soil waterlogging is a measure that can be applied with results against root rot. However, in places where the main source of water is rainfall, and if rainfall is abundant, it is essential to have an optimal horizontal and vertical drainage of the soil. (Erwin and Ribeiro, 1996). Plant extracts have been shown to be beneficial in preventing the growth of some *Phytophthora* species. According to reports, *Pinus radiata* bark applied to water and substrate inhibits the proliferation and pathogenicity of *P. cinnamomi* species. In culture, volatile extracts from the roots of *Acacia pulchella* species (*Fabaceae shrub*) hindered mycelial growth, restricted sporangia generation and germination, and reduced zoospore germination (Gerrettson-Cornell *et al.*, 1976).

- **biological control methods:** Biological control is the use of organisms, genes, or gene products (products of gene expression) to reduce a pathogenic species pathogen's inoculum, postpone or prevent infection, or promote host resistance mechanisms. Biological control, according to some authors, can include both the development of disease-resistant plants and the employment of cultural control approaches, which increase the activity of microorganisms in the soil and may result in the establishment of suppressive soils (Erwin and Ribeiro, 1996).

- **use of resistant rootstocks**: In recent years much work has been done to make the European chestnut more resistant to the pathogen responsible for the ink disease, while maintaining the properties that value (the fruit). According to Gouveia (1993), to improve the chestnut tree regarding the disease (to obtain plant material resistant to the disease) it is necessary to identify the sources of resistance identify the mechanisms of resistance linking them with the responsible genes to develop a methodology to transmit the resistance to the *Castanea sativa* species and to monitor resistance over time for different environmental conditions

- **chemical control methods:** The use of chemicals in the control of oomycetes has been a topic addressed in several investigations. Their taxonomic position in a kingdom that is different from the generality of fungi of fungi gives rise to a specific control strategy for this class. Thus, fungicides successfully used to control used successfully to control fungi may have no effect on oomycetes, as is the case with polyoxins that inhibit chitin synthesis, and substances that inhibit sterol biosynthesis such as benomyl (Erwinand Ribeiro, 1996). In Portugal, products with the active substances, copper oxychloride and copper hydroxide and fosetyl in the form of aluminium salt are approved by the Direção Geral de Alimentação e Veterinária (DGAV, 2019) for the treatment of chestnut ink disease.

The available control methods for chestnut ink disease have not, to date, efficiently and durably solved the health problems of crops infected by this parasite. Disease control is based on preventing infection and limiting the spread of the pathogen through cultural, biological, and chemical measures (Smith *et al*., 1992). The chemical treatments proposed are very diverse: spraying the canopy of infected trees with fungicides and foliar fertilizers, injections into the trunk of diseased trees and application of different fungistatic products to diseased roots and soil (Navarro *et al.*, 2004).

2. Trapping method for *Phytophthora* detection.

Biodetection approaches are based on *Phytophthora* species' specific pathogenicity to the tissue employed as a bio-trap (Eckert and Tsao, 1962), and selective culture mediums allow *Phytophthora* species to be isolated and grown in culture (Tsao, 1987; 1990).

There are many documents describing trap tissue methods for *Phytophthora*, however, there have been few systematic studies to determine the best trap tissue protocol to use (Burgess *et al*., 2021) there are also a variety of trap tissue methods currently recommended by agencies around the world. Martin *et al*. (2012) analyzed various detection methods and concluded that leaf-based trapping were preferred over fruits. Leaves of Camellia, Rhododendron, and Quercus spp. have been the most used in recent years (Jung *et al*. 2000, Themann *et al*. 2002, Fichtner *et al*. 2007, Ghimire *et al*. 2009, Sutton *et al*. 2009). For *P. cinnamomi*, Eucalyptus seedlings, conifer needles and lupines are among commonly used trapping tissues (Eden et al. 2000). Gouveia et al.

(2009) concluded that chestnut leaves were the best trap tissue for detecting *P. cinnamomi*, compared to camellia and holly leaves.

The trap tissue method is an indirect method of isolation that involves attracting motile zoospores released by sporangia to the trap tissue (Erwin and Ribeiro, 1996; Martin et al. 2012). These spores are negatively geotropic and exhibit chemotaxis (Hardham, 2005) by swimming upwards towards the trap tissue. This method is semi-selective as the other fungi present in the soil do not have swimming spores and are unlikely to be able to infect the trap tissue. Often, trap tissue will show highly specific symptoms or a lesion when infected (Cooke *et al*. 2007).

Successful soil isolation is often increased when soil is kept between 15 and 20°C and trap tissue is not injured, which discourages colonization by *Pythium* spp. and bacteria (Ferguson and Jeffers, 1999; Ghimire *et al*. 2009). False negatives can be avoided when soils are used in the trap tissue technique after air-drying, pre-wetting or there are cold storage practices that induce dormant spore germination (Jeffers and Aldwinckle, 1987; Davison and Tay, 2005; Tooley and Carras, 2011).

The efficiency of this technique depends largely on the susceptibility of the material used as a trap plant, the development of characteristic symptoms in these plant tissues and incubation conditions that maximize the chances of formation and release of zoospores, the primary propagules in the infection process (Tsao, 1987; Gouveia *et al*. 2009).

3. Identification of *Phytophthora* Species by Molecular Methods

Specificity, sensitivity, and the ability to obtain results in a time frame that allows for the deployment of suitable phytosanitary measures are all requirements for detection and diagnostic procedures. The classical methods of detection, which are based on parasite isolation techniques in pure culture, are a reference for specificity, allowing Kock's postulates to be used to establish the diagnosis. The efficacy of the isolation from plant tissues or soil is linked to sensitivity. The specificity of PCR-based detection methods is determined by the primers, the genomic area to be amplified, and the PCR reaction conditions. The sensitivity of detection is determined by the PCR reaction protocol, sampling procedure, and DNA template sample collection procedure. (Gouveia, 2004).

