

Involvement of the β -cinnamomin elicitin in infection and colonisation of cork oak roots by *Phytophthora cinnamomi*

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Accepted: 15 March 2010 / Published online: 15 April 2010
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Abstract The virulence of two wild type (PA45 and PA37) and two genetically modified (13C: hygromycin resistant; FATSS: hygromycin resistant and β -cin knock-down) *Phytophthora cinnamomi* strains towards cork oak (*Quercus suber*) was assessed via a quantitative evaluation of disease symptoms arising from a soil infestation assay, and by a histological analysis of root colonization. Comparison of virulence, as expressed by symptom severity, resulted in the following ranking: highly virulent (wild type strains),

medium virulence (strain 13C) and weakly virulent (FATSS). Both transgenic strains were compromised in their virulence, as expressed by symptom severity, but strain 13C was much less affected than FATSS. Microscopic observation showed that the FATSS strain was unable to effectively invade the root, while 13C and the two wild type strains were all able to rapidly colonize the whole root, including the vascular tissue. These results strengthen the notion that elicitins are associated, either directly or indirectly, with the infection process of *Phytophthora*.

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Keywords Elicitin · Pathogenicity · *Quercus*

Introduction

A number of species within the genus *Phytophthora* are known to be responsible for heavy losses in crop production, and are recognized to also be among the causative organisms for forest decline and dieback. Their impact in biodiversity and sustainability of forest ecosystems is further enhanced by the international trade of plants (Brasier 2008) and climate change (Brasier 1996; Bergot et al. 2004; Jung 2009). Thus, a better understanding of the biology and ecology of *Phytophthora* species is needed for the elaboration of effective control and management protocols for forest ecosystems.

The evergreen oak *Quercus suber* has a particular ecological role. Products provided by cork oak are

obtained from live trees in contrast to other forest species. Therefore, cork oak plantations are a permanent component of the landscape and a significant barrier against forest fires, a characteristic due to the non flammable attribute of the cork. Cork oak exhibits a high degree of edaphic and climatic adaptability. It can grow in different soil types except limestone, in regions with mean monthly temperature ranging from 3 to 24°C and mean annual rainfall ranging from 450 to 2,000 mm. It also provides an effective shelter against sun heat, prevents soil erosion and constitutes together with holm oak (*Q. ilex*, subsp. *rotundifolia*), a first line barrier against desertification in southern semi-arid regions characterised by poor soils exposed to adverse climatic conditions. Their disappearance would leave extensive areas highly unproductive, in particular in the central and southern regions of Portugal where *Q. suber* is the most representative component of the indigenous flora. As far as fauna is concerned, the cork oak ecosystem is one of the richest in Europe (Correia 1993; Joffre et al. 1999; Correia and Oliveira 2002).

Quercus suber has an important economic impact in the Mediterranean basin, and is suffering from a severe decline associated with its colonization by the root parasite *Phytophthora cinnamomi* (Brasier et al. 1993; Moreira-Marcelino 2001; Sanchez et al. 2002; Caetano 2007). This wide-range host necrotroph persists in the soil or on plant material in the form of chlamydospores, which can either form germ tubes able to infect living root tissue, or produce sporangia. Mature sporangia release motile zoospores, which in turn are chemotactically attracted to living roots, where they proceed to attach, encyst, form germ tubes and after penetration develop ramifying hyphae into the host plant tissue (Hardham 2005). Some cellular alterations in roots in response to their infection and colonisation by *Phytophthora* spp. have been described for *Eucalyptus* and *Acacia* spp. and *P. cinnamomi* (Cahill et al. 1989), *Citrus* spp. and *P. nicotianae* and *P. palmivora* (Widmer et al. 1998), and *Q. robur* and *P. quercina* (Brummer et al. 2002).

Phytophthora cinnamomi secretes abundantly cinnamomins, proteins from the elicitin family whose biological role remains challenging (for a revision see Ponchet et al. 1999). These proteins are thought to work as effectors and play a key role in the host-*Phytophthora* interaction (Kamoun 2007). In compatible interactions, effectors promote infection by suppressing defence

responses, enhancing susceptibility or inducing disease symptoms; in incompatible interactions they are recognized by the products of resistance genes of the plant, resulting in host cell death and an effective defence response.

Elicitins are encoded by complex gene families. Jiang et al. (2006) proposed a novel classification system for elicitin and elicitin-like genes known in various *Phytophthora* species. The elicitins sharing a highly conserved 98-amino acid domain with six cysteine residues and a typical elicitin type cysteine spacing pattern were classified as ELIs. Elicitin-like proteins possessing shorter or longer elicitin domains that are more diverse at the sequence level than the conserved domains in ELIs are classified as ELLs. In the expression overview made by Jiang et al. (2006) the conclusion was that overall, the expression levels of *eli* genes seem to be higher than those of *ell* genes.

