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A study on the susceptibility of the model legume plant *Medicago truncatula* to the soil-borne pathogen *Fusarium oxysporum*

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Abstract Fusarium wilt is a soil-borne disease caused by *formae specialis* of *Fusarium oxysporum* on a large number of cultivated and wild plants. The susceptibility of the model legume plant *Medicago truncatula* to *Fusarium oxysporum* was studied by root-inoculating young plants in a miniaturised hydroponic culture. Among eight tested *M. truncatula* lines, all were susceptible to *F. oxysporum* f.sp. *medicaginis*, the causal agent of Fusarium wilt in alfalfa. However, a tolerant line, F83005.5, and a susceptible line, A17, could be distinguished by scoring the disease index. The fungus was transformed with the GFP marker gene and colonisation of the plant roots was analysed by epifluorescence and confocal microscopy. A slightly atypical pattern of root colonisation was observed, with massive fungal growth in the cortex. Although colonisation was not significantly different between susceptible and tolerant plants, the expression of some defence-related genes showed discrimination between both lines. A study with 10 strains from various host-plants indicated that *M. truncatula* was a permissive host to *F. oxysporum*.

Keywords Defence · Gene expression · Fusarium wilt · Green fluorescent protein · Microscopy · Root colonisation

Abbreviations

ATMT	*Agrobacterium tumefaciens*-mediated transformation
dpi	days post-inoculation
Fom	*Fusarium oxysporum* f.sp. *medicaginis*
Fol	*Fusarium oxysporum* f.sp. *lycopersici*
Forl	*Fusarium oxysporum* f.sp. *radicis-lycopersici*
PDA	Potato Dextrose Agar
qRT-PCR	quantitative RealTime-PCR

Introduction

Fusarium oxysporum is an ubiquitous soil inhabitant, able to grow saprophytically on organic substrates. The species comprises plant endophytes that colonise roots without causing disease symptoms (Fravel et al. 2003) as well as plant pathogens causing two major plant diseases: Fusarium wilt and Fusarium root rot. The wilt-causing strains are specific with regard to their host plants and are classified into more than 120 *formae specialis* associated to one species or a narrow

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range of plant species. A well known example is *F.o.* f.sp. *lycopersici* (*Fol*) causing Fusarium wilt in tomato (Agrios 2005). The strains causing root rots are generally less specific and only in rare cases are they identified as a *forma specialis*, as in the case of the tomato root rot attributed to *F.o.* f.sp. *radicis-lycopersici* (*Forl*) (Jarvis and Shoemaker 1978).

Infection of roots may occur by conidia or mycelium living in the soil. Whereas in the case of root rot the whole root is colonised, Fusarium wilt involves a directed growth of the fungus towards the stele and a preferential colonisation of the xylem vessels where sporulation occurs (Di Pietro et al. 2003). Plugging of the vessels by masses of fungal hyphae and conidia then leads to the typical wilt symptoms. In tomato where the interaction with *F. oxysporum* is particularly well studied, race-specific resistance against *Fol* has been described. This resistance takes place in the endodermis and prevents passage of the fungus into the stele (Mes et al. 2000).

Fusarium wilt diseases may have severe impact on the culture of grain legume crops such as pea, bean or chickpea (reviewed by Infantino et al. 2006). Since soil-borne pathogens are difficult to control, the study of their biology and of their interaction with host plants is an important step for the development of crop protection strategies. In order to know more about this disease and defence mechanisms against it, we set up a fungal root pathosystem involving the model legume plant *Medicago truncatula*.

M. truncatula is a near relative of alfalfa (*M. sativa*), the world's economically most important forage legume. Due to its diploid small genome, self-fertility, amenability to genetic transformation and synteny with agronomically-important grain legumes, it has been selected as a model for studying the biology of legume plants (Cook 1999). In addition, legume plants are particularly suited for comparative studies of symbiotic and pathogenic interactions, which are not possible with *Arabidopsis thaliana*. *M. truncatula* is a host plant to several pathogenic microorganisms that may cause yield losses in alfalfa fields. The interaction with fungi that attack aerial parts, such as *Colletotrichum trifolii* (Torregrosa et al. 2004; Yang et al. 2008) or *Erisyphe pisi* (Ameline-Torregrosa et al. 2008), has been described, and resistant and susceptible lines of *M. truncatula* have been used for the genetic dissection of resistance mechanisms. Soil-borne pathogenic microorganisms such as the oomycete *Aphanomyces euteiches* (Nyamsuren et al. 2003; Pilet-Nayel et al. 2009), the bacterium *Ralstonia solanacearum* (Vailleau et al. 2007) and the necrotrophic fungus *Phymatotrichopsis omnivora* (Uppalapati et al. 2009) are also able to infect *M. truncatula* roots, and quantitative traits involved in resistance have been identified for the two first interactions.

In order to study Fusarium disease in *M. truncatula*, wilt-inducing strains of *F. oxysporum* f.sp. *medicaginis* (*Fom*) isolated from alfalfa as well as strains from other host plants were used to inoculate *M. truncatula* lines. The alfalfa strain *Fom* 179.29 was transformed with the *GFP* marker gene and colonisation of the root was studied by fluorescence microscopy. In addition the expression of some defence-related genes was assessed.

Materials and methods

Plants

Medicago truncatula Gaertn. seeds were obtained from plants grown in our greenhouse. The various lines are derived from natural populations of different geographical origins, such as France (F83005.5), Morocco (A20, A10), Israel (R108), Tunisia (TN1.11) and Algeria (DZA315.16); they are either parent lines involved in crosses for genetic studies (A17, A10, A20, F83005.5, DZA315.16) or have interest because of their amenability to stable genetic transformation (2HA, R108). Jemalong A17 (origin unknown) is the genotype which has been chosen for sequencing.

Scarification and germination of seeds, as well as in vitro culture of seedling on slanted Farhaeus agar medium was performed as described by Vailleau et al. (2007).

Fungal isolates

Fusarium oxysporum f.sp. *medicaginis* (*Fom*) strain 179.29 was purchased from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. The strains *F. oxysporum* f.sp. *raphani* (*For* 2), f.sp. *melonis* (*Fom* 24), f.sp. *lycopersici* (*Fol* 8), f.sp. *ciceris* (*Foci* 1), f.sp. *pisi* (*Fopi* 1), f.sp. *lini* (*Foln* 3) and the non-pathogenic strains *Fusarium oxysporum* *Fo*47 et *Fo*3 were all provided by C. Steinberg and N. Gautheron, INRA Dijon, France. *F. oxysporum* f.sp.

phaseoli (*Fop* SP6) was provided by J.M. Diaz-Minguez, Universidad de Salamanca, Spain, and *Fom* 605 and 606 by A.S. Alizivatos, Benaki Phytopathological Institute, Greece. All strains were grown on PDA at 24°C with a photoperiod of 12 h. Spore suspensions were obtained by flooding the Petri dish with sterile water and stored as glycerol stocks in 25% glycerol at −80°C.

Genetic transformation of *F. oxysporum* f.sp. *medicaginis*

Fom strain 179.29 was transformed with the GFP marker gene by *Agrobacterium tumefaciens*-mediated transformation (ATMT) as described by Mullins et al. (2001). *A. tumefaciens* strains AGL-1 and C58C1 were electroporated with the binary vector pBin-GFP-hph containing the GFP gene under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter of *Aspergillus nidulans* and a hygromycin B resistance marker (O'Connell et al. 2004). Spores of *Fom* 179.29 were obtained from 2-week-old cultures and the suspension was adjusted to a concentration of 10^7, 10^6 and 10^5 spores per ml respectively. Equal volumes of fresh bacterial and spore suspensions were mixed and 200 µl of this mixture were plated onto sterile cellophane sheets placed on induction medium containing 200 µM acetosyringone. After 2 days of incubation at 22°C in the dark, the cellophane sheets were transferred to selective PDA medium with 150 µg/ml hygromycin for transformant selection and 50 µg/ml cefotaximine and spectinomycin for elimination of the bacteria. After 2 days of incubation at 24°C the cellophane sheet was peeled off, and incubation of the Petri dishes continued. Colonies of the fungus appeared after 2 days of incubation and putative transformants were transferred to fresh PDA selective medium. Monosporic strains were obtained after sporulation on selective PDA medium.