To obtain specific and sensitive trustworthy procedures, immunoenzymatic detection and diagnosis techniques with a high degree of sensitivity and specificity in viruses, phytoplasmas, bacteria, and some fungi were applied to *Phytophthora*. The outcomes of these procedures even led to the marketing of diagnostic "kits" like the ones Miller *et al.* (1994). However, the specificity of these approaches is only at the genus level, not at the level of the *Phytophthora* species participating in the pathogenesis process, which would be preferable. Themann *et al*. (2002) described a similar issue when they developed specialized antisera for *P. cactorum* and *P. cinnamomi* for use in DASELISA - (Double - Antibody - Sandwich - Enzyme - Linked Immunosorbent - Assay).

The Figure 4 represents the rDNA genomic region and the diagram of the steps and techniques for molecular identification of *Phytophthora* species based on the ITS1, 5.8S and ITS2 rDNA regions. The study by Cooke *et al*. (2000) is a reference in the characterization of the ITS region in the genus *Phytophthora*. DNA was amplified by PCR using the universal primers ITS6 and ITS4. Primer ITS6 is like ITS5 of White *et al*. (1990) but modified by Cooke & Duncan (1997) according to the sequence of the sequence of the 18S rDNA region of *P. megasperma* to increase the specificity of amplification of this region in *Phytophthora.*

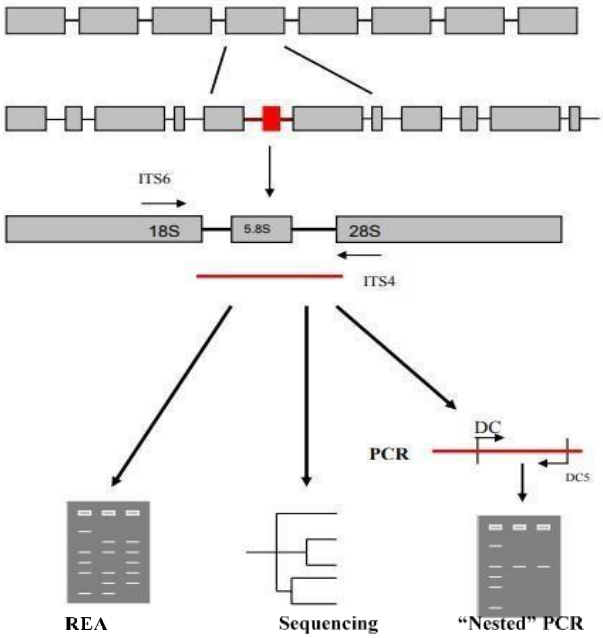

Figure 4 Representative scheme of rDNA organization and identification strategies of *Phytophthora* species of *Phytophthora* species based on the ITS1, 5.8S and ITS2 genomic region. (GOUVEIA, 2004).

Systematic and phylogenetic studies based on the sequencing of the genomic region that codes for ribosomal RNA (rDNA) in *Phytophthora* have made a significant contribution to the development of PCR-based identification, detection, and diagnostic tools (Cooke *et al.,* 2000). In addition to the genomic region that codes for ribosomes, the gene that codes for elicitins, holoproteins thought to be characteristic of the genus *Phytophthora* (Ricci, 1977), and "anonymous" regions of the genome obtained by cloning fragments of DNA thought to be characteristic of a given species, were used for the same purpose. a feature of a particular species.

The gap between coding regions of rRNA genes (ITS) is particularly useful for discriminating organisms at the species and genus level (Cooke *et al*., 1999), because the rate of mutation accumulation in this region often approaches the rate of speciation (Lee and Taylor, 1992), which is why many PCR molecular identification strategies in the genus *Phytophthora* are based on amplification of this region of the genome. The method consists of designing primers in conserved regions of the rDNA for amplification of characteristic regions that after sequencing or analysis by restriction enzymes allow the identification of the organisms under study.

4. OBJECTIVES

This work aims to develop molecular methods for the detection and identification of *Phytophthora* species in soils, providing higher sensitivity of detection and higher specificity of identification and allowing reliable and fast results.

5. MATERIAL AND METHODS

5.1. Detection and identification of *Phytophthora* associated with ink disease from natural soils

5.1.1. Soil origins

The soil used for the isolation of *Phytophthora* was taken from chestnut groves and from pots with infected soil in greenhouses and with the application of fungicides. In Carragosa (Bragança, Trás-os-Montes - Portugal) were recovered soils from 12 chestnuts infected with ink disease and from eight pots of natural soils collected from chestnuts with ink disease symptoms and were plants of chestnuts are growing in the greenhouse. Three repetitions of each sample were used for the tests.

5.1.2. Classical method of detection

The soil from the grove and pots was used for the isolation of *Phytophthora*, using the biological trap method with chestnut leaf discs (Figure 5) proposed by Gouveia et al. (2009). From each pot, 20 ml of soil was collected and placed in a 90 mm diameter Petri dish. In each plate, 8 ml of distilled water was added and where ten 0.5 cm diameter discs of chestnut leaves were placed. The plates were left at room temperature and after 72 hours, three leaves were removed from each plate and were placed to grow in PARPH selective culture medium (Pimaricin, 5 mg l^{-1}; Ampicillin, 250 mg l^{-1}; Rifanpicin, 10 mg l^{-1}; Hymexazole, 25 mg l^{-1}) or in PDA medium. The presence of *Phytophthora* was confirmed by microscopic observation, at the hyphae level.

Figure 5 Biological trap method with chestnut leaf discs.

After mycelial growth, the mycelium was transferred to PDA (Potato Dextrose Agar, Difco 39 g l^{-1}) culture medium for subsequent molecular identification of the species.

5.1.3. Identification of species

The identification of the different species of *Phytophthora* was based on morphological characteristics (Figure 6) and molecular methods by amplification and sequencing of the ITS region of the ribosomal DNA (rDNA) using the universal primers ITS4 and ITS6 (White *et al*., 1990) and compared with the sequences published in the databases of GenBank, BLAST (https://blast.ncbi.nlm.nih.gov/). DNA extraction from the fungi was performed using the REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich) (attachment 3). Sequencing of amplified region was performed by Stabvida Laboratories (Caparica, Portugal). Each species was taxonomically classified according to the MycoBank Database (www.micobank.org).