Elicitins belonging to clade ELI-1 obtained by the phylogenetic reconstruction of ELIs and ELLs (Jiang et al. 2006) were the first to be discovered and are the most studied. It is known that α -elicitin genes are down-regulated during the early stages of a successful infection of both potato by *P. infestans* (Kamoun et al. 1997) and tobacco by *P. parasitica* (Colas et al. 2001), although the expression of a *P. parasitica* α -elicitin was maintained throughout the compatible interaction with tomato (Colas et al. 2001). The silencing of the α -elicitin *infl* gene in *P. infestans* did not alter the ability of the pathogen to colonize potato leaves (Kamoun et al. 1998). Quercinin, a *P. quercina* β -elicitin, is produced by the pathogen during its growth in *Q. robur* infected root tissue; the protein is present within the hyphal cell wall, intercellular spaces and within the invaded host cells (Brummer et al. 2002). In *P. cinnamomi*, a gene cluster consisting of four elicitin genes has been identified by Duclos et al. (1998). It was recently shown that the β -cinnamomin gene (*β -cin*) is transcribed during the active growth of the pathogen when infecting newly germinated cork oak roots, and that the effect of silencing the *β -cin* also reduces the expression level of other elicitin genes in the cluster (Horta et al. 2008); furthermore, at the phenotypic level, it delays the *in planta* growth of the pathogen.

The objectives of the present research work were to describe in detail the cellular alterations induced by *P. cinnamomi* when it infects cork oak roots, and help to elucidate the role of elicitins, by comparing the

responses to infection by two wild type strains and two transgenic, hygromycin resistant strains, one of which has been β -*cin* silenced.

Materials and methods

Phytophthora cinnamomi strains

Phytophthora cinnamomi strains PA45 and PA37 were isolated from soil of the rhizosphere of cork oak trees in declining stands in the Algarve region (southern Portugal). Isolations were carried out using pieces of young leaves of *Q. suber* seedlings as baits floated over flooded soil (Moreira-Marcelino 2001). Infected brownish leaflets, which normally appeared after 3±5 days, were plated onto selective PARPH medium (Jeffers and Martin 1986) and incubated at 25°C in the dark. After 48 h, *Phytophthora* hyphae were transferred to V8 agar.

The strains were multiplied by growing in the dark at 25°C either on semi-solid V8 agar or in a liquid V8 medium. The V8 media were prepared by adding 4.5 g CaCO₃ to 330 ml V8 juice (Campbell Soup Co., Camden, N.J., U.S.A.) and stirring for 30 min. The mixture was transferred to 1,000 ml centrifuge flasks and centrifuged at 2,590 g for 15 min at 20°C. The supernatant was then poured into a new flask without disturbing the pellet. The cleared V8-juice was then diluted 10 fold with distilled water. V8 agar was prepared by adding 15 g agar per 1,000 ml V8. Both media were sterilized at 121°C for 20 min. The strains were identified by their morphological characters as well as by a colorimetric molecular assay (Coelho et al. 1997).

As *P. cinnamomi* proved to be difficult to transform (Horta et al. 2008), it was not possible to obtain a stable β -*cin* knock-down transformant and a hygromycin resistant control strain from the same wild type strain. Thus, the genetically transformed strains FATSS (derived from PA45) and 13C (derived from PA37) (Horta et al. 2008) were used here. Strain 13C is hygromycin resistant, while FATSS is both hygromycin resistant and β -*cin* silenced. The transgenic strains were grown in V8 media supplemented with 250 µg/ml hygromycin. Both the wild type and the transgenic strains were stored at the Mycological Library of the Laboratory of Molecular Biotechnology and Phytopathology, University of Algarve.

Histological studies of colonized root tissue

Histological sections were made from 2-months old *Q. suber* seedlings, grown from seeds collected from one tree located in Alentejo, Portugal. The acorns were surface-disinfested and germinated in sterile vermiculite in a growth chamber (300 µmol.m⁻².s⁻¹ light intensity over a 14 h light (20–25°C) / 10 h dark (10–15°C) photoperiod at 60–80% relative humidity). From germination until infection seedlings were grown in vermiculite irrigated with sterile water.

