Mycorrhiza (1999) 9:103–109

ORIGINAL PAPER

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Effect of ectomycorrhizal fungi on chestnut ink disease

Accepted: 20 May 1999

Abstract Seedlings of *Castanea sativa* were inoculated at transplanting time with four ectomycorrhizal (ECM) fungi, *Laccaria laccata*, *Hebeloma crustuliniforme, H. sinapizans* and *Paxillus involutus*. At the end of the first vegetative season, 7 months after sowing, half of the mycorrhizal and nonmycorrhizal seedlings were challenged with a zoospore suspension of *Phytophthora cambivora* and the other half with *P. cinnamomi.* Five months later, mycorrhizal plants infected with *P. cambivora* or *P. cinnamomi* showed no sign of pathogen infection. The ECM fungi increased plant biomass also in the presence of the pathogen. Mycorrhizal seedlings inoculated with the pathogens showed greater shoot and root development than nonmycorrhizal chestnut plants. All the fungi tested reduced the negative effect of the ink disease pathogens on the plant host in vivo. The mechanisms by which the ECM fungi protect chestnut seedlings are discussed.

Key words Ectomycorrhiza · *Castanea sativa* · *Phytophthora cambivora* · *Phytophthora cinnamomi*

Introduction

Ink disease is a widespread disease in chestnut (*Castanea sativa* Mill.) groves and is responsible for considerable annual losses of chestnut trees. It is associated with the soil-borne fungi *Phytophthora cambivora* (Petri) Buism. and *P. cinnamoni* Rands and induces root and collar rot with die-back of branches, defoliation, gradual decline and death of the infected plants. The two pathogens lead to major epidemics in North America and Europe (Crandall et al. 1945; Grente 1961 a,b). Recently, *P. cinnamomi* has been found associated with ink disease in Italy (Cristinzio 1986; Turchetti and Parrini 1993; Biocca et al. 1993). The disease cannot be controlled by chemical applications because of the cost of such treatments, their lack of effectiveness against soil-borne diseases and the environmental impacts of agrochemicals in the soil and ground water. The use of ink-resistant cultivars has produced limited results, depending on soil characteristics (Abreu et al. 1993). More recent studies have shown that an integrated approach to chestnut ink disease, involving the improvement of material quality, could lead to better control of the disease (Bounous and Abreu 1998).

Several ectomycorrhizal (ECM) fungi can exert a protective effect against root pathogens and thus can be used as biological control agents, either as an alternative to or integrated with application of fungicides or bacterial strains (Zak 1964; Marx 1972; Duchesne et al. 1989; Chakravarty et al. 1990, 1991; Hwang et al. 1995). Previous reports showed that early mycorrhizal inoculation of seedlings and micropropagated plants of chestnut improves the general condition of plants by increasing growth and survival during the nursery stage (Branzanti and Zambonelli 1986; Martins et al. 1996, 1997). Furthermore, some ECM fungi which are able to form ECM with chestnut in greenhouse experiments exhibited antagonistic activity against *P. cambivora* and *P. cinnamomi* in paired cultures on agar plates (Branzanti et al. 1994). The aim of the present study was to investigate whether an early infection by mycorrhizal fungi in vivo might protect chestnut seedlings against attacks by *P. cambivora* and *P. cinnamomi.*

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Materials and methods

Fungal inocula

Laccaria laccata (Scop. ex Fr.) Berk and Br. (an isolate from Dr. Molina, Corvallis, Ore.), *Paxillus involutus* (Batsch) Fr., *Hebeloma crustuliniforme* (Bull. ex Fr.) Quèl and *H. sinapizans* (Paulet ex Fr.) all isolates from the Mycological Centre, University of Bologna, Italy, were prepared according to Marx and Kenney (1982).