Inoculation and symptom scoring

Suspensions of microconidia were obtained as described above, and the concentration was adjusted to 10^6 spores per ml. The roots of 4-day-old *M. truncatula* plantlets grown on Farhaeus medium were submerged in the spore suspension for 30 min before being transferred into Copro vessels (Fig. 1a) filled with water which was replaced by Farhaeus nutritive solution (Farhaeus 1957) after 6 days. These hydroponic cultures were incubated in the growth chamber at 25°C with a 16 h photoperiod and a light intensity of 70 µM $m^{-2}s^{-1}$. Symptoms were rated on a scale from 1 to 5, corresponding to the following phenotypes: 1=cotyledon chlorosis, 2=cotyledon necrosis, 3=leaf chlorosis, 4=leaf necrosis, 5=dead plant. Each Copro vessel contained 4 plants of one line only, and at least 12 plants per line (3 Copro pots) were inoculated for each interaction, with at least 3 biological repetitions.

Microscopy

Roots were cut into 1 cm fragments at 1, 2 and 3 cm from the tip and the fragments were embedded in 5% low melting point agarose. Root sections of 90 and 400 µm thickness were obtained with a vibratome (Leica VT 1000 S) and were mounted on a glass slide with a drop of distilled water. For observations by confocal microscopy the sections were stained with Congo Red (0.05%, w/v) for 10 s.

Observations under an inverted microscope (DMIRBE, Leica, Rueil-Malmaison, France) were made to obtain images in bright field and fluorescence. The microscope was equipped with epifluorescence illumination (excitation filter, BP 450–490 nm, suppression filter LP 515) and with a CCD camera (colour Coolview, Photonic Science, Robertsbridge, UK). The images were treated by image analysis (Image Pro-Plus, Media Cybernetics, Silver Spring, MD, USA).

Confocal images were obtained with 400 µm thick root sections and were acquired with a spectral confocal laser scanning system (SP2 SE, Leica, Germany) equipped with an upright microscope (DM 6000, Leica, Germany). Observations were made using 10× (HC PL Fluotar, N.A. 0.3) and 40× (HCX PL APO, N.A. 0.8) dry and water immersion objectives, respectively. The 488 nm ray line of an argon laser was used to detect the GFP fluorescence emission collected in the range between 490 and 540 nm.

Gene expression

The terminal halves of the roots were harvested at 0, 3 and 6 dpi in liquid nitrogen and stored at −80°C until use. Total root RNA was extracted using the SV Total

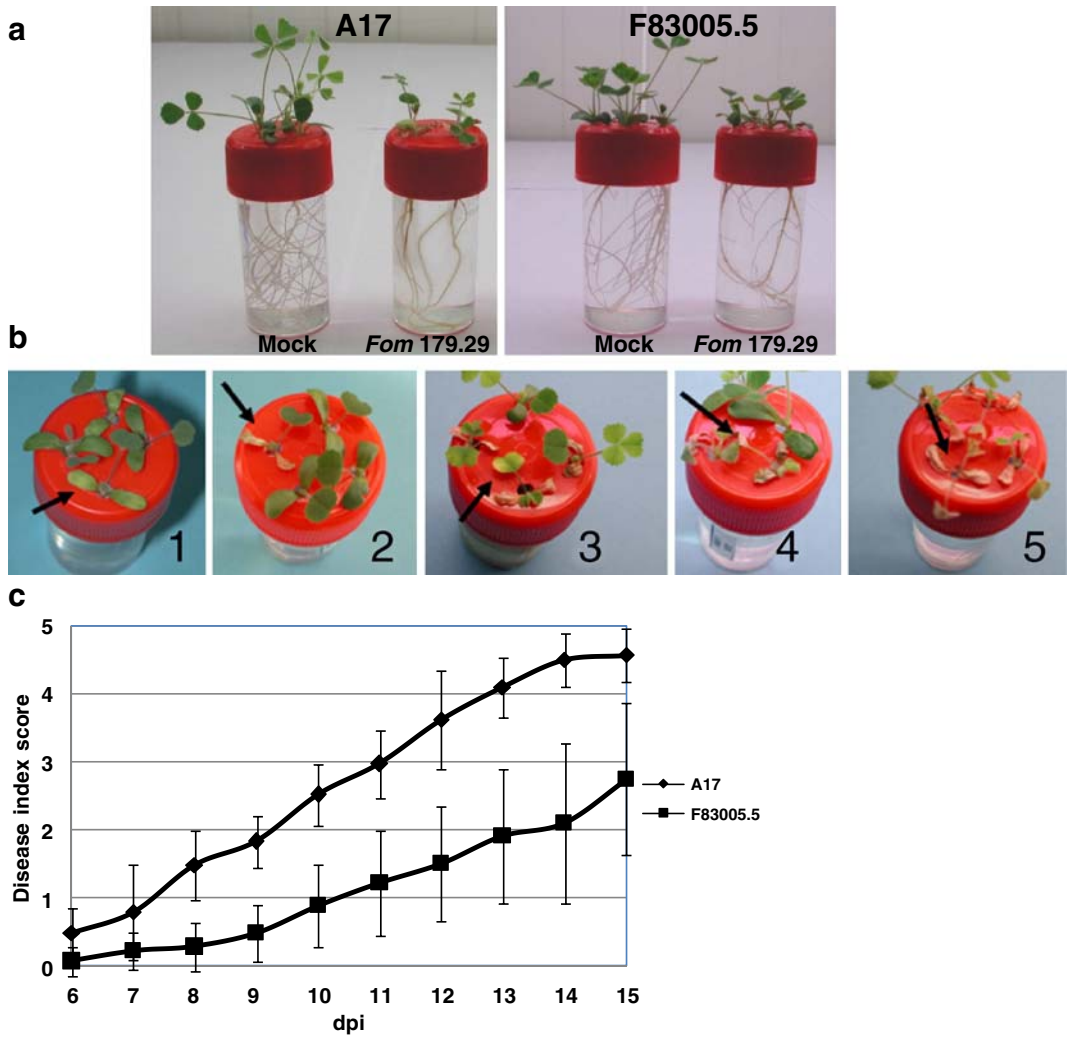

Fig. 1 Disease development in *M. truncatula* inoculated with *F. oxyxporum* f.sp. *medicaginis*. **a**. View on A17 (*left*) and F83005.5 (*right*) plantlets in Copro vessels, treated with water (*Mock*) or inoculated with *Fom* 179.29, at 12 dpi. Note the brown discoloration of roots, presence of fungal mycelium around the roots and reduced root growth of inoculated plants. **b**. Symptom scale used for scoring plant susceptibility in miniaturised hydroponic cultures: 1=cotyledon chlorosis, 2=cotyledon necrosis, 3=first leaf chlorosis, 4=first leaf necrosis, 5=death of the plant. **c**. Symptom development in *M. truncatula* lines A17 and F83005.5. Plants were root-inoculated with *Fom* 179.29 and then transferred to Copro vessels. Results represent the mean values from 10 biological repetitions with standard deviation, each experiment was conducted with at least 3 Copro vessels containing each four plants

RNA Isolation System kit (Promega), RNA concentration was determined with a NanoDrop Spectrophotometer (Thermo Scientific) and quality was checked by the Bioanalyzer 2100 (Agilent). cDNA was synthesised from 1 µg of RNA with the ImPromII™ Reverse Transcription System (Promega).