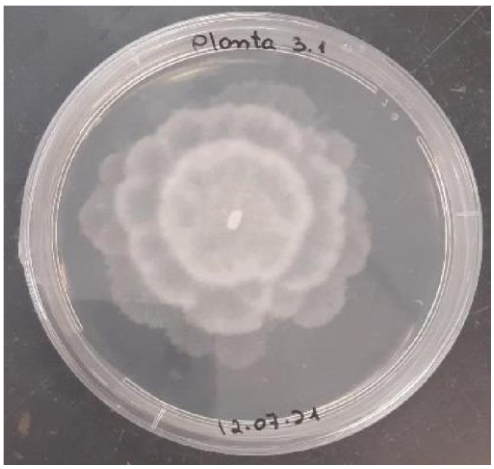

Figure 6 *Phytophthora* isolate.

5.1.4. PCR Detection of *Phytophthora* by total DNA extraction from soil

5.1.5.1. DNA extraction

The DNA extraction from soil was performed with the DNeasy® PowerSoil® Pro Kit (Qiagen). For the extraction, 250 mg of freshly collected soil was used and the extraction was performed following the manufactures recommended protocol.

Briefly, soil samples (0.25 g of soil each sample) were vortexed with Powerbeads and CD1 solution. The supernatant was transferred to Microcentrifuge tubes and CD2

solution was added. After centrifugation CD3 solution was added and the lysate was placed on MB Spin Columns and centrifuged for cleaning the pellet. After that was placed into a clean 2 ml collection tube and was added Solution EA and centrifuged. The Solution C5 was added and centrifuged again. After that the column was placed into a new 1.5 ml Elution Tube and C6 solution was added. After that DNA is ready for further applications (attachment 1).

5.1.5.2. PCR reaction

DNA from the region encoding for ribosomes was amplified by the universal primers ITS6 (5' GAA GGT GAA GTC GTA ACA AGG 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3'). Primer ITS6 is like ITS5 from White *et al*. (1990). ITS4 from White *et al*. (1990) is a universal primer in eukaryotes located at the 5' end of the 28S rDNA gene, and primer ITS6 (Cooke & Duncan, 1997) is located at the 3' end of the 18S rDNA.

PCR reactions were performed in a final volume of 25 μL. Chemical conditions were empirically adjusted, and consistent results were obtained under the following conditions: 100 μM of each dNTPs, 20 pmoles of each primer, 1.5 mM $MgCl_2$, enzyme buffer at final concentration 1×, one unit of Taq DNA polymerase (Promega), 1 μL of DNA, and sterile ultrapure water to the final reaction volume (Attachment 2).

PCR reactions were performed in a C1000 TouchTm Thermal Cycler (Bio-Rad). The reaction started with an initial denaturation cycle at 95 ºC for 3 minutes followed by 35 amplification cycles with the following parameters: 55 ºC for 30 seconds, 72 ºC for 60 seconds, and 94 ºC for 30 seconds. The reaction ended with a final extension period at 72 ºC for 10 minutes (Attachment 2).

5.1.5. Diagnostic PCR for detection of *Phytophthora cinnamomi*

Primers designed on the SCAR marker - OP120 (Gouveia, 2004), Cin1, Cin2 and Cin3 were tested in PCR reactions in the combination Cin2/Cin1 and Cin3/Cin1 to detect the species *P. cinnamomi*. This Diagnostic PCR was done using total DNA extracted from soil.

Cin1 - 5’TCGAATGGAGTACATATGCGTAT 3’

Cin2 - 5’GTTCAGATTAGAGTTTGTGGAGT 3’

Cin3 - 5’CTAGATAATCTTAAATAATCTGG 3’

The chemical conditions were the same in both primer pairs and has the following concentrations: 10 mM of each dNTPs, 25 mM of MgCl2, 10X enzyme buffer, 0.5 µM of each primer and one unit of Taq DNA polymerase (Promega).

The physical conditions were different in the two primer pairs. The amplification cycle with the Cin2/Cin1 primer pair was initiated with a period of denaturation of the DNA at 94 ºC for 60 seconds followed by 35 cycles of 94 ºC - 30 seconds; 50 ºC - 60 seconds; 72 ºC - 30 seconds, ending the reaction with a final extension period of 5 minutes at 72 ºC. For the primer pair Cin3/Cin1 the following amplification cycle was used: 94 ºC for 60 seconds followed by 39 cycles of 94 ºC - 30 seconds; 48 ºC - 40 seconds; 72 ºC - 60 seconds, ending the reaction with a final extension period of 5 minutes at 72 ºC.

5.2. Testing the method on soils where fungicides are applied

In order to test the detection method, a greenhouse trial was carried out where different formulations of metalaxyl-M were compared in the protection of chestnut plants and development of the pathogen in the soil. The objective of this study was to compare the PCR detection protocol developed by our laboratory with the baiting methods.

Figure 7 Bench in the greenhouses with the test.

5.2.1. Biological material

Chestnut plants with one year of age were used for the tests. The chestnuts were previously stratified in sand, kept moist for germination to occur. The germinated chestnuts were planted in trays with the dimension of 6 cm x 6 cm x 20 cm for the normal growth of the chestnut plants.

The chestnut plants were planted in pots containing the substrate and arranged on a bench, with weekly flood irrigation and mist irrigation at regular intervals during the daytime period. the pots were kept in the greenhouse for one year.

5.2.2. Pot substrate

Perlite was added to the field soil from symptomatic diseased chestnut trees at a ratio of 3:1 (v/v). The planting substrate was placed in 4 L capacity pots.

5.2.3. Fungicides

The fungicides used in the tests were Ridomil Gold R WG and Ridomil Gold SL. Ridomil Gold R WG is a contact systemic fungicide with 2% (w/w) metalaxil-M and Ridomil Gold SL is a systemic fungicide with 465 gl^{-1} of metalaxil-M.

The active substances were diluted in a glass beaker containing 500 ml of tap water, according to the concentrations and the content of each glass was poured directly into the soil of the pot around the plant. The first application was made on July 27, 2021.