Pathogen inoculum

Cultures of *P. cambivora* and *P. cinnamomi* were grown on V8 agar at 23 °C for 1 week. Inoculum was obtained by chopping the mycelial mat into very small pieces (1–2 mm^3 and suspending 1 ml of fragments in 9 ml of sterile distilled water. Aliquots (3 ml) of this inoculum were applied to 1-year-old chestnut seedlings. Inoculum of *P. cambivora* and *P. cinnamomi* zoospores was produced by the method of Chen and Zentmyer (1970): mycelial blocks cut from V8 agar plates were placed in 9-cm Petri dishes containing V8 broth and grown in darkness at 23 °C for 24 h. The young mycelial mat produced by actively growing hyphae was washed four times at 1 h intervals with 15–20 ml of a mineral salt solution (calcium nitrate 10 mM, potassium nitrate 5 mM, magnesium sulphate 4 mM, chelate iron in 1 l distilled water), incubated at 24 °C in the light, chilled at 5 °C and returned to 24 °C; sporulation began 8 h after the last washing. For the release of zoospores, the sporange-bearing mycelium was rinsed with three changes of sterile water, chilled at 5 °C for 15–20 min and returned to 24 °C after each washing. Zoospores were released within 1 h after chilling. Plants were infected by applying the zoospore suspension (3 ml of water containing 80×10^5 zoospores per ml).

Plant growth parameters and determination of the degree of infection

Leaf area of plants was measured with an area-meter 7 months after sowing and 5 months after pathogen inoculation in seedlings infected by *P. cambivora* and *P. cinnamomi*. Shoot and root dry weights were determined after drying plants at 80 °C for 48 h.

Mycorrhizal colonization was evaluated using the method of Grand and Harvey (1982): 100 short roots were randomly selected on 5 plants from each mycorrhizal treatment, examined and classified. The degree of mycorrhizal formation was expressed as the percentage of root tips colonized.

Infection by *P. cambivora* and *P. cinnamomi* on chestnut was assessed by checking visually for the presence of mycelia on root tips. Disease severity was expressed as the number of oospores per mm of root segment.

Roots for scanning electron mycroscopy were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 1 h and dehydrated through an aqueous ethanol series (10, 30, 50, 75, 95%) for 15 min each at 5 °C, then in 100% ethanol for a few minutes. The samples were then critical point dried, mounted on aluminium stubs with silver glue, coated with gold palladium film and observed under a Philips 515 microscope at 6–9 Kv.

Experiment 1

Chestnut seeds were surface sterilized with 3% sodium hypochloride for 20 min, rinsed several times with sterile distilled water, stratified in a sterilized peat-vermiculite mix (1:1) and kept at 5 °C for 4 months. Inoculation of the seedlings with mycorrhizal fungi (*L. laccata*, *H. crustuliniforme*, *H. sinapizans*, *P. involutus*) was carried out at planting time, mixing inoculum of each mycorrhizal isolate with the potting mixture in a ratio of 1:6. The potting substrate consisted of a sterilized mix of peat, sand and soil (6:1:1, v:v:v). Seedlings were planted into 1.5-l tube containers (Issapots, Istituto Sperimentale per la Selvicoltura, Arezzo, Italy) filled with this medium and grown for 4 months in controlled environmental conditions at 20 °C, 60% RH, natural photoperiod and no supplementary light. Pots were then transferred to an unheated greenhouse for 2 months and arranged in a completely randomized design: four ECM treatments and control (nonmycorrhizal treatment) with 20 replicates each. Six months after planting, 5 plants for each treatment were randomly selected and examined for mycorrhizal formation. Leaf area, root and shoot dry weights were determined.

Experiment 2

Chestnut seedlings were prepared and inoculated with mycorrhizal fungi as in experiment 1. Six months after planting, mycorrhizal and nonmycorrhizal plants were inoculated with *P. cinnamomi*.

The following treatments were applied:

1) control plants
2) plants inoculated with *P. cinnamomi*
3) mycorrhizal plants colonized by *L. laccata* and inoculated with *P. cinnamomi*
4) mycorrhizal plants colonized by *P. involutus* and inoculated with *P. cinnamomi*
5) mycorrhizal plants colonized by *H. crustuliniforme* and inoculated with *P. cinnamomi*
6) mycorrhizal plants colonized by *H. sinapizans* and inoculated with *P. cinnamomi*

Five plants were harvested for each treatment 5 months after pathogen inoculation. Leaf area and shoot and root dry weights were determined.

Experiment 3

This experiment was identical to experiment 2, except that the pathogen inoculated was *P. cambivora*.

Data analysis

Analysis of variance was performed and significant differences between treatments were calculated by Student Newman Keul's test.

