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Hydrogen peroxide scavenging mechanisms are components of *Medicago truncatula* partial resistance to *Aphanomyces euteiches*

Naceur Djébali · Haythem Mhadhbi · Claude Lafitte · Bernard Dumas · Marie-Thérèse Esquerré-Tugayé · Mohamed Elarbi Aouani · Christophe Jacquet

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Abstract The biochemical processes underlying the expression of resistance in the roots of *Medicago truncatula* against *Aphanomyces euteiches* infection was investigated, with emphasis on oxidative stress. The levels of H_2O_2, superoxide dismutase, peroxidase, ascorbate peroxidase, catalase, soluble phenolics and lignin were measured in the roots of two lines, A17 partially resistant and F83005.5 susceptible to *A. euteiches* at three infection stages; penetration of the epidermis (1 dpi), colonization of the cortex (3 dpi) and invasion of the root stele (6 dpi). A rapid and large decrease of the H_2O_2 levels in A17 roots occurred. However, in F83005.5 roots, the decrease in H_2O_2 levels was delayed until 3 dpi. In A17 roots, the activities of ascorbate peroxidase, peroxidase and catalase were induced as early as 1 dpi, whereas a general decrease in the activity of the four antioxidant enzymes was observed in F83005.5 roots. The levels of soluble phenolics and lignin were increased in A17 roots at 3 and 6 dpi, respectively. The H_2O_2 levels were negatively correlated to ascorbate peroxidase, catalase and lignin production at 1, 3 and 6 dpi, respectively in A17 roots. Physiological concentrations of H_2O_2 found in *M. truncatula* infected roots had no detrimental effect on the in vitro growth of this oomycete. Our data suggest that H_2O_2 does not have a direct antimicrobial effect on *M. truncatula* resistance to *A. euteiches*, but is involved in cell wall strengthening around the root stele, preventing pathogen invasion of the vascular tissues.

Keywords Antioxidant enzymes · Model legume · Oomycete · Partial resistance · Phenylpropanoid compounds · Reactive oxygen species

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
dpi	Day post inoculation
H_2O_2	Hydrogen peroxide
POX	Peroxidase
ROS	Reactive oxygen species
SOD	Superoxide dismutase

N. Djébali · H. Mhadhbi
Centre of Biotechnology of Borj Cedria,
Laboratory of Legumes,
BP 901, Hammam-Lif 2050, Tunisia

N. Djébali · C. Lafitte · B. Dumas ·
M.-T. Esquerré-Tugayé · C. Jacquet
Université de Toulouse, Surfaces Cellulaires
et Signalisation chez les Végétaux,
BP 42617, Auzeville 31326, Castanet-Tolosan, France

M. E. Aouani
NEPAD/North Africa Biosciences Network,
National Research Center,
El Buhouth St,
Cairo 12311, Egypt

Present Address:
N. Djébali (✉)
Centre of Biotechnology of Borj Cedria,
Laboratory of Molecular Physiology of Plants,
BP 901, Hammam-Lif 2050, Tunisia
e-mail: dnaceur@yahoo.fr

Introduction

Pea root rot, caused by *Aphanomyces euteiches* Drechs, is the most damaging root disease of pea in most pea-producing areas. The only means of controlling the disease currently available is to avoid using infested fields for pea crops. Therefore, the development of new strategies to control *A. euteiches* is necessary and will require a detailed knowledge of the mechanisms of resistance underlying this oomycete-plant interaction. Genetics may provide one way to control this disease. However, breeding for resistance to *A. euteiches* is difficult for two main reasons: only a few sources of partial resistance have been identified in pea germplasm, and the resistances identified are polygenic, with low heritability (Pilet-Nayel et al. 2002). Under field conditions, *A. euteiches* has an extremely wide host range (Grau et al. 1991). The model legume *Medicago truncatula*, another host for this pathogen with a conserved synteny to pea (Aubert et al. 2006), has the potential to serve as a surrogate for genetic analysis of disease resistance in pea and to study mechanism of resistance.

Plants have developed strategies to defend themselves against pathogen attacks. One of the earliest plant defence responses is the production of reactive oxygen species (ROS) after pathogen recognition (Bolwell and Daudi 2009). These oxygen intermediates (superoxide [$O_2^{\bullet -}$], hydrogen peroxide [H_2O_2] and the hydroxyl radical [$HO^{\bullet}$]) serve as signalling molecules and appear to form part of a signalling cascade, resulting in the activation of plant defence responses (Lamb and Dixon 1997). ROS also have direct antimicrobial activity, inhibiting spore germination of many fungal pathogens (Peng and Kuc 1992). H_2O_2 hinders micro-organism penetration in plant tissues because it contributes to wall strengthening by facilitating peroxidase reactions, catalyzing intra- and inter-molecular cross-links between structural components of cell walls and lignin polymerization (Ralph et al. 2004). The interaction between oomycete pathogens and plants leads to the production of ROS at early time points of the interaction. This oxidative burst seems to be effective in controlling pathogen infection in the case of incompatible interactions. However, only a transient and moderate production of ROS was detected in the case of compatible interactions that do not prevent pathogen spread (Lebeda et al. 2008). The species belonging to the genus *Aphanomyces* are considered as necrotrophs in the oomycete group (Latijnhouwers et al. 2003). Whereas the ROS-based defence system is highly effective against biotrophic pathogens (which are dependent on living host cells), necrotrophic pathogens can also colonize dead tissue and could therefore benefit from such a host defence response, suggesting that other roles exist for ROS, such as signalling (Govrin and Levine 2000). For necrotrophic oomycete pathogens relatively little research has attempted to elucidate the role of ROS in plant defence, especially at later stages of the interaction. The availability of *M. truncatula* lines differing in degree of resistance to *A. euteiches* root infection, i.e., line F83005.5 (susceptible) and line A17 (partially resistant) (Djébali et al. 2009), provides a possibility for examining the mechanisms of resistance in this model legume against *A. euteiches* attack from the viewpoint of oxidative stress.

