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Evaluation of root damage to English walnut caused by five *Phytophthora* species

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The pathogenicity of five species of *Phytophthora* to English walnut was studied in a greenhouse experiment. *Phytophthora cinnamomi* was the most aggressive species, causing severe root rot and seedling mortality. The other species tested, *P. cambivora*, *P. citricola*, *P. cactorum* and *P. cryptogea*, did not induce visible crown symptoms on seedlings 2 months after inoculation. Some strains of *P. cambivora* and *P. cactorum* also caused taproot damage to seedlings. All except one of the tested isolates caused significant necrosis of fine roots and a significant reduction of root weight compared with noninoculated seedlings. Reduction of above-ground plant development was not statistically significant. While *P. cinnamomi* is well known as an aggressive primary pathogen of English walnut, the other species of *Phytophthora* may act as predisposing factors to walnut decline, affecting root system development and increasing host vulnerability to environmental stress.

Keywords: English walnut, *Juglans regia*, pathogenicity, soilborne pathogens, walnut decline

Introduction

English (Persian) walnut (*Juglans regia*), is widely cultivated in Europe in pure and mixed plantations for wood and nut production and agro-forestry. The cultivation of English walnut is supported in Europe through EU regulation no. 2079/92 for reforestation of rural land. Plantations are established with 1- to 2-year-old seedlings or grafted plants with English walnut as rootstock.

A decline of this species has been reported recently in southern Europe. Symptoms range from slow decline to sudden wilt and are often associated with root and/or collar rot. Various species of *Phytophthora* have been associated with these symptoms in nurseries (Belisario *et al*., 1997) and plantations (Belisario *et al*., 2001; 2002): *P. cinnamomi* was most frequently recovered from trees showing sudden wilt. *P. cambivora*, *P. cactorum* and *P. citricola* were recovered from slowly declining trees, and *P. cryptogea* was recovered from soils in the rooting zone of declining walnut trees (Belisario *et al*., 2002). These observations suggest that different symptoms are associated with different *Phytophthora* spp.

Most of these species of *Phytophthora* have been shown to cause extensive and rapid mortality to English walnut and *J. hindsii* and Paradox rootstocks following prolonged and/or repeated flooding (Mircetich & Matheron, 1983; Matheron & Mircetich, 1985a; Belisario *et al*., 2001). However, prolonged and repeated flooding rarely occurs in walnut plantations in southern Europe due to limited precipitation and the use of sprinkler rather than flood irrigation. Soil saturation occurs occasionally following heavy rain or excessive irrigation. Based on field observations, it is hypothesized that, in the absence of frequent soil saturation, these species of *Phytophthora* are associated with different symptoms on English walnut.

The present study examines the effect of different species of *Phytophthora* on English walnut. In particular, the patterns of colonization of the different species and the impact of infection on host development are investigated in the absence of prolonged and repeated soil saturation conditions.

Materials and methods

Plant material and *Phytophthora* isolates

In April 2001, 130 1-year-old bare-root English walnut seedlings (approximately 50 cm tall and 2 cm in collar diameter) were transplanted from a Latium state nursery into 5 L pots containing steam-pasteurized potting mix (50% peat, 25% sand, 25% ground pumice). Pots were placed in a glasshouse and maintained at approximately 22°C (range 15–26).

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Table 1 Isolates of *Phytophthora* spp. used for pathogenicity tests to English walnut

Species	Isolate	Host	Mating type	Date of isolation	Source
P. cambivora	Pcamb1	*Juglans regia*	A2	2001	ISPaVe – Italy
P. cambivora	Pcamb3	*Juglans regia*	A2	2001	DIPROP – University of Tuscia – Italy
P. cactorum	Pcact1	*Juglans regia*	Homothallic	2001	DIPROP – University of Tuscia – Italy
P. cactorum	CN26a	*Castanea sativa*	Homothallic	2000	DIPROP – University of Tuscia – Italy
P. citricola	Vald1	*Castanea sativa*	Homothallic	2000	DIPROP – University of Tuscia – Italy
P. citricola	Pcit1	*Juglans regia*	Homothallic	2001	DIPROP – University of Tuscia – Italy
P. cryptogea	Ve4cry	*Juglans regia*	A2	2001	DIPROP – University of Tuscia – Italy
P. cryptogea	MF9	*Castanea sativa*	A2	2000	DIPROP – University of Tuscia – Italy
P. cinnamomi	F1Alcin	*Juglans regia*	A2	2001	ISPaVe – Italy
P. cinnamomi	G3Alcin	*Juglans regia*	A2	2001	ISPaVe – Italy