Results

Experiment 1

Six months after planting, mycorrhiza had developed on seedlings inoculated by mycorrhizal fungi (Table 1). *H. sinapizans* was the most effective colonizer of chestnut roots (57% of root tips infected), while *L. laccata* was the least infective (40%). Mycorrhiza morphology was as described by Agerer (1995) on *Fagus sylvatica*: ECM were initially simple and elongate (never dicotomous) then monopodial – pinnate and monopodial – pyramidal, depending on the fungal species, and covered with a plectenchymatous mantle. Mycorrhizae of *L. laccata* showed swollen round apices (pale violet initially to honey-brown) enveloped by a compact fungal sheath containing 2–3 layers of hyphae randomly arranged. *H. crustuliniforme* formed brownish mycorrhizae with swollen, pointed apices and surrounded by a

Table 1 Mycorrhizal colonization and growth response, 6 months after mycorrhizal inoculation of chestnut seedlings. For each column, values followed by different letters are significantly different ($P=0.05$)

Treatment	Leaf area (cm^2)	Root dry weight (g)	Shoot dry weight (g)	Root/Shoot ratio	Mycorrhizal development (% root tips colonized)
Control	12.3a	3.71a	3.64a	1.02a	0
Laccaria laccata	30.2b	6.83c	5.51b	1.24b	39.8a
Paxillus involutus	22.1ab	5.01b	4.47ab	1.12ab	42.8a
Hebeloma crustuliniforme	24.2ab	4.66a	3.86a	1.21b	53.6ab
H.sinapizans	28.7b	5.88bc	4.71b	1.25b	57.2b

highly dense mat of hyphae. Mycorrhizae of *H. sinapizans* had elongate-acuminate apices completely ensheathed by whitish, cottony mycelium with inner layers of hyphae arranged closely together and outer layers of loosely woven hyphae (Figs. 1, 2). *P. involutus* mycorrhizae appeared monopodial - pyramidal, dark brown with poor mycelium and warty mantle surface; loosely woven hyphae surrounded the apices.

The ECM fungi increased plant development and biomass: mycorrhizal plants were taller and had larger root systems (data not reported) than nonmycorrhizal plants. Leaf area, root and shoot dry weights were in general higher than those of nonmycorrhizal plants for seedlings colonized by *L. laccata*, *P. involutus* and *H. sinapizans* (Table 1). Chestnuts colonized by *H. crustuliniforme* showed no significant increase in growth over control plants.

Experiment 2

Phytophthora cinnamomi reduced the development and biomass of nonmycorrhizal chestnut seedlings. Nonmycorrhizal plants showed poor growth 5 months after pathogen inoculation, leaves were significantly smaller (43%) than those of uninoculated plants and had large chlorotic areas. The root system was decreased by 48% compared with the control plants and root tips were poorly developed. Dry weights of roots and shoots of plants infected with *P. cinnamomi* were significantly lower than in the control plants (Table 2).

In contrast, the growth of mycorrhizal plants was not affected by the pathogen. At the end of the experiment, the leaf areas of plants colonized by ECM fungi and inoculated with *P. cinnamomi* were always higher than those of nonmycorrhizal plants infected with the pathogen. Dry weights of roots and shoots were also significantly greater than those of the infected nonmycorrhizal plants and no chlorotic areas were observed on leaves of mycorrhizal plants (Table 2).

The biomass of mycorrhizal plants 5 months after inoculation with *P. cinnamomi* was not significantly different to control plants.

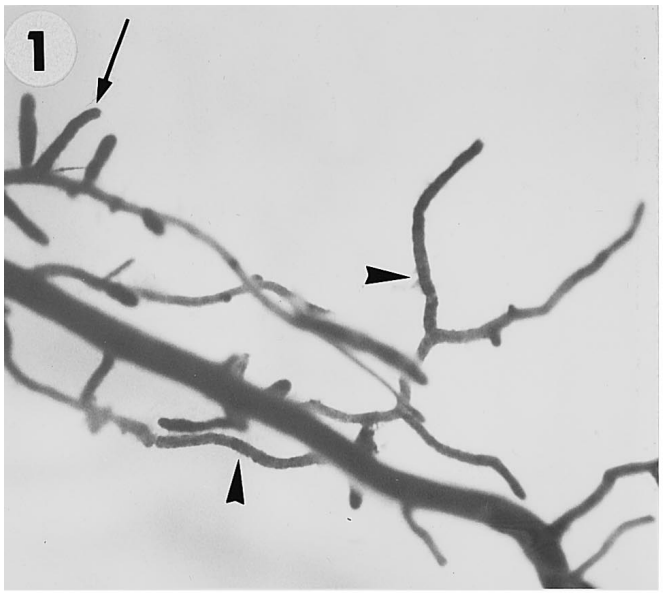
Infection by *P. cinnamomi* was observed only in nonmycorrhizal plants. The hyphae of the pathogen covered the infected roots damaging the epidermal layer, provoking cell sloughing and penetrating the cells (Fig. 3). In infected roots, the number of oospores ranged from 40 to 70 per mm root (Fig. 4). In mycorrhizal chestnut seedlings, no pathogen penetration was observed. In roots colonized by *L. laccata*, some hyphae of *P. cinnamomi* were found but only in nonmycorrhizal segments. Plants inoculated with *P. involutus* and *H. crustuliniforme* had ungerminated zoospores on the surface of the loose mantle and rare hyphae of *P. cinnamomi* were localized in the zone behind the mycorrhizal root tips. No infection of the pathogen (either hyphae or zoospores) occurred in roots colonized by *H. sinapizans* (Fig. 5).