The following fungicide concentrations were tested: Ridomil Gold R WG - 1.67 gl^{1}, 3.33 gl^{-1} and 6.67 gl^{-1}; Ridomil Gold SL - 0.47 gl^{-1}, 0.94 gl^{-1} and 1.39 gl^{-1}.

5.2.4. Physiological parameters evaluated

Measurements of plant growth, year growth and diameter at collar level were taken on all plants on July 27, 2021, and on December 9, May 6, 2022, June 3 and July 15.

5.2.5. Soil DNA extraction and *Phytophthora* detection by PCR

Three samples of each concentration of both fungicides were chosen for detection by PCR. DNA extraction from soil was done as described in 5.1.5.1 and the PCR reaction is described in 5.1.5.2.

6. RESULTS AND DATA ANALYSIS

6.1. Conventional method of detection

Data of baiting test are representing in Table 1. In the tissue trap assay, positive isolations of *Phytophthora* were obtained in both soil samples that were collected in Carragosa and in the soil samples from pots of the greenhouses. From the 12 natural soil samples of chestnut orchard (Carragosa-Bragança) were obtained positive isolations of *Phytophthora* in eight samples (67% of samples) and from the eight soil samples from soils of pots were obtained positive isolations of *Phytophthora* in six samples (75% of the samples).

The percentage of positive *Phytophthora* isolates, obtained from baiting tissues, ranged from 20% to 100%. The variation in the effectiveness of isolation was associated in most situations to the irregularity of *Phytophthora* distribution in the soil. It should be noted that in Carragosa all the trees showed symptoms of ink disease.

Table 1 Positive isolation of Phytophthora on PARPH medium obtained from chestnut leaf discs after 72 hours.

Samples	Chestnut orchard			Soil from pots		
	1	2	3	1	2	3
1	1/5	0/5	0/5	5/5	5/5	1/5
2	2/5	3/5	3/5	0/5	0/5	0/5
3	0/5	0/5	0/5	3/5	2/5	2/5
4	3/5	1/5	0/5	5/5	2/5	4/5
5	3/5	5/5	3/5	1/5	1/5	1/5
6	0/5	0/5	0/5	0/5	3/5	0/5
7	0/5	0/5	0/5	0/5	0/5	0/5
8	1/5	2/5	0/5	0/5	1/5	0/5
9	2/5	0/5	2/5			
10	0/5	0/5	0/5			
11	3/5	1/5	1/5			
12	1/5	0/5	2/5			

6.2. Identification of *Phytophthora* species

From the isolates of *Phytophthora* obtained from the leaf discs trap method from natural soils of symptomatic chestnut trees, five isolates were selected for molecular species identification (Figure 8, Table 2).

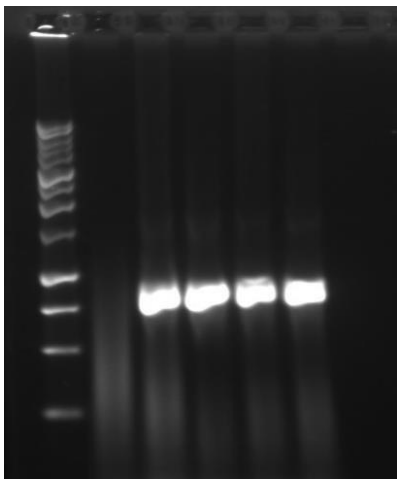

Figure 8 Agarose gel electrophoresis of the PCR products. DNA marker 1kb (Promega) Column (2-5) – samples of Phytophthora. 1.5% agarose gel, GelRed staining and visualisation by UV light.

As can be seen in the Figure8, one isolate did not result in amplification products. Thus, only four isolates were used for identification. (Table 2).

Table 2 Samples and FASTA (Consensus sequence)

1+ITS1
aaataaacaagcgggggngnattccgtnggtgaacctgcggattgcataatcaataagcggaggaagattccgtaggtgaacctgc gggatatgcatatcaataagcggaggaagattccgtaggtgaacctgcggatatgcatatcaggggcggaggaatattccctctctttt taaacccattctgtagtactgaacatactgtggggaccaaagtctctgcttttaactagatagcaactttcagcagtggatgtctaggctcg cacatcgatgaagaacgctgcgaactgcgatacgtaatgcgaattgcaggattcaatgagtcatcgaaattttgaacgcatattgcactt ccgggttagtcctgggagtatgcctgtatcagtgtccgtacatcaaacttggctctcttccttccgngtgatcngtggatggaggtgcca gacgtgaggtgtcttgcgggcggtcttcagactggctgtgagtcccttgaaatgtactgaactgtacttctctttgctcgaaaagcgtgac gttgctggttgtggaggctgcctgtatggccagtcggcgaccggtttgtctgctgcggcgtttaatggaggagtgttcga
2+ITS1

