Silva Lusitana, ***29****(2): 115- 131, 2021*

Comparing Intraspecific Aggressiveness in *Phytophthora cinnamomi* Isolates

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Abstract. *Phytophthora cinnamomi* is a major plant destructive pathogen with a wide range of hosts and is considered one of the main causes of *Quercus* spp. decline in the Southwest of the Iberian Peninsula. This study compares two inoculation tests, using simple and fast methodologies, in order to select the most aggressive isolate to use in future *Quercus suber* inoculation experiments. To evaluate *P. cinnamomi* intraspecific variability, cork oak excised shoots were inoculated with five isolates, obtained from *Q. suber*, *Quercus rotundifolia* and *Castanea sativa* stands. These isolates were used for *in vitro* inoculation of a *Trifolium subterraneum* cultivar available in Portugal in order to assess its usefulness as a "model plant" in rapid screening tests. This study revealed intraspecific variability of *P. cinnamomi* isolates to cause lesions in *Q. suber* excised shoots and in the number of *T. subterraneum* developing lesions on stem and leaves. However, there was no correspondence between the aggressiveness of the isolates in subterranean clover and in the cork oak. Only one isolate showed consistency in aggressiveness regardless of the host species. In conclusion, *T. subterraneum* is not a good "model plant" for the evaluation of *P. cinnamomi* isolates aggressiveness.

Key-words: *Quercus suber*; *Trifolium subterraneum*; isolates screening; root rot disease

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Article available at https://silva-lusitana.edpsciences.org or https://doi.org/10.1051/silu/20212902115

Avaliação da Agressividade Intraespecífica em Isolados de *Phytophthora cinnamomi*

Sumário. *Phytophthora cinnamomi* é um importante agente fitopatogénico com uma ampla gama de hospedeiros e que é considerado uma das principais causas do declínio de *Quercus* spp. no Sudoeste da Península Ibérica. Este estudo compara dois testes de inoculação, usando metodologias simples e rápidas, de forma a selecionar o isolado mais agressivo para usar em futuros ensaios de inoculação de *Quercus suber*. Para avaliar a variabilidade intraespecífica de *P. cinnamomi*, rebentos excisados de sobreiro foram inoculados com cinco isolados, obtidos em povoamentos de *Q. suber*, *Quercus rotundifolia* e *Castanea sativa*. Uma cultivar de *Trifolium subterraneum* usada em Portugal foi inoculada *in vitro* com os mesmos isolados de *P. cinnamomi* para avaliar sua utilidade como "planta modelo " numa triagem rápida. Este estudo revelou variabilidade intraespecífica entre os isolados de *P. cinnamomi* testados para causar lesões em rebentos excisados de *Q. suber* e no número de lesões desenvolvidas no caule e folhas de *T. subterraneum*. No entanto, não houve correspondência entre a agressividade dos isolados no trevo subterrâneo e no sobreiro. Apenas um isolado apresentou consistência na agressividade independentemente da espécie hospedeira. Em conclusão, *T. subterraneum* não é útil como "planta modelo" para a avaliação da agressividade de isolados de *P. cinnamomi*.

Palavras-chave: *Quercus suber*; *Trifolium subterraneum*; seleção de isolados; declínio do montado

Comparaison de L'agressivité Intraspécifique des Isolats de *Phytophthora cinnamomi*

Résumé: *Phytophthora cinnamomi* est un important pathogène des plantes avec un large nombre d'hôtes et est considérée comme l'une des principales causes du dépérissement de *Quercus* spp. au Sud-Ouest de la Péninsule Ibérique. Dans cette étude on a utilisé des méthodologies simples et rapides pour comparer deux tests d'inoculation avec l'objectif de sélectionner l'isolat le plus agressif pour utiliser dans futures testes d'inoculation de *Quercus suber*.

Pour évaluer la variabilité intraspécifique de *P. cinnamomi* chez *Q. suber*, des pousses excisées de chêne-liège ont été inoculées avec cinq isolats obtenus en *Q. suber*, *Q. rotundifolia* et *Castanea sativa*. Un cultivar de *Trifolium subterraneum* commercialisée au Portugal a également été soumis à inoculation similaire.

