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Resistance reaction of *Medicago truncatula* genotypes to *Fusarium oxysporum*: effect of plant age, substrate and inoculation method.

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Running Title: Fusarium wilt resistance of *Medicago truncatula*

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Summary Text for the Table of Contents

Fusarium wilt is one major constraint of crop and pasture legume production worldwide. Here we characterized the response of barrel medic (Medicago truncatula) to the disease according to plant age, plant substrate and inoculation method and established an inoculation method suitable for the molecular characterization of the fusarium wilt-legume interaction. This will be very useful to unravel the resistance mechanisms for an efficient control of the disease.

Abstract

Fusarium wilt caused by several formae speciales of Fusarium oxysporum is an important disease of most crop and pasture legumes including pea, chickpea, alfalfa and barrel medics. Medicago truncatula is an important pasture legume and a model legume species. Thus it can be used to increase our knowledge on the resistance mechanisms efficient to block F. oxysporum infection provided that its response to the disease is characterized. Here we evaluated the physiological and susceptibility response to the disease of two contrasting M. truncatula genotypes, and the effect of several cultural conditions known to affect the disease incidence such as plant age at infection time, growth substrate and the method of inoculation. Our results indicated that the A17 accession harbours a moderate level of resistance to the disease. We also showed that the method of inoculation strongly affected fusarium wilt disease development in this model species while it was not significantly altered by the plant age or the inorganic growth substrate tested. In addition we describe a rapid change in leaf temperature after infection that can be used as indirect parameter to confirm fungal infection at a very early stage of the interaction.
Keywords

Barrel Medic, Fungal pathogen, Disease resistance, Infra-red imaging system, Phenotyping
Introduction

Barrel medic, *Medicago truncatula* is an important pasture legume (Crawford *et al.* 1989) cultivated on over 4.5 million hectares in Australia alone (Hill and Donald 1998). It is of particular importance in southern Australian cereal-livestock zone where it provides feed for livestock and plays an important role in crop rotation. It contributes to improve soil fertility due to its capacity to fix atmospheric nitrogen through symbiosis and provides a disease break for various cereal root pathogens (Nair *et al.* 2006). Beside its agronomic importance, barrel medic is also an important model plant species for structural and functional genomic due to its small diploid genome and short life cycle (Cook 1999; Frugoli and Harris 2001). As such, it has been widely used to increase our understanding of the symbiotic interaction and legume disease resistance (Rispail *et al.* 2010).

Fusarium wilt caused by the soil-borne ascomycete *Fusarium oxysporum* is a recurrent disease that causes important losses in many crops including alfalfa, pea, chickpea and tomato (Gordon and Martyn 1997; Rubiales *et al.* 2015). Barrel medic can be also susceptible to fusarium wilt caused by *F. oxysporum* f. sp. *medicaginis* (*Fome*). While both monogenic and quantitative resistance mechanisms have been described in different legumes (Hijano *et al.* 1983; Infantino *et al.* 2006; Bani *et al.* 2012), only monogenic resistance has been used in breeding programmes which are at risk of breakdown by the emergence of new races and/or pathotypes (Sharma *et al.* 2010). This is in part due to the difficulty to include the quantitative resistance mechanisms within current breeding programmes for which a better understanding of disease resistance mechanisms at molecular level are needed.
The large post-genomic resources established for *M. truncatula* may serve to better understand the *F. oxysporum*-legume interaction. However, before large-scale “-omics” analyses could be applied, the disease response of *M. truncatula* accessions, and in particular of the A17 accession for which most post-genomic resources have been developed, should be well-characterised.

Previous works have described the A17 genotype as either susceptible or moderately resistant to *Fome* (Ramirez-Suero et al. 2011; Rispail and Rubiales 2014). The controversy of these data could have arisen by differences in the plant age, plant substrate or in the inoculation method used since the severity of fusarium wilt disease is influenced by host, pathogen and environmental factors (Ben-Yephet and Shtienberg 1997). Among them, the pathogenic strain, the concentration of inoculum and the temperature are the most limiting factors (Ben-Yephet and Shtienberg 1997). However, under optimal conducive conditions, plant age at infection, plant growth substrate and the inoculation methods can also greatly influence the establishment and intensity of fusarium wilt disease (Nywall and Haglund 1976; Swanson and van Gundy 1985; Latin and Shell 1986; Sedra and Besri 1994; Cohen et al. 2008).