Isolates of *P. cinnamomi*, *P. citricola*, *P. cambivora*, *P. cactorum* and *P. cryptogea* used for pathogenicity tests are listed in Table 1. At least one of the isolates of each species was recovered from walnut tissues with symptoms or soil at the base of declining trees in Europe. Isolates were maintained on carrot agar (CA) (Brasier, 1969) at 20°C in darkness and subcultured at 4-week intervals.

Pathogenicity tests

Pathogenicity of *Phytophthora* spp. to walnut was evaluated by the soil infestation method (Vettraino *et al*., 2001). Seedlings were randomly divided into two groups of 65 plants. Within each group, inoculations were carried out in a completely randomized design with five replicates for each isolate, apart from *P. cinnamomi* where 10 plants were used for each isolate. There were five uninoculated control plants for each group. *Phytophthora* inoculum was prepared by growing cultures for 4–6 weeks at 20°C on sterilized millet seeds thoroughly moistened with V8 broth (V8 juice, 200 mL; $CaCO_3$, 3 g; distilled H_2O, 800 mL). The inoculum was repeatedly rinsed with sterile water to remove unassimilated nutrients and then added to the potting mixture at the rate of 25 mL inoculum/1000 mL potting mixture. Uninoculated control plants received sterilized millet seeds.

The relative soil moisture was measured with a TDR probe PG2 (Trime® System, MESA Systems Co., Medfield, MA, USA) on 10 randomly chosen pots immediately before and 6 h after each irrigation. The potting mixture matric potential (ψ_m) was estimated with a soil moisture curve determined by the filter paper method (Hamblin, 1981). The experiment was initiated in early summer when the soil in the pots (of both inoculated and control seedlings) was saturated by maintaining the level of water 2–3 cm below the soil surface for 24 h. After that, plants were watered to field capacity every other day (70·2% soil moisture value; ψ_m–28 kPa). The average value for the period was 31% (ψ_m–210 kPa) at 22°C (40% maximum soil moisture, ψ_m–259 kPa; 22·4% minimum soil moisture, ψ_m–333 kPa). Seedling height was measured twice a week. Seedlings that died during the experiment were immediately harvested and scored for damage as described below. Seedlings in the first group were harvested after 1 month. Root systems were carefully washed and examined to assess their development and the presence of necrotic lesions. Isolation of *Phytophthora* spp. from inoculated and uninoculated (controls) root systems was attempted by plating tissues from each pot onto PARBhy selective medium (Robin *et al*., 2001). To determine the survival of *Phytophthora* in soil, 50 g of soil from each pot for each isolate of *Phytophthora* was flooded with water and baited separately with 15 rhododendron leaf disks, 1 cm in diameter.

Visual damage to tap (TDI) and lateral roots (LDI) of seedlings was assessed on a scale of 0–4, as follows: 0, no damage; 1, up to 25% damage; 2, 25–50% damage; 3, 50–75% damage; 4, 75–100% damage. The mean value for each seedling × isolate combination was calculated using the following formula: $\Sigma(g \times n)/N$, where g is the damage score, n is the number of samples belonging to each class, and N is the total number of inoculated plants. Roots and shoots were separated, lateral roots were separated from the taproot, and necrotic lateral roots were separated from healthy lateral roots. This material was dried at 60°C and weighed at 24 h intervals until no reduction in weight was obtained between two subsequent measurements. Damage to lateral roots was scored as a percentage of necrotic lateral roots to the total dry weight of lateral roots (NLW). The effect of pathogen inoculation on relative growth of the plant was calculated as the dry weight of the above-ground portion (AGW) and of the total root system (RW) (sum of necrotic and healthy lateral roots, and taproot).