L'étude a confirmé la variabilité intraspécifique des isolats de *P. cinnamomi* concernant l'agressivité chez *Q. suber* et *T. subterraneum*. Cependant, aucune correspondance n'a été démontrée entre l'agressivité des isolats en *T. subterraneum* et en *Q. suber*.

Un seul isolat a montré une agressivité constante indépendante de l'hôte. Donc *T. subterraneum* n'est pas utile comme « plante modèle » pour évaluer l'agressivité de *P. cinnamomi*.

Mots-clés: *Quercus suber*; *Trifolium subterraneum*; sélection d'isolats; maladie de la pourriture des racines

Introduction

Cork oak (*Quercus suber* L.) is associated with agro-silvo-pastoral systems, being of high importance due to its potential multifunctionality. Although this tree is worldwide known due to cork production once over one third of the world's total area of cork oak stands is located in Portugal (736 775 ha) (APCOR, 2016) it is also associated with a wide range of activities, such as forage and pasture crops production, livestock production and hunting or even leisure activities. Derived from these multiple activities, the cork oak stand, known as "montado" in Portugal and as "dehesa" in Spain, also has a social impact, contributing to the employment of thousands of people. It contributes to soil conservation and improvement of water infiltration and storage, favours carbon sequestration and may constitute a fire barrier, becoming a key element in preserving life biodiversity and improving the quality of life of human populations (ARONSON *et al.*, 2009; COELHO *et al*., 2012).

From 1980's onwards, an increasing number of dead or declining trees have been registered (CABRAL *et al*., 1992). This oak decline seems to result from an interaction of several factors, yet since the 90's several authors (BRASIER *et al*., 1993; BRASIER, 1996; BRASIER, 1999; MOREIRA and MARTINS, 2005; TAPIAS *et al*., 2006; CAMILO-ALVES *et al*., 2013) have been associating *Phytophthora cinnamomi* Rands with the high mortality of cork oaks, the former being considered one of the main causes of *Quercus* spp. decline in the Southwest of the Iberian Peninsula and may in the future even threaten these species survival.

The genus *Phytophthora* comprises over 100 species and contains major plant destructive pathogens. Most species survive in soil for long periods, even in the absence of hosts, and affect parts of the plant in contact with soil, thus inducing root system destruction (ERWIN and RIBEIRO, 1996; AGRIOS, 2005; THINES, 2013). Although the first symptoms are difficult to detect, fine roots rotting is considered a primary symptom, with consequent interference in transpiration and, plant vigour (chlorotic leaves and leaf fall) (HARDHAM, 2005). *P. cinnamomi* often causes stem cankers, exudations and necrosis and young shoots dieback. Fine roots rotting may lead to the host death, by preventing the absorption of water and nutrients (BRASIER *et al.*, 1993).

Phytophthora cinnamomi has a wide range of hosts beyond cork oak, infecting close to 5 000 species of plants worldwide (HARDHAM and BLACKMAN, 2018) including fruit species such as avocado (*Persea americana* Miller) in Latin America, as well as woody species, such as eucalyptus (*Eucalyptus* spp.) in Australia and chestnut in Italy, Spain and Portugal (BRASIER, 1999; HARDHAM

and BLACKMAN, 2018). It is complex and difficult to control due to its high survival capacity under adverse conditions and to rapid dispersion and infection capacity (ERWIN and RIBEIRO, 1996; CAMILO-ALVES *et al.*, 2013). It has a wide geographic distribution among 80 countries, including the United States, the Mediterranean region and some Central and Northern European countries such as Germany, Netherlands, United Kingdom and Russia (EPPO, 2020).

The intraspecific variability in *P. cinnamomi* has been recognized for decades in different host species (ZENTMYER and GUILLEMET, 1981; DUDZINSKI *et al.*, 1993; ROBIN and DESPREZ-LOUSTAU, 1998; LINDE *et al.*, 1999). A significant effect among *P. cinnamomi* isolates was demonstrated when comparing the lesion length of *Q. suber* and *Q. ilex* L. taproots with 10-cm-length (ROBIN *et al.*, 1998).

Results reported by ROBIN and DESPREZ-LOUSTAU (1998) comparing *P. cinnamomi* isolates in soil contamination tests *versus* stem and bark inoculation, of chestnut and different oak species, led to the election of the excised shoots inoculation method in cork oak tests.