nnnnnnnnngcggnagaagantccgtaggtgacctgcggatatgcatatcaataagcggaggaagattccgtaggtgaacctgcg gatatgcatatcaataagcggaggaagattccgtaggggcgagcgtttgggtccctctcgggggaactgagctagtagcctctctttta aacccattctgtaatactgaacatactgtggggacgaaagtctctgcttttaactagatagcaactttcagcagtggatgtctaggctcgc acatcgatgaagaacgctgcgaactgcgatacgtaatgcgaattgcaggattcagtgagtcatcgaaattttgaacgcatattgcacttc cgggttagtcctgggagtatgcctgtatcagtgtccgtacatcaaacttggctctcttccttccgtgtagtcggtggatggaggtgccaga cgtgaggtgtcttgcgggcggtcttcggactggctgtgagtcccttgaaatgtactgaactgtacttctctttgctcgaaaagcgtgacgtt gctggttgtggaggctgcctgtatggccagtcggcgaccggtttgtctgctgcggcgtttaatggaggagtgttcgattcgcggtatggt tggcttcggctgaacaaagcgcttattggatgttcttcctgctgtggcggtacggatcggtgaaccgtagctgtgctaggcttggcgtttg aaccggcggtgttgttgcgaagtagggtggcggcttcggctgtcgagggtcgatccatttgggaactctgtgtctctgcggcgcacttg tgtgcttgtggtggcatctcaattggacctgatatcaggcaagattacccgctgaacttaagcatatcaataaggcggaggaaa
3+ITS1
nnnnngnnnnnnnnnnncnnnnggnngantccgtaggtgacctgcggatatgcatatcaataagcggaggaagattccgtaggt gaacctgcggatatgcagatgaataagcggaggaggattccgtacgggcgagcgtttgggtccctctcgggggaactgagctagta gcctctcttttaaacccattctgtaatactgaacatactgtggggacgaaagtctctgcttttaactagatagcaactttcagcagtggatgt ctaggctcgcacatcgatgaagaacgctgcgaactgcgatacgtaatgcgaattgcaggattcagtgagtcatcgaaattttgaacgca tattgcacttccgggttagtcctgggagtatgcctgtatcagtgtccgtacatcaaacttggctctcttccttccgtgtagtcggtggatgga ggtgccagacgtgaggtgtcttgcgggcggtcttcggactggctgtgagtcccttgaaatgtactgaactgtacttctctttgctcgaaaa gcgtgacgttgctggttgtggaggctgcctgtatggccagtcggcgaccggtttgtctgctgcggcgtttaatggaggagtgttcgattc gcggtatggttggcttcggctgaacaaagcgcttattggatgttcttcctgctgtggcggtacggatcggtgaaccgtagctgtgctagg cttggcgtttgaaccggcggtgttgttgcgaagtagggtggcggcttcggctgtcgagggtcgatccatttgggaactctgtgtctctgc ggcgcacttgtgtgcttgtggtggcatctcaattggacctgatatcaggcaagattacccgctgaacttaagcatatcaataagcggagg aaa
4+ITS1
nnnnnnnnnnnnnnnnnngnnnnngnngnnnngnacgggttgacctgcggatatgcatatcaataagcggaggaagattccgt aggtgaacctgcggatatgcatatcaataagcggaggaagattccgtaggtgaacctgcggatatgcatatcaataagcggaggaata ttcccctcgtttttaaacccattctgtaatactgaacatactgtggggacgaaagtctt

The molecular identification by comparing with the sequences published in the databases of GenBank, BLAST (https://blast.ncbi.nlm.nih.gov/) showed that three isolates belonged to the species *Phythophthora cinnamomi*, and the nucleotide identity of the ITS region of the isolates ranged from 98 to 100% (Table 3). Isolate 4 was not possible to identify.

Table 3 Species identification and percentage of nucleotide identity of each identified species.

Samples	Origin of Detention	Species	Identity (%)
1	Pots	*Phythophthora cinnamomi*	98.23%
2	Pots	*Phythophthora cinnamomi*	99.87%
3	Pots	*Phythophthora cinnamomi*	99.87%

6.3. PCR Detection of *Phytophthora* by total DNA extraction from soil

The DNA extraction method provided DNA in quantity to use in the PCR reaction (Figure 9).

DNA was used in this study without cleaning of proteins or other inhibiting substances.

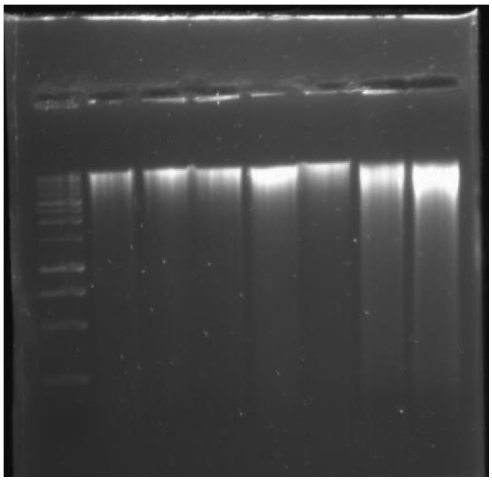

Figure 9 Genomic DNA extracted from samples soil. Column 1 - DNA marker 1kb (Promega) Column (2-8) – samples of soil. 0.8% agarose gel, Gel red staining and visualisation by UV light.

The PCR protocol, after optimization of the physical and chemical conditions and with the specific primers - ITS6 and ITS4, provided the amplification of a single band with approximately 900 bp (Figure 10) in the *Phytophthora* species.

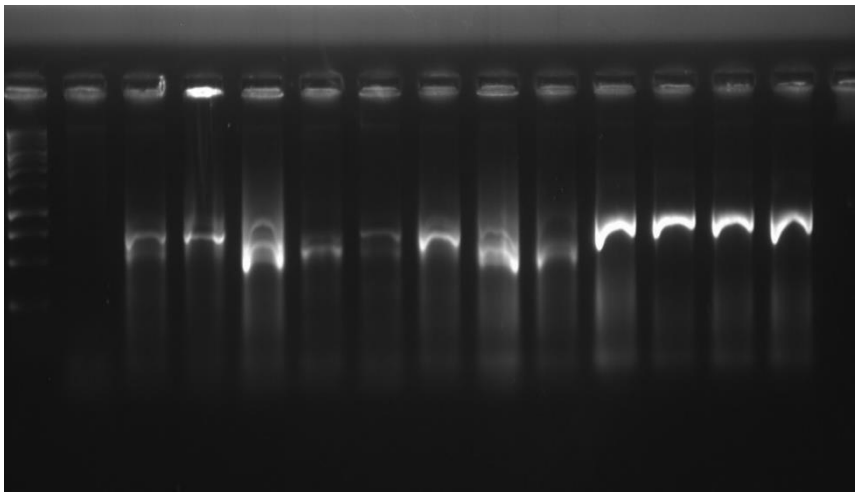

Figure 10PCR amplification using genomic DNA of Phytophthora isolates, pathogenic on chestnut with primers ITS6 (Cooke & Duncan, 1997), ITS4 (White et al., 1990). DNA marker 1kb (Promega) Column (2-14) – samples of soil. 1.5% agarose gel, GelRed

staining and visualisation by UV light

In some soil samples, the PCR reaction worked irregularly and sometimes did not amplify any DNA fragment, a situation that was associated with the presence of high amounts PCR inhibitors or with extemporaneous DNA degradation. In these circumstances, it has been opted for the repetition of the protocol of DNA soil extraction.