Primer sets used in qRT-PCR assays are shown in Table 1. They correspond to genes encoding Pathogenesis-related protein 10 (PR10), basic chitinase (CHIT), phenylalanine ammonia lyase (PAL), chalcone flavanone isomerase (CFI), proteinase inhibitor 20 (PI20), and elongation factor 1-α (EF1-α). The qRT-PCR was performed on an ABI PRISM® 7900HT (Applied Biosystems). Two technical replicates were conducted for each gene in a total volume of 10 µl containing 5 µl 2xSYBR green, 5 µM of each primer and 1 ng of cDNA, with the following PCR regime: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95ºC for 15 s and 60ºC for 1 min. The generated data were analysed using SDS 2.2.2 software

Table 1 Primers used for q RT-PCR

ID*	Gene	Primers
TC118987	Pathogenesis-related protein 10 (PR10)	F: 5′-CAGTGGAGAAGATCTCCTTTGAGTCT-3′
		R: 5′-GCTTTGCAATGGATCCTCCAT-3′
TC106842	Basic chitinase (CHIT)	F: 5′-AAGAGGACAGGATGGAAGAGTG-3′
		R: 5′-TAGCTAGGTGTCATGGAGTTG-3′
TC113890	Phenylalanine ammonia lyase (PAL)	F: 5′-TCGGCGGTGAGACACTTACC-3′
		R: 5′-ACACCACTATCATGGGAAGCAA-3′
TC140281	Chalcone flavanone isomerase (CFI)	F: 5′-CCCAGCGGTGGTTACATCTC-3′
		R: 5′-CTGCGCCACCGAGGAA-3′
TC121286	Proteinase inhibitor 20 (PI20)	F: 5′-CACACAATACACACAGCAAAATACAGTAA-3′
		R: 5′-GCCCAAATCAGACGAGGTATGT-3′
TC67846	Elongation Factor 1-α (EF1-α)	F: 5′- ATGAACCACCCTGGACAGAT-3′
		R: 5′-GTCCAAGAGGAGGATATTCAGC -3′

*TIGR *M. truncatula* Gene Index (MtGI)

and the relative levels of gene expression were determined following the Comparative Ct Method or $2^{-\Delta\Delta Ct}$ method where ΔCt=Ct value of the gene under study−Ct value of the internal reference gene EF1 α, and ΔΔCt=ΔCt inoculated sample−ΔCt mock-inoculated sample.

Results

Susceptibility of *M. truncatula* to *F. oxysporum* f.sp. *medicaginis*

A miniaturised hydroponic culture was developed in order to study disease and resistance responses in *M. truncatula* towards *Fom*. Young plantlets of lines A17, 2HA, R108, F83005.5, A10, A20, DZA315.16, TN1.11, were root-dip inoculated with *Fom* 179.29 and symptoms were scored on their aerial parts during 3 weeks. Early symptoms were visible on cotyledons, then the first leaves turned chlorotic and dried, and at the end of the experiment most of the plants were dead. Fungal mycelium developed massively on the root surface and root growth was reduced (Fig. 1a). Dark-brown discoloration of the tap root and emerging secondary roots was observed after 6 to 7 days. All the lines developed symptoms, no resistant line has been identified (data not shown). Two isolates from Greece, *Fom* 605 and 606 were equally pathogenic when tested on lines A17 and F83005.5 (data not shown). Hence, fungal strains reported to cause Fusarium wilt disease in cultivated alfalfa were also able to infect the model plant *M. truncatula*, and susceptibility to *Fom* was not limited to one or two lines. F83005.5 showed some degree of tolerance during this preliminary screen, and a disease index scale based on the sequential symptoms was established in order to quantify tolerance to *Fom* 179.29 (Fig. 1b). Based on the time course of disease index evolution, the tolerance of F83005.5 was confirmed, compared to the susceptibility exhibited by A17 (Fig. 1c). The time of appearance of first symptoms was delayed and the rate of symptom development and final scores were significantly lower in the tolerant line.

Transformation of *F. oxysporum* f.sp. *medicaginis*

In order to study the penetration of *Fom* into the host's root and its subsequent colonisation, the fungus was transformed by ATMT with the coding sequence of the green fluorescent protein (GFP) under control of a fungal promoter. Transformation was carried out with two bacterial strains, and different concentrations of bacteria and spores were tested during co-culture. Colonies appeared after 4 days of culture on selective medium in the case of transformation assays, none were observed for negative controls (co-culture in the absence of acetosyringone). Strain AGL-1 was about ten times more efficient than C58:C1, yielding 1500 transformants per 10^6 spores. The spore concentration used for co-culture with the bacteria was found to be

important, only suspensions of 10^5 spores per ml yielded transformants, higher concentrations of 10^6 and 10^7 sp.ml^{-1} were not efficient. The same effects of bacterial strain and spore concentration were observed when a different protocol for ATMT described by Tkacz et al. (2004) was tested (data not shown). Monosporic isolates were obtained for more than 20 colonies, and a strain exhibiting strong fluorescence without modification of its phenotype and pathogenicity was selected for microscopic studies, referred to as *Fom*-GFP in this work. Stability of the transformation was checked by three cycles of subculture in the absence of selective antibiotic; no loss of hygromycin resistance or of green fluorescence was observed (data not shown).

Colonisation of *M. truncatula* roots by *F. oxysporum* f.sp. *medicaginis*

We made use of the GFP-expressing strain to characterise penetration and further root colonisation by *Fom* in the susceptible *M. truncatula* line A17, and to compare it with that of the tolerant line F83005.5.

Young plantlets of A17 and F83005.5 were dip-inoculated on their roots with *Fom*-GFP and then maintained in hydroponic culture. Roots were harvested at various times after inoculation and either observed directly by epifluorescence microscopy or embedded in agarose for observations of root sections. Two days after inoculation germinated conidia were observed at the root surface and hyphae first grew on the root surface in the grooves between epidermal cells (Fig. 2a, b). The root hair zone was the preferential site of initial fungal development, and most hyphae were observed entangled between them at early times of the interaction. At later stages the mycelium covered the whole root surface forming a dense layer around the roots (data not shown). Penetration occurred directly without the formation of specialised structures such as appressoria, along the root surface. The presence of a hypha in a root hair was observed once (Fig. 2c, d), but this was not a common penetration mechanism.

After 3–4 days, hyphae were seen in the first layer of cortical cells, growing inter- and intracellularly. Colonisation of the cortex continued, with hyphae growing centripetally towards the stele as well as vertically along the root axis. Observation of A17 by confocal laser scanning microscopy allowed the visualization of the colonisation process inside the root (Fig. 3). Between 9 and 10 days after inoculation the fungus reached the central cylinder and colonised