In order to carry out root inoculation tests using a fast growing species and considering that *Trifolium* spp. are reported to be susceptible to *Phytophthora* spp. (GREENHALGH and TAYLOR, 1985; YOU and BARBETTI, 2017) we chose *Trifolium subterraneum* L. as a possible model plant species for *in vitro* inoculation. However, we took into account the preliminary results of MORALES-RODRIGUEZ *et al.* (2013), under undetailed publishing, which refers to three cultivars of *T. subterraneum* resistant to *P. cinnamomi*.

This study aims to evaluate the aggressiveness of different *P. cinnamomi* isolates in cork oak and in subterranean clover (*T. subterraneum*), using two simple and fast methodologies, with the objective of selecting the most aggressive isolate for use in future *Q. suber* inoculation experiments. At the same time, the possibility of using subterranean clover as a model plant for the selection of the most virulent isolates was evaluated.

Materials and Methods

Phytophthora cinnamomi isolates

Five *P. cinnamomi* isolates were used in this study (Table 1). Pure cultures were maintained in Difco™ potato dextrose agar (PDA) at 24 °C.

The mycelial growth was evaluated in two culture media, PDA and

Murashige and Skoog medium (MMS, MURASHIGE and SKOOG, 1962), using five replications per isolate. Mycelium plugs (5-mm-diameter) from the edges of 3-day-old colonies were placed at the centre of 90-mm-diameter Petri dishes and incubated at 24°C in darkness. The mycelium growth of isolates was evaluated in two perpendicular colony diameters every day during 7 days.

Table 1 - List of *Phytophthora cinnamomi* isolates

***P. cinnamomi* isolate**	**Year of isolation**	**Host**	**Location**	**Accession Sequence ID***
PH107 (CS)	1990	*Castanea sativa* L.	From UTAD - IMI 340340 (Portugal)	OL901253
PH194 (QS)	2010	*Quercus suber* L.	Setúbal (Portugal)	OL901222
PH98_13 (QS)	2013	*Quercus suber* L.	Coruche (Portugal)	OL901221
PH1247 (QS)	2012	*Quercus suber* L.	Montemor-o-Novo (Portugal)	OL901223
PH4005 (QR)	2017	*Quercus rotundifolia* Lam.	Barrancos (Portugal)	OL901224

* Accession Sequence ID in GenBank.

Excised shoots inoculation test

Season shoots were collected on March 21, 2018, from an apparently healthy adult cork oak tree (*Q. suber* L.) located in a "montado" stand next to Herdade Experimental da Fataca, Southern region of Portugal. Budburst had not happened yet and buds were completely enclosed on protective scales.

After collection, 10 cm-length excised shoots with one to three leaves were prepared (Figure 1). Thirty shoots were used for each *Phytophthora* isolate (Table 1) and for the control. The shoots were randomly distributed between three blocks.

A 5-mm-diameter mycelial plug (Figure 2A) was placed on the top of each shoot, with the mycelium in contact with the plant material. To obtain a humidity chamber-like environment and to prevent the excised shoots from drying, tops were covered with wet cotton and aluminium foil (Figure 2B). Shoots were placed in boxes with moist vermiculite (Figure 2C). To maintain a humid atmosphere and prevent shoots dehydration, the boxes were placed in transparent plastic bags and remained for two weeks in a constant temperature chamber at 24°C±1°C with natural light.

Figure 1 - Illustration of an excised shoot from *Q. suber*.

Two weeks after inoculation the excised shoots were sliced longitudinally and lesions length were measured. Analysis of *P. cinnamomi* isolates aggressiveness was based on quantification of lesion length.

Figure 2 - *Quercus suber* excised shoots test: preparation of mycelial plug (A); shoot covered with wet cotton after inoculation (B); container with moist vermiculite covered with plastic bag to maintain a humid atmosphere (C)

In vitro inoculation of T. subterraneum

Subterranean clover cultivar Claire commercial seeds were obtained in *Fertiprado* (Monforte, Portugal). Seeds were disinfected for 10 min with a 5% (w/v) calcium hypochlorite solution. Seeds pre-germination occurred for two days on agar medium (7 g L^{-1} agar and 1 g L^{-1} glucose) at 26°C±2°C, in the dark. When radicles reached 2 cm in length, plantlets were placed in MMS medium.