Seedlings were removed from vermiculite and the roots washed in sterile water. The lateral and fine roots of four plants per pathogen strain were inoculated by placing a 2 cm² V8 agar plug containing actively growing mycelium in contact with the root surface at a distance of 5 mm from the root tip for 3 days.

Infected roots (five root pieces per plant) were cut into 2–3 mm³ fragments and fixed for 16 h at 4°C in 0.1 M cacodylate buffer pH 7.2 containing 4% w/v paraformaldehyde and 0.5% v/v glutaraldehyde. (Lherminier et al 2003) The root material was then dehydrated by passing it through an ethanol series, embedded in acrylic embedding agent LR White (London Resin Company) and polymerized for 24 h at 57–58°C (Roland and Vian 1991).

From each strain, 20 root fragments were sectioned by microtome into 2–3 µm thick slices, which were stained in 0.5% aqueous toluidine blue. The sections were studied by light microscopy. In total, 400 sections per sample were analysed.

Soil infestation assay

Young (12–18 months old) *Q. suber* plants were obtained from a nursery (Direção Geral de Florestas, Montegordo, Portugal) for pathogenicity assessment of the strains by using a soil infestation assay.

As the genetically transformed strains have an impaired ability to produce zoospores (Horta et al. 2008), chlamydospore production was evaluated in order to be used as initial inoculum in soil infestation assays. The strains were grown in the dark at 25°C in clarified 10% V8 agar during 4 months. Fifteen ml of sterile water were added to each Petri dish (three per strain) to cover the mycelium. With an L-shaped rod, 360° rotations were done repeatedly until the mycelia were reduced into small pieces, to yield free

chlamydospores. This suspension was transferred to a 50 ml falcon and centrifuged at 2,590 g for 10 min. The supernatant was discharged keeping 500 μ l. This concentrated suspension was homogenised and the number of chlamydospores was determined using a Fuchs-Rosenthal counting chamber (Hausser Scientific Company, Horsham, USA). Microscopic counting showed that all strains produced similar numbers of chlamydospores (from 5×10^3 to $1,5 \times 10^4$ chlamydospores/ml). Thus, these propagules were used as inoculum in the soil infestation test.

Phytophthora cinnamomi liquid inoculum (Sanchez et al. 2002) was prepared by growing separately the strains in Petri dishes ($\varnothing=92$ mm) (36 plates per strain) containing 20 ml V8 liquid medium at 25°C in the dark for 30 days. At the end of this period, the medium was filtered and the mycelium washed. For each strain, the content of three Petri dishes was added to 100 ml sterile water and homogenised for 3 min. This inoculum suspension was distributed evenly into the

root ball of each plant. Each plant was then transferred to a pot containing 4 l of substrate (a non sterile 4:1 mixture of peat and sand).

Twelve plants per strain were inoculated while another twelve plants were used as control, by the addition of water instead of the inoculum suspension. All the plants were transferred to a greenhouse, grouped into separate trays to avoid cross-contamination and waterlogged 2 days per week during 3 months.

At the end of the experiment, each plant was removed from its pot, and roots washed to remove soil particles. The severity of foliar symptoms, ie. chlorosis, necrosis or abscission was assessed on a 0–4 scale, with 0 representing 0% symptomatic leaves, and 1–3 representing 1–33%, 34–66%, and 67–99% symptomatic leaves, respectively, while 4 denoted a dead plant (Sanchez et al. 2002). Root damage was quantified according to the same 0–4 scale, reflecting the abundance of roots and the percentage of roots showing necrotic symptoms. An analysis of variance was performed for both foliar