Currently the best way to check successful fusarium wilt infection involves the isolation of the fungus from root or stem tissues which requires the destruction of the sample (Zhou and Everts 2004). Thus, an efficient non-destructive and fast phenotyping method suitable for large scale analysis is required to confirm plant infection. The recent development of imaging-based phenotyping such as the evaluation of superficial temperature by infra-red imaging system or the measurement of chlorophyll
fluorescence may offer an alternative to detect the pathogenic infection. This method has been applied to detect the presence of a few plant diseases both in fields and laboratory including virus, downy mildew, cercospora leaf spot and verticilium wilt (Chaerle et al. 2004; Chaerle et al. 2006; Stoll et al. 2008; Calderón et al. 2013). F. oxysporum inoculation has been recently shown to increase superficial leaf temperature in banana and cucumber (Dong et al. 2012; Wang et al. 2012). Thus, monitoring the changes in superficial leaf temperature by infra-red imaging may be an adequate method to confirm fusarium wilt infection.

The impact of plant age at inoculation, inorganic growth substrate and inoculation method on the disease response of M. truncatula accessions to Fome is still unknown. Thus, we aimed, here, to determine their effects on the fusarium wilt disease severity in contrasting M. truncatula accessions including the reference genotype A17. We also explore the possibility to use infra-red imaging system as a fast phenotyping method to confirm the successful infection at an early stage of the interaction. Since the commonly used root dipping method may induce uneven and uncontrolled stress to the plants, it may be inappropriate for highly sensitive purposes such as advanced imaging-based phenotyping or molecular studies of resistance, that are strongly affected by slight changes in plant physiology, the possibility to use alternative inoculation methods for such applications is discussed.

Material and Methods

Fungal isolate and culture conditions
Fome isolate 605 (MIAE00930), was obtained from the Microorganismes d’Intérêt Agro-Environmental (MIAE) collection of UMR1347 Agroecology, INRA Dijon, France (http://www2.dijon.inra.fr/umrmse/spip.php?rubrique47) and was used for all experiments. The fungal strain was routinely stored as microconidial suspensions at –80 °C with 30 % glycerol. For microconidia production, cultures were grown in potato dextrose broth (PDB; Difco, Detroit, MI) at 28 °C with shaking at 170 rpm.

Plant material and growth conditions

M. truncatula genotypes, PI 577607 and A17, were used in all experiments to determine the impact of inorganic substrate, plant age at inoculation and the inoculation methods on disease development. The accessions PI 516927 and PI 577600 were also used in additional experiments to confirm the influence of the inoculation method on fusarium wilt disease development. PI 577607 and PI 516927 were highly susceptibles, PI 577600 was resistant while the A17 showed moderate resistance to this Fome isolate (Rispail and Rubiales 2014). All PI accessions were kindly provided by the Agricultural Research Service of the United States Department of Agriculture (USDA-ARS, USA) germplasm collection while the A17 genotype was provided by the Institut National de Recherche Agronomique (INRA, France).

In all cases, M. truncatula seeds were scarified with sulphuric acid solution for 5 minutes and surface-sterilized for 2 minutes with a 3 % chlorine solution and left to imbibe in sterile water before being plated onto water-agar plates. Then, they were stratified for 2 days at 4 °C in the dark and incubated at 20 ± 2 °C until germination still in the dark. Once germinated, seedlings were transferred to pots (36 cm² x 8 cm) filled with sterile inorganic substrate and grown in a controlled environment chamber under a
16/8 h light-dark photoperiod at constant 26 °C ± 2 °C temperature regime with 200
µmol m⁻² s⁻¹ of illumination before inoculation. Plants were watered every three days
with tap water and weekly with Hoagland nutrient solution (Hoagland and Arnon 1950).

To test the impact of plant substrate on disease development, plants were grown either
in sterile fine grade vermiculite (1-3 mm Ø) or in a sterile mixture of sand: perlite at 1:3
proportion (V/V) both medium commonly used for *M. truncatula* growth. To define the
level of susceptibility of the contrasting *M. truncatula* accessions, plants were grown in
vermiculite For all subsequent experiments, *M. truncatula* plants were grown in the
sand:perlite mixture.

**Inoculation and disease assessment**

To estimate the impact of plant age on disease development, the response of *M.
*truncatula* to the disease was evaluated after inoculating 10 days old young seedlings
(2-3 leaves) or 30 days old established plants (10 leaves). To define the level of
susceptibility of the contrasting *M. truncatula* accessions and determine the impact of
plant substrate, plants were inoculated after 10 days of growth. In all additional
experiments, plants were inoculated after 30 days of growth.

