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RESEARCH PAPER

Aggressiveness and host range of *Phoma medicaginis* isolated from *Medicago* species growing in Tunisia

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Summary. Aggressiveness of 14 *Phoma medicaginis* isolates obtained from *Medicago truncatula* (barrel medic) and *M. ciliaris* (ciliate medic) growing in Tunisia was measured after inoculation on leaves and roots of *M. truncatula*. The ability of one isolate to cause disease on *M. sativa* (alfalfa), *Cicer arietinum* (chickpea), *Pisum sativum* (pea), *Lens culinaris* (lentil) and *Phaseolus vulgaris* (common bean) was also tested. The pathogen caused dark lesions that enlarged and coalesced causing yellowing and premature abscission of leaves, resulting in decreased shoot fresh weight in barrel medic plants. All *P. medicaginis* isolates infected barrel medic roots causing collar rot, brown root discoloration, yellowing of cotyledons and reduced shoot and root development. The pathogen colonized the cortex and the stele of plants and produced fertile pycnidia on infected roots. Symptoms on leaves allowed for greater discrimination in aggressiveness among isolates in comparison to symptoms on roots. No correlations were observed between the parameters measured on leaves and roots suggesting organ specialization in this pathogen. *Phoma medicaginis* infected leaves of alfalfa, pea, common bean and chickpea causing necrosis and tissue yellowing at 15 d post inoculation (dpi). Pycnidium production was observed on dead and dying foliar tissues of alfalfa, pea and common bean, but not on chickpea. The pathogen caused symptoms of collar rot and brown root discoloration on alfalfa, chickpea, pea and common bean, but did not cause symptoms on leaves or roots of lentil at 15 dpi. *Phoma medicaginis* was more pathogenic on barrel medic, the host of origin, in comparison to the other legumes, suggesting that these species are likely to be secondary hosts for this pathogen.

Key words: grain legumes, host specificity, *Medicago truncatula,* pathogenicity, spring black stem and leaf spot.

Introduction

Phoma medicaginis Malbr. & Roum., the causal agent of spring black stem and leaf spot on perennial (*Medicago sativa* L.) (Gray *et al.*, 1990) and annual *Medicago* species (Barbetti 1995), occurs under a wide range of environmental conditions. Distributed worldwide, these diseases are common on *Medicago* species in North America, Europe, Australia (Tivoli *et al.*, 2006) and North Africa, particularly in Tunisia (Djébali and Aouani, 2004). *Phoma medicaginis* is a major cause of yield loss and stand decline in alfalfa. The fungus also causes losses in forage quality and it is more destructive in irrigated fields of alfalfa than dryland crops. Black lesions are frequently observed on leaves and stems of alfalfa. The pathogen also causes black stem on annual *Medicago* spp. and is reported to be pathogenic to a wide range of legumes (Edmunds and Hanson, 1960; Mead and Cormack, 1961). However, Ellwood *et al.* (2006) suggested that *P. medicaginis* has a more restricted host range, limited to *M. sativa* and *M. truncatula*. *Phoma medicaginis* is mainly known to infect the shoots of plants and few studies reported it as a primary pathogen of *Medicago* roots (Rodriguez and Leath, 1992). *Phoma* spp. are also considered to be root pathogens of other legumes (Barbetti, 1984). However, the reports on the pathogenicity of *P. medicaginis* to *Medicago* and legume roots are inconsistent and no study has demonstrated colonization of the roots at the cellular level.

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Some studies reported *P. medicaginis* to be a secondary pathogen of roots, since it was always associated with other fungi and bacteria in the crown and root rot complex of alfalfa (Lukezic *et al.*, 1983). In other studies only slight to moderate rot symptoms were observed in inoculated alfalfa roots in the field (Cormack, 1945).

The objectives of the research outlined in this paper were to determine the aggressiveness of a number of *P. medicaginis* isolates, isolated from annual *Medicago* species, on leaves and roots of *M. truncatula.* In addition, the pathogenicity of one isolate on leaves and roots of five legumes was evaluated in order to determine its host specialization.

