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RESEARCH PAPERS

Experimental minimum threshold for *Phytophthora cinnamomi* root disease expression on *Quercus suber*

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Summary. *Quercus suber* seedlings were potted in soils infested with increasing concentrations of *Phytophthora cinnamomi* chlamydospores and submitted to weekly flooding for 3 months to favour root infections. Increasing quantities of chlamydospores led to an exponential increase in their ability to germinate. Root symptoms (necrosis and/or absence of feeder roots) were significantly more severe than those recorded in uninfested soil only for plants potted in soils infested with 61 cfu g^{-1} or more. Although generated using potting mix, this minimum threshold represents a tool for checking the potential infectivity of infested soils or to assess the effectiveness of some control methods to reduce soil inoculum. However, a low level of root infection was recorded even at 3 cfu g^{-1}. Therefore, long-term disease risk may be present whenever the pathogen is detectable in oak forest soils.

Key words: chlamydospores, cork oak, infection, inoculum.

Introduction

Quercus is a genus especially threatened by *Phytophthora cinnamomi* in areas of Mediterranean climate, including Spain (Sánchez *et al*., 2002), California (Garbelotto *et al.*, 2006) and Italy (Scanu *et al*., 2013). Highly variable *P. cinnamomi* soil inoculum densities have been reported for diseased oak ecosystems in southern Spain, ranging from 4–49 colony forming units (cfu) g^{-1} dry soil (Romero *et al.*, 2007) to 25-2500 cfu g^{-1} (Gómez-Aparicio *et al*., 2012). Knowledge of the minimum threshold of *P. cinnamomi* inoculum provides an experimental reference point to check the potential infectivity of infested soils or to assess the effectiveness of control methods aimed at reducing soil inoculum (green or mineral amendments, biofumigation). The aim of the research reported here was to determine the threshold of inoculum necessary to cause root disease in cork oak plants under highly favourable experimental conditions for disease development.

Materials and methods

Experiment 1

Two *P. cinnamomi* isolates (PE90 from holm oak, and PA25 from cork oak) were used to obtain inocula at different concentrations. The mother inoculum was prepared following Sánchez *et al*. (2002) and consisted of a water suspension of chlamydospores from both isolates adjusted to 1.5×10^4 chlamydospores mL^{-1} (Romero *et al.*, 2007). Additional inocula of 1500, 150 and 15 chlamydospores mL^{-1} were prepared by successive water dilutions of the original suspension. Four containers, each with 30 L of soil (sand-peat 1:1 vol., 22.5 kg, pH = 6.36) were homogeneously infested with 1 L of each inoculum suspension and a fifth container (control) was prepared by adding 1 L of water (0 concentration). Three 15 mL-soil samples per container (replicates), were plated on NARPH medium as described in Romero *et al.*

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(2007). Individual *Phytophthora cinnamomi* colonies, each derived from at least one viable chlamydospore, were identified and counted. After sampling, each soil was distributed into ten free-draining 3 L capacity plastic pots and 18 month-old *Q. suber* seedlings (replicates) were planted (one per pot) after removing most soil without damaging them. The pots were each placed in a plastic tray without drainage (57 × 41 × 9 cm) and placed in an air-conditioned greenhouse (daily cycle of 25 ± 2°C for 12 h and 10 ± 2°C for 12 h) in a randomized block design. For 2 d a week for the next 3 months the trays were partially filled with tap water, to periodically flood the soil (Serrano *et al.*, 2012). After this time, root rot symptoms were assessed according to the percentage of root necrosis or root absence on a 0–4 scale (0 = 0% necrotic roots, 1 = 10–33%, 2 = 34–66%, 3 = more than 67% necrotic roots, 4 = 100% dead root) (Serrano *et al.*, 2012).

Experiment 2

To narrow the concentration range of inoculum necessary to cause significant root disease, a new mother inoculum suspension was prepared as described above, adjusted to 3×10^3 chlamydospores mL^{-1} and diluted with water to obtain inocula containing 2×10^3, 1.5×10^3, 10^3, 500 or 50 chlamydospores mL^{-1}. Soil mix (above) was infested and processed as described above, and six 18 months-old *Q. suber* seedlings (replicates) were individually potted, incubated, harvested and assessed, as described above.