Several mechanisms by which plant cells regulate their intracellular ROS level have been described. Plants possess a number of enzymatic and non-enzymatic mechanisms of ROS detoxification. Superoxide dismutase (SOD) catalyses the dismutation of O_2^- to H_2O_2, catalase (CAT) scavenges H_2O_2 to oxygen and water, and ascorbate peroxidase (APX) reduces H_2O_2 to water by utilizing ascorbate (ASC) as specific electron donor. Differently from APX, peroxidases (POX) do not use ASC as their direct electron donor, but they oxidize other molecules, mainly of phenolic nature. These are considered the main enzymatic systems for protecting cells against oxidative damage (De Gara et al. 2003). In addition to this enzymatic system, phenolics (especially flavonols and hydroxycinnamic acids) can be oxidized by peroxidase, and a H_2O_2 scavenging function for ascorbic acid/phenolics/peroxidase systems has been proposed (Takahama 2004). Some phenylpropanoids (hydroxycinnamyl alcohols) are constituents of lignin and these phenolics are oxidized by peroxidase during lignin formation at the expense of H_2O_2 (Ralph et al. 2004).

The current study aims to enhance understanding of the infection process of *A. euteiches* in *M. truncatula* roots and the biochemical processes underlying the expression of resistance in the roots of this legume against *A. euteiches* infection, with emphasis on oxidative stress. We focused on H_2O_2

and four anti-oxidant enzyme systems (POX, APX, SOD and CAT) at three infection stages of *A. euteiches* in *M. truncatula* roots. Also, the levels of soluble phenolics and lignin were determined in the roots of the lines A17 and F83005.5, together with histochemical localization of peroxidases. In addition, the effect of H_2O_2 on *A. euteiches* growth was performed by an in vitro sensitivity assay.

Materials and methods

Plant and pathogen material

Seeds of lines F83005.5 and A17 were provided by J. M. Prosperi (Institut National de la Recherche Agronomique SGAP Laboratory, Mauguio, France). *M. truncatula* seed germination took place as described by Djébali et al. (2009). *A. euteiches* (strain ATCC201684) culture conditions and inoculum preparation were performed using the protocol described by Badreddine et al. (2008).

Plant growth, inoculation and sampling dates

After germination, 5 seedlings were placed on M medium (Bécard and Fortin 1988) without sucrose (pH 5.5) in square Petri dishes (12 cm×12 cm) and grown vertically in an incubator (FirLabo SP-BVEHF, France) at 22°C/18°C day/night and 16 h photoperiod of fluorescent light (60 μE). For biochemical and microscopic analyses, the whole roots of 15-day old plants were inoculated with a zoospore suspension (10^5 ml^{-1}) containing 0.01% Tween 20. Control plants were inoculated with sterile spring mineral water (Volvic, Auvergne, France) containing the same amount of Tween 20. According to the *A. euteiches* infection progress in *M. truncatula* roots, three time points were studied: infection of the root epidermis (1 dpi), invasion of the root cortex (3 dpi) and beginning of invasion of the stele in the susceptible line F83005.5 (6 dpi).

H_2O_2 extraction and measurements

H_2O_2 measurements were performed using a chemiluminescence method according to Warm and Laties (1982). Roots excised from the plants were immediately ground in liquid nitrogen with a mortar and pestle, weighed and placed in an Eppendorf tube. The roots of approximately 10 to 15 plants were pooled for each extraction. Per 100 mg of fresh weight (FW), 300 μl of a mixture (9:1, v/v) of methanol: 0.5 M EDTA (pH 8) was used to extract H_2O_2. After 15 min centrifugation (13,000 × g) at 4°C, the supernatant was transferred to a fresh Eppendorf tube. For H_2O_2 chemiluminescence measurement, the reaction mixture contained 20 μl 3-aminophthalhydrazide (luminol) (0.11 μM) and 20 μl extract. The reaction was started by the addition of 20 μl potassium ferrycianide ($K_3[Fe(CN)_6]$) (14 mM). The luminol is oxidized by the H_2O_2 in a basic medium to form the 3-aminophthalate accompanied by a light emission which is correlated to the H_2O_2 concentration (Yuan and Shiller 1999). Chemiluminescence was measured continuously using a Sirius single tube luminometer (Berthold detection system, Germany) for 2 min. The values represent the maximum intensity of chemiluminescence reached in 30 s, which were converted in nmol H_2O_2 g^{-1} root FW on the basis of a standard curve made with known concentrations of H_2O_2 (50%, w/v, Acros Organics).

Enzyme extraction and activity assays

To maintain enzyme activities, all operations were carried out at 4°C. Frozen roots of 10 to 15 plants were crushed to a fine powder with 5% (w/w) PVPP in a mortar with liquid nitrogen. Soluble proteins were extracted by re-suspending 500 mg of the powder in 2 ml extraction buffer containing 50 mM potassium phosphate buffer, (pH 7.8), 0.1 mM EDTA, 1 mM PMSF and 10 mM DTT, to which was added 5 mM ascorbate for studies of APX activity. The homogenate was centrifuged at 14,000 × g for 30 min. Enzyme extracts were stored at −80°C until use. Protein concentration was measured by the Bradford method (Bradford 1976) using a Protein Assay Kit (Biorad) with bovine serum albumin as a standard (Sigma).

POX [EC1.11.1.7] activity was determined according to the method described by Lin and Kao (1999). The reaction mixture consisted of 976 μl potassium phosphate buffer (50 mM, pH 7), 10 μl guaiacol (900 mM), 20 μl H_2O_2 (500 mM) and 3 μl enzyme extract. The reaction was started by adding H_2O_2 and the oxidation of guaiacol was determined by measuring the increase in absorbance at 470 nm. Enzyme

activity, expressed as μmol min^{-1} mg^{-1} protein of oxidized guaiacol, was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} for guaiacol.

APX [EC.1.11.1.11] activity was determined by measuring the decrease in absorbance of oxidized ascorbate at 290 nm, according to Nakano and Asada (1980). The reaction mixture consisted of 4 μl H_2O_2 (50 mM) and 20 μl enzyme extract in 976 μl phosphate buffer (50 mM, pH 7) containing 0.5 mM ascorbate (5 mg ascorbate in 50 ml phosphate buffer). Enzyme activity, expressed as μmol min^{-1} mg^{-1} protein of oxidized ascorbate, was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbate.