Materials and methods

Plant material and growth conditions

Six legumes were used in this study: barrel medic (*Medicago truncatula* L., line F83005.5); alfalfa (*Medicago sativa* L.; cv. Gabès); chickpea (*Cicer arietinum* L.; cv. Chetoui); common bean (*Phaseolus vulgari*s L.; cv. Coco Blanc); pea (*Pisum sativum* L. ; cv. Lincoln) and lentil (*Lens culinaris* L.; unknown cultivar). The seeds of chickpea, pea and common bean were provided by Mohamed Kharrat, Institut National de la Recherche Agronomique de Tunis. The line F83005.5 of *M. truncatula* was used because it was described as highly susceptible to *P. medicaginis* attack (Djébali *et al.*, 2007).

The seeds of *M. truncatula* and alfalfa were extracted from pods and immersed in pure sulfuric acid for 7 min and 3 min, respectively, and then washed five times with autoclaved distilled water. Seeds of chickpea, pea, lentil and common bean were surface sterilized by immersion in 70% alcohol for 30 s and then in 0.2% $HgCl_2$ for 2 min and extensively washed with autoclaved distilled water. Imbibed seeds were transferred to agar plates (0.9%) and incubated at 25°C in darkness until radicle emergence. Plants were cultivated in pots (250 mL capacity) containing autoclaved perlite in a growth chamber at 25°C with 16 h daily photoperiod, and were irrigated with a nutrient solution (Vadez *et al.*, 1996) modified by Mhadhbi *et al.* (2005).

Field sampling and fungal isolation

Three regions in Northern Tunisia were surveyed (Table 1) in order to isolate *P. medicaginis* that naturally infected annual *Medicago* species in this country. Sampling of *M. truncatula* and *M. ciliaris* plants was carried out during roadside and field surveys conducted from March to May during 2002 to 2005. Where symptoms suggestive of fungal infection were observed, at least three plants were collected. Samples of diseased material were placed between filter paper at room temperature to dry for subsequent microbiological examination.

For the isolation of fungi, symptomatic stem and leaf samples were washed with autoclaved distilled water, placed on filter paper soaked with sterilized distilled water and incubated in a humid chamber at 25°C with 16 h daily photoperiod (100 μE) for 24 to 72 h until pycnidia formed. Pure cultures were obtained as described by Djébali *et al.* (2009). The fungal isolates were maintained on potato dextrose agar (PDA) plates (Pronadisa, Madrid, Spain) in the above conditions. The morphological identifications of fungi were based on microscopic (Olympus BX41; Olympus Optical Co. Ltd., Tokyo, Japan) examination of conidial length, width, and shape after mounting in clear lactophenol.

Morphology of Phoma medicaginis *in culture*

The description of each *P. medicaginis* culture was made from visual examination after 4 weeks of culture on Sanderson & Srb medium (Dhingra and Sinclair, 1995) at 25°C and 16 h daily photoperiod. Conidium dimensions (length and width) were measured using a micrometric ruler under a microscope (Olympus BX41).

Inoculum production and inoculation tests

Phoma medicaginis was grown on the specific sporulation Sanderson & Srb medium at 25°C and 16 h daily light. Conidium suspensions were prepared by flooding the plates with autoclaved distilled water and manual disruption of the culture. The conidium suspensions used for inoculations were prepared from 1-month-old cultures, and were applied at a concentration of 10^6 conidia mL^{-1}. The pathogenicity tests of the fungal isolates were made on legume plants according to the Koch's rules (Agrios, 1988).

Aggressiveness tests on leaves and roots of M. truncatula

One-month-old plants were spray inoculated with the conidium suspension until run-off and were covered with transparent plastic bags for 15 d to maintain high humidity to stimulate infection.

Table 1. List of the studied *Phoma medicaginis* isolates, and their sites (in Tunisia) and hosts of origin.

Fungal isolate	Site of origin		Host of origin
BrMt0302Ph1 (Pm1)	Bulla Regia (36°33′N8°45′E)	North-West	*Medicago truncatula*
BrMt0404Ph2 (Pm2)			
BrMt0404Ph3 (Pm3)			
BrMt0404Ph4 (Pm4)			
BrMt0404Ph5 (Pm5)			
BrMt0505Ph6 (Pm6)			
BrMt0505Ph7 (Pm7)			
BrMt0404Ph8 (Pm8)			
SoMc0505Ph9 (Pm9)	Soliman (36°41′N10°32′E)	North-East	*Medicago ciliaris*
SoMc0505Ph10 (Pm10)			
SoMc0505Ph11 (Pm11)			
SoMc0505Ph12 (Pm12)			
MaMc0505Ph13 (Pm13)			
TuMt0402Ph14 (Pm14)	Tunis (36°49′N10°10′E)		*Medicago truncatula*

The control plants were inoculated with autoclaved distilled water. Plants were placed in a growth chamber at 25°C with 16 h daily photoperiod (100 μE). The percentages of leaves with necrosis (%LN), leaves with chlorosis (%LC), defoliation (%DfL), of dead leaves (%DL), and the shoot fresh weight (SFW) were determined at 15 d post inoculation (dpi). The experiment was repeated twice, with five replicates per treatment in a randomized complete block design.