Four 150-mm-diameter Petri dishes with ten plantlets each were used for each *Phytophthora* isolate and control modality (without pathogen inoculation). Pre-germinated seeds were positioned according to a 90-mm-diameter circle

with the radicles placed towards the centre (Figure 3A). Petri dishes remained seven days in a bioclimatic chamber with a mean temperature of 23°C±2°C and 16h of light. After, mycelial 5-mm-diameter plugs of *P. cinnamomi* isolates collected from the margins of a 7-day-old culture were placed next to the primary root apex of each plantlet (Figure 3B). The number of stems showing symptoms on leaves (Figure 3C) was recorded at day six, seven, eleven and twelve after inoculation.

At the end of the experiment, root, stem and leaf portions from inoculated subterranean clover were fixed in formaldehyde - acetic acid - ethanol (1:1:18 v:v:v) and dehydration was done through a progressive ethanol series. Plant material was clarified using *Histo-Clear* and then embedded in paraffin. The embedded samples were sectioned with a *Leika RM2255* rotary microtome using a 10 µm thickness. Transverse sections were stained with *AstraBlue*. Slides were observed under a *Olympus BX51* microscope and image acquisition was performed with *Olympus DP-Soft* software.

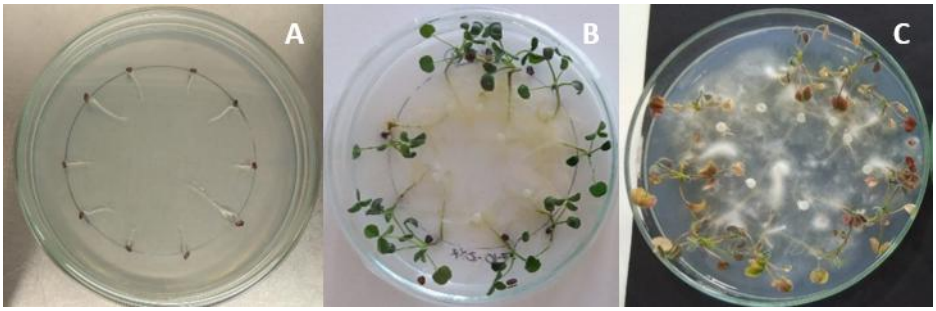


Figure 3 - Sequence of the *T. subterraneum* test: aspect of germinated seed radicles directed towards the centre (A), aspect of seedlings after inoculation with *P. cinnamomi* (B), and final aspect 12 days after inoculation (C).

Statistical analyses

Data from *T. subterraneum* experiment were subjected to a nested analysis of variance, with the Petri dishes nested within isolates. Data from *Q. suber* excised shoots experiment was analysed using a two-way ANOVA, using isolate and block as factors. Tuckey tests were used for multiple comparisons of the means. Data were analysed in R (R Core Team, 2018) with RStudio software (RStudio Team, 2018) and the level of significance applied was always of 95% ($P<0.05$).

Results

Phytophthora cinnamomi isolates

Phytophthora grew well in both PDA and MMS, tough the growth rate was higher in the PDA medium. Total radial growth at day seven of *P. cinnamomi* isolates on both media are presented in Figure 4.

Isolates PH1247 and PH4005 demonstrated the highest radial growth while isolate PH98_13 had the lowest growth at both media used. In MMS medium the growth of isolate PH107 is close to isolates PH1247 and PH4005.