Total SOD [EC1.15.1.1] activity was assayed based on the method of Yu and Rengel (1999) by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT). The reaction mixture consisted of 100 μl Hepes (500 mM), 100 μl EDTA (1 mM), 100 μl Na_2CO_3 (500 mM, pH 10.4), 100 μl methionine (130 mM), 100 μl Triton X-100 (0.25% w/v), 10 μl NBT (7.5 mM) (Sigma), 10 μl riboflavin (200 μM) (Sigma) and 50 μl enzyme extract in 430 μl of phosphate buffer (50 mM, pH 7). The reaction was started by adding riboflavin, and A_{560} was measured after 10 min incubation under continuous fluorescent light. One SOD unit was defined as the amount of enzyme (volume of enzyme extract) that inhibited the rate of NBT reduction by 50% (Beauchamp and Fridovich 1971).

CAT [E.C. 1.11.1.6] activity was determined by following the decline of absorbency (decomposition of H_2O_2) at 240 nm (Aebi 1984). The reaction mixture consisted of 20 μl H_2O_2 (500 mM) and 10 μl enzyme extract in 970 μl phosphate buffer (50 mM, pH 7). CAT activity, expressed as μmol min^{-1} mg^{-1} protein, was calculated using the extinction coefficient of 36 M^{-1} cm^{-1} for H_2O_2.

Soluble phenolics extraction and quantification

Root samples (300 mg FW) were crushed to fine powder in a mortar with liquid nitrogen and homogenized with 1 ml 80% aqueous methanol. The homogenized samples were incubated for 5 min at room temperature, and then centrifuged 2 min at 10,000 × g. The supernatant was transferred to a fresh tube. The residue was homogenized again with 1 ml 80% aqueous methanol and after centrifugation, the supernatant was added to the first collected supernatant. The 2-ml extract was dried using a SpeedVac and the residue re-suspended in 1 ml 50% aqueous methanol. Samples were used immediately for phenolic determination or stored at −20°C. Total soluble phenolics levels were determined using the Folin-Ciocalteu reagent and the absorbance was measured at 760 nm according to the method of Singleton and Rossi (1965). The content of the soluble phenolics was calculated from a standard curve obtained with different concentrations of ferulic acid (1 to 30 μg) (Sigma-Aldrich).

Lignin extraction and quantification

M. truncatula infected and non-infected (control) roots (700 mg FW) were crushed to fine powder in a mortar with liquid nitrogen. The powder was transferred to a test tube and washed twice in 5 ml phosphate buffer (0.1 M, pH 6.8) at room temperature. After 10 min centrifugation at 2,000 × g, the resulting residue was washed twice with double-distilled water. The pellet was then incubated two times in 5 ml of a methanol:chloroform (2:1; v/v) mixture for 15 min each time, to remove lipids and pigments from cell wall. After centrifugation (2,000 × g, 10 min) the residue was dried by acetone and then placed at 50°C for 48 h. Five ml of NaOH (0.5 M) was added to the dried residue and incubated for 1 h at 90°C to remove proteins and phenolics from cell wall. The NaOH was then eliminated by washing the cell walls three times with double-distilled water. The finally obtained material was dried, as previously described, and weighed. The lignin content was determined by the acetyl bromide/acetic acid method (Morrison and Stewart 1995). The absorbance was measured at 280 nm. The amount of lignin was calculated from a linear calibration curve (0–0.1 mg) with lignin alkali, 2-hydroxypropyl ether (Sigma-Aldrich).

In vitro H_2O_2 sensitivity assay

H_2O_2 (50%, w/v) was added at different concentrations (0, 8, 21, 300 μM and 20 mM) to plates containing autoclaved Peptone Glucose Agar (PGA) medium (Soya Peptone 20 g, Glucose anhydrous 5 g,

Agar-agar 15 g per 1 l distilled water). The concentrations 8 and 21 μM was the highest physiological concentration found in A17 infected and control roots respectively in this study. The concentration 300 μM was found by García-Pineda et al. (2010) in avocado roots inoculated with the oomycete *Phytophthora cinnamomi* at 4 dpi. In order to know the limit of tolerance of *A. euteiches* to H_2O_2, the concentration of 20 mM was tested, which was reported to partially inhibit the in vitro growth of *P. cinnamomi* (García-Pineda et al. 2010) and totally inhibit the in vitro growth of the fungal pathogen *Botrytis cinerea* (Schouten et al. 2002). Plates were inoculated with plugs of agar containing 15-day old mycelium of *A. euteiches* with the mycelium facing down and incubated at 24°C in darkness. Mycelial growth on these plates was determined every 24 h by measuring colony diameter. The fungicide Propinebe (Antracol 70WP; Bayer®) used to control plant diseases caused by oomycete pathogens was added as a negative control for *A. euteiches* mycelia growth at the manufacture recommended rate (2.5 g l^{-1} of Antracol 70WP).

Microscopy

Observations of A. euteiches structures in inoculated roots

To study the infection biology of *A. euteiches* in *M. truncatula* roots, infected organs were fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 24 h at 4°C. For bright field microscopy, fixed root samples were rinsed in the buffer without glutaraldehyde, dehydrated in a series of aqueous solutions of increasing ethanol concentration (10, 30, 50, 70, 80, 95, 100%, 2 h each) and infiltrated step-wise (25, 50, 75 and 100%, in ethanol, 12 h each at 4°C) with Spurr's epoxy resin (Oxford Agar, Oxford, UK). Infiltrated samples were embedded in moulds and allowed to polymerize for 24 h at 70°C. Semi-thin (1 μm thickness) sections were prepared using an UltraCut E ultramicrotome (Reichert-Leica Germany). The sections were mounted on glass slides and stained with Toluidine Blue O (0.5%, w/v in an aqueous solution of 2.5% sodium carbonate, pH 11). For electron microscopy, intact fixed roots were mounted on metal stubs and observed without staining in a Scanning Electron Microscope (Hitachi S 450, Singapore) at 15 KV. For each treatment, at least 20 infection sites were examined.