In the gel represented below, the first five columns were from infected soil from Carragosa and the others from soil from the greenhouse. The results of *Phytophthora* detection by PCR are in Table 4.

Table 4 *Phytophthora* detection by PCR of soil samples from Carragosa and from pots (greenhouse) with primers ITS6 (Cooke & Duncan, 1997), ITS4 (White et al., 1990).

Samples	Chestnut orchard (Carragosa)	Soil from pots (greenhouse)
1	+	-
2	+	+
3	-	+
4	+	+
5	-	+
6	+	+
7	-	-
8	+	-
9	+	
10	-	
11	+	
12	-	

(+) detected, when detected in one of the three repetitions; (-) not detected

In Carragosa was detected *Phytophthora* in seven samples (58% of positive isolates) and from greenhouse infected pots it was detected *Phytophthora* in five samples (63% of positive isolates).

6.3.1. Diagnostic PCR for detection of *Phytophthora cinnamomi*

The primers under study (Cin1/Cin2, Cin1/Cin3) did not result in amplification products (Table 5).

Table 5 Phytophthora cinnamomi detection by PCR of soil samples from Carragosa and from pots (greenhouse).

Samples	1	2	3	4	5	6	7	8	9	10	11	12
Chestnut orchard	-	-	-	-	-	-	-	-	-	-	-	-
Soil from pots	-	-	-	-	-	-	-	-				

(-) not detected

6.4. Testing the method on soil where fungicides are applied

The growth of the chestnut plants was recorded at the end of the trial, after 1 year of growth. The values of height and diameter at the collar level of the chestnut plants, expressed in centimeters, in the different modalities are shown in Table 6.

Table 6 Year growth (cm) and diameter at collar level (cm) in the tested modes (Ridomil Gold R WG and Ridomil Gold SL) at the end of the trial.

Ridomil Gold R WG				**Ridomil Gold SL**		
Concentration (g/L)	Pots	Height (cm)	Diameter at collar level (cm)	**C**oncentration (g/L)	Height (cm)	Diameter at collar level (cm)
1.67	1	48.4	0.81	0.47	54.5	0.78
	2	43.0	0.85		55.0	0.66
	3	38.8	0.77		35.5	0.54
	4	33.0	0.55		44.5	0.74
	5	43.1	0.81		47.2	0.72
3.33	1	28.4	0.56	0.94	49.0	0.76
	2	40.0	0.84		39.0	0.84
	3	37.2	0.34		70.0	0.87
	4	33.5	0.78		46.5	0.82
	5	26.0	0.31		65.5	0.78
6.67	1	51.1	0.63	1.39	73.5	0.82
	2	53.0	0.82		47.5	0.87
	3	63.5	0.90		52.0	0.78
	4	63.5	0.86		53.5	0.54
	5	64.0	0.87		49.5	0.71

The greatest height was obtained in plants grown at a concentration of 6.67 gl^{-1} of Ridomil Gold R WG (64.0 cm) and at a concentration of Ridomil Gold SL of 1.39 gl^{-1} (73.5 cm).

The highest average growth obtained at the end of the trial in the test with Ridomil Gold R WG was obtained in plants with application of 6.67 gl^{-1} (59.02 cm), and in plants with Ridomil Gold SL was obtained in plants with 1.39 gl^{-1} (55.20 cm) as can see in Table 7.

Table 7 Year growth (cm) and diameter at collar level (cm) in the tested modes (Ridomil Gold R WG and Ridomil Gold SL) at the end of the trial.

	Ridomil Gold R WG		
	1.67g/L	**3.33g/L**	**6.67g/L**
Growth of the year	41.26±5.74	33.02±5.85	59.02±6.40
Diameter at collar level	0.70±0.21	0.56±0.24	0.82±0.10
	Ridomil Gold SL		
	0.47g/L	**0.94g/L**	**1.39g/L**
Growth of the year	47.34±8.03	54.00±13.18	55.20±10.49
Diameter at collar level	0.69±0.09	0.81±0.04	0.74±0.13

Plants treated with Ridomil Gold R WG at a concentration of 6.67 gl^{-1} showed a larger diameter at collar level (0.82±0.10 cm) than the other concentrations where the product was applied. For plants where Ridomil Gold SL was applied the larger diameter was obtained for the concentration of 0.94 gl^{-1} (0.81±0.04 cm).

6.5. Detection of *Phytophthora* by total DNA extraction from soil from greenhouse pots

In this study, no *Phytophthora* was identified in the total DNA extracted from the potting soil by primers ITS6 (Cooke & Duncan, 1997) and ITS4 (White *et al*., 1990).

Table 8 Phytophthora detection by PCR of soil samples from pots (greenhouse) where fungicides were applied.

Samples	Ridomil Gold R WG	Ridomil Gold SL
1	-	-
2	-	-
3	-	-

7. DISCUSSION AND CONCLUSION

By conventional methods of microbiology, *P. cinnamomi* is detected from soil by placing soil in distilled water containing susceptible plant material as a trap tissue (e.g., vegetable leaf or fruit). This susceptible plant material is subsequently placed in selective culture medium (agar with antibiotics and fungicides), and identification of *Phytophthora* is done by morphological characteristics and/or DNA amplification-based methods (Eckert and Tsao, 1962; Jeffers and Martin, 1986). Baiting or trapping methods are generally considered specific, reliable, and efficient for *Phytophthora* detection in natural and complex soil samples because propagules density is generally low in natural environments and the volume of soils used for baiting can be increased to improve sensitivity. Diagnostic protocols of OEPP/EPPO, PM7/26 - *Phytophthora cinnamomi* is based on baiting principles and establish that positive diagnosis is obtained when the pathogen is isolated by baiting soil with susceptible plant parts and isolation baits, followed by morphological (or molecular identification) of the pathogen in pure culture (OEPP, 2004).

However, conventional baiting is time consuming, and isolates must be transferred to new media to produce isolates for identification. Although many methods exist, there is no standardization among *Phytophthora* detection methods. According to Burgess *et al*. (2021) many of the comparative methods studied do not include positive methods and negative controls, are performed at only one station, and there is no standardization of media, time, bait material, volume/ratio, temperature, or shape of container to be used.