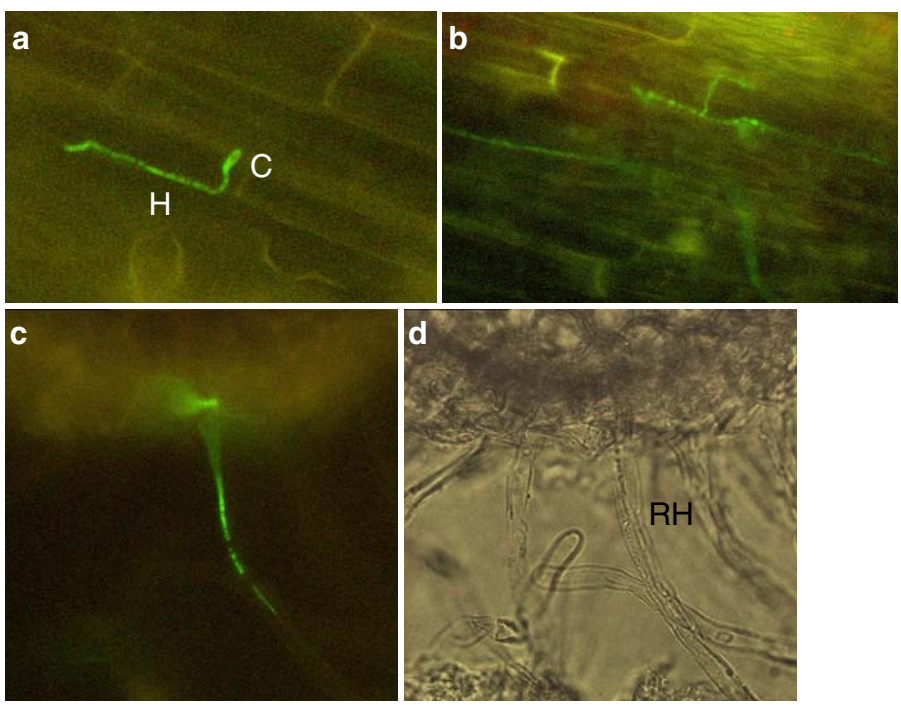

Fig. 2 Early steps of *M. truncatula* root colonisation by *Fom*-GFP. Observation of roots or root sections by epifluorescence microscopy or under bright field (× 400). Pictures show **a** a germinated microconidia (**C**) and hypha (**H**) on the root surface of A17 at 2 dpi; **b** hyphae growing on the surface of A17 at 3 dpi; **c**, **d** a hypha growing in a root hair (RH) of A17 at 3 dpi, observed under UV and bright field respectively. Roots were observed directly on a slide (**a**, **b**) or after embedding in 5% agarose and sectioning with a vibratome (**c**, **d**)

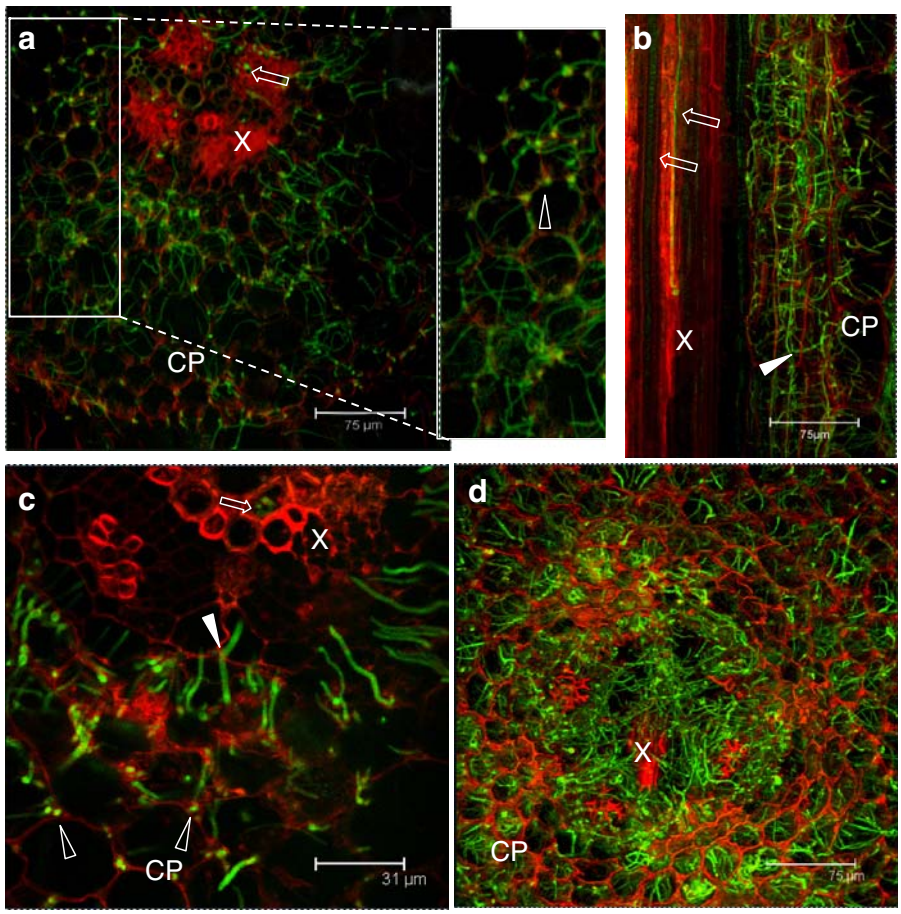

Fig. 3 Laser scanning confocal microscopy of *M. truncatula* root colonisation. Transverse (**a**, **c**, **d**) and longitudinal (**b**) root sections observed by confocal laser scanning microscopy, after cell wall staining with Congo red. Sections were prepared from inoculated roots of A17 at 10 dpi (**a**, **b**), 11 dpi (**c**) and 17 dpi (**d**). Note fungal growth in intercellular cavities (*empty arrowheads*), through cell walls between cells (*full arrowheads*) and inside xylem vessels (*empty arrows*). X=Xylem, CP= cortical parenchyma

the xylem vessels (Fig. 3a–c). Hyphal growth was observed inter- and intracellularly, but intercellularly growing hyphae were mainly seen along the root axis in the cavities between cortical parenchyma cells (Fig. 3a with inset, b, c). At the final disease stage the cells of cortex and stele were massively filled with fungal hyphae and the central cylinder seemed completely macerated (Fig. 3d). Observations by epifluorescence microscopy of roots of A17 and F83005.5 showed that in both lines the fungus colonised the cortex and the stele, without significant difference in the time course (data not shown). Hence the delay in symptom development and reduction of disease index in F83005.5 was not correlated to a delay in root colonisation or exclusion from the central cylinder.

Responses of *M. truncatula* roots to *F. oxysporum* f.sp. *medicaginis* inoculation

In order to study putative defence mechanisms in roots of tolerant and susceptible plants, we focused on genes that have been found to be induced during the interaction of *M. truncatula* with *R. solanacearum* in our laboratory (unpublished results). Roots were harvested for RNA extraction at the onset of the experiment (0 dpi) and at 3 and 6 dpi, which corresponded to time points at the onset of the experiment and where the fungus enters the first cell layer or colonises the cortex, respectively. Only the lower halves of the roots were used since this corresponds to the zone of preferential attachment and penetration. Expression of genes encoding PR10, PAL, CFI, CHIT and PI20 was studied by qRT-PCR using EF1-α as internal standard.

As shown in Fig. 4, the gene expression follows different patterns. A group of 3 genes (PAL, PR-10 and CFI) is induced significantly and with similar induction ratios in both lines; PAL and PR10 increased at 3 dpi and stayed at the same level until 6 dpi, and CFI increased at 6 dpi. A different pattern was observed for chitinase which was induced early at 3 dpi in F83005.5, and only later at 6 dpi in A17