At the end of both experiments, root segments from plants potted in infested or control soils were plated on NARPH medium for re-isolation of the pathogen.

Data analyses

Inoculum concentration data were transformed to $[(\text{cfu } g^{-1}) + 0.5]^{1/2}$ for ANOVA analysis. A regression curve was performed with data from Experiment 1, to establish the relationship between the amount of chlamydospores added to the soils and the number of viable chlamydospores recovered. Data obtained from root symptom assessments were tested for homocedasticity by the Bartlett's test, and when heterogeneity was detected, angular (Experiment 1) or logarithmic (Experiment 2) transformations were applied to the data. ANOVA was performed for root symptoms and mean values compared by the Tukey's HSD test at $P<0.05$. Statistix 8.0 (Analytical Software) was used for data analyses.

Results and discussion

In Experiment 1, significant (DF = 5, F = 33.67, $P<0.0001$) differences in viable chlamydospores (cfu g^{-1}) were detected depending on the chlamydospore concentration added to the soils, following an exponential relationship: $y = 0.0416\ e^{2.0893x}$ ($R^2 = 0.9393$) (Figure 1). Increasing quantities of chlamydospores in soil led to an exponential increase in their ability to germinate, while for other soilborne pathogens, such as *Fusarium oxysporum* f. sp. *lini*, chlamydospore viability suffers a slight decrease at high initial chlamydospore densities (Couteaudier and Alabouvette, 1990). Only root symptoms recorded in plants growing in soils infested with 1.5×10^4 or 1500 chlamydospores mL^{-1} (256.5 and 54.7 cfu g^{-1}) were significantly (DF = 4, F = 6.29, $P<0.0001$) more severe than those recorded for plants potted in soils infested with the two lowest chlamydospore concentrations and the control soil (Figure 2). Because of the constraints imposed by the pots and by the frequent flooding, all the experimental controls developed low levels of root necrosis; hence such controls need to be included for a correct determination of disease symptoms.

Average numbers of viable chlamydospores obtained from infested soils in Experiment 2 fitted well with the quantities expected according to the relationship presented in in Figure 1. This resulted (on average, and respectively) in 3, 21, 41, 61 and 82 cfu g^{-1} from the initial 50, 500, 10^3, 1.5×10^3, and 2×10^3 chlamydospores mL^{-1} applied. Root symptoms of seedlings potted in soils infested with 61 or 82 cfu g^{-1} were significantly greater (DF = 5, F = 9.48, P=0.0001) than those recorded for plants potted in control soil, while lowest concentrations of viable inoculum did not cause disease (Figure 2). This threshold of 61 cfu g^{-1} was the minimum required to cause cork oak root disease in the highly favourable conditions artificially provided in this experiment. This value is similar to that obtained for *P. capsici*, which requires 41 oospores g^{-1} to produce 50% mortality in pepper plants (Bowers and Mitchell, 1991).

Phytopthora cinnamomi was re-isolated from necrotic roots of plants potted in soil infested with low inoculum concentrations (2.7 cfu g^{-1} in Experiment 1,

or 3, 21 and 41 cfu g^{-1} in Experiment 2), resulting in 14–39% of positive isolations. The pathogen was never recovered from control roots or from soil infested with 0.7 cfu g^{-1} (Experiment 1). These results indicate that infections caused by these low amounts of inoculum lack the ability to progress into significant root mortality. It is possible, however, that in time, these lesions may lead to significant symptoms or new infections. Mitchell (1978) concluded that only 0.6 or 0.9 chlamydospores g^{-1} of *P. citrophtora* or *P. palmivora* were needed to infect, respectively, *Morrenia odorata* or *Carica papaya*, in growth chamber studies, although some of these results pertained to aerial infections. Additionally, these limited infections may allow for persistent survival of the pathogen in a site, as suggested by recrudescence of disease in absence of stringent inoculum eradication (Dunstan *et al*., 2010). Notwithstanding the role played by infections caused by low levels of inoculum, our results suggest that 61 cfu g^{-1} represents a consistent threshold of inoculum capable of inducing significant root infection, at least in the experimental conditions applied in the present study.