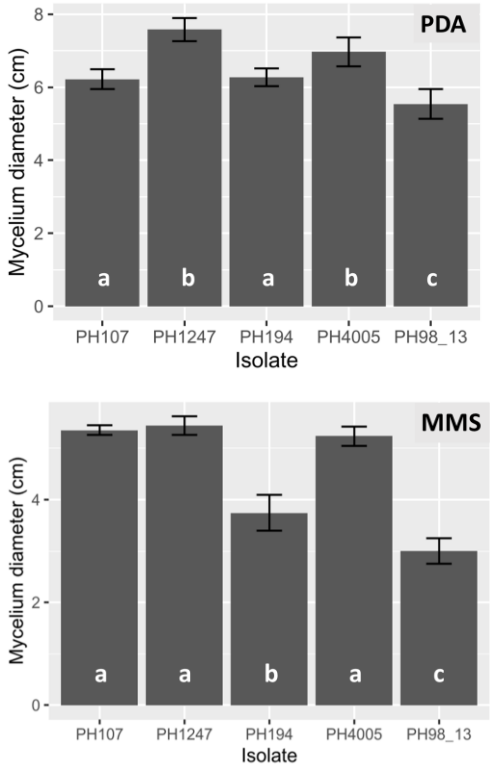


Figure 4 - Means of mycelium diameters of isolates PH107, PH1247, PH194, PH4005, PH98_13 (n=5) and respective standard deviation, at day seven in PDA medium (PDA) and in MMS medium (MMS). Different letters indicate significant differences ($p<0.05$)

Excised shoots inoculation test

Lesion length was the variable selected to analyse the aggressiveness of *P. cinnamomi* isolates in cork oak excised shoots. This variable was analysed two weeks after inoculation.

Inoculated excised shoots had darker and drier brownish areas (Figure 5A) whereas in control shoots the tissues remained relatively green (Figure 5B), exhibiting only dehydration at the top. There were significant differences between the control and the five isolates and a significant effect of the isolates in lesion length (Figure 6).

Isolates PH98_13 (3.96 cm), PH107 (3.64 cm) and PH194 (3.15 cm) led to lesions lengths greater than 3 cm, not differing significantly between them. These isolates showed similar aggressiveness to *Q. suber* excised shoots.

Isolates PH98_13 and PH107 caused significantly higher lesions length than isolates PH1247 and PH4005. Isolate PH4005 led to the smallest lesion length (2.15 cm), but it didn't differ significantly from PH1247 (2.42 cm).

Figure 5 - Longitudinal lesion in *Q. suber* shoots: inoculated with *P. cinnamomi* isolate PH194 (A) and non-inoculated (B)

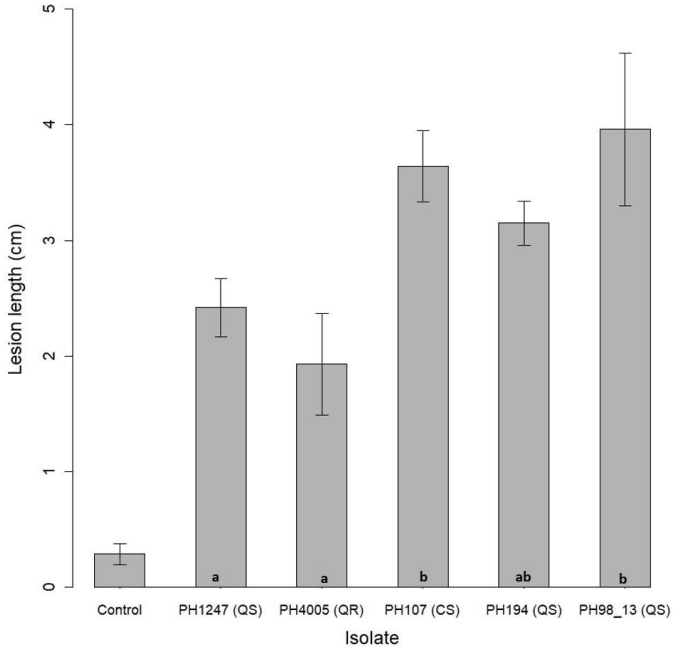


Figure 6 - Lesion length average caused by the five *P. cinnamomi* isolates under study (PH1247, PH4005, PH107, PH194 and PH98_13) on *Q. suber* excised shoots 14 days after inoculation and on control. Bars represent standard errors of the means. Significant differences between means have different letters ($P<0.05$) (n=30)

In vitro inoculation of T. subterraneum

At the first registration day (six days after inoculation), all *P. cinnamomi* isolates caused damages in subterranean clover, such as yellowing and wilting, while no symptoms were observed in control plants (Figure 7).

The presence of *P. cinnamomi* hyphae was confirmed in histological sections of root, stem and leaf from an inoculated plant, associated with degradation of cell walls and cells collapse (Figure 8).