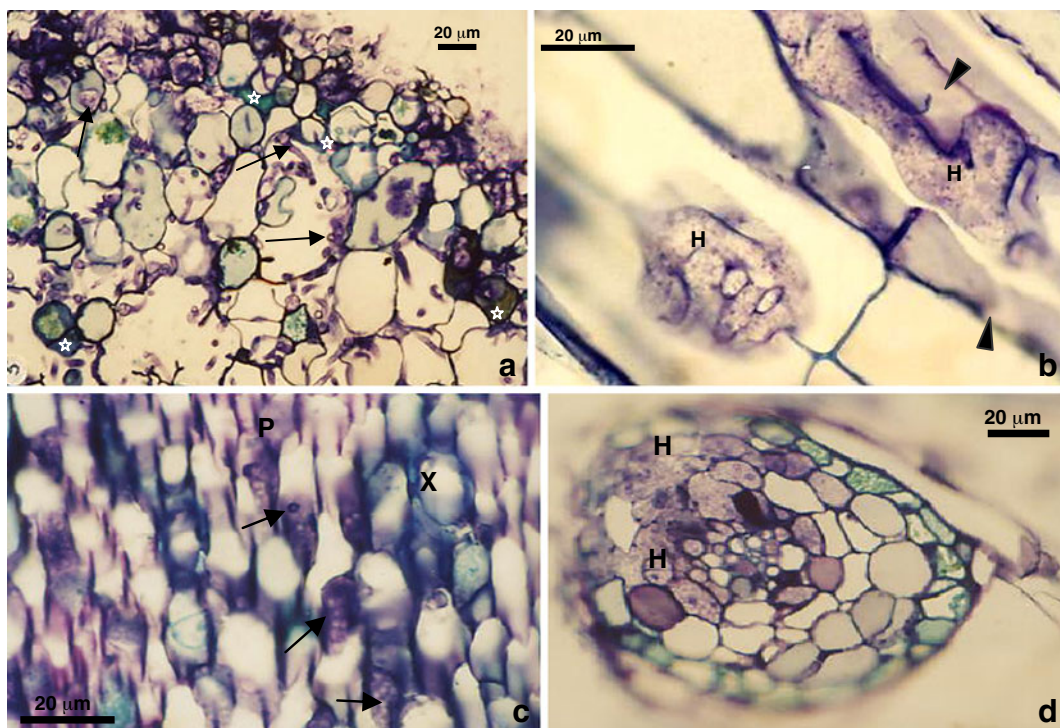


Fig. 1 Light microscopy sections of *Q. suber* roots inoculated with *P. cinnamomi* strain PA45 (*wild type*). **a** A large number of hyphae (arrows) has penetrated the epidermal and sub-epidermal cell layers and invaded the cortex (* *intercellular* and *vacuolar phenol-like materials*). **b** Hyphae (*H*) growing

actively within the cortical parenchyma and host cell destruction (*arrow-head*). **c** Colonization of the vascular cylinder (arrows), P–Phloem, X–xylem. **d** Cross section of a lateral root branch. The pathogen (*H*) has invaded the cortex and the vascular tissues

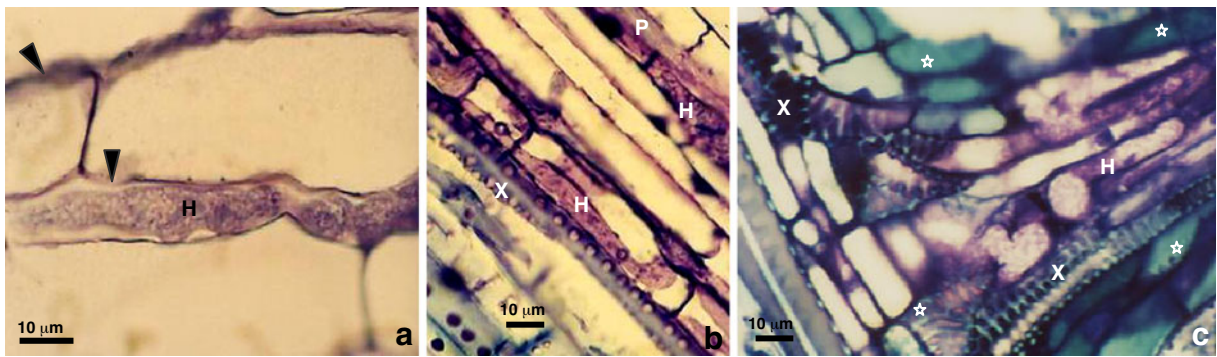


Fig. 2 Light microscopy sections of *Q. suber* roots inoculated with *P. cinnamomi* strain PA37 (wild type). **a** Active intercellular hyphal growth (H) within the cortical parenchyma and host cell destruction (arrow-head). **b** Colonization of the

vascular cylinder. The hyphae have invaded the phloem (P) and xylem (X). **c** Longitudinal section of a lateral root branch. Several hyphae (H) have invaded the xylem (* intercellular and vacuolar phenol-like materials)

and root symptom severity, and mean values were compared to each other by the Fisher’s protected LSD test (Steel and Torrie 1985), using SPSS v14.0 software (SPSS for Windows 2001).