For the aggressiveness tests of the fungal isolates on the roots of *M. truncatula* plants, an *in vitro* inoculation method was performed to allow the monitoring of the pathogen progression in the root tissues in a real time manner. Five *M. truncatula* seedlings (1 d after germination) were placed on M medium (Bécard and Fortin 1988) plates in square Petri dishes (12 cm × 12 cm × 1.3 cm) and grown for 2 d in a growth chamber at 25°C and 16 h daily photoperiod. Inoculation of each plant was performed by depositing 10 μL of conidium suspension on roots (3 mm from the root apex). For the control plants, 10 μL of autoclaved distilled water were deposited on roots. Symptom development was recorded at 15 dpi. The percentage of root and hypocotyl showing brown discoloration (%BR), yellow or dry area on cotyledons (%YC), plant death (%DP) and the percentage of roots covered with pycnidia (%RP) were recorded for each inoculated plant.

Pathogenicity tests

In order to study the pathogenicity of *P. medicaginis* to leaves and roots of five additional legumes, one isolate (Pm8) was inoculated on 1-month-old plants by spraying on leaves or by placing 10 µL of the conidium suspension (10^6 conidia mL^{-1}) on roots at the base of the stem of each plant. Control plants were inoculated with autoclaved distilled water. After shoot inoculation, the presence and the level of necrosis and pycnidium formation on leaves were recorded at 15 dpi. After root inoculation the presence and the level of brown discoloration and pycnidium formation on the collar and roots of each plant were recorded at 15 dpi.

Microscopic analysis

For optical microscopy, 250 μm sections of fresh samples were made from roots embedded in 5% low melting point agarose, using a vibratome (Leica VT1000S, Nussloch, Germany), mounted on glass slides in a drops of distilled water and observed by bright field microscopy using an inverted microscope (DMIRBE, Leica, Rueil-Malmaison, France). Sections were labeled for 30 min with WGA-FITC (50 μg mL^{-1} in phosphate-buffered saline solution at pH 7.2), to highlight *P. medicaginis* hyphae using epifluorescence illumination (excitation filter, BP 450–490 nm). Images were acquired using a CCD camera (colour Coolview, Photonic Science, Robertsbridge, UK).

Statistical analyses

All experiments were repeated twice. The data collected were subjected to analysis of variance (ANOVA) with comparison of means using STATISTICA software version 5.1H (StatSoft, France). Mean values of each treatment were compared using the LSD test at 5% probability. Correlations between measured parameters were estimated on the basis of the means of three replicates using the Pearson coefficient, and the level of significance was adjusted for multiple comparisons by Bonferroni corrections.

Results

Morphology of *Phoma medicaginis* in culture

The macroscopic observation of *P. medicaginis* cultures on Sanderson medium showed flat, grey, felty mats that darkened gradually, sectorially or in concentric zones, until carbonaceous (Figure 1a). Numerous large pycnidia were scattered over the mats. Crystals (15 to 33 crystals per 9 cm Petri dish) appeared in the medium after about 3 to 4 months of culture at 25°C and 16 h daily light (Figure 1b and c). The number of crystals produced differed between isolates (data not shown). Microscopic examination revealed that the isolates produce primarily non-septate conidia (Figure 1d) with a low proportion of one-septate conidia (Figure 1e). The conidium sizes ranged from 4.9 to 7.1 μm in length and between 2.0 to 2.9 μm in width.

Aggressiveness of *Phoma medicaginis* isolates on *Medicago truncatula*

Aggressiveness on leaves

Inoculation of *P. medicaginis* isolates on leaves of *M. truncatula* line F83005.5 caused small dark lesions (necrosis) each surrounded by a yellow margin (chlorosis). As the spots enlarged and coalesced, infected leaves turned yellow and abscised prematurely. Pycnidia of the fungus developed on dead tissues at the margin of the necrosis. The fungus was consistently recovered from inoculated leaves. No necrosis or chlorosis symptoms developed on the control plants.