Experiment 3

In nonmycorrhizal chestnut plants, growth parameters were reduced after 5 months by *P. cambivora* inoculation. As in experiment 2, significant differences were detected in biomass between infected and noninfected plants (Table 3). Root and shoot dry weights were reduced by 45% compared with the control plants. In mycorrhizal seedlings inoculated with the pathogen, no

Table 2 Effect of *Phytophthova cinnamomi* on mycorrhizal and nonmychorrhizal chestnut seedlings 5 months after pathogen inoculation. For each column, values followed by different letters are significantly different ($P=0.05$)

Treatment	Leaf area (cm^2)	Root dry weight (g)	Shoot dry weight (g)	Root/Shoot ratio
Control	21.3b	4.70bc	4.63c	1.01a
L. laccata + *P. cinnamomi*	27.79c	5.13c	4.20c	1.22ab
P. involutus + *P. cinnamomi*	18.79ab	3.21b	3.00b	1.07a
H. crustuliniforme + *P. cinnamomi*	22.03b	3.08b	2.65ab	1.16ab
H. sinapizans + *P. cinnamomi*	27.70c	5.47c	4.56c	1.20b
P. cinnamomi	12.06a	2.45a	2.50a	0.98a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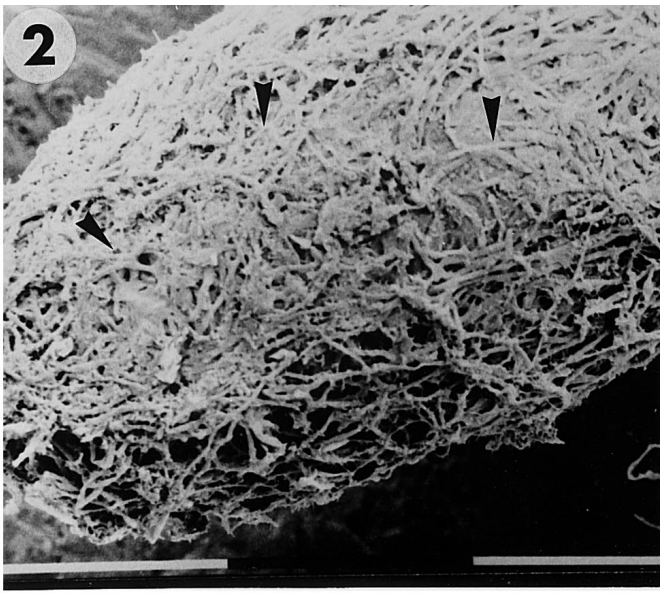
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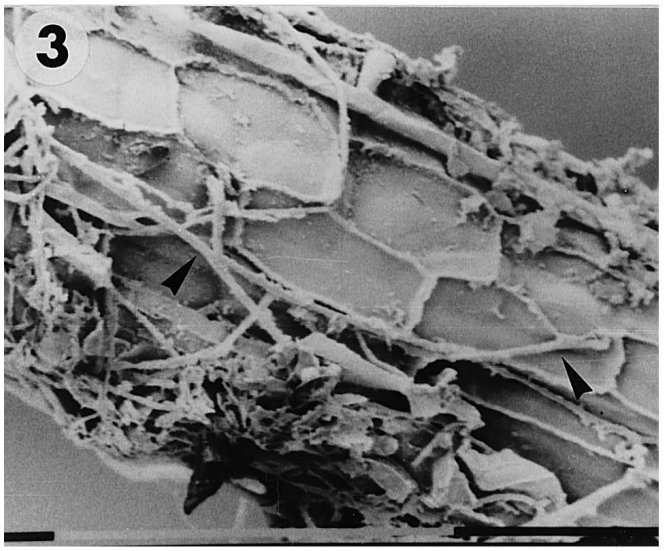
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Table 3 Effect of *Phytophthova cambivora* on mycorrhizal and nonmychorrhizal chestnut seedlings 5 months after pathogen inoculation. For each column, values followed by different letters are significantly different ($P = 0.05$)