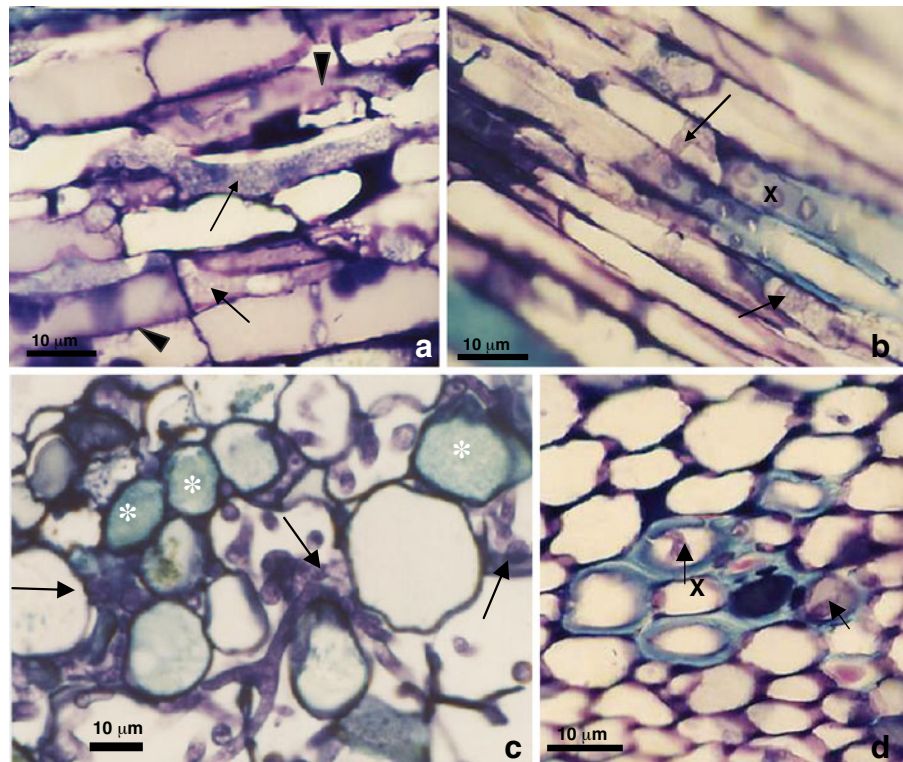
Fragments of roots from inoculated and control plants were washed in running tap water during 90 min and plated on PARPH medium for re-isolation of *P. cinnamomi* (Caetano 2007).

Results

Histological studies of colonized root tissue

All the inoculated roots appeared necrotic at the inoculation point. There was no noticeable difference between the apparent infectivity of strains PA45, PA37 and 13C, all being able to rapidly invade the

Fig. 3 Light microscopy sections of *Q. suber* roots inoculated with *P. cinnamomi* strain 13C (transgenic, hygromycin resistant). **a** A large number of hyphae (arrows) have colonized the cortical parenchyma. Host cell destruction (arrow-head). **b** Colonization of the vascular cylinder, longitudinal section. **c** (* intercellular and vacuolar phenol-like materials), and large number of hyphae (arrows). **d** Colonization of the vascular cylinder, cross section. Several hyphae (arrows) have penetrated the xylem (X)



cortical parenchyma both inter- and intracellularly (Figs. 1, 2 and 3). A large number of hyphae were visible in epidermic, sub-epidermic and inner cortical cell layers. Active growth of the pathogen in the cytoplasm and host cell destruction was observed. Many hyphae ramified throughout the cortex penetrating xylem and phloem vessels in the samples observed. About 10% intercellular spaces and 25% cell vacuoles turned a brilliant blue green when stained with toluidine blue.

In contrast, the β -*cin* silenced strain FATSS appeared as a weakly aggressive pathogen (Fig. 4). Only scarce hyphae were visible on the roots, and they appeared degraded in *ca* 90% of the sections. A weak colonization of the outer cortical parenchyma was noted in *ca* 10% of the sections, with the hyphae showing symptoms of degradation, such as cytoplasmic agglutination and cytoplasmic rarefaction. No FATSS hyphae were observed within the inner cortex or inside the vascular cylinder, even at the lateral root branching points, which represent the most vulnerable part of the root. No brilliant green precipitate was seen in any of the FATSS sections. In general, these roots had a similar aspect to the non-inoculated roots used as control (Fig. 5)

Soil infestation assay

The results of the soil infestation assays are presented in Figs. 6 and 7. The aerial symptoms of plants infected with wild type strains PA45 (Fig. 6b) and PA37 (Fig. 6d) consisted of leaf yellowing and wilting, reduced plant growth and leaf abscission. The mean scores of foliar symptoms for the two strains were 2.64 and 2.30, respectively. Functional fine roots were scarce, and those few which remained were necrotic (overall root scores of 3.01 for PA45 and 3.18 for PA37). Symptoms induced by strain 13C (Fig. 6e) scored 1.35 (aerial) and 2.39 (roots), while the FATSS strain (Fig. 6c) was less virulent (1.02

aerial, 1.45 root symptoms). Although roots were still present in plant (e), most of them presented necrotic lesions and were no longer suited to feed the plant. The waterlogging induced some degree of stress