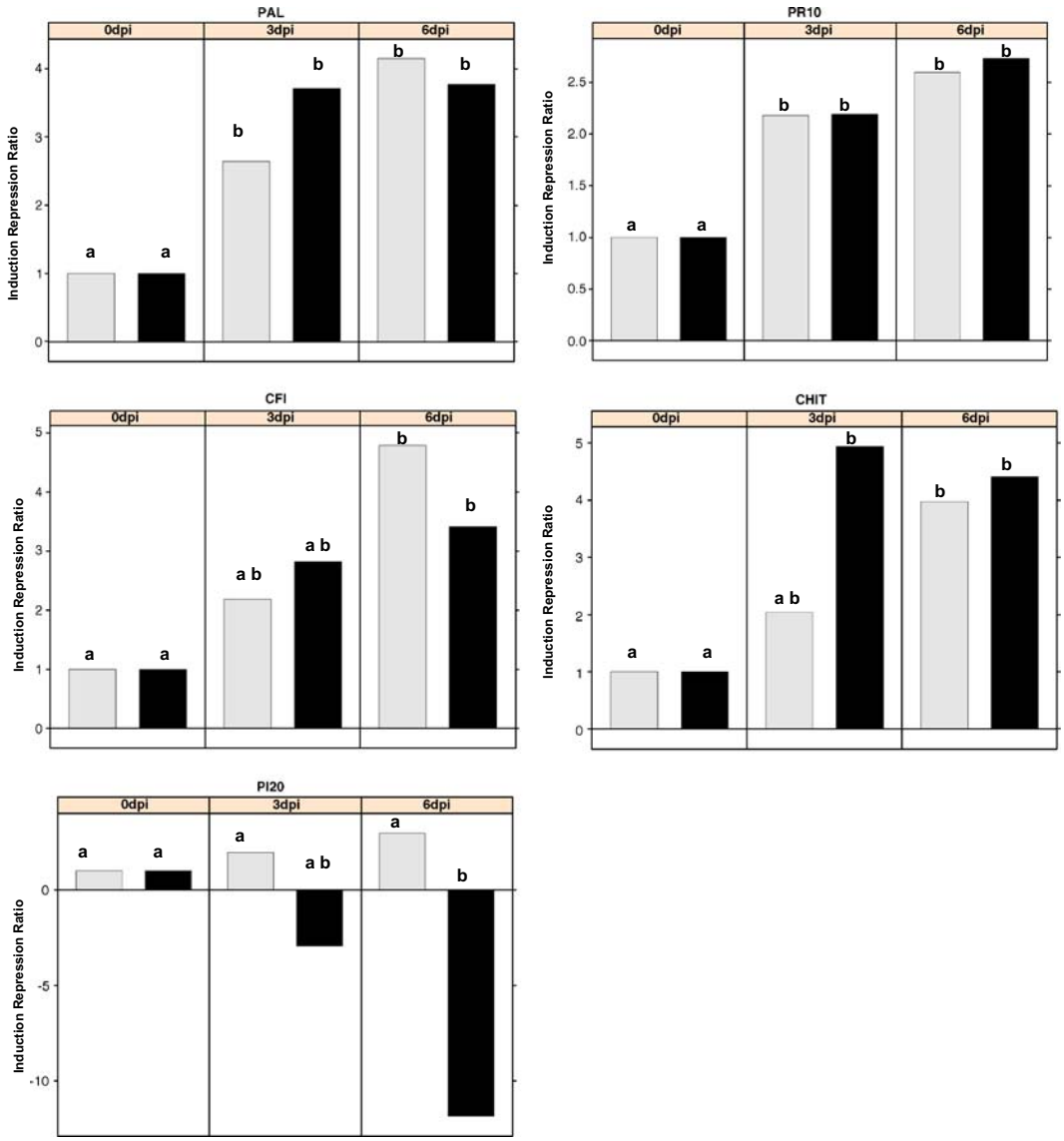

Fig. 4 Defence gene expression in roots of *M. truncatula* A17 and F83005.5 inoculated with *Fom*. Gene expression in roots of A17 (*grey bars*) and F83005.5 (*black bars*) was assessed by qRT-PCR. The results represent induction factors or repression factors of inoculated versus mock-inoculated plants at 0 dpi, 3 dpi and 6 dpi. The genes tested are Phenylalanine ammonia lyase (PAL), Pathogenesis-related Protein 10 (PR10), chalcone flavanone isomerase (CFI), chitinase (CHIT), and proteinase inhibitor (PI20). Expression at 0 dpi was set to 1, and values are the means of 4 biological repetitions. ANOVA analysis was performed with the R 2.7.0 software. Means with the same letter are not significantly different ($P<0.05$)

reaching a similar level as in F83005.5. A third expression pattern was exhibited by PI20 which did not change significantly in A17 but was strongly repressed in F83005.5 at 6 dpi. This first expression study with a limited number of genes shows that although the colonisation pattern in both susceptible and tolerant lines was similar, differences could be detected in their defence reactions early during the interaction.

Susceptibility of *M. truncatula* to *F. oxysporum* strains from different host plants

In search for interactions between *M. truncatula* and *F. oxysporum* that would show a stronger differential response between A17 and F83005.5, *F. oxysporum* strains isolated from various plants were obtained for inoculation assays (Table 2). Among the additional

Table 2 *Fusarium oxysporum* isolates used in this work

Fusarium isolates		Origin	Isolated from
F. oxysporum	Fo 3	Greece	cucumber*
F. oxysporum	Fo 47	France	Chateaurenard´s soil*
F.o. f.sp. *ciceris*	Foci 1	Spain	chickpea
F.o. f.sp. *lini*	Foln 3	France	flax
F. o. f.sp. *lycopersici*	Fol 8 race 1	France	tomato
F.o. f.sp. *medicaginis*	Fom 179.29	Unknown	alfalfa
*F.o.*f. sp. *melonis*	Fomel 24	France	melon
F.o. f.sp. *phaseoli*	Fosp 6	Spain	bean
F.o. f.sp. *pisi*	Fopi 1	Unknown	pea
F.o. f.sp. *raphani*	For 2	Netherlands	radish

*non-pathogenic strain

strains, 3 were isolated from legume plants (f.sp. *ciceri*, f.sp. *phaseoli* and f.sp. *pisi*), 4 from various other plant families (f.sp. *lini*, f.sp. *lycopersici*, f.sp. *raphani*, f.sp. *meloni*) and two were non-pathogenic strains isolated from soil (*Fo*47) and cucumber (*Fo*3). Both lines were inoculated with spore suspensions from these strains under the same conditions as above, and symptom development was scored.

Figure 5 shows the disease index at 9, 12 and 15 days in A17 and F83005.5. It appeared that all strains were able to induce disease symptoms in *M. truncatula* lines, even those described as non-pathogenic, and that strains from legume plants were not more pathogenic than others. In general, A17 was more susceptible than F83005.5, but none of the strains gave a better difference in susceptibility between both lines than did strain *Fom* 179.29 (Fig. 1). For most strains the difference between the two lines was only one point in the disease index scale, in contrast to a difference of 2 points with *Fom* 179.29. Taken together, the results suggest that *M. truncatula* is a permissive host plant for *F. oxysporum*, at least under hydroponic culture conditions.

Discussion

In the present work we describe a first study of the interaction between *Medicago truncatula* and the soil-borne fungus *F. oxysporum* f.sp. *medicaginis*, with the aim to establish a fungal root pathosystem for the study of disease and resistance in this model legume plant. Inoculation of *M. truncatula* in a miniaturised hydroponic system was found to reproduce symptoms observed in the field, such as reduction of root growth and chlorosis of leaves followed by drying and death of the plants. In our system the roots can be observed continually, and it gives access to roots for studying colonisation and gene expression without wounding of the tissues. In most studies on *F. oxysporum* and their respective host plants, the inoculation protocol involves cutting the lower part of the roots (Alves-Santos et al. 1999; Altier et al. 2000; Infantino et al. 2006), which allows a fast entry of spores and is supposed to mimic the situation in the field where pathogen attack is favoured by natural wounds within the root system (Stutz et al. 1985). Such a method has also been described for inoculation and assessment of susceptibility of *M. truncatula* with *Fom* (Lichtenzveig et al. 2006). In our system, induction of disease symptoms did not necessitate prior cutting of the roots. However, we cannot exclude that microscopical wounding occurred during transfer of the plants between Farhaeus medium, spore suspension and Copro vessels.

Among the eight *M. truncatula* lines tested we did not identify a completely resistant line, but moderate tolerance was found in F83005.5. The various *formae specialis* of *F. oxysporum* attack many legume crops, and dominant resistance genes have been identified for example in bean and chickpea (Schneider et al. 2001; Halila et al. 2008). *M. truncatula* is a model plant for grain and forage legumes and crosses between lines from different geographical origin are available for genetic studies. Notably the cross between the reference line A17 and F83005.5 is well mapped and a population of recombinant inbred lines has been used for genetic analyses of resistance to *C. trifolii* and *E. pisi* (Ameline-Torregrosa et al. 2008; Yang et al. 2008) and *R. solanacearum* (Vailleau et al.

