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Assessing *Phytophthora* Zoospore Activity to Enhance Disease Management and Promote Ecological Surveillance of Chestnut Ink Disease

M.E. Gouveia[a] and L.F. Nunes
CIMO Centro de Investigação de Montanha
Instituto Politécnico de Bragança
Campus de Santa Apolónia, Apartado 1172
530-855 Bragança
Portugal

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Abstract

***Phytophthora cinnamomi* and *P. cambivora* are soil borne *oomycetes* that cause Chestnut Ink Disease, a lethal and widespread disease of the European chestnut (*Castanea sativa* Mill.). Soil moisture is a key factor for the onset of *Phytophthora* root rot epidemics. Zoospores are the main infective propagules that reach the roots by swimming in liquid environments, become encysted and after that infect the host. Considering this biological uniqueness, we studied zoospore release and environmental conditions that promote zoospore production and host infection. Growing nursery media, previously infested with *P. cinnamomi*, were tested with different host plants (*Castanea sativa*, *Camellia japonica*, *Ilex aquifolium*) and different time-spans of flooding. Data analysis, made by nonparametric Kruskal-Wallis test and followed by multiple comparisons of mean ranks, found that infection of *P. cinnamomi* is significantly higher ($p<0.001$) on *C. sativa*. No significant differences were detected by a nonparametric two-way ANOVA analysis on studied environmental conditions. In natural soils, collected around the canopy of diseased chestnut trees, *Phytophthora* has a similar pattern of zoospore activity as on growing potting mix used as positive control. Variability between samples from the same tree was associated with physiographic and soil site conditions. This successful, simple and rapid methodology enables *Phytophthora* ecological surveillance and prompt implementation of sanitary management practices.**

INTRODUCTION

Decline and death of European chestnut has been reported in Portugal since the middle of the 19th century. At that time the disease was already present in all countries in Mediterranean and Central Europe. Chestnut Ink Disease (CID) is still actually a great threat for the European chestnut which invariably causes chestnut tree death. In Portugal, CID nearly eliminated the European chestnut in the NW and has reduced the chestnut area by half in the NE. *P. cinnamomi* Rands and *P. cambivora* (Petri) Buisman, are associated with CID in Portugal (Pimentel, 1947; Fernandes, 1966; Gouveia et al., 2005). *P. cambivora* has a limited number of host plants; chestnut and beech in Europe, maple in North America, and some root rot complexes of fruit trees (Erwin and Ribeiro, 1996). It is also geographically restricted to temperate regions. On the contrary, *P. cinnamomi* is considered as one of the most ubiquitous and destructive plant pathogens, the most widely distributed and the species with the largest host range (Zentmyer, 1980). Liquid environments are necessary for the formation of sporangia and zoospore discharge, dispersal and active swimming to reach the roots (Hardham, 2001, 2005; Van West et al., 2003; Walker and Van West, 2007; Jeger and Patausso, 2008). The primary role of zoospores is the transmission of the pathogen (Walker and Van West, 2007). Zoospores,

[a] egouveia@ipb.pt

which are single nucleated and wall-less cells, should not be considered a weak link in the life cycle of zoosporic plant pathogens. Zoospore genesis and zoospore dispersal are milestone events for the rapid spread of infective inoculum (Jeger and Pautasso, 2008) that promote multiple sites of infection which weaken the plant's defenses, even in strong roots of old chestnuts trees.

Soilborne *Phytophthora* are difficult to detect in decayed plant tissues (Tsao, 1990), the most frequent situation in root and collar rot diseases, and in naturally infected soils (Tsao, 1987, 1990). Detection was improved by means of specific bioassays which link the selective pathogenicity of *Phytophthora* species to the plant tissue used as bait. The efficiency of these techniques depends on the plant tissues used as bait as well as environmental conditions. Despite the developed new methods for diagnosis of soilborne *Phytophthora*, the bioassay is still a useful and necessary step in immunological and molecular diagnostic methods (Hardham, 2005). Spread of soilborne *Phytophthora* in the chestnut ecosystem can occur on a local scale by soil-splash, water movement and run-off in soil drainage. This work was developed to assess *Phytophthora* zoospore activity in soils of chestnut orchards for CID diagnosis and to obtain data on the potentially threatening events in order to implement early management measures.