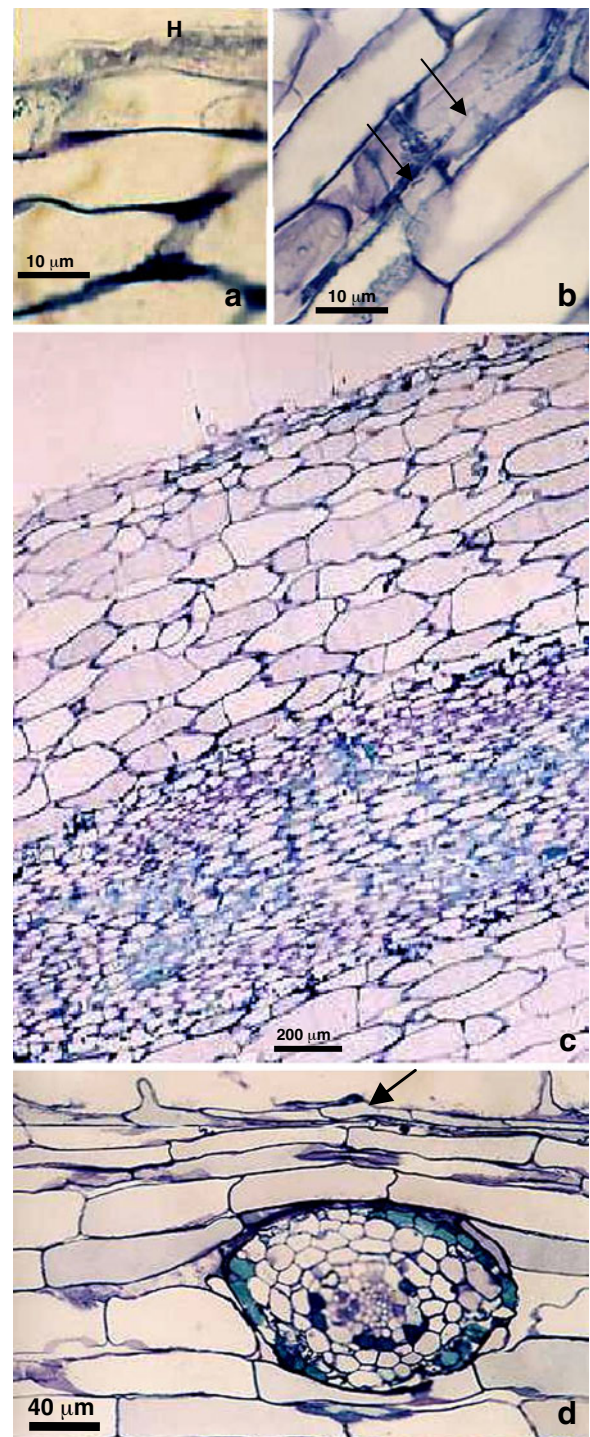


Fig. 4 Light microscopy sections of *Q. suber* roots inoculated with *P. cinnamomi* strain FATSS (transgenic, hygromycin resistant and β -*cin* knock-down). **a** Disorganized hypha (*H*) on the epidermis. **b** Damaged hyphae in the outer cell layers of the cortex. The cytoplasm is heavily agglutinated (*arrows*) or shows rarefaction. **c** Non-invaded cortex and vascular cylinder. **d** A longitudinal section shows a cross section of a lateral fine root branch and a damaged hypha (*arrow*) on the epidermis. FATSS is not observed within the vascular cylinder