Quantification of oospores in inoculated roots

The method described by Colditz et al. (2005) was used, for *in planta* staining of *A. euteiches* oospores at 6 and 21 dpi with some modifications. The inoculated roots were incubated for 30 min in KOH (10%, w/v) at 90°C. The roots were stained with a solution of Blue Ink (2%, v/v) and acetic acid (7%, v/v) for 20 min at 90°C, and then rinsed three times with de-ionized water. Twelve roots from each parental line were examined. The counting was done on a length of 5 mm for each root in a bright field microscope.

Histochemical localization of POX activity

Intact roots were incubated for 15 min in succinate buffer (25 mM succinic acid, 3.5 mM EDTA, pH 4), containing 19 mM H_2O_2 (50%, w/v) and 4-chloro-1-naphthol (0.6 mg ml^{-1}) (Sigma-Aldrich) as staining reagent. H_2O_2 is used by endogenous peroxidases that oxidize 4-chloro-1-naphthol, causing the formation of a precipitate that turns dark blue. After staining, roots were sectioned using a vibratome (Leica VT1000S). Sections, 300 μm in thickness, were observed using an inverted microscope (DMIRBE, Leica, Rueil-Malmaison, France). For each treatment, measurements were done on root sections from 12 plants.

Statistical analyses

Data were subjected to an analysis of variance using the line, the treatment and the sampling date as factors (three-way complete model) in the Statistica software version 5.1 (www.statsoft.com) and means were compared with Duncan's multiple range test. The level of significance was set to 0.05 and adjusted for multiple comparisons of means by Bonferroni corrections. Correlations between measured parameters were estimated by computing Pearson's correlation coefficient (r) based on the means of each line per treatment and per sampling date combination separately. All experiments were repeated twice. The results are presented as averages of two experiments.

Results

First steps of *A. euteiches* infection process in *M. truncatula* roots

Observations of the oomycete structures on and inside the roots of *M. truncatula* were made using scanning electron (Fig. 1a) and bright field microscopy (Fig. 1b-e) respectively, to gain information on the first steps of infection. No differences in the development of the oomycete on the root surfaces of the two *M. truncatula* lines, F83005.5 and A17, were observed. The biflagellate zoospores of *A. euteiches* lost their flagellas and encysted to form spherical cysts (Fig. 1a). The germination of a cyst started from 1 dpi, giving rise to a germ tube that penetrated the root (Fig. 1a). No specialized appressorium-like structures were observed where the pathogen penetrated the epidermis directly. At 3 dpi, coenocytic hyphae of *A. euteiches* invaded the root cortex (mainly extracellularly) of the two *M. truncatula* lines, but with a difference in speed of colonization and oospore production within root tissues. Indeed, in the intercellular spaces and in the plant cell compartments, there were numerous visible hyphae (Fig. 1b). Oospores were produced in line F83005.5 in large numbers and some areas of the cortical tissues were strongly damaged. However, in A17 sections (Fig. 1c) only few oospores were observed within the roots in comparison to line F83005.5. The number of oospores was more than 2.5 fold higher in roots of F38005.5 compared to A17 at 6 dpi (Fig. 2). The epidermal and cortical cell layers were intact in A17 infected roots in comparison to the susceptible line F83005.5. At 6 dpi, oospores were already present within the stele of F83005.5 roots, whereas infection was not observed in the stele in the partial resistant line A17 (Fig. 1e).

H_2O_2 measurements by chemiluminescence

The level of H_2O_2 in infected and non-infected roots of the two lines showed reductions of 62.3, 55.5 and 53.3% in A17 infected roots compared to the controls at 1, 3 and 6 dpi, respectively (Table 1). In the roots of the susceptible line, no difference in H_2O_2 level was seen between inoculated and control plants at 1 dpi. At 3 and 6 dpi, infected roots of the susceptible line showed a decrease of 24.1 and 54.3% in H_2O_2 levels respectively, compared to the control (Table 1).

Fig. 1 Microscope observations of early steps of *A. euteiches* infection in roots of two *M. truncatula* lines, F83005.5 (susceptible) and A17 (partially resistant) to the pathogen. **a**: Scanning electron microscopy of germinating cyst at the root surface. **b-c**: Thin transverse sections of (**b**) F83005.5 and (**c**) A17 roots stained with Toluidine Blue O at 3 dpi. **d-e**: Thin transverse sections of (**d**) F83005.5 and (**e**) A17 roots stained with Toluidine Blue O at 6 dpi. Large arrows (**b**, **d**), indicate damaged tissues and *black* and *red arrows* indicate oospores in the cortical tissues and in the stele. PP: penetration point, Cy: cyst, GCy: germinated cyst. Co: cortical tissues, St: stele of the root. Figure 1a: Bars=10 μm. Figure 1b-e: Bars=100 μm