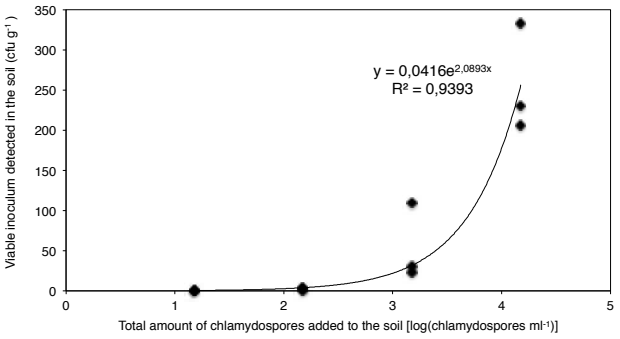

Figure 1. Relationship between numbers of *Phytophthora cinnamomi* chlamydospores added to soil [log (chlamydospores ml^{-1})] and viable chlamydospores detected (cfu g^{-1}). Dots are the obtained values and the line the adjusted exponential curve

Although the minimum threshold determined here can be useful as an experimental reference for checking the potential infectivity of infested soils, or to assess the effectiveness of some control methods to reduce soil inoculum, we are also aware that such a threshold may be different in natural forest soils. It is likely that in natural soils the threshold may be greater than the one determined here, thus our results may be valuable under this precautionary principle. We emphasize that these types of experiments are useful when monitoring disease spread in sites already known to be infested. When assessing risk, a site should be regarded "at risk" whenever the pathogen is detectable in the soil (Dunstan *et al*., 2010).

We conclude that presence/absence of *P. cinnamomi* needs to be accurately monitored at the large geographic scale to identify all cork oak sites at risk and all sites that may be a source of new infestations. Inoculum loads may be calculated to track disease expression and efficacy of disease management approaches. Results from inoculum load studies may indicate which sites may be more at risk and where

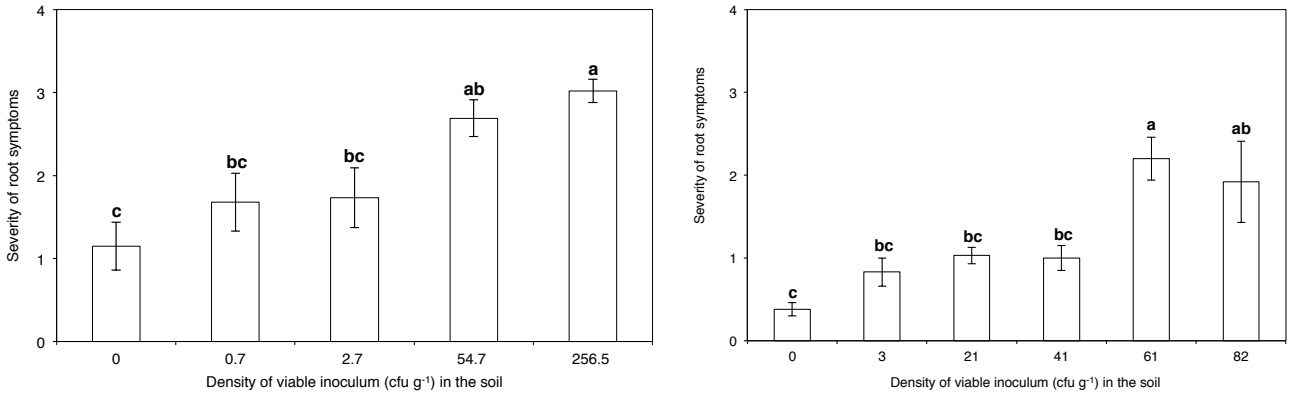

Figure 2. Mean severity of root disease symptoms recorded for cork oaks growing in soils infested with different numbers of *Phytopthora cinnamomi* chlamydospores. Lines are standard errors of ten replicates (Experiment 1, left chart) or six replicates (Experiment 2, right chart). Bars with different letters differ significantly according to Tukey´s HSD test ($P<0.05$).

control measures may be more efficient. This will provide a way to prioritize choices when dealing with the widespread presence of *P. cinnamomi* in oak forest soils in southwestern Spain and southern Portugal (Romero *et al*., 2007), to indicate threats to the survival of oak forests in the region.

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