The analysis of variance showed that all measured parameters, except shoot fresh weight, differed significantly among the 14 *P. medicaginis* isolates (Table 2). The percentage of leaf necrosis exhibited the greatest discrimination among the isolates contributing 26% of the observed variability, followed by the percentage of dead leaves (23%), leaf chlorosis (22%) and leaf abscission (20%) (Table 2).

The comparison of means showed that the *P. medicaginis* isolates Pm1 to Pm11 exhibited statistically similar levels of aggressiveness (Table 3). The isolates Pm12, Pm13 and Pm14 were less aggressive in comparison to the other isolates for the four measured parameters on leaves (Table 3).

Aggressiveness on roots

The rot symptom caused by *P. medicaginis* when inoculated on *M. truncatula* roots was dark brown, dry necrosis often accompanied by collapsed root tissue at or around the infection site (Figure 2a). The infection extended from the principle to the lateral roots (Figure 2a) and up to the base of stems at the level of cotyledons (Figure 2b) causing a reduction in shoot and root development leading to plant death (Figure 3a and b). Pycnidia of the fungus appeared on the dead root tissues (Figure 2c) which produced masses of pycnidiospores (Figure 2d). The pathogen was able to enter intact roots and colonize the cortex and the stele of individual roots of *M. truncatula* (Figure 2g and h). Chlorosis of foliage occurred on plants with severely diseased roots. The fungus was consistently recovered from infected roots. No symptoms developed on the roots of the control plants (Figure 2a and f).

The analysis of variance showed that the percentage of brown discoloration on roots and the percentage of the root surface covered with pycnidia discriminated between the 14 *P. medicaginis* isolates

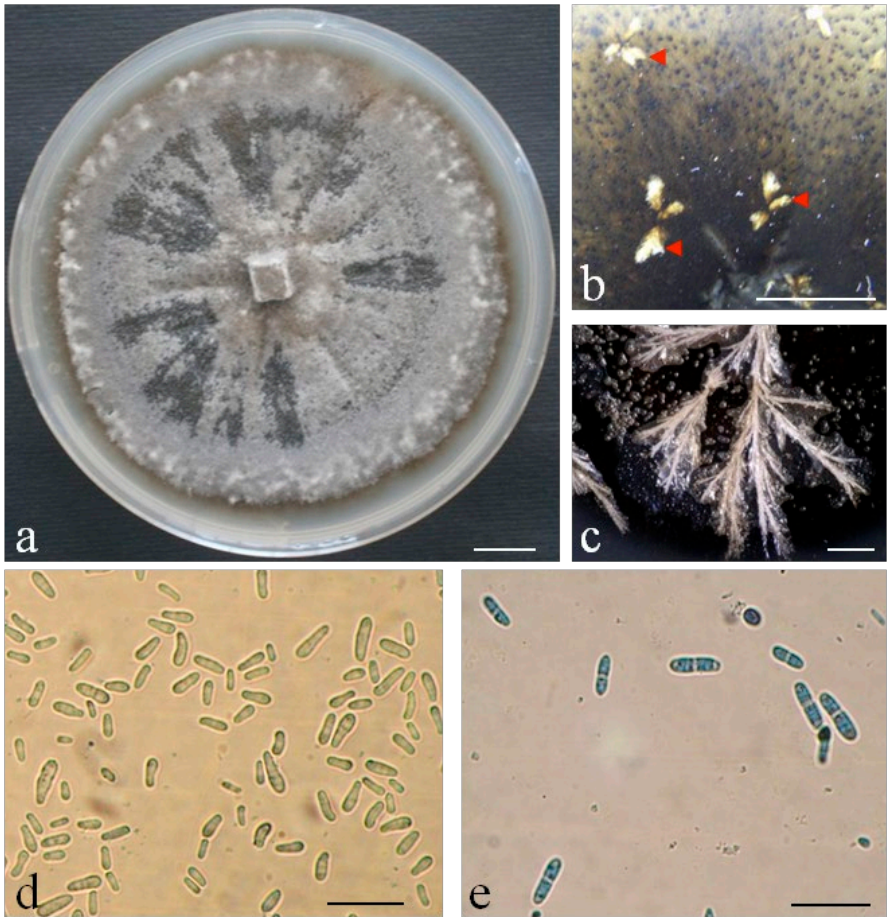

Figure 1. (a) Colony morphology of *Phoma medicaginis* 1 month after plating on Sanderson & Srb medium at 25°C and 16 h daily photoperiod. (b–c) Production of crystals (red arrow) by *P. medicaginis* on the same medium (b) and their magnification (c). (d–e) Non-septate (d) and one-septate (e) pycnidiospores of *P. medicaginis*. Bars correspond to 1 cm (a–b), 1 mm (c) and 10 μm (d–e)