Antioxidant enzyme activities

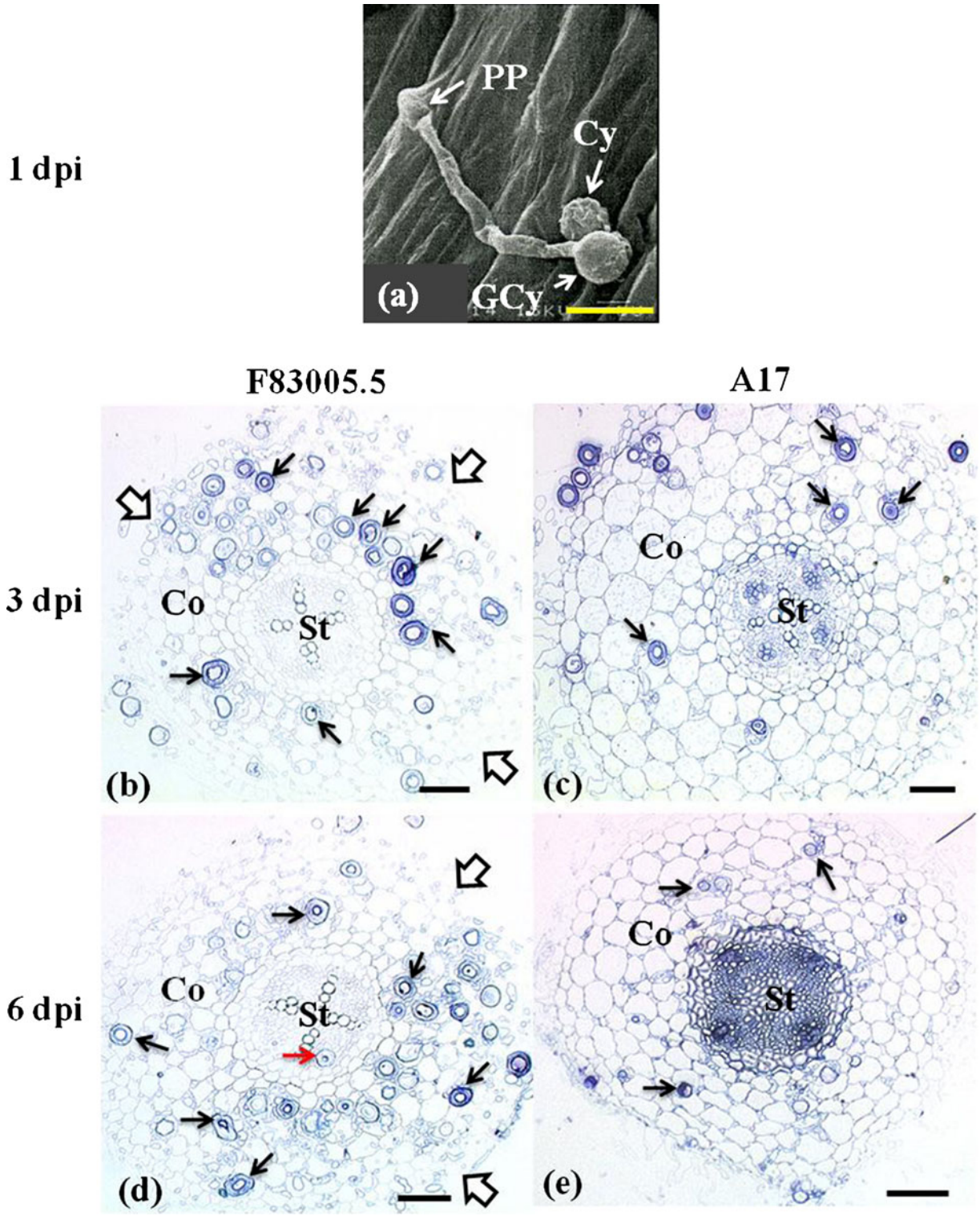
In F83005.5, the APX activity was unchanged at 1 dpi in infected roots and then significantly decreased at 3 and 6 dpi compared to control roots (Table 1). In A17 roots, the APX activity was significantly enhanced after infection by *A. euteiches* at 1 dpi, but unchanged at 3 and 6 dpi (Table 1). The POX activity decreased significantly in infected F83005.5 roots at 1 dpi, but increased significantly in infected A17 roots at the same sampling time. There were no significant changes in POX activity at 3 and 6 dpi in either line (Table 1). In infected F83005.5 roots, the CAT activity showed a decrease about 37, 80 and 56% at 1, 3 and 6 dpi, respectively. By contrast, the CAT activity increased about 300, 650 and 300% at 1, 3 and 6 dpi, respectively, in A17 infected roots (Table 1). The SOD activity decreased significantly in F83005.5 inoculated roots at 1 and 3 dpi. The SOD activity decreased significantly in A17 infected roots at 1 dpi and then remained unchanged (Table 1).

Soluble phenolics and lignin quantification

The concentration of phenolic compounds was significantly increased in infected A17 roots at 3 and 6 dpi. However, it was unchanged in F83005.5 infected roots at all the three sampling dates (Table 1). The lignin level was increased in infected A17 roots at 6 dpi, but was not changed in the infected roots of F83005.5 at the three sampling dates (Table 1).

Histochemical detection of POX activity

Histochemical detection of POX activity was achieved by incubation of roots in succinate buffer containing H_2O_2 and the chromogenic substrate 4-chloro-1-naphthol. The POX activity is detected by the formation of a dark blue precipitate consequently



to the oxidation of 4-chloro-1-naphthol by endogenous peroxidases at the expense of H_2O_2. Control roots of lines A17 and F83005.5 did not show any dark blue precipitate (Fig. 3a and b), as well as

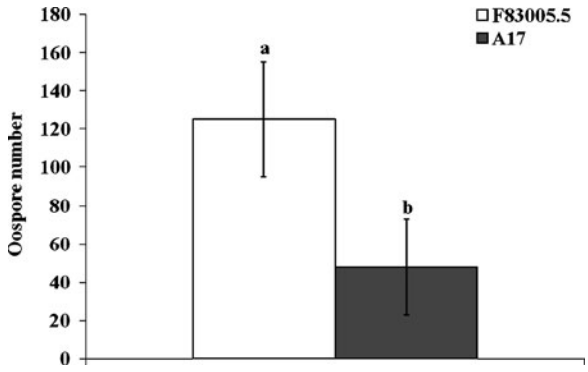


Fig. 2 Number of *A. euteiches* oospores on the surface of the roots of two *M. truncatula* lines at 6 dpi. Bars marked with different letters are significantly different at $p \leq 0.05$ (Duncan's multiple range test)

inoculated F83005.5 roots at 1 dpi (Fig. 3c). There was an early accumulation of POX at 1 dpi in A17 root epidermis (Fig. 3d). Contrary to F83005.5 roots (Fig. 3e), A17 roots showed an accumulation of POX activity in the epidermis and the first cell layers of the cortex at 3 dpi (Fig. 3f). In F83005.5 roots, no POX staining was observed, at 6 dpi (Fig. 3g and i). Additionally, few cells undergoing phenolics oxidation, if any, were observed in F83005.5 roots (Fig. 3g and i). At 6 dpi, POX activity was observed in A17 cortical root cells adjacent to those undergoing phenolic oxidation which were distinguished by a brown colour (Fig. 3h and j).