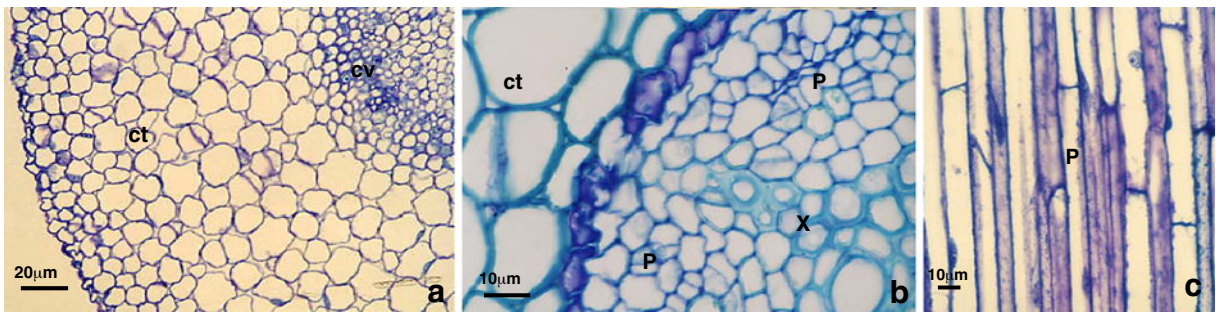


Fig. 5 Light microscopy sections of non-inoculated roots of *Q. suber* (control). **a** and **b** Cross sections. Cortex (*ct*) and vascular cylinder (*cv*), phloem (*P*) xylem (*X*). **c** Longitudinal section of phloem (*P*)

response in the control plants (Fig. 6a), as reflected by the symptom scores of 0.96 (aerial) and 0.91 (roots).

Root symptom severities as measure of virulence to cork oak showed significant differences between strains which could be grouped into classes I (highly virulent, PA 37 and PA45), II (medium virulence, 13C), and III (weakly virulent, FATSS). The strains fell into three classes on the basis of crown symptoms, namely I (high virulence, PA37 and PA45), II (weak virulence, 13C), and non virulent (FATSS).

In comparison to the mean root scores of the wild type strains, root symptom severities caused by the hygromycin resistant strain 13 C and the hygromycin resistant, β -*cin* silenced strain FATSS were reduced by 22.9% and 53.2%, respectively.

All strains of *P. cinnamomi* were successfully re-isolated from symptomatic roots while no *Phytoph-*

thora could be recovered from roots of non-inoculated control plants.

Discussion

Histological studies of root sections showed both the wild type and the transgenic 13C strains of *P. cinnamomi* were very aggressive, able to rapidly colonize the root tissues of cork oak, with hyphae proliferating both inter- and intra-cellularly through the epidermis, cortical parenchyma and vascular cylinder. This mode of invasion conforms to that observed in other woody hosts (Cahill et al. 1989; Widmer et al. 1998; Brummer et al. 2002; Pires et al. 2008). However, *Q. suber* appears to be much more affected by *P. cinnamomi* than *Q. robur* by *P.*



Fig. 6 Overall appearance of *Q. suber* 12–18-month-old plants at the conclusion of greenhouse pathogenicity test with *P. cinnamomi* strains. **a** Control plant. **b** Plant infected with wild type strain PA45. **c** Plant infected with the hygromycin

resistant, β -*cin* knock-down strain FATSS. **d** Plant infected with wild type strain PA37. **e** Plant infected with the hygromycin resistant strain 13C

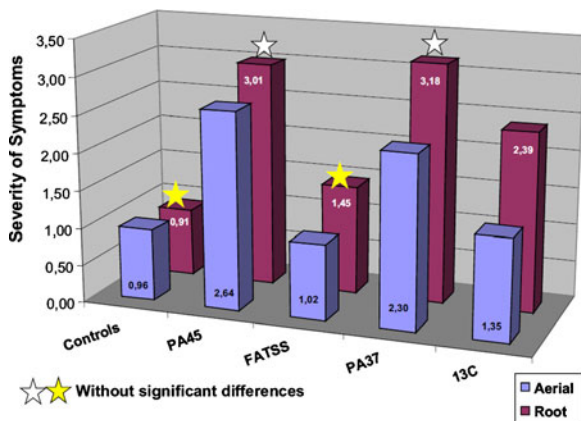


Fig. 7 Mean symptom severity in the shoot and root of cork oak plants infected with wild type and transgenic strains of *P. cinnamomi*

quercina since in the later case hyphae were only rarely observed inside xylem vessels (Brummer et al. 2002).

The wild type and the transgenic 13C strains induce brilliant green precipitates within the intercellular spaces and cells of the stained roots suggesting the formation of phenol/tannin-like compounds associated with the plant defence response (Cahill et al. 1989; Picard et al. 2000; Lherminier et al. 2003). However, despite the accumulation of those compounds, they did not prevent root invasion by *P. cinnamomi*. Thus, infection of *Q. suber* roots by wild-type and the transgenic 13C strains induce defence mechanisms not efficient enough to stop the root invasion and the subsequent disease symptom development. The same was observed in the interaction of *P. cinnamomi* with *Eucalyptus marginata* (Cahill et al. 1989).