(Table 2). The percentage of the root surface covered with pycnidia exhibited the greatest discrimination between the isolates contributing 58% to the observed variability, followed by the percentage of brown discoloration on roots (23%) (Table 2).

All *P. medicaginis* isolates caused brown discoloration of roots and yellowing of cotyledons at 15 dpi, except for Pm13 (Table 3). Eleven of the 14 *P. medicaginis* isolates caused *M. truncatula* plant death when inoculated on roots. The isolates Pm2, Pm6 and Pm11 caused the greatest percentage of death at 15 dpi after root inoculation (over 80%), followed by the isolates Pm7, Pm8 and Pm10 which caused 50–70% dead plants. The remaining isolates caused less than 40% plant death. Ten out of 14 *P. medicaginis* isolates were able to sporulate on roots of *M. truncatula* at 15 dpi. The isolates Pm7 and Pm2 showed the greatest percentage of pycnidium production on roots of inoculated *M. truncatula* plants.

Correlations between the aggressiveness parameters measured on leaves and roots

The analysis of correlations between the different aggressiveness parameters measured on F83005.5 leaves at 15 dpi showed that all measured traits were positively correlated with each other except for shoot fresh weight (Table 4). For the parameters measured on roots, the percentage of yellow cotyledons was positively correlated with the percentage of brown discoloration on roots. The number of leaves per

Table 2. Analysis of variance of measured parameters on *Medicago truncatula* shoots and roots 15 days post inoculation with 14 *Phoma medicaginis* isolates.

Treatment	Parameter	Effect		Error		F[c] (%)
		df[a]	MS[b]	df	MS	
Shoot inoculation	Leaf necrosis (%)	13	825.502	43	180.191	26*
	Leaf chlorosis (%)	13	904.636	43	234.118	22*
	Leaf abscission (%)	13	644.98	43	181.103	20*
	Dead leaves (%)	13	662.451	43	165.758	23*
	Shoot fresh weight (g)	13	0.066	43	0.043	9 ns
Root inoculation	Yellow cotyledon (%)	13	279.447	34	260.416	5 ns
	Brown discoloration on roots (%)	13	0.096	34	0.019	23*
	Number of leaves per plant	13	1.318	34	1.142	5 ns
	Dead plants (%)	13	0.383	34	0.205	9 ns
	Pycnidia on roots (%)	13	1.306	34	0.102	58*

* Highly significant ($P<0.001$).
ns, not significant ($P>0.05$).
[a] df, degrees of freedom.
[b] MS, means square.
[c] F, index of Fisher Snedecor.

plant was negatively correlated with the percentage of dead plants and the percentage of roots covered with pycnidia. The percentage of dead plants was positively correlated with the percentage of roots covered with pycnidia (Table 4). The parameters measured on leaves were not correlated with those measured on roots (Table 4).

Specificity of *P. medicaginis* infection

Specificity on leaves

The results of inoculation of *P. medicaginis* isolate Pm8 on leaves of six legumes gave severe necrosis on leaves of barrel medic (Figure 3b) and slight to moderate necrosis was observed on the lower leaves of alfalfa, chickpea, pea and common bean plants at 30 dpi (Figure 3; Table 5). No necrosis on lentil leaves was observed (Table 5). The infected leaves of barrel medic, alfalfa, pea, common bean and chickpea turned yellow and died (Figure 3b, e, h, k and n). Leaf abscission occurred after inoculation of barrel medic, alfalfa and common bean. *Phoma medicaginis* was able to produce pycnidia on the leaves of barrel medic, alfalfa, pea and common bean (Figure 3c, f, i and l), while no pycnidia were observed on chickpea or lentil leaves (Table 5). Pycnidia formed abundantly on dead and dying leaves of barrel medic, alfalfa, pea and common bean (Table 5). The fungus was consistently recovered from leaves showing pycnidium production. No necrosis and chlorosis symptoms developed on the control leaves (Figure 3a, d, g, j and m).