The baiting trap in this work was based on the methodology proposed by Gouveia *et al*. (2009) which proved to be an efficient method for the detection of *Phytophthora* from soils where there were chestnut trees with symptoms of ink disease. The method is a positive test and confirm the presence of *Phytophthora* if one of the three replications of

the test is positive. Overall, of the 12 sampled natural soils, eight samples were screened as positive for *Phytophthora* by the conventional baiting method and on soils of greenhouse, six samples out of eight are screened as positive for *Phytophthora.*

DNA Amplification-based methods for *Phytophthora* detection provide similar results and seven out of 12 samples natural soils screening positive for *Phytophthora* and five out of eight soil samples from the pot soils of greenhouse were *Phytophthora* positive. Only one sample is negative in bait method and positive in DNA amplification -based method.

In Portugal, two species of *Phytophthora* have been associated with chestnut tree, *P. cinnamomi* and *P. cambivora*. However, *P. cinnamomi* is the species most detected from soil samples and plant tissue from chestnut stands and it is considered the predominant pathogen in the development of ink disease (Fernandes, 1979; Gouveia, 2004), which agrees with the results of this study. Similarity between nucleotides sequences was 98% to 100% from *P. cinnamomi* isolates obtained from the soil of pots.

The development of the PCR technique for DNA amplification has altered molecular diagnostics and supported highly specific diagnostics of pathogen detection from small amounts of plant tissue. With properly designed amplification primers, highly specific diagnosis can be developed using conventional PCR techniques. This approach was developed by Schena *et al*. (2008) based on a region of the ras related protein gene *Ypt1* for the identification of 15 *Phytophthora* species that damages forests and trees and was named PCR-based "molecular toolbox". In this method using existing methods for rapid extraction and purification of DNA, single-round amplification was appropriate for detection of target *Phytophthora* species in leaves, but nested PCR was required for soil and water samples. The authors considered one great advantage of the Ypt1gene that the gene is structured in conserved and variable regions and enables the development of nested PCR with a first round using the genus-specific primer and a second round with a species-specific primer. However, the use of nested PCR implies a great risk of false positives arising from cross contaminations as well as increasing the time and technical requirements of the procedure.

Utilizing the exposed primers designed strategy, Senna *et al*. (2018) using direct soil DNA extraction none of the samples were positive for *P. cinnamomi* with the primers Ycin3F and Ycin4R targeting a 300-bp segment of the Ypt1 gene. As noted by Sena *et al*. (2018), this could be due to the limited capacity of the extraction kit, which only extracts

DNA from small amounts of soil samples (about 0.25 g of soil) and the concentration of *Phytophthora* DNA in the soil may be below the limit of detection by the PCR method.

In this work utilizing the SCAR methodology (Gouveia, 2004) for primers designing for specific detection of *P.cinnamomi* we also haven't detected *P. cinnamomi* from any of the soils tested.

The extraction of good quality DNA and free of PCR inhibitors are conditions to have a good PCR diagnosis. Commercial kits, easier to use and with the potential for better results, have replaced traditional chloroform/phenol DNA extraction with different amounts of PCR inhibitors that can lower the sensitivity of the diagnostic test by this method (Martin *et al*. 2012). The DNeasy® PowerSoil® Pro Kit used in this study to obtain DNA of natural and pot soil samples contains streamlined Inhibitor Removal Technology (IRT) to eliminate the challenging inhibitors commonly found in soil and environmental samples in even less time. This kit is more effective than alternative methods (8-fold higher DNA yield) with inhibitor-free DNA for direct use in NGS applications (The DNeasy® PowerSoil® Pro Kit (Qiagen), (www.qiagen.com). Using this kit, we obtained DNA of high quality that result in PCR amplification with the primers ITS4 and ITS6 in all tested samples.

Many plant diseases that are associated with soil borne *Phytophthora* species involve a complex of several species that reveal the need to use a community approach in studies of the disease and not only study single pathogens currently associated with the plant host. The new High Throughput Sequencing (HTS) of amplicons has supported studies of microbial community with a very high resolution and has changed fungal and bacterial ecology studies, but very few studies included oomycetes metabarcoding have been carried out and with highly variable results, (Sapkot and Nicolaisen, 2016). Riit *et al*. (2016), citing different authors, consider that has been identified as suitable barcoding for oomycetes the cytochrome c oxidase subunit 1 (cox1), the internal transcribed spacer (ITS) and the cytochrome c oxidase subunit 2 (cox2) and they have developed new PCR primers from oomycete-specific ITS regions with improved specificity for metabarcoding and identification of oomycetes to validate an alternative method for metabarcoding of oomycetes in complex substrates as natural soils. The modified and newly developed forward primers are in the end of the conserved fragments that reduces the size of amplicons, which is of great importance for HTS platforms producing short fragments such as Illumina and ion Torrent (Riit *et al*. 2016). The same authors also considered that

when combined with universal reverse primers, these oomycete-specific primers could be used in multiplex with other specific forward primers to address several taxonomic groups of pathogens simultaneously, without adding the cost of multiple barcoded reverse primers.

Castanea sativa is highly susceptible to soil borne *Phytophthora* and the ink disease of chestnut globally threats the chestnut ecosystem. *P. cambivora* and *P. cinnamomi* are associated with the disease in Portugal, even though some other *Phytophthora* species as *P. cambivora*, *P. citricola*, *P. cactorum* and *P. gonapodyides* are present in soils of diseased trees (Vitraino *et al*. 2001). Early detection of pathogens, at species level, as well as interactions and dynamics in the oomycete community in the complex natural soils are important steps for successful disease management and evaluation of different strategies to control the ink disease of chestnut.

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ATTACHMENTS

Attachment 1

DNeasy® PowerSoil® Pro Kit (Qiagen) Protocol:

The CD2 solution should be stored at 2-8°C on arrival. All other reagents and kit components should be stored at room temperature (15-25°C).