The ability of the FATSS strain to invade the root tissue was substantially reduced. FATSS hyphae appeared to be degraded, probably because of their inability to synthesize pathogenic factors such as β -cinnamomin, required for the colonization process, and a determinant for the aggressiveness of the pathogen. The lack of any brilliant green deposit into the host cells infected by FATSS indeed suggests that either the low root colonization level of the FATSS strain was not able to induce the formation of phenol/tannin like compounds or the host did not recognize the presence of the pathogen because of the absence of β -cinnamomin. However, although infection of *Q. suber* roots by the β -*cin* mutant does not induce defence mechanisms, colonization of roots does not take place.

The *P. cinnamomi* strains fell into three classes (highly, medium and weakly virulent) on the basis of root symptoms induced in cork oak plants and into three classes (high virulence, weak virulence and non-virulent) on the basis of crown symptoms, in agreement with Moreira-Marcelino (2001) and Caetano (2007) who showed that the shoot damage does not always directly reflect the extent of root damage. This is clearly apparent in plants infected with strain 13C.

The transgenic strains 13C and FATSS have an impaired capacity to produce zoospores (Horta et al. 2008). Other reports have also noticed that *Phytophthora* strains transformed to become hygromycin resistant were compromised in their ability to produce zoospores, and it has been suggested that this failure may be due to the expression of the antibiotic resistance itself (Érsek et al. 1994; Gaulin et al. 2002). The quantitative reduction of zoospore inoculum of both transgenic strains, implying a reduced number of infections, most probably led to a lower colonisation rate and caused a decrease of symptom severity of both 13C (22,9% reduction) and FATSS (53,2% reduction) strains when compared to wild type strains. Differences in virulence between 13C and FATSS are explained by the lower aggressiveness of FATSS (reduced ability to colonize plant tissue, as observed by histological sectioning). Thus, the virulence of *P. cinnamomi* is not independent of its ability to colonize roots.

The silencing of β -*cin* gene drastically reduced the colonization of seedling roots of *Q. suber* in direct contact with *P. cinnamomi* mycelium suggesting that β -cinnamomin plays a key role in the invasion process, acting as an aggressiveness factor. Quercinin, another β -elicitor was also shown to be produced during the invasion process of *Q. robur* roots by *P. quercina* (Brummer et al. 2002), indicating a role for this group of elicitors in the early stage of tissue colonization. On the other hand, α -elicitors do not appear to be directly involved in the early steps of colonization as their expression is repressed at this stage. Their function seems to be related to later stages of infection, in particular sporulation and/or pathogen survival under saprophytic conditions. They also act as avirulence factors in specific *Phytophthora*/plant interactions (Kamoun et al. 1997; Kamoun et al. 1998; Colas et al. 2001). Jiang et al. (2006) have provided evidence that the elicitor family diversified prior to speciation and that variation is maintained by selective purifica-

tion. This strongly suggests that each elicitor group has its own distinct set of functions. The differential expression of each type of elicitor, with highly variable isoelectric points (pI) may be an adaptive response of the pathogen to regulate interactions within different surrounding environments, regardless of whether their function is distinct or not.

It cannot be excluded that the transformation process itself generated some collateral disruption of the pathogen genome, which may have altered physiological features of the genetically transformed strains, such as reducing the aggressiveness of the FATSS strain or the competitiveness of both transformed strains within the non-sterile environment used for the infestation test (Erwin and Ribeiro 1996, Zentmyer 1980). Moreover, *HAE- α -cin* transcripts are also inhibited in FATSS (Horta et al. 2008) and thus it can not be excluded that this protein is involved in the colonization process. However, HAE elicitors have not yet been isolated and studied. To clarify these issues it would be useful to generate other elicitor-knock down transformants; unfortunately this is a major technical challenge as *P. cinnamomi* has proven to be rather recalcitrant to transformation (Horta et al. 2008).

The molecular events associated with variation in pathogenicity of *Phytophthora* spp. remain the object of intensive research. In the present work we were able to show that the pathogenicity of *P. cinnamomi* is associated with the production of β -cinnamomin acting as an aggressiveness factor. The high virulence of *P. cinnamomi* to cork oak roots is primarily a consequence of its high aggressiveness although it cannot be excluded that virulence factors can also be involved.

Acknowledgements This research was financed by the EC—III Framework Programme for Research and Technological Development, co-financed by the European Social Fund (ESF) and by national funding from the Portuguese Ministério da Ciência e do Ensino Superior (MCES) (PTDC/AGR-AAM/67628/2006). M. Horta thanks Fundação para a Ciência e a Tecnologia (FCT) and ESF (EC—III Framework Programme) for her grant (SFRH/BD/1249/2000).

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