MATERIAL AND METHODS

Phytophthora cinnamomi Zoosporic Activity

Inoculum of *P. cinnamomi* was prepared by growing the parasite on humidified vermiculite which was autoclaved for one hour (121°C, 1.5 atm) twice. Growing nursery medium (Kekkila Iberica) was infested with the inoculum of *P. cinnamomi*. The infested growing nursery medium was placed in pots and then three chestnut seedlings were planted in each pot. As soon as above ground symptoms of CID were evidenced on chestnut seedlings, the growing media was used for the assay. In the laboratory, sub-samples were flooded with sterile deionised water in the proportion of 1:4 (v/v) and baited with the host bait tissues. In the assay three host baits, European chestnut (*Castanea sativa*), camellia (*Camellia japonica*) and holly trees (*Ilex aquifolium*), were used. Positive detection of *P. cinnamomi* was evaluated, considering the effect of time-span of flooding (24, 48, 72 and 96 h) and the effect of incubation conditions. These included sterile deionised water or sterile deionised water amended with (50 $\mu g\ ml^{-1}$) penicillin and pimaricin. Normal laboratory conditions (temperature and light) and constant temperature (22-24°C) in the dark were tested. In each replication, 20 leaf discs with 5 mm diameter were placed to float in the container for each modality. Five discs, per host bait were removed from each modality and replication after 24, 48, 72 and 96 hours of the assay. Results were expressed in percentage of positive isolation (number of positive cases in five). The presence of *Phytophthora* was evaluated in the selective agar media (PDA, 39 g L^{-1} Difco®, amended ($\mu g\ ml^{-1}$) with pimaricin (10), Penicillin G sodium salt (250), pentachloronitrobenzene (100), Iprodiona (100) Rovral, Bayer Cropscience and hymexazol (50) Tachigarem, 36% (p/v) Comercial Química Massó SA). *P. cinnamomi* was then confirmed by microscopic observation of hyphal swellings and swollen vesicles that formed on the mycelium that grew into the selective agar media.

Zoosporic *Phytophthora* Activity in Natural Soils

Once the test conditions were established, soils were sampled in old chestnut orchards with symptoms of CID. Around the canopy of each tree, six samples of soil were taken at a distance of 1-1.5 m from the base of each selected tree. Individual samples were systematically taken considering physiographic and natural water flow and always beginning at the top. Soil samples were collected in September, kept in normal laboratory conditions and processed as promptly as possible. Each soil sample was carefully mixed and expanded into six sub-samples and tested for *Phytophthora*. Detection was considered positive when characteristic *Phytophthora* mycelium was identified from the chestnut leaf discs. As a positive control, infested *P. cinnamomi* growing nursery media

was included.

All statistical analysis was performed using 16.0 SPSS (SPSS Inc.). After initial explanatory data analysis, it was determined that nonparametric statistics were required since homogeneity of variance and normality of data was not met. Data of *Phytophthora* detection by host baits were analysed using the nonparametric Kruskal-Wallis test followed by multiple comparison of mean ranks at $\alpha = 0.05$ significance level. A two-way non parametrics ANOVA using the Scheirer-Ray-Hare technique (Sokal and Rohlf, 1995) was adopted to study the effects of environmental conditions, time span of bait and host bait on the percentage of the positive detection of *Phytophthora*.

RESULTS

Phytophthora cinnamomi Zoospore Activity

General analysis of data showed that after 24 hours of flooding, *P. cinnamomi* was detected in *C. sativa.* This revealed that zoosporogenesis occurred, the zoospores reached the host tissues and infection was established. On *C. japonica* and *I. aquifolium* the infection process was also detected but only after 96 hours of flooding. Positive detection of *Phytophthora* was higher on *C. sativa* in all studied conditions. Constant temperature, however, provided higher infection on *C. sativa* but more variability occurred during the time span of flooding. With *C. japonica*, there was an increase in *P. cinnamomi* detection while with *I. aquifolium* no positive detection was achieved. Biocides in the water also provided a slight increase in the positive isolations but a great variability of detection occurred during the flooding time, particularly at constant temperature. The presence of biocides in the water allowed an increase in positive detection of *P. cinnamomi* when *C. japonica* and *I. aquifolium* were used as bait tissue. This is rather obvious when they are used at room temperature (Fig. 1).