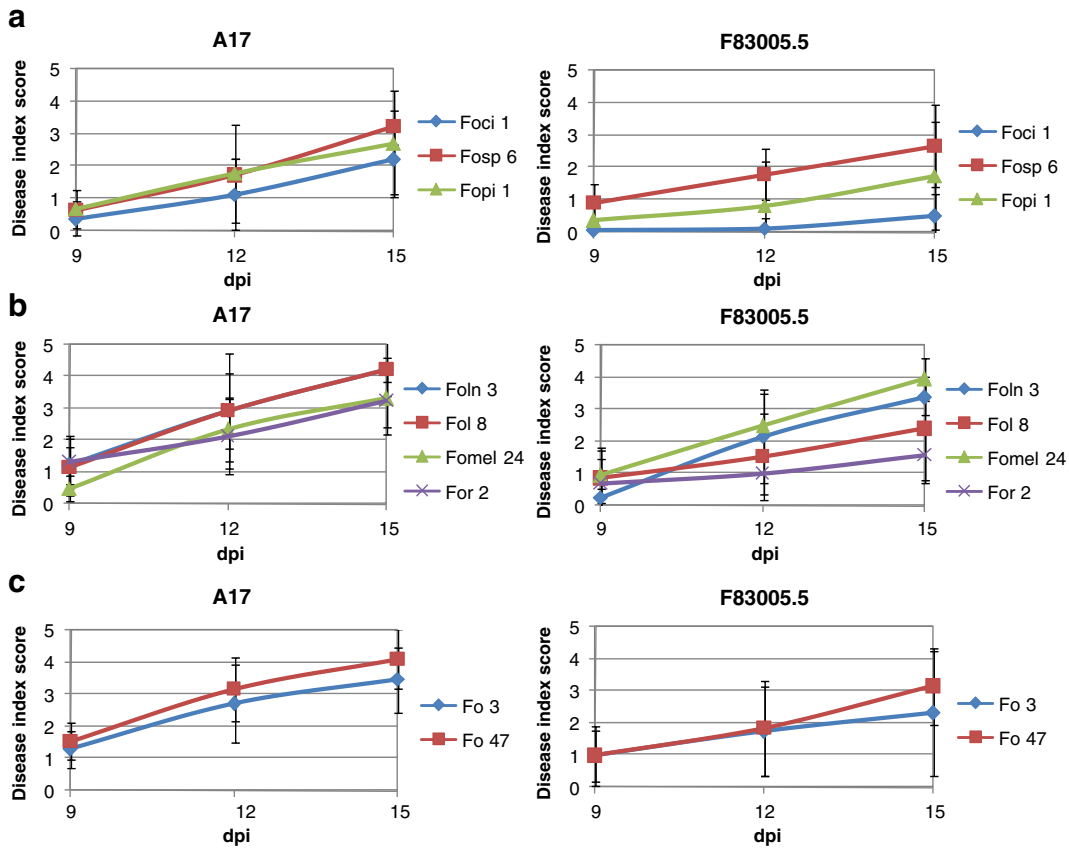

Fig. 5 Disease development in *M. truncatula* A17 and F83005.5 inoculated with various *F. oxysporum* strains. Disease index in A17 (*left panels*) and F83005.5 (*right panels*) after dip-inoculation of roots with **a** strains isolated from legume plants, **b** strains isolated from non-legume plant families, **c** non-pathogenic strains. Three Copro vessels each containing four plants were prepared for each interaction. Results show the arithmetical means of the disease index at 9, 12 and 15 dpi of 4 biological repetitions, with standard deviation

2007). Furthermore, it has been reported that resistance to *C. trifolii* could be transferred from *M. truncatula* to cultivated alfalfa (Yang et al. 2008), demonstrating that results obtained with a model plant were useful for crop improvement. However, the degree of tolerance observed in F83005.5 towards *Fom* 179.29, and the weak robustness of the phenotype as shown by the standard deviation of disease index scores (Fig. 1c) did not encourage further genetic analysis.

In order to characterise root colonisation by *Fom*, the fungus was transformed with the *GFP* marker gene by ATMT. This method is now widely used in filamentous fungi to introduce genes as a vital marker (O'Connell et al. 2004; Eckert et al. 2005), or for the creation of random insertional mutants (Huser et al. 2009). As described for other fungal species (Tkacz et al. 2004), the bacterial strain as well as the balance between bacterial and fungal growth strongly influenced the success of transformation. These parameters have to be adjusted for each species and strain. The transformation efficiency of *Fom* was comparable with published data (Mullins et al. 2001; Huser et al. 2009) showing the usefulness of ATMT for random insertional mutagenesis of this fungus, and the transformant phenotype was stable in the absence of selective pressure.

Although sources and types of resistance against *F. oxysporum* are widely studied in legume plants, (Infantino et al. 2006), there are no recent investigations of the colonisation process in the literature. In pea, penetration into the root has been reported to occur preferentially through the apex and secondary roots, and further colonisation until invasion of the xylem vessels was intra- or intercellular (Kraft 1994). The different steps of infection of tomato by *Fol and*

Forl are well described in the literature, notably by detailed microscopy studies and the use of transgenic strains expressing marker genes or affected in pathogenicity genes (Olivain and Alabouvette 1999; Di Pietro et al. 2001; Lagopodi et al. 2002). Hence the tomato pathosystem is often considered as representative for Fusarium wilt.

Colonisation of the host root by *Fom-GFP* started with growth on the root surface and penetration into the epidermis without the formation of specialised structures, as has been described for other ff.spp. (Olivain and Alabouvette 1999; Lagopodi et al. 2002). *Fol* has been reported to penetrate through root hairs in tomato (Olivain and Alabouvette 1999), and our observation confirms this mode of infection although it is not of great importance in the *M. truncatula-Fom* interaction. After penetration, *Fom-GFP* colonised first the cortex and then the central cylinder, by growing between and inside the cells, and finishing with a complete invasion of the tissues. In this regard, the colonisation pattern is somewhat different from what is described for wilt-inducing *F. oxysporum* f.sp. where fungal growth is restricted to the xylem vessels after having crossed the cortex with intercellularly growing hyphae (Di Pietro et al. 2003; Agrios 2005). The pattern in *M. truncatula* is more similar to the occasional colonisation of the cortex and intracellular fungal growth reported for *Fol*-inoculated tomato and which was associated with severe decay of cortex and stele (Olivain and Alabouvette 1999). However, our observations showed also that the tissue structures, with the exception of the parenchyma in the central cylinder at final infection stages, were well preserved in colonised roots. The form of the cells was regular and adhesion between cells did not seem affected, indicating that the cell walls have not been strongly degraded in *M. truncatula* roots, although *F. oxysporum* is known to produce a quantity of cell wall-degrading enzymes *in vitro* and *in planta* (Di Pietro et al. 2003). This might be correlated to the fact that intercellular growth of *Fom* was observed mainly in the natural cavities between cells.

In tomato inoculated with *Fol*, it has been described that susceptible and resistant cultivars differed by the fact that the fungus did not penetrate into the stele of resistant plants (Gao et al. 1995). In *M. truncatula*, the central cylinder was colonised by *Fom* in both susceptible and tolerant lines A17 and F83005.5, and the colonisation rates were not significantly different. These observations might be an illustration of fundamental differences between mechanisms of tolerance and resistance, and/or be related to the phylogenetic differences between tomato and *M. truncatula.* In summary, colonisation of *M. truncatula* roots by *Fom* followed a pattern which shows similarities and differences to described interactions, emphasising the need for detailed studies in legume plants, and more generally in each new pathosystem.