Sensitivity of *A. euteiches* to H_2O_2

To analyze the sensitivity of *A. euteiches* to H_2O_2, mycelium was grown in Petri dishes in the presence of four externally added H_2O_2 concentrations, i.e., 8 µM, 21 µM, 300 µM and 20 mM. Concentrations of 8 and 21 µM H_2O_2 increased *A. euteiches* growth in vitro. At 300 µM H_2O_2, *A. euteiches* growth was comparable to the control. Total inhibition of growth was observed with 20 mM H_2O_2 and Propinebe, a fungicide to control plant diseases caused by oomycete pathogens (Table 2).

Correlations between H_2O_2 levels, antioxidant enzymes, soluble phenolics and lignin content in roots

Calculation of correlations between H_2O_2 levels, the activities of the four antioxidant enzymes and the concentration of soluble phenolics and lignin, indicated

Table 1 Amount and activity of H_2O_2, ascorbate peroxidase (APX), peroxidase (POX), catalase (CAT), superoxide dismutase (SOD), soluble phenolics and lignin in uninoculated controls and *A. euteiches*-inoculated roots of the two *M. truncatula* lines F83005.5 (susceptible) and A17 (partially resistant) to this Oomycete at 1, 3 and 6 days post inoculation (dpi)

	F83005.5						A17					
	Control			Inoculated			Control			Inoculated		
	1 dpi	3 dpi	6 dpi	1 dpi	3 dpi	6 dpi	1 dpi	3 dpi	6 dpi	1 dpi	3 dpi	6 dpi
H_2O_2 (µmol g^{-1} FW)	18.64bc	20.79ab	19.71abc	20.79b	15.77d	9.0f	20.93a	18.06c	11.71e	7.88f	8.03f	5.46g
APX (µmol min^{-1} mg^{-1} protein)	4.62cd	5.06c	4.9c	5.83bc	3.77e	2.75f	4.56cd	4.71bc	4.87cd	7.87a	6.28b	3.97de
POX (µmol min^{-1} mg^{-1} protein)	54bc	58.52ab	61.14ab	36.67f	52.89bc	51.6bcd	40.34ef	47.14cde	53.56bc	50.81cd	44.88de	50.39cd
CAT (µmol min^{-1} mg^{-1} protein)	55.07b	53.62b	21.48de	34.83c	9.91f	9.47f	23.32d	10.48f	17.49e	71.76a	69.12a	51.83b
SOD (U mg^{-1} protein)	68.29a	58.42b	48.75c	37.46d	32.39de	44.99c	45.33c	37.78de	38.54de	33.88de	30.77e	30.65e
Soluble phenolics (µg mg^{-1} DW)	8.93e	10.5 cd	7.48f	9.45de	11.5bc	8.1ef	9.1e	9.33de	8.8ef	9.05e	13.45a	12.23ab
Lignin (µg mg^{-1} DW)	17.08cdef	14.7f	19.00bc	15.35ef	15.78def	18.43bc	18.33bcd	17.45bcde	17.33bcde	17.43bcde	19.88b	25.48a

FW fresh weight, *DW* dry weight, Comparison of means can be made only horizontally and means marked with different letters are statistically different after using Bonferroni corrections (α=(0.05/12=0.0041)