Specificity on roots

Inoculation of *P. medicaginis* isolate Pm8 on roots at the base of stems caused severe collar rot and brown discoloration on roots of barrel medic and alfalfa at 15 dpi (Table 5). While the inoculation of *P. medicaginis* caused slight to moderate tissue maceration and brown discoloration on the collar and roots of pea, chickpea and common bean, no symptoms were observed on lentil (Table 5). *Phoma medicaginis* produced pycnidia on the collars and roots of barrel medic and alfalfa at 15 dpi (Table 5).

Discussion

The Tunisian *P. medicaginis* isolates had similar appearance in culture, delayed production of crys-

Table 3. Measurement of the symptoms of infection by *Phoma medicaginis* isolates on shoots and roots of *Medicago truncatula* (line F83005.5) 15 days post inoculation. Comparison of means can be made only vertically and means marked with different letters are different at $P \leq 0.05$ (LSD test).

	Shoot inoculation					Root inoculation				
***Phoma* isolate**	**Leaf necrosis (%)**	**Leaf chlorosis (%)**	**Dead leaves (%)**	**Leaf abscission (%)**	**Shoot fresh weight (g)**	**Brown discoloration on roots (%)**	**Yellow cotyledon (%)**	**Number of leaves per plant**	**Dead plants (%)**	**Pycnidia on roots (%)**
Pm1	66.57 a	46.07 ab	33.28 abc	42.25 a	0.45 cd	100.00 a	100.00 a	1.33 ab	33.33 ab	33.33 cd
Pm2	58.79 abc	43.71 ab	20.09 bcdef	27.99 abc	0.58 bcd	100.00 a	100.00 a	0.00 b	100.00 a	83.33 a
Pm3	65.04 ab	43.86 ab	36.68 ab	44.85 a	0.57 bcd	100.00 a	100.00 a	1.33 b	0.00 b	0.00 e
Pm4	60.46 abc	44.27 ab	29.63 abcd	39.57 ab	0.68 abc	100.00 a	100.00 a	1.33 ab	0.00 b	0.00 e
Pm5	48.26 bcd	21.67 cd	16.11 def	21.67 bcd	0.66 abc	100.00 a	100.00 a	1.66 ab	33.33 ab	16.66 de
Pm6	49.87 abcd	32.86 bc	7.42 efg	28.31 abc	0.58 bcd	100.00 a	95.83 a	0.50 b	83.33 a	58.33 b
Pm7	57.62 abc	40.48 abc	8.81 efg	28.81 abc	0.57 bcd	100.00 a	100.00 a	0.33 b	66.66 ab	100.00 a
Pm8	46.80 cd	42.17 ab	19.93 cdef	31.28 abc	0.70 ab	100.00 a	100.00 a	0.33 b	50.00 ab	50.00 bc
Pm9	65.28 ab	54.17 a	38.33 a	38.33 ab	0.54 bcd	100.00 a	100.00 a	1.00 ab	33.33 ab	50.00 bc
Pm10	57.47 abc	44.59 ab	22.63 abcde	35.69 abc	0.49 bcd	100.00 a	100.00 a	0.33 b	66.66 ab	50.00 bc
Pm11	56.25 abc	42.67 ab	33.54 abc	39.44 ab	0.49 bcd	100.00 a	100.00 a	0.00 b	100.00 a	50.00 bc
Pm12	28.43 de	2.94 de	0.00 fg	2.94 def	0.94 a	100.00 a	100.00 a	1.66 ab	0.00 b	0.00 e
Pm13	32.35 de	20.66 cd	8.13 efg	17.72 cde	0.35 d	33.33 b	66.66 b	1.33 ab	33.33 ab	0.00 e
Pm14	18.06 e	2.81 de	0.00 fg	1.56 ef	0.42 cd	100.00 a	83.33 ab	1.66 ab	33.33 ab	16.66 de
Control	0.00 f	0.00 e	0.00 g	0.00 f	0.87 a	0.00 c	12.50 c	2.16 a	0.00 b	0.00 e