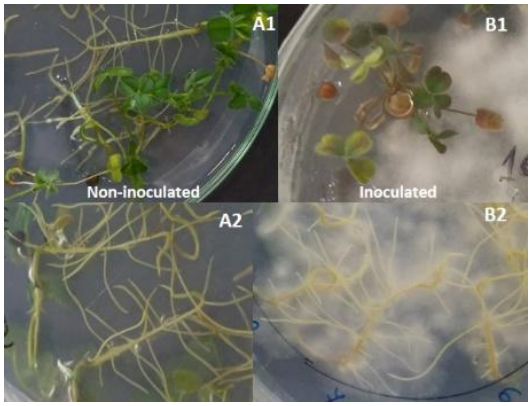


Figure 7 - Aerial part (A1) and roots (A2) of non-inoculated clover (control) and aerial part (B1) and roots (B2) of clover 7 days after inoculation with *P. cinnamomi*

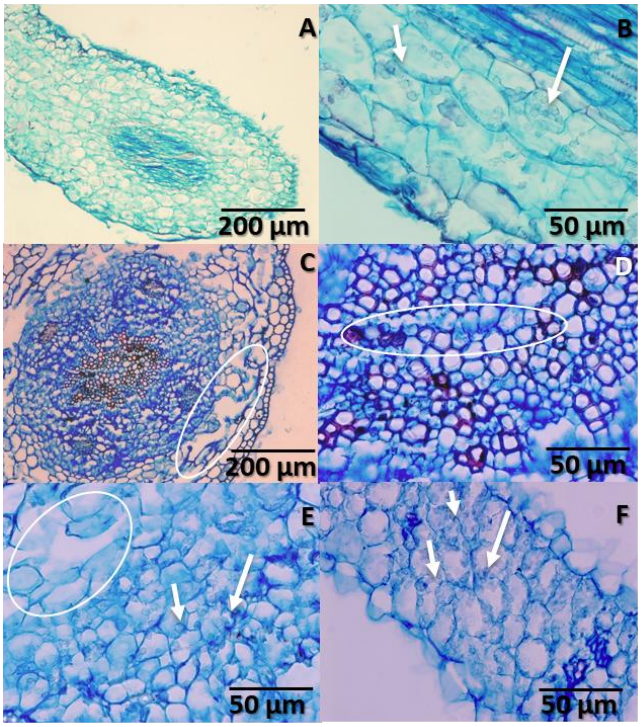
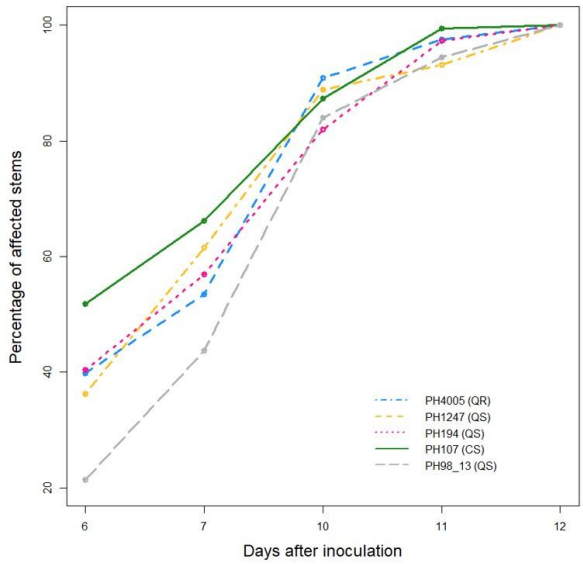


Figure 8 - Cross sections of root, stem and leaf from *P. cinnamomi* inoculated clovers with external symptoms seven days after inoculation: A - Root general structure; B- Hyphal aggregations in roots cells; C- Stem general structure; D, E- Cell degradation on stem; F- Hyphal aggregations on leaf cells. Arrows point to hyphae and circles surround damaged areas

Figure 9A shows the evolution of stems with chlorotic or necrotic leaves over time following inoculation, for each one of the five *P. cinnamomi* isolates. At day twelve after inoculation all isolates induced 100% of affected stems at all plantlets.