Treatment	Leaf area (cm^2)	Root dry weight (g)	Shoot dry weight (g)	Root/Shoot ratio
Control	21.3ab	4.70bc	4.63c	1.01a
L. laccata + *P. cambivora*	28.09b	5.26c	4.08c	1.29c
P. involutus + *P. cambivora*	19.54ab	3.31b	2.80b	1.18b
H. crustuliniforme + *P. cambivora*	21.98ab	3.27b	2.64a	1.23bc
H. sinapizans + *P. cambivora*	28.13b	5.53c	4.73c	1.17b
P. cambivora	15.59a	2.59a	2.56a	1.01a

significant reduction was observed in any treatment. Root and shoot dry weights of mycorrhizal plants were always significantly higher than those of nonmycorrhizal plants, except for plants inoculated with *H. crustuliniforme*. The ECM fungi were always associated with an increase in root biomass, even in the presence of the pathogen (Table 3). There was no sign of infection by *P. cambivora* in mycorrhizal plants: no pathogen mycelium occurred on the surface of root tips colonized by the ECM fungi or penetrated the mantle and only rare ungerminated zoospores surrounded by the ECM mantle were observed (Fig. 6). Feeder roots of nonmycorrhizal plants inoculated with *P. cambivora* appeared to be covered by hyphae of the pathogen, which penetrated the epidermal layer. Both germinated and ungerminated zoospores were observed on the surface of the root system. Roots infected by *P. cambivora* had 38–62 oospores per mm root.

Discussion

The results from experiment 1 show that early mycorrhizal inoculation has a benefical effect on the growth of chestnut seedlings. The developmental differences between nonmycorrhizal and mycorrhizal plants 6 months after inoculation with ECM fungi resulted in differences in dry mass. Root dry weight was significantly enhanced by mycorrhizal treatments, except for seedlings inoculated with *H. crustuliniforme* and the root/shoot ratio was modified by ECM colonization.

In experiments 2 and 3, the development of mycorrhizal plants was not negatively influenced by the pathogens but the growth behaviour of the host-plant was again affected by the ECM species. *L. laccata* and *H. sinapizans* produced a significant increase in the biomass of mycorrhizal plants. Chestnut seedlings precolonized with ECM fungi and subsequently inoculated with *P. cinnamomi* or *P. cambivora* showed no change in biomass compared with control plants, whereas root and shoot dry weights decreased in nonmycorrhizal plants infected with the pathogens. Mycorrhizal plants infected with the pathogens always showed greater development than nonmycorrhizal chestnuts. Thus ECM fungi appear to reduce or eliminate negative effects of the pathogens on the plant host in vivo.

Mycorrhizal development on roots appeared to prevent pathogen penetration and the fungal mantle was also a mechanical barrier. Chestnut roots with a complete fungal mantle were not infected by either *P. cinnamomi* or *P. cambivora*; hyphae and rare ungerminated zoospores were only detected in nonmycorrhizal segments where the hyphal network was absent. Nonmycorrhizal roots of control plants were heavily infected and showed widespread damage of epidermal cells (see also Marx 1970). This is the first report that *L. laccata* and *H. sinapizans* can deter ink disease in chestnut seedlings. The results agree with those of Marx and Davey (1969 a,b), who reported that naturally mycorrhizal roots of shortleaf pine were not susceptible to infection by zoospores of *P. cinnamomi* cortex cells enclosed by Hartig net were not infected by the pathogen.

The mechanical effect of the Hartig net may be a factor involved in disease suppression, in addition to mechanisms such as a chemical barrier, antibiosis, antifungal compound production or competition which may protect mycorrhizal plants against phytopathogenic fungi. Disease protection by ECM fungi has been associated with several mechanisms that may act in synergy (Zak 1964; Marx 1973; Wichlacz et al. 1998).