Notes before you begin

- Ensure that the PowerBead Pro tubes rotate freely in the centrifuge without rubbing
- If the CD3 solution has precipitated, heat at 60°C until the precipitate dissolves.
- Perform all centrifugation steps at room temperature (15-25°C).
-

1. Lightly spin the tubes (PowerBead Pro Tube) to ensure that the beads have settled to the bottom. Add up to 250 mg of soil and 800 µl of CD1 Solution. Vortex briefly to mix.

2. Attach the PowerBead Pro tube horizontally to a vortex adapter for 1.5-2 ml tubes (Cat. no. 13000-V1-24). Vortex at maximum speed for 10 minutes. Nota: Se utilizar o Adaptador Vortex para mais de 12 preparações em simultâneo, aumente o tempo de vortex em 5-10 minutos.

3. Centrifuge the PowerBead Pro Tube at 15,000 x g for 1 min.

4. Transfer the supernatant to a clean 2 ml Microcentrifuge Tube (provided).

Note: 500-600 µl are expected. The supernatant may still contain some soil particles.

5. Add 200 µl of CD2 Solution and vortex for 5 s.

6. Centrifuge at 15,000 x g for 1 min at room temperature. Avoiding the pellet, transfer about 700 µl of supernatant to a clean 2 ml Microcentrifuge Tube (provided). Note: 500-600 µl are expected.

7. Add 600 µl of CD3 Solution and vortex for 5 s.

8. Place 650 µl of the lysate on an MB Spin Column and centrifuge at 15,000 x g for 1 min.

9. Discard the flow (what comes out of the MB Spin Column into the tube) and repeat step 8 to ensure that all of the lysate has passed through the MB Spin Column.

10. Carefully place the MB Spin Column into a clean 2 ml collection tube (provided). Avoid splashing any flux onto the MB Spin Column.

11. Add 500 µl of Solution EA to the MB Spin Column. Centrifuge at 15,000 x g for 1 minute.

12. Discard the flow and place the MB Spin Column back in the same 2 ml collection tube.

13. Add 500 µl of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x g for 1 minute.

14. Discard the flush and place the MB Spin Column into a new 2 ml collection tube (provided).

15. Centrifuge at up to 16,000 x g for 2 min. Carefully place the MB Spin Column into a new 1.5 ml Elution Tube (provided).

16. Add 50-100 µl of C6 solution to the center of the white filter membrane.

17. Centrifuge at 15,000 x g for 1 minute. Discard the MB centrifugation column. The DNA is now ready for further applications.

Note: We recommend storing the DNA frozen (-30 to -15°C or -90 to -65°C) because C6 solution does not contain EDTA. To concentrate the DNA, please refer to the Troubleshooting Guide.

Attachment 2

PCR conditions with primers ITS4 + ITS6

H2O	14,25µL
sNTP's	2,5 µL
MgCl2	2,5 µL
Primer ITS4	1,0 µL
Primer ITS6	1,0 µL
Buffer 10x	2,5 µL
DNA	1,0 µL
Taq	0,25 µL
Total	25 µL

Attachment 3

REDExtract-N-Amp™ Plant PCR Kit (protocol)

(https://www.sigmaaldrich.com/technical-documents/protocols/biology/redextract-nampplant-protocol.html)

Storage

The Extraction Solution, Dilution Solution and REDExtract-N-Amp PCR ReadyMix can be stored at 2-8 °C for short-term; –20 °C for long-term. Do not store in a "frost-free" freezer.

Procedure

All steps are carried out at room temperature unless otherwise noted.

A. DNA extraction

1. Rinse the paper punch and forceps in 70% ethanol prior to use and between handling different samples.
2. Punch a 0.5 to 0.7 cm disk of leaf tissue into a 2 ml collection tube using a standard one-hole paper punch. If frozen plant tissue is used, keep the leaves on ice while punching disks.
3. Add 100 µL of Extraction Solution to the collection tube. Close the tube and vortex briefly. Make sure the disk is covered by the Extraction Solution.
4. Incubate at 95 °C for 10 minutes. Note that leaf tissues usually do not appear to be degraded after this treatment.
5. Add 100 µL of Dilution Solution and vortex to mix.
6. Store the diluted leaf extract at 2-8 °C. It is not necessary to remove the leaf disk before storage.

B. PCR amplification

The REDExtract-N-Amp PCR ReadyMix contains JumpStart Taq antibody for specific hot start amplification. Therefore, PCR reactions can be assembled at room temperature without premature Taq DNA polymerase activity.

Typical final primer concentrations are ~0.4 µM each. The optimal primer concentration and cycling parameters will depend on the system being used.

1. Add the following reagents to a thin-walled PCR microcentrifuge tube:

Reagent	Volume
Water, PCR reagent	x µL
REDExtract-N-Amp PCR ReadyMix	10 µL
Forward primer	y µL
Reverse primer	y µL
Leaf disk extract	4 µL*
Total volume	20 µL

***Note:** The REDExtract-N-Amp PCR ReadyMix is formulated to compensate for components in the Extraction and Dilution Solutions. If less than 4 µL of leaf disk extract is added to the PCR reaction volume, use a 50:50 mixture of Extraction:Dilution Solutions to bring the volume of leaf disk extract up to 4 µL.

2. Mix gently and briefly centrifuge to collect all components at the bottom of the tube.
3. For thermal cyclers without a heated lid, add 20 µL of mineral oil to the top of each tube to prevent evaporation.
4. The amplification parameters should be optimized for individual primers, template, and thermal cycler.

Common cycling parameters:

Step	Temperature	Time	Cycles
Initial Denaturation	94 °C	3 min.	1
Denaturation	94 °C	0.5-1 min	30-35
Annealing	45 to 68 °C	0.5-1 min	
Extension	72 °C	1-2 min (~1 kb/min)	
Final Extension	72 °C	10 min.	1
Hold	4 °C	Indefinitely	

5. The amplified DNA can be loaded directly onto an agarose gel after the PCR is completed. It is not necessary to add a separate loading buffer/tracking dye.

Note: PCR products can be purified, if desired, for downstream applications, such as sequencing, with the **GenElute™ PCR Clean-Up Kit, Catalog No. NA1020**.