The second group of seedlings was harvested 2 months after inoculation. The survival of *Phytophthora* was checked as described above, but the seedlings were assessed only for visual root damage (TDI and LDI) and above-ground symptoms.

Data analysis

Data were analysed with the Graphpad Instat® software (San Diego, CA, USA). One-way ANOVA (parametric) and Kruskal–Wallis test (nonparametric ANOVA) were used as appropriate. The Kolmogorov–Smirnov method was used for testing Gaussian distribution of data sets. Bartlett's test was used to evaluate differences in standard deviation (SD) among data sets.

Results

Phytophthora spp. were isolated from all inoculated seedlings and soil at both harvests. Sudden wilt of seedlings inoculated with *P. cinnamomi* isolates began 26 days after inoculation. Seedlings inoculated with isolate F1Alcin and G3Alcin caused 80 and 60% mortality of seedlings after 1 month, respectively, and 100% mortality after 2 months. Seedlings inoculated with isolates of *P. cambivora*, *P. cactorum*, *P. citricola* and *P. cryptogea* did not show any visible crown or stem symptoms 1 or 2 months after inoculation. These isolates caused visible symptoms on the root system. There was no significant difference in TDI and LDI between the two harvests. There was a significant effect of species on TDI ($P < 0{\cdot}0001$).

Phytophthora cambivora, *P. cactorum* and *P. cinnamomi* produced more severe taproot damage compared with the control (Fig. 1). There were no differences between isolates of the same species, apart from *P. cactorum*; isolate P. cact1, from walnut, was significantly ($P = 0{\cdot}03$) more aggressive to taproots than isolate CN26a, from chestnut. Taproot damage caused by *P. citricola* and *P. cryptogea* was negligible and necroses were always observed at the insertion point of lateral roots (Fig. 2).

There was a significant effect of *Phytophthora* spp. on NLW ($P < 0{\cdot}0001$). Each species caused significant damage to lateral roots compared with the control. *P. cinnamomi* caused significantly more damage than the other species (Fig. 3). Isolates of the same species did not differ significantly in their ability to damage lateral roots.

Visual assessment of lateral root damage (LDI) was correlated (Spearman $r = 0{\cdot}85$, $P = 0{\cdot}001$) with the percentage of root dry weight that was necrotic (NLW).

The effect of *Phytophthora* spp. on AGW is shown in Fig. 4. There was no significant effect of *Phytophthora* species on AGW ($P = 0{\cdot}1$). There was a significant effect of *Phytophthora* spp. on RW ($P = 0{\cdot}003$). Each species, apart from *P. citricola*, caused a significant reduction in root development compared with the control (Fig. 5).

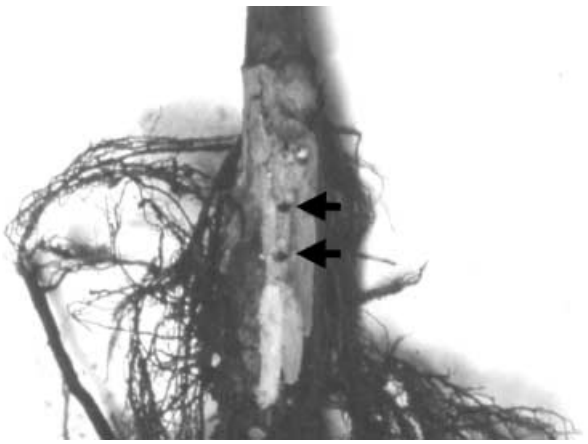

Figure 2 Localized taproot necroses on English walnut caused by *Phytophthora citricola* are restricted to the insertion point with dead lateral roots (arrows). *Phytophthora citricola* was re-isolated from these necrotic lesions.