In all experiments, *M. truncatula* plants were inoculated with the standard root dipping
method as described previously (Rispail and Rubiales 2014). Briefly, the plants were
removed from the substrate, cleaned gently under tap water and submerged in a Fome
spore solution for 30 min. Then the plants were transplanted to new pots containing
sterile vermiculite and maintained in the above mentioned growth chamber.
To establish the impact of the method of inoculation on disease development, plants were also inoculated with two alternative watering methods. For these methods, the inoculum was supplied either by adding 50 ml of inoculum at the base of the plants in each pot (pot watering method) or by placing the pots in a tray containing the spore inoculum until complete absorption (pot dipping method). Two days before watering inoculation, water supply of these plants was stopped to avoid the washing through of fungal spores at inoculation and to maximise the contact of these spores with the host root. In all cases, the inoculum consisted on a solution of $5 \times 10^6$ *Fome* spores ml$^{-1}$ in water and control plants were maintained non-inoculated by replacing the spore inoculum by sterile water. Only one control was used for both watering methods. For all experiments, five plants were used per treatment and each experiment was repeated twice independently.

*Fome* symptoms were rated every three days from 7th to 30th day post-inoculation (dpi) by estimating the percentage of leaves with symptoms (leaf yellowing, necrosis and wilting all together) per plant (Bani *et al.* 2012). These data were used to calculate the area under the disease progression curve (AUDPC) value as described previously (Bani *et al.* 2012).

**Infection bioassays**

To confirm the pathogen presence within inoculated plants, the fungus was re-isolated from root and stem tissues of two inoculated plants per genotype and treatment after 10 dpi as described previously (Bani *et al.* 2012).
In addition, infrared images of *M. truncatula* plants were obtained with a Thermovision A40M (FLIR, USA) thermal camera equipped with a 43° FOV lens and connected to computer via IEEE-1394 protocol. The image sensor was a Focal Plane Array (FPA) based on uncooled microbolometers with a resolution of 320 × 240 pixels, a spectral response in the range 7.5–13 µm, with 0.08 °C sensitivity at 30 °C and 0.1 mm minimal focus distance. Digital thermograms were acquired with the temperature range set between +10 - +55°C with the spectral rainbow color scheme with FSCAP software (FLIR, USA). The leaf temperature for each plant was determined by calculating the mean temperature of three 0.3 mm spots placed on three distinct leaves. Leaf temperature was monitored at 4 and 10 dpi. To take into account natural variation in the superficial leaf temperature, 10 plants were used per genotype and inoculation method.

**Statistical Analysis**

All experiments followed a completely randomized design and was repeated independently twice. Preliminary one-way ANOVA was conducted to compare the results between independent repeats. No significant differences were detected and the results of both replicates were combined before further analysis. Percentage of symptom values was subjected to an angular transformation to normalise the data and stabilize the variances before being subjected to the analysis of variance (Baird *et al.* 2002). The significance of the differences in leaf number, disease symptoms and superficial leaf temperature according to the treatment or genotype was estimated by one and two-Way ANOVA. All statistical analyses were performed using SPSS statistics v.22 software (IBM Corp., USA).
Results

Response to Fusarium oxysporum inoculation

Clear differences in disease ratings were observed between A17 and PI 577607 genotypes when 10 day old plants grown in vermiculite were inoculated according to the traditional root dipping method (Fig. 1). Thus, whereas PI 577607 plants were dead after 15 dpi, plants of A17 genotype remained alive until the end of the experiment although it was moderately affected by the pathogen (Fig. 1).

Effect of plant substrate on disease severity

To determine the influence of inorganic plant substrate, the overall plant growth and disease ratings were compared between plants grown in vermiculite or in a sand:perlite (1:3, v/v) mixture. A more profuse plant growth was obtained with the sand:perlite medium in which plants reached around 70 leaves after 55 days whereas the PI 577607 and A17 plants only grew up to 18 and 30 leaves in vermiculite respectively (Fig. 2a). However, both media were similarly conducive to the disease when 10 day old seedlings were inoculated with the root dipping method. Inoculated plants growing in both substrates showed clear Fome symptoms after 7 and 10 dpi for the PI 577607 and A17 genotypes respectively. Although the level of disease was significantly different between genotypes, the symptoms developed by inoculated plants were similar for both substrates reaching AUDPC values of 2126.7 and 1902.1 in sand:perlite and vermiculite, respectively for the susceptible accession PI 577607 and of 948.2 and 1122.3 for the A17 genotype ($P > 0.05$, Fig. 2b).