Results of percentage of stems with chlorotic or necrotic leaves at day 6 and 7 after inoculation are presented in Figure 9B. At day six after inoculation isolate PH107 caused the maximum percentage of lesions (51.75%), differing significantly from the other isolates. Isolate PH98_13 showed the lowest aggressiveness, with 21.33% of lesions, differing significantly from isolates PH1247, PH194, PH107 and PH4005. There were no significant differences among isolates PH194 (40.33%), PH4005 (39.75%) and PH1247 (36.25%).

A



B

Days after inoculation \ Isolate	Percentage of stems with lesions				
	PH1247	**PH4005**	**PH107**	**PH194**	**PH98_13**
6	36.25% ± 3.14 **a**	39.78% ± 3.27 **a**	51.75% ± 2.31 **b**	40.33% ± 2.41 **a**	21.33% ± 2.37 **c**
7	61.50% ± 3.83 **ab**	53.50% ± 3.26 **bc**	66.21% ± 3.1 **a**	56.92% ± 3.22 **ab**	43.67% ± 3.24 **c**

Figure 9 - Evolution of stem with chlorotic or necrotic leaves in *T. subterraneum*, previously inoculated with *P. cinnamomi* isolates under study (PH107, PH194, PH1247, PH4005 and PH98_13 at days 6, 7, 10, 11 and 12 after inoculation (n=40) (A). Means and respective standard errors of the percentage of stems affected at days 6 and 7 (B). Different letters at each day (after inoculation) indicate significant differences (P<0.05)

As on day six after inoculation, at day seven isolate PH107 (66.21%) led to the highest value of lesions and isolate PH98_13 (43.67%) to the lowest damage in the aerial parts. There were significant differences between PH98_13 (lowest damages) and isolates PH107, PH1247 and PH194, but there were no significant differences between PH107 and PH1247 (61.50%), PH194 (56.92%). This indicates that the aggressiveness of isolates PH107, PH1247 and PH194 may be similar in *T. subterraneum*.

Considering the significant differences between PH98_13 and the remaining isolates, this isolate was the least aggressive to *T. subterraneum* of the five isolates.

Comparing the aggressiveness of isolates in the two tests, it seems that there is no correspondence between the aggressiveness of isolates in the cork oak excised shoots and in the subterranean clover experiments (Figure 10).

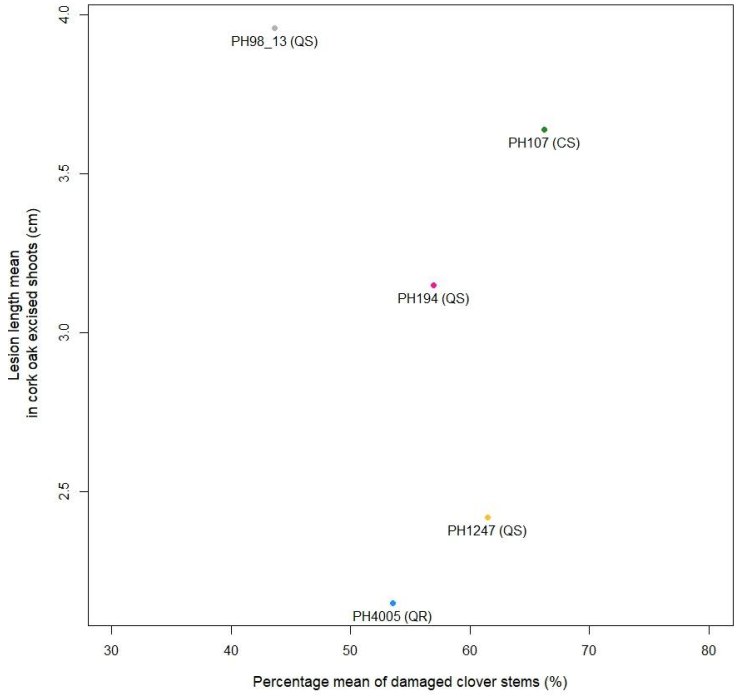


Figure 10 - Comparison of lesion length means in *Q. suber* shoots 14 days after inoculation with the percentage means of damaged clover stems in day seven after inoculation, for *P. cinnamomi* isolates under study (PH107, PH194, PH1247, PH4005 and PH98_13)

Discussion

This study was setup to evaluate the aggressiveness of different *P. cinnamomi* isolates to cork oak and subterranean clover, comparing simple and fast methodologies and evaluate the use of *T. subterraneum* as a plant model for future screening of the most virulent isolates.