Statistical data analysis based on a two-way non parametrics ANOVA using the Scheirer-Ray-Hare technique (Sokal and Rohlf 1995) showed a significant host bait effect (X^2_{kw} (2) = 111.6; $p<0.0001$; N=192) on positive recovery of *Phytophthora*. Multiple comparison of means ranks (α level = 0.05) found a significantly higher positive isolation of *Phytophthora* by the host bait *C. sativa* (Fig. 2). Note that values of 0% of Phytophthora detection with *C. japonica* and *I. aquifolium* were frequently recorded. The symbol * in the box plot diagram shows values of positive isolation from *I. aquifolium* and *C. japonica*. After statistically verifying the effectiveness of *C. sativa* for positive isolation of *P. cinnamomi*, we studied the effect of incubation conditions, with 4 levels, and the time-span of baiting also with 4 levels (time-span) on the positive isolation of *Phytophthora* in *C. sativa*. A non-parametric two-factor ANOVA (Scheirer-Ray-Hare extension of the Kruskal-Wallis test on ranked data) was used (Sokal and Rohlf, 1995). Results by this statistical methodology found that the conditions of incubation (H=2.424, df=3, P=0.489) the time-span of baiting (H=3.860, df=3, P=0.277) and their interaction were not statistically significant.

Statistical analysis and laboratory conditions for the assay advise general room conditions to perform the test.

Phytophthora Zoospore Activity in Natural Soils

Reliability of the assay for detection of *Phytophthora* activity was performed on soils collected as multiple samples under the canopy of diseased CID trees. *Phytophthora* was detected in every, except one, tested soil of chestnut trees (Table 1). A great number of positive detections was obtained and a total of 265 *Phytophthora* isolates were found. Positive detection of *Phytophthora* varied among trees. For instance, all soil samples were positive in tree VN4, none was positive in soil samples of VN2 and only one sample was found positive in tree VN1. Great variability also occurred between sub-samples of the same soil sample. Sometimes all tests were positive in one sub-sample (*Phytophthora* was detected in all bait discs) and no positive isolation occurred in any other sub-sample of the same soil. Positive control for *Phytophthora* isolation was performed on infested pot mix

as previously described and 90% of baits detected positively for *Phytophthora*. During the isolation on selective agar media, some *Pythium* spp., *Sporothrix* spp. and bacteria species hinder *Phytophthora* detection. *Pythium* spp. and bacteria are frequently present, which not only hinder growth and identification of *Phytophthora* but also hamper purification of *Phytophthora* isolates.

Sampling of natural soils or growing media in the nursery is of great importance for positive detection. Soil borne *Phytophthora* are not uniformly distributed in natural soils or nursery substrates and biological activity of parasites can be different, which in turn can modify the detection ability of the test. Statistical analysis was performed considering sample site criterion and only considering the six trees with positive detection in this study. Kruskal-Wallis test showed that the sampling site had a significant effect on the number of positive isolates (X^2_{kw}=40.488 *df*=5 p<0.05; N=251). Multiple comparison of mean ranks found that sites 4 and 5 had a value of positive isolation significantly higher than the other sites (Fig. 3). Note that sampling points 3, 4 and 5 correspond to the downward slope and natural drainage flow of soil water. Soils in these sites should retain moisture longer, which provides more favorable conditions for the maintenance and survival of the parasite. Nevertheless, trees on a flat physiographic location (VN4, VN6) showed a generalized presence of *Phytophthora*, regardless of the sampling site.

DISCUSSION

Phytophthora is a notable genus in plant pathology. All known species, in terrestrial environments, are aggressive plant pathogens. They have narrow (*P. infestans*, *P. sojae*) or broad (*P. cinnamomi*, *P. ramorum*) host ranges that cause devastating diseases on numerous plant species, having an enormous impact on agriculture, forestry and natural ecosystems. *P. cinnamomi* and *P. cambivora* are specific parasites of roots and lower stem and their life cycle occurs entirely in the complex and competitive microbial below-ground ecosystems. They produce a wide range of reproductive spores (chlamydospores, sporangia, zoospores) during different life stages and under different environmental conditions (Zentmyer, 1980; Weste, 1987; Hardham, 2005). Zoospore formation is considered as one of the fastest developmental processes in any biological system. It occurs within minutes (Walker and Van West, 2007) and reveals the rapid capacity of *Phytophthora* to adapt to drastic environmental changes. As was stated by Jeger and Patausso (2008) zoospore behaviour is essential in order to understand and predict epidemic development.