Effect of plant age on disease severity
M. truncatula seedling establishment in growth substrate is a very long process that require about a month in a controlled environment. During this period, M. truncatula seedling grows very slowly reaching 2-3 leaves after 10 days and 10 leaves only after 30 days of growth in inorganic substrate (Fig. 2a). Once established, approximately 30 days after sowing, M. truncatula grows exponentially producing profuse ramifications each harbouring several leaves and flower buds. Thus we determined the response of the contrasting M. truncatula genotypes to Fome when inoculated with the root dipping method at seedling stage (10 days after sowing) or after plant establishment (30 days after sowing). Significant differences (P < 0.05) between genotypes were detected at both plant ages, with the disease ratings of the susceptible accessions PI 577607 reaching 2127 ± 88 and 2022 ± 29 when inoculating 10 and 30 days old plants respectively while it only reached 891 ± 56 and 789 ± 45 for the A17 accession (Fig. 3). By contrast, the differences detected in the AUDPC values of each genotype when comparing plants inoculated at 10 or 30 days after sowing were not statistically different (P > 0.05, Fig. 3).

Effect of the inoculation method on disease severity

To determine the impact of the inoculation method on fusarium wilt disease development on contrasting M. truncatula genotypes, we tested the pot watering and the pot dipping method as alternative to the standard root dipping method. Following the three inoculation methods, we detected the characteristic wilt symptoms on susceptible plants. However, the root dipping method induced faster and stronger symptoms development (Fig. 4). Compared to the fast symptoms appearance at 7 dpi, symptoms induced by pot watering initiated at 15 dpi in both accessions reaching 28 % and 65 % of the leaves at the end of the experiment for the A17 and PI 577607 genotypes.
respectively (Fig. 4c and d). Thus, although both genotypes showed a lower level of
disease it was possible to distinguish between healthy and inoculated plants. It was also
possible to distinguish the genotypes since the A17 genotype showed a moderately
resistant phenotype as previously observed while the PI 577607 genotype was
susceptible (Fig. 4c). By contrast, the pot dipping method induced milder and delayed
symptoms on inoculated plants (Fig. 4e and f). While it was still possible to distinguish
between accessions ($P < 0.001$), it was not possible to discriminate between healthy and
inoculated A17 plants following this inoculation method ($P > 0.05$) (Fig 4e).

Inoculation of susceptible accessions PI 516927 with these methods showed very
similar disease ratings as the susceptible reference PI 577607 while a resistant accession
PI 577600 remained symptomless in all conditions tested (Fig. 5).

**Validation of Fome infection**

*Fome* was easily re-isolated from stems of both genotypes when inoculated with the
root dipping and pot watering methods, however, few colonies could be re-isolated
from stems of plants inoculated with the pot dipping method, confirming the low
efficiency of this method of inoculation (Fig. 6a). In all cases, lower numbers of *Fome*
colonies were isolated from the stems of A17, confirming its partial resistance.

*Fome* inoculation led to changes on the leaf surface temperature on both PI 577607 and
A17 accessions (Fig. 6b and c). However, differences could be detected according to the
genotype and the method of inoculation. Overall, *Fome* inoculation led to a rapid and
significant ($P = 0.02$) increase in leaf temperature of about 1 °C for the A17 genotype
and of 0.5 °C for the PI 577607 accession at 4 dpi with the root dipping method (Fig.
6c). Thereafter, the leaf temperature continuously rose to reach a significant difference
of 3 °C at 10 dpi for both accessions ($P < 0.001$; Fig. 6c). By contrast, inoculation by
pot watering and pot dipping led to long-lasting differences between PI 577607 and A17 accessions. For PI 577607 plants both methods of inoculation led to a rapid and significant temperature increase of approximately 1 °C at 4 dpi that was maintained at 10 dpi. For the moderately resistant A17 genotype, this rapid temperature increase was also observed at 4 dpi for both methods of inoculation (Fig. 6c), but it was only transient and it reduced in successive days (Fig. 6c), albeit slightly faster for the pot dipping method. Thus, although the susceptible and the moderately resistant genotypes followed a different trend at longer times following inoculation, both accessions showed a significant temperature increase at the early stages of the infection process ($P < 0.001$ and $P = 0.003$ for PI 577607 and A17 genotypes, respectively) that could be used to check the successful plant infection by $Fome$ before appearance of the first visible symptoms.