Inoculation with *Fom* modified defence-related gene expression in *M. truncatula* roots, in a variable way and at early time points before the appearance of symptoms. Genes encoding PAL, PR-10 and CFI were induced similarly in both A17 and F83005.5, whereas chitinase was induced in both lines but earlier in the tolerant line F83005.5. PAL and CFI are enzymes of the phenylpropanoid pathway leading to the synthesis of a large variety of compounds such as lignin, coumarins and antimicrobial flavanone phytoalexins (Dixon et al. 2002). Genes related to flavonoid biosynthesis were reported to be transiently induced in *M. truncatula* roots 3 days after inoculation with the necrotrophic fungus *Phymatotrichopsis omnivora,* and it was proposed that this basal defence was downregulated by the pathogen (Uppalapati et al. 2009). Their induction as well as that of PR-10 might be part of the basal defence response in susceptible and tolerant plants. Members of the PR-10 family have been reported to be expressed in response to pathogen attack. During compatible and incompatible interactions of *M. truncatula* with *Aphanomyces euteiches,* a group of PR-10 proteins was induced to higher levels in roots of the susceptible line F83005.5, compared to the tolerant line F83005.9 (Colditz et al. 2005). During the interaction of *M. truncatula* with *Phytophthora medicaginis*, PR-10 gene expression was induced in susceptible and moderately tolerant lines but not in a resistant line (D'Souza 2009), and in alfalfa PR-10 gene expression was induced by inoculation but did not correlate with resistance to *Colletotrichum trifolii* (Truesdell and Dickman 1997). Silencing of 6 members of the PR-10 family in *M. truncatula* led to increased tolerance towards *A. euteiches* (Colditz et al. 2007). It was proposed that this phenotype was related to antagonistic induction of PR-5b in the silenced lines. The exact biological function of PR10 proteins is still not well understood

and more studies are necessary to get insight into its role in plant defence.

Induction of the same CHIT gene was also reported for *P. omnivora*-inoculated roots, and its earlier induction in *Fom*-inoculated F83005.5 might represent reactions that contribute to tolerance. Although invasion by the fungus was not stopped in roots of the tolerant *M. truncatula* line, degradation of chitin in its cell walls might weaken the pathogen and interfere with its aggressiveness. Expression of PI20 was regulated specifically in the tolerant line where it was strongly suppressed upon inoculation. Proteinase inhibitors, notably those inhibiting serine proteases, are known to be induced in plants by wounding and pathogen attack (Koiwa et al. 1997). Through their capacity to inhibit proteases of fungal or animal origin they take part in the defence mechanism against insects and pathogens. PI20 belongs to the family of serine protease inhibitors and was found to be induced in *M. truncatula* roots by *R. solanacearum* inoculation (A. Yamchi, unpublished results). Hence the contrasting response to *Fom* indicates that in the same organ clear differences may exist between defence mechanisms against different pathogens. The results obtained on this small scale study show that early responses in defence gene expression are regulated differently in the tolerant and the susceptible line and encourage us to continue on a larger scale in order to understand the mechanisms underlying tolerance to this pathogen.

It was interesting to note that most strains and notably the two non-pathogenic strains *Fo*47 and *Fo*3 induced symptoms in *M. truncatula*. Non-pathogenic strains are considered as good candidates for biocontrol of Fusarium wilt and root rot, mostly by competition when colonising the root surface (Olivain and Alabouvette 1999; Bolwerk et al. 2005; Fravel et al. 2003). However, such strains were defined as non-pathogenic with regard to tomato and a limited range of plants on which they have been inoculated, and it was not excluded that they might be pathogenic on plant species that have not been tested; the same is true for the pathogenic *formae specialis* (Fravel et al. 2003; Recorbet et al. 2003). In addition to putative not yet identified host plants, the conditions of culture might have some influence on pathogenicity. However, our hydroponic culture conditions allowed to reproduce resistance and susceptibility of *M. truncatula* to the root pathogen *Ralstonia solanacearum* previously shown under different growth conditions in Jiffy pots or on agar medium (Vailleau et al. 2007) and to *Verticillium albo-atrum* (unpublished results). They were also quite similar to those for studies conducted on tomato with *Fol,* and on flax with *Fo* 47 and a pathogenic *F. oxysporum* strain (Olivain and Alabouvette 1999; Olivain et al. 2003). This suggests that hydroponic culture conditions have neither an effect on the general resistance ability of the plant nor on the biology of the fungus, and that *M. truncatula* is a permissive host to various *F. oxysporum* isolates.

To our knowledge, the present study describes for the first time the interaction between *M. truncatula* and *Fom*. Our microscopical observations have shown that colonisation of *M. truncatula* by *Fom*-GFP followed a slightly atypical pattern for Fusarium wilt, notably that later colonisation steps were not restricted to the xylem vessels. It can be hypothetised that characteristics of the *M. truncatula* root cells are responsible for this pattern and that maybe this atypical pattern is related to the permissive nature of the plant. Hence it will be interesting to study if the other *F. oxysporum* strains found to be pathogenic in the present work, and notably the well described non-pathogenic strain *Fo*47*,* colonise *M. truncatula* in the same way. Our results on the interaction between *M. truncatula* and *F. oxysporum* have shown for the first time that this model plant is a permissive host for a group of root pathogens. This opens new ways for the studies of acceptance and rejection of pathogens by a plant in legumes. If we understand what makes a plant permissive, this might also lead us to understand the mechanisms of basal resistance, such an approach is complementary to ongoing work with resistant and susceptible lines.

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References

Agrios, G. N. (2005). *Plant pathology* (5th ed.). USA and UK: Elsevier Academic.

Altier, N. A., Ehlke, N. J., & Rebuffo, M. (2000). Divergent selection for resistance to *Fusarium* root rot in birdsfoot trefoil. *Crop Science, 40*, 670–675.

Alves-Santos, F. M., Benito, E. P., Eslava, A. P., & Díaz-Mínguez, J. M. (1999). Genetic diversity of *Fusarium oxysporum* strains from common bean fields in Spain. *Applied and Environmental Microbiology, 65*, 3335–3340.

Ameline-Torregrosa, C., Cazaux, M., Danesh, D., Chardon, F., Cannon, S. B., Esquerré-Tugayé, M. T., et al. (2008). Genetic dissection of resistance to anthracnose and powdery mildew in *Medicago truncatula*. *Molecular Plant-Microbe Interactions, 21*, 61–9.

Bolwerk, A., Lagopodi, A. L., Lugtenberg, B. J. J., & Bloemberg, G. V. (2005). Visualization of interactions between a pathogenic and a beneficial *Fusarium* strain during biocontrol of tomato foot and root rot (2005). *Molecular Plant-Microbe Interactions, 18*, 710–721.

Colditz, F., Braun, H. P., Jacquet, J., Niehaus, K., & Krajinski, F. (2005). Proteomic profiling unravels insights into the molecular background underlying increased *Aphanomyces euteiches*—tolerance of *Medicago truncatula*. *Plant Molecular Biology, 59*, 387–406.

Colditz, F., Niehaus, K., & Krajinski, F. (2007). Silencing of PR-10 like proteins in *Medicago truncatula* results in an antagonistic induction of other PR proteins and in increased tolerance upon infection with the oomycete *Aphanomyces euteiches*. *Planta, 226*, 57–71.

Cook, D. R. (1999). *Medicago truncatula*—a model in the making! *Current Opinion in Plant Biology, 2*, 301–304.

Di Pietro, A., Garcia-Maceira, F. I., Meglecz, E., & Roncero, M. I. G. (2001). A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. *Molecular Microbiology, 39*, 1140–1152.

Di Pietro, A., Madrid, M. P., Caracuel, Z., Delgado-Jarana, J., & Roncero, M. I. G. (2003). *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. *Molecular Plant Pathology, 4*, 315–325.

Dixon, R. A., Achnine, L., Kota, P., Liu, C., Reddy, M. S. S., & Wang, L. (2002). The phenylpropanoid pathway and plant defence—a genomics perspective. *Molecular Plant Pathology, 3*, 371–390.