In this study, zoosporic activity was measured by positive isolation of *P. cinnamomi* which indicated that all events for pathogenesis: sporangia formation, zoosporogenesis and discharge of zoospores, zoospores swimming to the host tissue, fixation, encystment, germ-tube elongation and colonization of host tissues, had occurred. *P. cinnamomi* was detected on C. sativa leaf tissue within 24 hours in all studied environmental conditions. With *C. japonica* and *I. aquifolium*, which are perennial species included in host list of *P. cinnamomi* (Zentmyer, 1980), infection was only detected after a 96 h time span of flooding, which emphasises the importance of the host used as bait for successful detection of specific *Phytophthora* species. The assay, when applied in a sequential and temporal order, can detect variations in pathogen activity and consequently establish the critical infection periods and the length of time during which physical conditions are conductive to infection.

This assay, which is a qualitative test, has a good performance on natural soils. Variability among samples of tested soils was detected, but in some cases the test performed just as well as in the positive control. Differences in detection capacity were associated with soil conditions and a more complex microbial environment in natural soils. The bioassay is a conclusive test when detection is achieved but, if *Phytophthora* is not detected, it does not confirm that the soil is free from the parasite. Negative detection may be read as being inefficient in culturing the parasite because of environmental conditions and/or the presence of fast growing soil microorganisms. Bacteria, *Pythium* spp. and *Sporothrix* spp. were the most frequent microorganisms that hampered

Phytophthora recovered from the performed test.

In chestnut ecosystems of Europe and based on baiting, Vettraino et al. (2005) found *P. citricola*, *P. cactorum* and *P. gonapodyides* from the rhizosphere of chestnut trees but their role on CID disease outbreak or severity is unknown. In forest ecosystems, recent *Phytophphora* surveys revealed the existence of a variety of *Phytophthora* species associated with tree crown decline. In a large spatial survey of forest soils on oak ecosystems in North America, Balci et al. (2007) found six different *Phytophthora* species which revealed a general pattern of a single species from a site and only a species from each soil sample. A more complex situation can be envisaged on fruit tree crops where more than one species are frequently reported in root rot *Phytophthora* diseases (Erwin and Ribeiro, 1996; Graham and Menje, 2000). The generalized detection of *Pythium* spp., the other genus of the oomycete *Phytheacea* family, could foresee a more complex and yet unknown oomycete microbial interaction in soil environment.

The reliability and simplicity of the bait technique will contribute to studying the epidemiology, pathways of dissemination and the identification of critical points as well as enhance disease management in chestnut groves and stands.

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Tables

Table 1. Positive detection of *Phytophthora* species from soil samples of seven trees of *Castanea sativa* with symptoms of Ink Disease.

Chestnut tree	VN1	VN2	VN3	VN4	VN5	VN6	VN7	Control*
Positive samples per tree	1 (6)	0 (6)	3 (6)	6 (6)	3 (6)	5 (6)	3 (6)	1** (1)
Positive sub-samples per tree	3 (36)	0 (36)	13 (36)	31 (36)	3 (36)	10 (36)	12 (36)	6** (6)
Positive tests per tree	15 (180)	0 (180)	30 (180)	125 (180)	12 (180)	45 (180)	38 (180)	27** (30)

Numbers in brackets are the maximum possible positive detections of *Phytophthora* per tree in each case.
*Growing nursery media infested with *Phytophthora cinnamomi*.
**Positive detection from growing nursery media infested with *Phytophthora cinnamomi*.