Discussion

$Fusarium$ wilt is a major constraint of most legume crop worldwide including pea, chickpea, lentil and alfalfa (Rubiales et al. 2015). The use of model plants such as $M. truncatula$ may contribute to improve our understanding of the $F. oxysporum$ – legume interaction and legume resistance. To this aim we characterised the response to the disease of two genotypes reported to have contrasting levels of resistance. Although A17 has been adopted as the reference genotype by $M. truncatula$ researchers worldwide (Choi et al. 2004; Young et al. 2005; Mun et al. 2006; Kamphuis et al. 2007) contradictory reports have been given on its actual response to the same isolate of $Fome$ (Ramirez-Suero et al. 2011; Rispail and Rubiales 2014). As for other species, $F. oxysporum$ disease severity on $M. truncatula$ might be affected by different parameters.
such as the growing substrate or plant age at time of inoculation (Swanson and van Gundy 1985; Cohen et al. 2008). For instance, the use of organic substrate such as peat and compost has been shown to affect disease severity leading to highly variable results mainly depending on the microorganism they contains (Cohen et al. 2008). By contrast inorganic substrates such as sand, perlite or vermiculite had often little influence on disease severity observed on susceptible accessions although they may lead to slight variations for partially resistant accessions (Ben-Yepthet and Shtienberg 1997; Cohen et al. 2008). In addition, these alternative substrates may sustain *M. truncatula* growth differently (Barker et al. 2006). Thus, we tested their influences on the disease response of two contrasting *M. truncatula* genotypes. Our results indicated that the A17 accession harbours a moderate level of resistance to the disease (Figs. 1 and 2). Although the plant growth was affected by the substrate, the disease developed similarly on both substrates and the A17 genotype was in all cases moderately resistant to *Fome* compared with the response of the susceptible genotype PI 577607 (Fig. 2).

It has been also reported that older plants may possess higher resistance levels to fusarium wilt compared with younger ones (Nyvall and Haglund 1976). Adult plant resistance has been frequently reported against airborne diseases (Fondevilla et al. 2006; Sánchez-Martín et al. 2011; Bansal et al. 2014) and has been detected for some crops against fusarium wilts (Nyvall and Haglund 1976; Cirulli and Ciccarese 1982; Latin and Snell 1986) but not for others such as cowpea or some tomato cultivars (Cirulli and Ciccarese 1982; Swandon and van Gundy 1985). At the ages tested in this study, *M. truncatula* plants showed no differences in their response to the infection and A17 and PI 577607 showed a similar response to that observed in the above mentioned experiments (Fig. 3). Thus, our data on these genotypes are in accordance to the results
found for other legumes in which no adult plant resistance against *F. oxysporum* was observed (Swanson and van Gundy 1985). The differences detected in the susceptibility of the A17 in previous studies (Rámirez-Suero *et al.* 2011; Rispail and Rubiales 2014) might be hence more likely due to the methods of inoculation used than to the plant age. On the other hand, the fact that *M. truncatula* susceptibility to *Fomes* is not dependent on plant age at inoculation time opens the possibility to use older plants when high amount of plant tissue is needed for genomic, proteomic or metabolomics studies. Simultaneous high through-put molecular studies require large amount of material and early sampling time after inoculation. Given the very slow growth of *M. truncatula* seedlings before establishment, many *M. truncatula* young seedlings would be required for each time point considering the needs of biological replicates which may not be possible for space limitation in most growth rooms. Thus, the use of established *M. truncatula* plants (30 days old) at inoculation may contribute to reduce the number of plants needed to establish the molecular basis of fusarium wilt resistance with such high through-put molecular approaches while the time of 10 days for inoculation may be optimal for other applications when no large samples are required (Barker *et al.* 2006; Rispail and Rubiales 2014). 