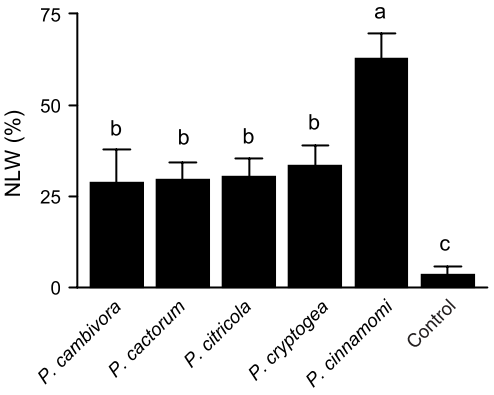

Figure 3 Damage to lateral roots caused by *Phytophthora* spp. to English walnut seedlings 1 month after inoculation expressed as a percentage of the total lateral root dry weight (NLW). Vertical bar represents SEM. Values with the same letter are not different according to the Mann–Whitney test.

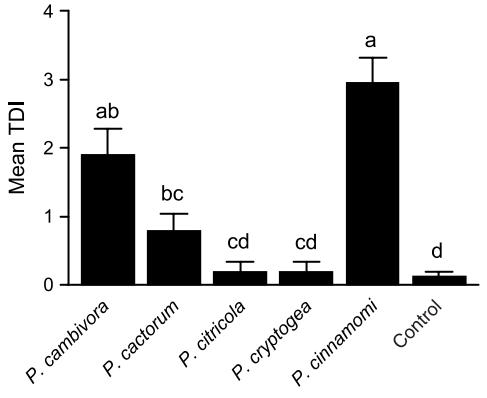

Figure 1 Damage to taproot (TDI) caused by *Phytophthora* spp. to English walnut seedlings 1 month after inoculation. The damage was evaluated with a 0–4 visual scale (see 'Materials and methods'). Vertical bar represents SEM. Values with the same letter are not different according to the Mann–Whitney test.

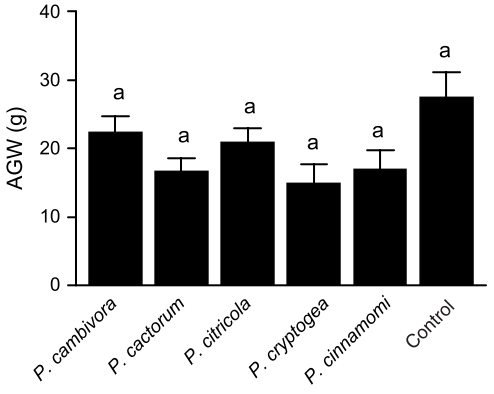

Figure 4 Dry weight of the above-ground part of seedlings inoculated with *Phytophthora* spp. (AGW) 1 month after soil infestation. Vertical bar represents SEM. Values with the same letter are not different according to the unpaired *t*-test.

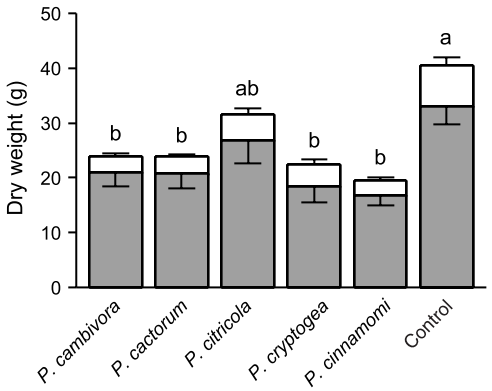

Figure 5 Dry weight of the taproot (grey bars) and lateral root (white bars) of seedlings (RW) challenged with *Phytophthora* spp. 1 month after soil infestation. Vertical bars above and below represent SEMs of lateral root and taproot dry weights, respectively. Values with the same letter are not different according to the unpaired *t*-test.

Table 2 Percentage of lateral root necrosis (NLW), taproot disease index (TDI), dry weight of the above-ground part (AGW) and height increment of *P. cinnamomi*-inoculated seedlings distinguished as dead or alive 1 month after inoculation

	Seedlings		
	Inoculated (20)[a]		
Damage score	Dead (14)	Alive (6)	Control (5)
NLW (%)	80·2a[b]	48·9b	3·6c
TDI	3·9a	1·3b	0·1c
AGW (g)	14·8a	26·2a	27·4a
Height increment (cm)	1·7a	3·0a	2·8a

[a]Numbers in brackets indicate the number of seedlings analysed.
[b]Numbers with the same letter along rows do not differ ($P = 0{\cdot}05$) according to the Tukey post-test of ANOVA (NLW, AGW, height increment) and the Mann–Whitney test of nonparametric ANOVA (TDI).