Previous experiments (Branzanti et al. 1994) have shown that *L. laccata* and *H. sinapizans* have antagonis-

Fig. 1 Segment of *Castanea sativa* root system with *Hebeloma sinapizans* mycorrhizae showing elongated-acuminate root apices (*arrow*) and cottony hyphal mantel (*arrowheads*). × 0.3

Fig. 2 SEM. Scanning electron micrograph of *C. sativa* root apex. *H. sinapizans* hyphae densely arranged on root apex (*arrowheads*). *bar* 0.1 mm

Fig. 3 Scanning electron micrograph of a damaged root of a nonmycorrhizal chestnut seedling infected with *Phytophthova cinnamoni arrowheads. bar* 0.1 mm

Fig. 4 Light micrograph of oospores of *P. cinnamoni* in nonmycorrhizal chestnut roots (*arrowheads*). × 18

Fig. 5 Scanning electron micrograph of a chestnut seedling root tip precolonized by *H. sinapizans* and inoculated with *P cinnamoni*. Note the absence of pathogen hyphae and the presence of the ECM fungus with its typical hyphal clamp connections (*arrowheads*), *bar* 0.1 mm

Fig. 6 SEM. Scanning electron micrograph of a *C. sativa* root tip preinoculated with *Laccaria laccata* and infected with *P. cambivora*, showing rare ungerminated zoospores (*arrowheads*) enveloped by ECM funged hyphae, *bar* 0.1 mm

tic activities in paired cultures on agar plates, reducing or inhibiting *P. cinnamomi* and *P. cambivora* mycelial growth. Consequently, the pathogen deterrent effect in experiments 2 and 3 may be related to the formation of mycorrhizae and the ability of the ECM fungi to inhibit zoospore germination. Furthermore, root fragments excised from chestnuts inoculated with *L. laccata* and *H. sinapizans* were unable to attract zoospores of *P. cinnamomi* (unpublished data), whereas they were attracted by nonmycorrhizal chestnut roots. This result disagrees with that of Marx (1972), who reported that *P. cinnamoni* zoospores were not strongly attracted to either nonmycorrhizal or ECM roots of shortleaf and loblolly pine seedlings. Zoospores were observed to germinate faster on nonmycorrhizal than on mycorrhizal roots after their encystment on the root surface. This suggests that the poor or failed germination of zoospores is rather due to the failure of ECM to chemically stimulate germ-tube growth. The same mechanism could be responsible for the protective effect of ECM fungi against *P. cambivora* and *P. cinnamomi* observed on chestnut seedlings.

In conclusion, our results show that ECM colonization reduces *P. cinnamomi* and *P. cambivora* infection of chestnut roots. In our experiments, the protective effect of ECM fungi against ink disease in greenhouse conditions seems to be related to the formation of mycorrhiza and subsequently to the lack of penetration of the pathogens. This is in agreement with Duchesne (1994), who reported that the protective effect of ECM fungi is expressed before the onset of the disease, and that the time of inoculation with ECM fungi influences the response of the host plant to the pathogen.

The ECM fungi tested in the present study have also shown suppressive effects against *Fusarium solani* damping off in *Pinus nigra* seedlings in in vivo experiments before mycorrhiza formation (Branzanti and Zambonelli 1994; Zambonelli et al. 1995). Their deterrent effect was ascribed to the production of host-plant exudates that stimulated antifungal activity, as observed by Duchesne et al. (1988). We may hypothesize a similar mechanism in the protection of chestnut seedlings from ink disease. Further investigations are required in vivo to determine whether the protective effect is expressed before mycorrhiza formation or when mycorrhizal inoculation is performed simultaneously or after pathogen infection.

Acknowledgements This research was supported in part by MURST ex 60% project "Studi ultrastrutturali sull'interazione ospite-patogeno in natura". Our sincere thanks to Prof. G. Cristinzio, Istituto di Patologia Vegetale, University of Napoli "Federico II" (Portici) for providing the isolates of *Phytophthora cambivora* and *P. cinnamoni*. We are also grateful to Prof. Manuela Giovannetti of the Dipartimento di Chimica e Biotecnologie Agrarie of the University of Pisa for a critical review of the manuscript and to Mr. Gianfranco Filippini of Istituto di Patologia Vegetale, U.C.I. S.T.A.A. of the University of Bologna for his technical assistance with electron microscopy.

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