In most plant species including *M. truncatula*, pea, tomato and melon, the standard method for *F. oxysporum* inoculation is the root dipping approach with or without root trimming that favours *F. oxysporum* root colonization through wounds (Latin and Snell 1986; Di Pietro and Roncero 1998; Lichtenzveig *et al.* 2006; Bani *et al.* 2012; Rispail and Rubiales 2014). *F. oxysporum* infection in other species have also been performed by transplanting or sowing healthy plants in inoculated substrate obtained by mixing plant substrate with either infected seeds or fungal material (Kraft *et al.* 1994; Riccioni
Alternatively, inoculum may also be applied to the plant as a liquid solution by supplying the spores to each pot at the base of the plants or by dipping plant pots or trays in the spore solution (Latin and Snell 1986; Baya et al. 1995; Schreuder et al. 2000; Riccioni et al. 2003). For rapid screening, continuous dipping methods involving the transfer of seedlings to flask or vials containing diluted spore solution has also been proposed (Freemand and Rodriguez 1993; Chikh-Rouhou et al. 2010). Since the inoculation method may led to variation of the severity of fusarium wilt according to the plant species considered (Latin and Snell 1986; Sedra and Besri 1994; Bayaa et al. 1995), we compared the response to the disease of the contrasting *M. truncatula* accessions obtained after inoculating the plants with the root dipping methods without root trimming or two alternative watering–based methods of inoculation. Both methods reduced the level of symptoms observed on the susceptible and partially resistant accessions indicating a lower efficiency of the inoculation compared to the standard root dipping method. Differences between these watering-based methods were also detected (Fig. 4). *F. oxysporum* enters host root preferentially through the root epidermis (Di Pietro et al. 2003). It has been also reported to enter through wounds and by crack entry at site of lateral root emergence (Michielse and Rep 2009). The root dipping method wounds the root system and allows direct contact with the pathogenic spores leading to massive entry of the pathogen directly in the xylem through these wounds. In addition, by entering through wounds, the pathogen might escape defence mechanisms acting at the root epidermis or cortex such as the formation of papilla-like structures that otherwise would prevent fungal penetration (Michielse and Rep 2009). For these reason, the root dipping method might induced faster and stronger symptom development than the watering methods. Recent studies have also shown that normal penetration through root epidermis is preceded by proliferation of the fungus along the root system.
(Czymmek et al. 2007) suggesting that the fungus should reach a certain density before root infection. This would delay further the host colonization when inoculated with the watering methods. It may also explain the differences we observed in the efficiency of both watering-based inoculation methods. Indeed, we reckon that a lower number of *Fomy* spores may reach the root system by the tray watering method than with the pot watering method and thus infection with the pot dipping method would require additional delays to reach the minimum density of mycelium before root penetration.

Although it induces delayed and milder symptoms on both susceptible and partially resistant accessions, the pot watering method still allow differencing the susceptible accessions PI 577607 and PI 516927 from the A17 accession that maintained its moderate resistance. By contrast with the pot dipping method, the A17 accessions remained symptomless similarly to the resistant accession PI 577600 while only mild symptoms were detected on the susceptible accessions (Figs. 4 and 5). Thus the use of the pot dipping method for resistance screenings may led to the erroneous classification of the A17 accession as resistant and PI 577607 and PI 516927 as partially resistant accessions. From all the parameters evaluated here, the inoculation method is the only one that affected the severity of fusarium wilt in *M. truncatula*. Thus, the difference in the A17 susceptibility to *Fomy* previously detected may thus be explained by the differences in inoculation method or the pathogenic strain used. The A17 was previously shown to be susceptible when inoculated with the strain 179.29 by root dipping in a hydroponic system (Ramirez-Suero et al. 2011) while it presented a partial resistant when inoculated with the strain 605 in our conditions (Fig. 1; Rispail and Rubiales, 2014). Previous comparison of both strains indicated that the strain 605 was also pathogenic on the A17 accessions (Ramirez-Suero et al. 2011) and more virulent.
than the strain 179.29 (Rispail and Rubiales 2014). Thus, it is unlikely that the
difference in the susceptibility of the A17 to Fome was due to differences in strain
virulence. Rather, this difference in susceptibility might be due to the experimental
conditions. Altogether, this would indicate that the optimal method for resistance
screening of M. truncatula collection to Fome is the traditional root dipping method.
This method was specifically developed for resistance screening or to assess the
efficiency of pathogen control methods (Haglund 1989; De Cal et al. 1999; McPhee et
al. 1999; Wang et al. 1999; Chikh-Rouhou et al. 2010; Bani et al. 2012; Rispail and
Rubiales 2014). This method that involve uprooting seedlings from their substrate and
dipping the root system in a solution of F. oxysporum spore before transplanting to new
pot (Di Pietro and Roncero 1998) generally allowed a rapid and strong infection as
shown here for M. truncatula. However, it does not take into account the general host
welfare and induces additional stresses to the plant at inoculation even when plant roots
were not trimmed at inoculation. Thus it may not be adequate for more sensitive
purposes such as advanced imaging-based phenotyping or molecular studies of
resistance, that are strongly affected by slight changes in plant physiology. Although the
pot watering method induced delayed and reduced disease symptoms, it provided
reproducible and homogeneous infection, maintained the phenotypic difference between
accessions and did not involved any plant manipulation at inoculation. Thus it may be
an adequate alternative to the root dipping method to elucidate the molecular basis of F.
oxysporum resistance in legume.