There was no difference between isolates of the same species, apart from *P. citricola*, where isolate P. cit1 (from walnut) caused a significant ($P = 0{\cdot}02$) reduction in root development compared with isolate Vald1 (from chestnut).

A comparison between seedlings that died following inoculation with *P. cinnamomi*, and those that were alive after 1 month, is shown in Table 2. NLW and TDI differed significantly, but there was no difference in AGW and plant height.

Discussion

A comparison of root damage caused to English walnut by the most common species of *Phytophthora* associated with walnut decline and root rot in Europe is presented here. These species are confirmed as pathogenic to English walnut under the conditions used. The extent and type of damage was different depending on the species.

Of the five species studied, *P. cinnamomi* was the most pathogenic species, colonizing the root system of English walnut and causing sudden wilting and death. Death of seedlings occurred when more than 50% of lateral roots were necrotic. In field surveys conducted in Italy, this species has been consistently isolated from trees showing sudden wilt (Belisario *et al*., 2001). Both *P. cambivora* and, to a lesser extent, *P. cactorum* acted as slow colonizers of large roots. In plantations in Italy, these two species were isolated from collar lesions and large roots of slowly declining trees (Belisario *et al*., 2002). These species also damaged lateral fine roots. *P. cambivora* has been considered the main cause of walnut root rot (Curzi, 1933; Crandall, 1936; Petri, 1937), but confusion between *P. cambivora* and *P. cinnamomi*, as suggested by Crandall (1950), may have led to the conclusion that *P. cambivora* was more aggressive than *P. cinnamomi*. *Phytophthora cactorum* has been frequently isolated from symptomatic seedlings in nursery stocks in Italy (Belisario *et al*., 1997).

Under the studied conditions, *P. citricola* and *P. cryptogea* caused lateral root rot but not rotting of the taproot. The former species is reported to colonize the large roots and collars of walnut trees (Matheron & Mircetich, 1985b); observations in the present study suggest that the infection by *P. citricola* spreads from fine roots to larger roots. There is no evidence supporting the role of *P. cryptogea* as a large root and collar pathogen of English walnut in Europe. Vettraino *et al*. (2002b) recovered this species only from feeder-root rich soils at the base of trees exhibiting symptoms.

Phytophthora cinnamomi must be considered a serious threat to English walnut in the absence of waterlogging, as well as to Paradox rootstocks and *J. hindsii* (Matheron & Mircetich, 1985a). Infection of English walnut by other *Phytophthora* spp. may result in chronic decline.

The inhibition of root system development and the necrosis of lateral and taproot by other *Phytophthora* spp. changed the root-shoot weight ratio of the host plant. Some trees can survive root reduction without appreciable crown symptoms (Crombie *et al*., 1987), even though their water relations and nutrition are affected (Labanauskas *et al*., 1976; Portela *et al*., 1999; Maurel *et al*., 2001) as well as the accumulation of compounds required for cambial growth (Bréda, 1994). Consequently, it may be difficult to establish infected walnut seedlings after transplanting, whilst adult trees challenged with *Phytophthora* spp. and exposed to drought and nutritional stresses may develop a slow decline.

These species of *Phytophthora* are widespread in Europe in natural and seminatural ecosystems (Brasier & Ferraz, 1993; Erwin & Ribeiro, 1996; Jung *et al*., 1996; Hansen & Delatour, 2000; Vettraino *et al*., 2002a,b). Their wide host range (Matheron & Mircetich, 1985b; Robin & Desprez-Lousteau, 1998; Brasier & Kirk, 2001) increases the likelihood of walnut becoming infected in plantations and nurseries. Infected plants may be unrecognized because they do not show foliar symptoms.

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