Figures

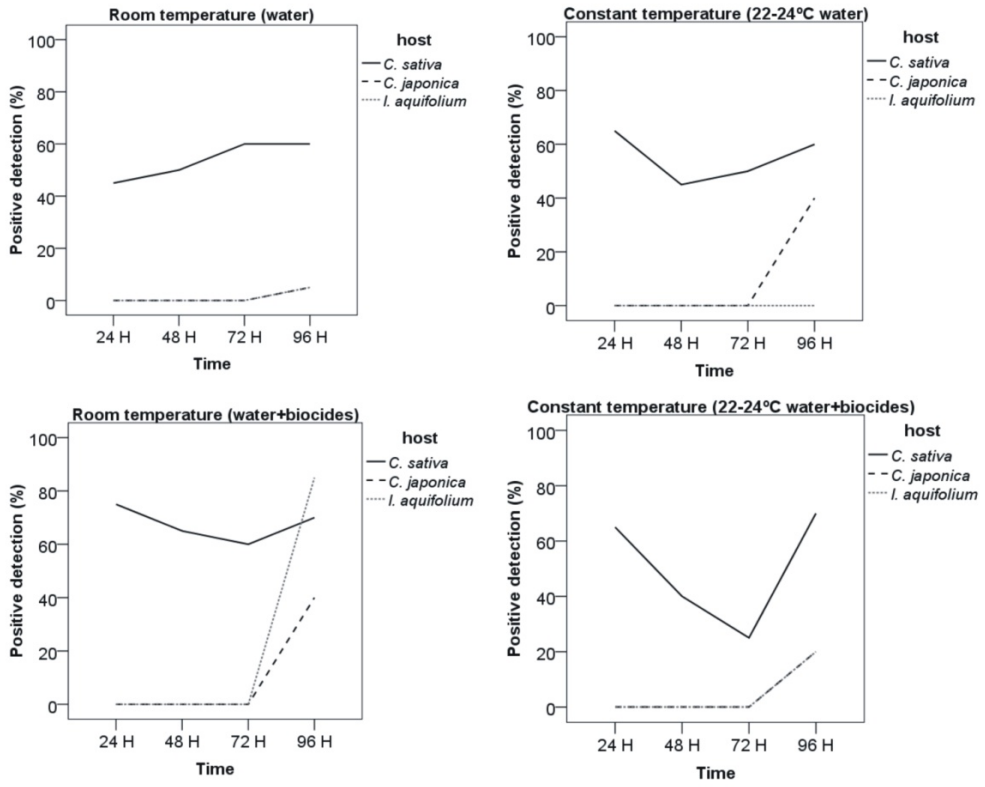

Fig. 1. Average positive detections of *Phytophthora cinnamomi* (%) by host baits on selective agar media in each environmental condition and time span of baiting (Time). Values represent the mean of four replicates.

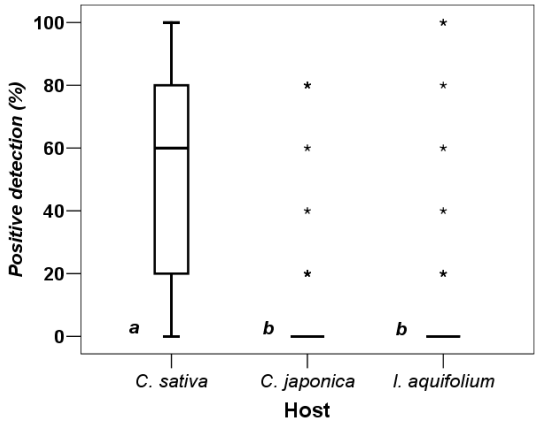

Fig. 2. Positive detection (%) of *Phytophthora cinnamomi* on *Castanea sativa*, *Camellia japonica* and *Illex aquifolium*. Cross-line in boxes is the median of positive detection. Different letters indicate significant (p<0.001) differences between host baits by the multiple comparison of mean ranks at α=0.05 significance level, after the Kruskal-Wallis test. Symbol * in the boxplot diagram are values of positive isolation (different from zero) from *I. aquifolium* and *C. japonica*.

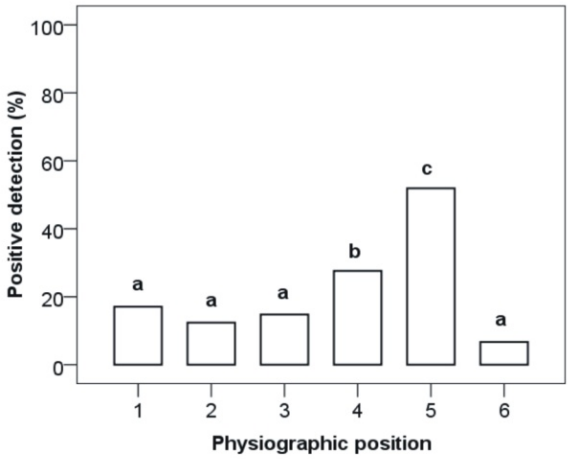

Fig. 3. Positive detection of *Phytophthora* (%) by C. sativa considering physiographic position (Kruskal-Wallis test followed by multiple comparisons of means ranks). Different letters in bars indicate significant differences (p<0.001) between physiographic sites. Point 3, 4 and 5 are the down slope, whereas points 1, 2 and 6 are the upper physiographic positions of each tree.