The recent development of imaging-based phenotyping such as the evaluation of
superficial temperature by infra-red imaging system is revolutionizing agriculture and
plant science. Infra-red thermal imaging is a non-contact, non-destructive and rapid
technique which provides a temperature map of the targeted material or plant. Our results showed that infra-red imaging might be used for controlling the successful infection of plants which is critical for large scale molecular analysis (Fig. 6). Thus, by infra-red imaging we could confirm the infection by *F. oxysporum* of *M. truncatula* plants as with the traditional, destructive and time-consuming re-isolation method. In general, plant superficial temperature is dependent on transpiration rates. At high transpiration rate, the leaf temperature is cooling while at lower transpiration rate, this temperature rises. The increase of temperature following *F. oxysporum* infection suggest a closure of stomata in the *M. truncatula* plants, which is a feature also observed upon infection with *F. oxysporum* (Dong *et al.* 2012; Wang *et al.* 2012) and other fungal or bacterial plants infection (Melotto *et al.* 2006; Prats *et al.* 2006; Prats *et al.* 2007; Mur *et al.* 2013). Stomata are important regulator of the interaction between plants and their environment. Stomata movement controls plant transpiration, thus stomata closure under drought stress limit water losses by the plant. While the function of stomata closure after challenge by pathogen is still unclear, it have been often described as a direct consequence of plant defence or pathogen action (Mur *et al.* 2013). In the case of *F. oxysporum*, the increase in leaf temperature and associated stomatal closure might reflects the perturbation of the water flux in infected plants due to the vessel plugging induced by the intensive fungal growth within xylem cells and the plant defence and/or the production of fusaric acid (Mepsted *et al.* 1995; Dong *et al.* 2012; Wang *et al.* 2012; Wang *et al.* 2013).

Independently of the mechanism controlling stomatal movement during plant infection, monitoring superficial temperature is a good indicator of plant stresses. On the other hand, the relation between stomatal closure and resistance is still unclear and depends
on the plant species, the pathogen and the resistance mechanisms involved at least for aerial pathogens (Prats et al. 2006; Prats et al. 2007; Mur et al. 2013). Here, by monitoring the temperature changes induced by infection at 4 days post inoculation it was possible to discriminate between genotypes since the temperature increase was slightly higher in the partially resistant A17 than in the susceptible PI 577607 (Fig. 6c).

Thus monitoring changes in superficial temperature might be useful not only to check for Fome infection but also to screen for resistance to F. oxysporum although further studies with a larger number of discriminating genotypes would be needed to confirm the usefulness of infra-red imaging as an alternative screening method.

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References


Chaerle L, Pineda M, Romero-Aranda R, Van Der Straeten D, Baron M (2006) Robotized thermal and chlorophyll fluorescence imaging of pepper mild mottle


Di Pietro A, Roncero MIG (1998) Cloning, expression, and role in pathogenicity of pg1 encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen 
Fusarium oxysporum.
Molecular Plant-Microbe Interaction 11, 91-98.


Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. California Agricultural Experimental Station Circular V. 347, the College of Agriculture, University of California-Berkeley, CA.


Fusarium oxysporum f. sp. cucumerinum. Plant Physiology and Biochemistry 61, 153-161.


Figure Legends

**Fig. 1.** Clarification of the response of *M. truncatula* A17 accessions to *F. oxysporum* f. sp. *medicaginis*. Comparison of the susceptibility of the PI 577607 (circle symbols) and A17 (square symbols) genotypes grown in vermiculite and inoculated by *F. oxysporum* f. sp. *medicaginis* with the root dipping method (closed symbols/bars) or maintained non-inoculated (open symbols/bars). The susceptibility was determined by plotting the progression over time of disease symptoms estimated with a visual scale ranging from 1 (healthy) to 10 (completely dead plants) and the corresponding Area Under the Disease Progression Curve (AUDPC) histogram. Vertical bars are standard errors for n = 10.