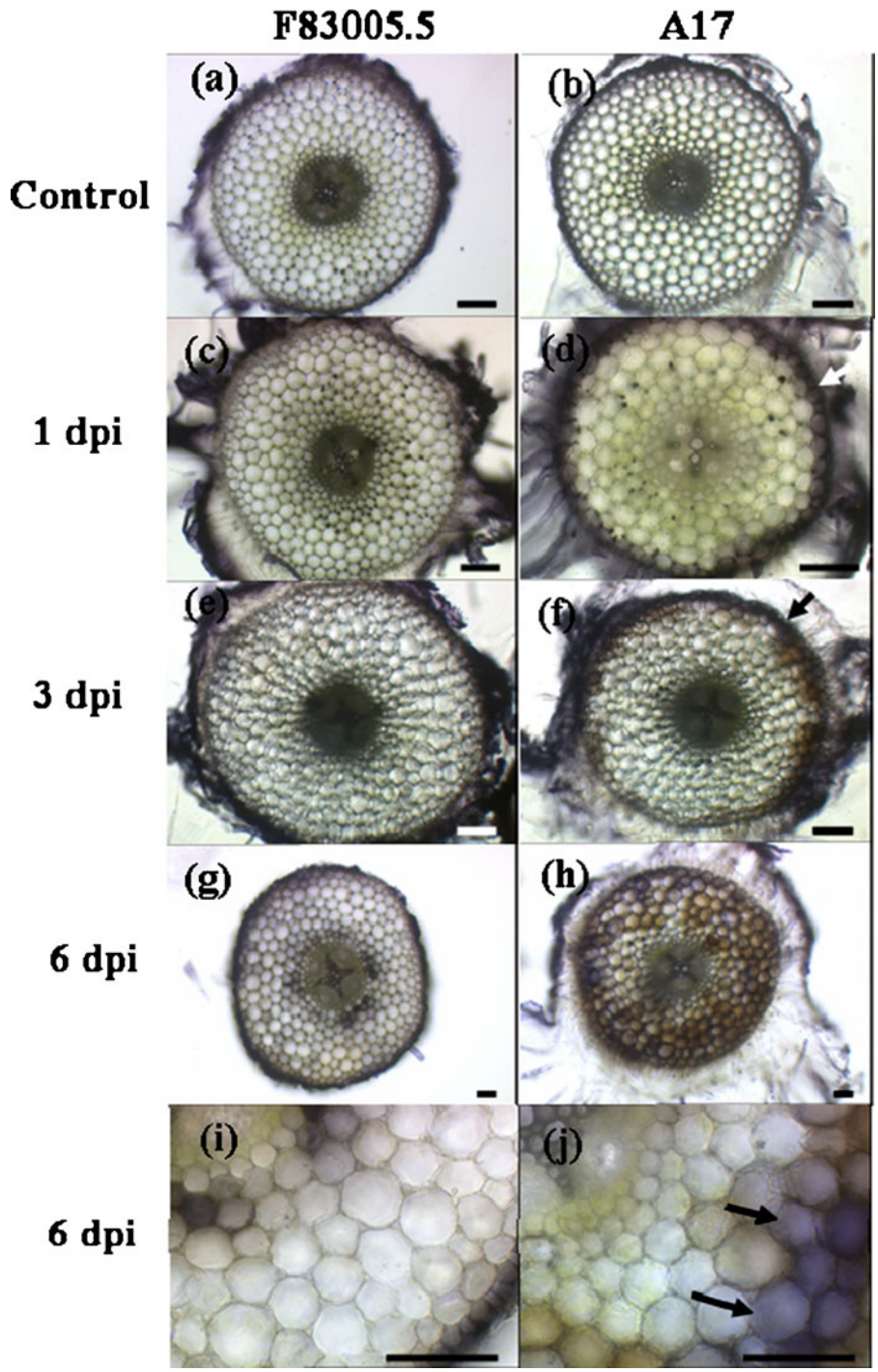


Fig. 3 Histochemical detection of peroxidase activity in the roots of two *Medicago truncatula* lines F83005.5 (susceptible) and A17 (partially resistant) either inoculated with *Aphanomyces euteiches* or left untreated. Roots were incubated in succinate buffer containing the 4-chloro-1-naphthol as a staining reagent. **a**-**b** Root sections of non-infected plants (control) of lines F83005.5 and A17, respectively. **c**, **e**, **g** F83005.5 infected roots at 1, 3 and 6 dpi, respectively. **d**, **f** and **h** A17 infected roots at 1, 3 and 6 dpi, respectively. At 1 and 3 dpi, POX activity (*arrow*) was detected only in epidermal cells of A17 infected roots. (**i**) and (**j**) show details of (**g**) and (**h**), respectively. Note absence and presence of POX activity (*arrow*) in cortical cells of F83005.5 and A17 infected roots at 6 dpi, respectively. Bars=100 μm

that no significant correlations were found in F83005.5 roots at the three sampling dates (Table 3). However, in A17 roots, H_2O_2 concentration was negatively correlated to APX activity, CAT activity and lignin content at 1, 3 and 6 dpi, respectively (Table 3).

Discussion

This study reports for the first time the infection process of *A. euteiches* in roots of the plant model *M. truncatula*. The inoculation of *M. truncatula* roots

Table 2 Effect of H_2O_2 on *Aphanomyces euteiches* mycelial growth

H_2O_2 concentration	Day after treatment							
	1	2	3	4	5	6	7	8
	Colony diameter (mm)							
0 μM H_2O_2	2.3b	12.1c	26.6b	41.1a	56.2a	66.4b	68.9b	71.9b
8 μM H_2O_2	6.1a	18.7a	31.6a	44.4a	58.7a	69.1ab	73.9a	75.6a
21 μM H_2O_2	7.1a	19.5a	31.9a	44.4a	60.3a	71.1a	75.3a	76a
300 μM H_2O_2	2.5b	15.6b	29.9a	44.1a	59.7a	70.1ab	75a	75ab
20 mM H_2O_2	0.0c	0.0d	0.0c	0.0b	0.0b	0.0c	0.0c	0.0c
Propinebe	0.0c	0.0d	0.0c	0.0b	0.0b	0.0c	0.0c	0.0c

Comparison of means can be made only vertically and the means marked with different letters are significantly different at $p \leq 0.05$ (Duncan's multiple range test)

with *A. euteiches* zoospores led to infection-like processes, as are known for plant-pathogenic root-infecting oomycetes (Kamoun 2006). *A. euteiches* entered the root epidermis at 1 dpi without formation of appressoria as previously reported by Trapphoff et al. (2009) in *M. truncatula* cell suspension inoculated with *A. euteiches* zoospores. By comparison to the infection process of *A. euteiches* in pea roots (Cunningham and Hagedorn 1962), we noted that our *A. euteiches* strain takes longer time to invade *M. truncatula* roots. In fact, in their work Cunningham and Hagedorn (1962) observed that *A. euteiches* invaded the pea root stele within 61 h. On the other hand, the invasion of the root stele of *M. truncatula* F83005.5 line occurred at 6 dpi (144 h). This difference could be explained in part by the fact that our *A. euteiches* strain was isolated from pea, so *M. truncatula* is not the natural host for this pathogen isolate. Indeed, physiologic specialization of *A. euteiches* isolates to several legume hosts was reported by Holub et al. (1991). Nevertheless, the implication of the inoculum concentration, plant age and isolate virulence cannot be excluded to explain this difference.

The effective concentration of ROS in plant tissues is a result of a dynamic equilibrium between the rates of their production and scavenging. SOD, POX, APX and CAT are known to be involved in the regulation of H_2O_2 levels in plant tissues (De Gara et al. 2003). Antioxidant enzyme activities in roots of both *M. truncatula* lines were influenced by *A. euteiches* infection. Line F83005.5 showed a general decrease in the activity of the four antioxidant enzymes after infection, which were not correlated to H_2O_2 levels at

Table 3 Correlations between H_2O_2 concentrations and levels of the antioxidant enzymes APX, POX, SOD and CAT, soluble phenolics and lignin in roots of two *M. truncatula* lines F83005.5 (susceptible) and A17 (partially resistant) to *A. euteiches* at 1, 3 and 6 days post inoculation (dpi)

		H_2O_2					
		F83005.5			A17		
		1 dpi	3 dpi	6 dpi	1 dpi	3 dpi	6 dpi
APX	r	ns	ns	ns	−0.97	ns	ns
	p				0.002		
POX	r	ns	ns	ns	ns	ns	ns
	p						
SOD	r	ns	ns	ns	ns	ns	ns
	p						
CAT	r	ns	ns	ns	ns	−0.98	ns
	p					0.003	
Soluble phenolics	r	ns	ns	ns	ns	ns	ns
	p						
Lignin	r	ns	ns	ns	ns	ns	−0.93
	p						0.005