In *Q. suber* excised shoots experiment, and despite the use of only one genotype (so genetic variability could be excluded), the variability within isolates may be attributed to anatomic differences in shoots tissues, since it was noticed that the presence of knots slowed *Phytophthora* progression conducting to smaller lesions or lesions deviation.

There were significant aggressiveness differences among *P. cinnamomi* isolates in both experiments. Intraspecific variability in *P. cinnamomi* has been previously confirmed by several authors (ZENTMYER and GUILLEMET, 1981; DUDZINSKI *et al.*, 1993; LINDE *et al.*, 1999) and even Rands, its classifier, mentioned this fact as early as in 1922 (ZENTMYER and GUILLEMET, 1981). ROBIN and DESPREZ-LOUSTAU (1998), who evaluated the aggressiveness of forty-eight *P. cinnamomi* isolates in *Castanea sativa* Miller, *Quercus rubra* L., *Pinus pinaster* Ait. and *Eucalyptus gunnii* Hook. f., concluded that all isolates were pathogenic, but with several levels of aggressiveness. ROBIN *et al*. (1998) also confirmed that twenty one isolates obtained from soil and plants collected in declining oak stands showed variable aggressiveness.

This work demonstrates the aggressiveness of *P. cinnamomi*, independently of the isolate origin (host). Isolate PH107, isolated from *C. sativa* and already tested in chestnut (SANTOS, 2017), proved and confirmed, in our study, its aggressiveness for both *T. subterraneum* and *Q. suber.* Thus, the high aggressiveness of the isolate PH107 in these three species, confirms its potential use as a positive control. Once the relative stability on pathogenicity among isolates independently of the host species was also suggested by DUDZINSKI *et al.* (1993) and ROBIN and DESPREZ-LOUSTAU (1998) we also suggest that isolate PH107 should continue to be used as a reference in future aggressiveness tests.

No correspondence was found between the aggressiveness of isolates in the cork oak excised shoots and in the subterranean clover experiments. The different tissues and structures (branches/root) of both species, one woody and the other herbaceous, could partly explain the discrepancy of results obtained in the two tests. Additionally, the choice of different registration periodicities, based on the different tissues, may have had some influence in our conclusions.

The different anatomy and composition of excised shoots and roots can also influence the isolates progress. Therefore, a new test should be carried out comparing the aggressiveness of the different isolates in cork oak seedlings, ensuring that the entry of the pathogen takes place by the roots, as most commonly occurs in *Phytophthora* spp.

We found a relation between growth (*in vitro*) and aggressiveness levels, as isolates PH107 and PH1247 had the major growths on MMS medium and led to the highest lesion values (on day seven), while isolate PH 98_13 had the minor growth on MMS and caused the lowest lesion values in *T. subterraneum* test. A similar conclusion has already been observed by LINDE *et al*. (1999) reporting a positive correlation between *in vitro* growth and aggressiveness levels.

We may also conclude that *P. cinnamomi* inoculation by stem incision may be a good method to assess its pathogenicity to *Quercus*, already suggested by ROBIN and DESPREZ-LOUSTAU (1998), and better than using *T. subterraneum* as host. The same authors reported similar results when the inoculum is placed in the stem incision and with inoculated substrate.

This study confirmed the intraspecific variability of *P. cinnamomi* isolates regarding aggressiveness in *Q. suber* and *T. subterraneum*. However, the choice of *T. subterraneum* as "plant model" is not the most appropriate way to select the most aggressive isolates to *Q. suber*. A range of *T. subterraneum* cultivars selected among the most commonly used in agriculture should be tested to better clarify its resistance to *P. cinnamomi*.

Acknowledgments

This work was supported by national funds from FCT - Fundação para a Ciência e Tecnologia through the research units UID/AGR/04129/2013 (LEAF), UIDB/04551/2020 and UIDP/04551/2020 (GREEN-IT - Bioresources for Sustainability).

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