**Fig. 2.** Effect of growth substrate on *Medicago truncatula* growth and susceptibility to *F. oxysporum* f. sp. *medicaginis*. (a) Comparison of the overall growth, estimated as the total number of leaves per plants over time, of the accessions PI 577607 (circle symbols) and A17 (square symbols) grown on vermiculite (open symbols) or on a mixture of sand:perlite (1:3, V/V) (closed symbols) as determined by the number of leaves. (b) Comparison of the susceptibility of the PI 577607 (closed bars) and A17 genotypes (open bars) grown in vermiculite or a sand:perlite mixture to *F. oxysporum* f. sp. *medicaginis* as determined by their AUDPC values. Inoculation was performed on 10 days old seedlings with the root dipping methods. Vertical bars are standard errors for n = 10.
Fig. 3. Effect of plant age on *M. truncatula* susceptibility to *F. oxysporum* f. sp. *medicaginis*. Comparison of the susceptibility of the PI 577607 (closed bars) and A17 (open bars) genotypes inoculated after 10 days or 30 days of growth by *F. oxysporum* f. sp. *medicaginis* with the root dipping method as determined by their AUDPC values. Vertical bars are standard errors for n = 10.

Fig. 4. Effect of inoculation methods on *M. truncatula* susceptibility to *F. oxysporum* f. sp. *medicaginis*. Comparison of the susceptibility of the moderately resistant A17 (a, c, e) and the susceptible PI 577607 (b, d, f) accession inoculated 30 days after sowing by *F. oxysporum* f. sp. *medicaginis* (closed symbols) or maintained non-inoculated (open symbols). Plants were inoculated with the root dipping (a, b), the pot watering (c, d) or the pot dipping (e, f). The susceptibility was determined by plotting the progression over time of disease symptoms estimated as the percentage of leaves with symptoms per plants. Vertical bars are standard errors for n = 10.

Fig. 5. Comparison of the susceptibility of different *M. truncatula* genotypes according to the method of inoculation. The histogram compares the AUDPC values obtained for the resistant accession PI 577600, moderately resistant accession A17 and the susceptible accessions PI 577607 and PI 516927 inoculated 30 days after sowing by *F. oxysporum* f. sp. *medicaginis* according to the method of inoculation. Vertical bars are standard errors for n=10.

Fig. 6. Verification and physiological characterisation of *M. truncatula* plant infection by *F. oxysporum* f. sp. *medicaginis* according to the method of inoculation. (a)
Detection of *F. oxysporum* f. sp. *medicaginis* presence by re-isolation of the fungus from inoculated stems. Photographs compare the extension of *F. oxysporum* f. sp. *medicaginis* colonies growing out of plant tissue from the susceptible PI 577607 and the moderately resistant A17 accessions control non-inoculated plants or inoculated by the root dipping, the pot watering or the pot dipping method. (b) Characterisation of the leaf temperature of *M. truncatula* control non-inoculated plants or inoculated by *F. oxysporum* f. sp. *medicaginis* with the root dipping, the pot watering or the pot dipping method as determined by infra-red imaging thermographic measurement at 4 and 10 dpi. (c) Evolution of the leaf temperature changes induced by the method of inoculation in the susceptible PI 577607 (closed symbols) or the moderately resistant A17 (open symbols) as determined by the difference in infra-red thermographic measurement between inoculated and control non-inoculated plants. Vertical bars are standard errors for n=10.
Fig. 1

Proportion of symptoms (\text{%})

Time post-inoculation (Days)

Accessions

AUDPC

0 1 2 3 4 5 6 7 8 9 10

0 100 200 300 400 500 600 700 800 900 1000

0 500 1000 1500 2000 2500

Fig. 1
Fig. 2

(a) Leaf number vs. Plant age (Days)

(b) AUDPC vs. Growth Substrate

Growth Substrate

- Sand:Perlite
- Vermiculite
Fig. 3
**Fig. 5**

**Medicago truncatula** genotypes

The chart shows the AUDPC (Area Under the Disease Progress Curve) for different treatments and genotypes. The treatments include Control, Pot dipping, Pot watering, and Root dipping. The genotypes are identified by their PI codes (R, PR, S) and are sorted by their response to the treatments.
Fig. 6