APX ascorbate peroxidase, *POX* peroxidase, *CAT* catalase, *SOD* superoxide dismutase, r: correlation coefficient, *p*: significance level, ns: not significant after using Bonferroni corrections $\alpha = (0.05/6 = 0.0083)$

the three sampling dates. So possibly the observed decrease in H_2O_2 levels in F83005.5 infected roots was due to the antioxidant enzyme activity of the pathogen. This idea is supported by the high level of F83005.5 root colonization by this pathogen and the previous findings by Madoui et al. (2007), i.e., the presence of several genes with antioxidant function in a cDNA library of *A. euteiches* mycelium grown in contact with root tissues of *M. truncatula*. In the partially resistant line, the activities of APX, POX and CAT were induced as early as 1 dpi in infected roots. This finding confirm the results of Trapphoff et al. (2009) which found that peroxidases and catalases were induced in *M. truncatula* cell cultures at early *A. euteiches* inoculation time points. Induced activities of catalases and peroxidases are also associated with resistance in other pathosystems, such as *Cicer arietinum* L.—*Fusarium oxysporum* f. sp. *ciceris* (García-Limones et al. 2002). The increase in antioxidant enzyme activities in infected A17 roots resulted in a decrease in H_2O_2 levels. Indeed, analyses of correlations showed that the concentrations of H_2O_2 were negatively correlated to those of APX and CAT activities in A17 infected roots. APX and CAT constitute the main H_2O_2 scavenging enzyme systems in plants (De Gara et al. 2003). Histochemical detection of POX activity showed the accumulation of this enzyme only in A17 infected root epidermis at 1 dpi and subsequently in cortical cells adjacent to those undergoing phenolic oxidation (cells with brown colour). Contrary to APX, POX does not use ascorbate as its direct electron donor, but oxidizes other molecules, mainly of phenolic nature (Takahama 2004). Thus, with the induced production of soluble phenolics in roots of the partially resistant line A17, presumably a reaction of oxidation by POX occurs which lead to the observed browning in A17 cortical root cells at 3 and 6 dpi. Browning of root tissues were observed in response to *A. euteiches* in three resistant lines DZA45.5, A17, and DZA315.16, whereas the roots tissues of the susceptible line F83005.5 showed a faint honey-gray colour (Djébali et al. 2009). Resistance to several fungal plant pathogens has been ascribed to the accumulation of phenolic oxidation products that can inhibit pathogen development (Lebeda et al. 2008). Moreover, this oxidation is a H_2O_2 consuming reaction (Takahama 2004), which likely contributes to lowering ROS levels in infected A17 roots.

We observed an increase of lignin content only in the roots of the partially resistant line at 6 dpi. This result is in concordance to our previous microscope observations of phloroglucinol stained root sections which showed that lignin-like polymers accumulate around the pericycle in the A17 infected roots at 6 dpi (Djébali et al. 2009). Lignification is a well known strategy that have developed many plants to prevent pathogen attack (Bhuiyan et al. 2009). In our pathosystem, the deposition of lignin polymers around the pericycle likely contributes (1) directly to the protection of the stele in the A17 roots by making the pericycle cell wall more resistant to mechanical pressure and cell-wall degrading enzymes applied by *A. euteiches* during colonization of the root tissues and (2) indirectly by preserving the flow of water and nutrients through the vascular tissues between roots and shoots which make the plant more resistant to the pathogen attack. Lignin deposition on the outer pericycle cell wall was already observed in the non-host interaction between the parasitic plant broomrape (*Orobanche crenata*) and *M. truncatula* roots (Lozano-Baena et al. 2007). Lignin synthesis in A17 infected roots was negatively correlated to H_2O_2 levels, so the lignification observed in the A17 roots likely contributes with the antioxidant enzymes to lowering of the H_2O_2 concentration. It is well known that lignin polymerization consumes H_2O_2 via peroxidase-mediated cross-linking of phenolic compounds (Ralph et al. 2004). In addition, this negative correlation highlights the implication of H_2O_2 in the observed lignification around the pericycle and consequently in the resistance to *A. euteiches* invasion of the root stele in line A17.

Partial resistance in line A17 was accompanied by a rapid decrease in H_2O_2 concentration in infected roots. However, in other plant-pathogen interactions, H_2O_2 levels increased in the host after pathogen infection (Bolwell and Daudi 2009). It is possible that our analyses were not done when such changes occurred, i.e., at early time points in the plant-oomycete interaction. In fact, Trapphoff et al. (2009) showed that H_2O_2 production in A17 cell cultures reached its maximum value at 15 min after inoculation with *A. euteiches* zoospores followed by a decline. Nevertheless, the decrease in H_2O_2 levels in the roots of the partial resistant line at 6 dpi could be due to utilization of this compound by the plant for defence, but also possibly by the pathogen for root

invasion even though the involvement of the pathogen probably was less important due to its low infection level in the A17 inoculated roots. This hypothesis is supported by the negative correlation found between lignin synthesis and H_2O_2 level in A17 roots at 6 dpi and the increase of *A. euteiches* mycelium growth on PGA medium supplemented with physiological concentrations of H_2O_2 (8 and 21 μM). *A. euteiches* was able to tolerate H_2O_2 in vitro up to 300 μM and was even stimulated by it. The in vitro tolerance to higher concentrations of H_2O_2 has also been observed for the oomycete *Phytophthora cinnamomi,* which causes root rot disease in avocado plants (García-Pineda et al. 2010). This behaviour likely contributes to the ability of these oomycete pathogens to colonize root tissues and cause disease in plants as suggested by García-Pineda et al. (2010). These results suggest that both plant and pathogen use H_2O_2, for defence and pathogenicity, respectively. Indeed, H_2O_2 is used by the plant to reinforce the cell walls around the stele and is also used by the pathogen to enhance development as demonstrated by the increase of the in vitro growth of *A. euteiches* when subjected to physiological concentrations of H_2O_2 found in *M. truncatula* roots.

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