D'Souza, N. K. (2009). Pathosystem development, characterization and genetic dissection of the soil pathogen *Phytophthora medicaginis* and the model legume *Medicago truncatula*: A view to application of disease resistance in susceptible legume species. PhD dissertation, Murdoch University, Perth, Australia, 330 pages. http://wwwlib.murdoch.edu.au/adt/pubfiles/adt-MU20090423.105414/01Front.pdf

Eckert, M., Maguire, K., Urban, M., Foster, S., Fitt, B., Lucas, J., et al. (2005). *Agrobacterium tumefaciens*-mediated transformation of *Leptosphaeria* spp. and *Oculimacula* spp. with the reef coral gene DsRed and the jellyfish gene gfp. *FEMS Microbiology Letters, 253*, 67–74.

Farhaeus, G. (1957). The infection of clover root hairs by nodule bacteria studied by a simple glass technique. *Journal of General Microbiology, 16*, 374–381.

Fravel, D., Olivain, C., & Alabouvette, C. (2003). *Fusarium oxysporum* and its biocontrol. *New Phytologist, 157*, 493–502.

Gao, H., Beckman, C. H., & Mueller, W. C. (1995). The rate of vascular colonisation as a measure of the genotypic interaction between various cultivars of tomato and various formae or races of *Fusarium oxysporum*. *Physiological and Molecular Plant Pathology, 46*, 29–43.

Halila, I., Cobos, M. J., Rubio, J., Millán, T., Kharrat, M., Marrakchi, M., et al. (2008). Tagging and mapping a second resistance gene for *Fusarium* wilt race 0 in chickpea. *European Journal of Plant Pathology, 124*, 87–92.

Huser, A., Takahara, H., Schmalenbach, W., & O'Connell, R. (2009). Discovery of pathogenicity genes in the crucifer anthracnose fungus *Colletotrichum higginsianum*, using random insertional mutagenesis. *Molecular Plant-Microbe Interactions, 22*, 143–156.

Infantino, A., Kharrat, M., Riccioni, L., Coyne, C. J., McPhee, K. E., & Gruenwald, N. J. (2006). Screening techniques and sources of resistance to root diseases in cool season food legumes. *Euphytica, 147*, 201–221.

Jarvis, W. R. & Shoemaker, R. A. (1978). Taxonomic status of *Fusarium oxysporum* causing foot and root rot of tomato. *Phytopathology, 68*, 1679–1680.

Koiwa, H., Bressan, R. A., & Hasegawa, P. M. (1997). Regulation of protease inhibitors in plant defense. *Trends in Plant Science, 2*, 379–384.

Kraft, J. M. (1994). *Fusarium* wilt of peas (a review). *Agronomie, 14*, 561–567.

Lagopodi, A. L., Ram, A. F. J., Lamers, G. E. M., Punt, P. J., Van den Hondel, C. A. M. J. J., Lugtenberg, B. J. J., et al. (2002). Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. *Molecular Plant-Microbe Interactions, 15*, 172–179.

Lichtenzveig, J., Anderson, J., Thomas, G., Oliver, R. & Singh, K. (2006). Inoculation and growth with root pathogenic fungi. In *Medicago truncatula Handbook* version 2006. http://www.noble.org/MedicagoHandbook

Mes, J. J., van Doom, A. A., Wijbrand, J., Simons, G., Cornelissen, B. J. C., & Haring, M. A. (2000). Expression of the *Fusarium* resistance gene *I-2* colocalizes with the site of fungal containment. *The Plant Journal, 23*, 183–193.

Mullins, E. D., Chen, X., Romaine, P., Raina, R., Geiser, D. M., & Kang, S. (2001). *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. *Phytopathology, 9*, 173–180.

Nyamsuren, O., Colditz, F., Rosendahl, S., Tamasloukht, M. B., Bekel, T., Meyer, F., et al. (2003). Transcriptional profiling of *Medicago truncatula* roots after infection with *Aphanomyces euteiches* (oomycota) identifies novel genes upregulated during this pathogenic interaction. *Physiological and Molecular Plant Pathology, 63*, 17–26.

O'Connell, R., Herbert, C., Sreenivasaprasad, S., Khatib, M., Esquerré-Tugayé, M. T., & Dumas, B. (2004). A novel *Arabidopsis-Colletotrichum p*athosystem for the molecular dissection of plant–fungal interactions. *Molecular Plant-Microbe Interactions, 17*, 272–282.

Olivain, C. & Alabouvette, C. (1999). Process of tomato colonization by a pathogenic strain of *Fusarium oxysporum* f.sp. *lycopersici*. *New Phytologist, 141*, 497–510.

Olivain, C., Trouvelot, S., Binet, M. L., Cordier, C., Pugin, A., & Alabouvette, C. (2003). Colonization of flax roots and early physiological responses of flax cells inoculated with pathogenic and nonpathogenic strains of *Fusarium oxysporum*. *Applied and Environmental Microbiology, 69*, 5453–5462.

Pilet-Nayel, M. L., Prospéri, J. M., Hamon, C., Lesné, A., Lecointe, R., Le Goff, I., et al. (2009). AER1, a major gene conferring resistance to *Aphanomyces euteiches* in *Medicago truncatula*. *Phytopathology, 99*, 203–208.

Recorbet, G., Steinberg, C., Olivain, C., Edel, V., Trouvelot, S., Dumas-Gaudot, E., et al. (2003). Wanted: pathogenesis-related marker molecules for *Fusarium oxysporum*. *New Phytologist, 159*, 73–92.

Schneider, K. A., Grafton, K. F., & Kelly, J. D. (2001). QTL analysis of resistance to *Fusarium* root rot in bean. *Crop Science, 41*, 535–542.

Stutz, J. C., Leath, K. T., & Kendall, W. A. (1985). Wound-related modifications of penetration, development, and root rot by *Fusarium roseum* in forage legumes. *Phytopathology, 75*, 920–924.

Tkacz, J. S., Dahl-Roshak, A. M., Ibrahim, N., & Paress, P. S. (2004). *Agrobacterium*-mediated transformation of filamentous fungi. In Z. An (Ed.), *Handbook of industrial mycology.* USA: CRC. ISBN 082475655X, 9780824756550.

Torregrosa, C., Cluzet, S., Fournier, J., Huguet, T., Gamas, P., Prosperi, J. M., et al. (2004). Cytological, genetic and molecular analysis to characterise compatible and incompatible interactions between *Medicago truncatula* and *Colletotrichum trifolii*. *Molecular Plant-Microbe Interactions, 17*, 909–920.

Truesdell, G. M. & Dickman, M. B. (1997). Isolation of pathogen/stress-inducible cDNAs from alfalfa by mRNA differential display. *Plant Molecular Biology, 33*, 737–743.

Uppalapati, S. R., Marek, S. M., Hee-Kyung, L., Nakashima, J., Tang, Y., Sledge, M. K., et al. (2009). In the interaction of *Medicago truncatula* with *Phymatotrichopsis omnivora*, global gene expression profiling reveals a role for jasmonic acid, ethylene and the flavonoid pathway. *Molecular Plant Microbe Interactions, 22*, 7–17.

Vailleau, F., Sartorel, E., Jardinaud, M. F., Chardon, F., Genin, S., Huguet, T., et al. (2007). Characterization of the interaction between the bacterial wilt pathogen *Ralstonia solanacearum* and the model legume plant *Medicago truncatula*. *Molecular Plant-Microbe Interactions, 20*, 159–167.

Yang, S., Gao, M., Xu, C., Gao, J., Deshpande, S., Lin, S., et al. (2008). Alfalfa benefits from *Medicago truncatula*: the RCT1 gene from *M. truncatula* confers broad-spectrum resistance to anthracnose in alfalfa. *Proceedings of the National Academy of Sciences USA, 105*, 12164–12169.