Figure 2. (a–b) Symptoms of *Phoma medicaginis* infection on *Medicago truncatula* roots and collar (line F83005.5) 15 days post inoculation. (c–e) Pycnidia of *P. medicaginis* on F83005.5 infected root. (f–h) Micrographs of sections of *M. truncatula* infected roots with *P. medicaginis* taken with light (f–g) and epifluorescence (h) microscopes. The infection appeared on the principle and lateral roots (green arrows in a) and on the collar (yellow arrow in b). Pycnidia were produced on dead roots (red arrow in d and e) which lead to the production of pycnidiospores (blue arrows in e). Cross-sections of inoculated roots were stained with wheat germ agglutinin-fluorescein isothiocyanate to visualize fungal mycelia (green color in h). Bars correspond to 5 cm (a), 1 cm (b–c), 1 mm (d–e) and 100 μm (f–h).

tals and produced non-septate conidia with a low percentage of one-septate pycnidiospores (Figure 1). The same cultural characteristics were described by Boerema *et al.*, (1965) for *P. medicaginis* (cultural type I) isolates collected from North America and in the Netherlands. The dimensions of conidia of the Tunisian *P. medicaginis* isolates (4.9–7.1 × 2.0–2.9 μm) are comparable to those indicated by Leath (1990) for *P. medicaginis* Malbr. & Roum var. *medicaginis* Boerema (4–15 × 2.4 μm).

All *P. medicaginis* isolates gave similar symptoms on the leaves of *M. truncatula*, characterized by dark lesions surrounded by yellow margins which extended to the whole infected leaves that turn yellow and abscise prematurely (Figure 3). Comparable symptoms of infection with *P. medicaginis* on alfalfa have been described previously (Angevain *et al.*, 1983; Wang *et al.*, 2004). All isolates were able to infect roots with brown discoloration symptoms on the main and lateral roots causing reductions in shoot and root development. Comparable symptoms on roots were noted by Rodriguez *et al.* (1990) in their study of the pathogenicity of *P. medicaginis* to roots of alfalfa.

The susceptible *M. truncatula* line F83005.5 proved to be useful in discriminating between the *P. medicaginis* isolates (Table 2). Susceptible host lines were successfully used in other studies for aggressiveness

Table 4. Correlations between the measured disease parameters on leaves and roots of *Medicago truncatula* 15 days post inoculation with *Phoma medicaginis*.

		Shoot inoculation					Root inoculation				
		Leaf necrosis (%)	Leaf chlorosis (%)	Leaf abscission (%)	Dead leaves (%)	Shoot fresh weight (g)	Yellow cotyledon (%)	Brown discoloration on roots (%)	Number of leaves per plant	Dead plants (%)	Pycnidia on roots (%)
Shoot inoculation	Leaf necrosis (%)	1									
	Leaf chlorosis (%)	0.909 *	1								
	Leaf abscission (%)	0.903 *	0.926 *	1							
	Dead leaves (%)	0.818 *	0.794 *	0.834 *	1						
	Shoot fresh weight (g)	-0.028 ns	-0.147 ns	-0.252 ns	-0.075 ns	1					
Root inoculation	Yellow cotyledon (%)	0.621 ns	0.483 ns	0.442 ns	0.458 ns	0.548 ns	1				
	Brown discoloration on roots (%)	0.349 ns	0.248 ns	0.230 ns	0.224 ns	0.372 ns	0.869 *	1			
	Number of leaves per plant	-0.393 ns	-0.543 ns	-0.342 ns	-0.184 ns	-0.093 ns	-0.349 ns	-0.198 ns	1		
	Dead plants (%)	0.198 ns	0.286 ns	0.119 ns	-0.054 ns	-0.146 ns	0.122 ns	0.113 ns	-0.835 *	1	
	Pycnidia on roots (%)	0.352 ns	0.461 ns	0.252 ns	-0.029 ns	-0.138 ns	0.335 ns	0.333 ns	-0.761 *	0.762 *	1

*, Significant after using Bonferroni correction (α= 0.05 / 25= 0.002).
ns, not significant.

Table 5. Host and organ specialization of *Phoma medicaginis* inoculated on six legume species.

Legume host	Cultivar	Shoot inoculation		Root inoculation	
		Necrosis on leaves	Pycnidia on leaves	Collar rot and root brown discoloration	Pycnidia on collar and roots
Barrel medic (*Medicago truncatula*)	F83005.5	+++	+++	+++	+++
Alfalfa (*Medicago sativa*)	Gabes	+++	+++	+++	++
Chickpea (*Cicer arietinum*)	Chetoui	+	-	+	-
Pea (*Pisum sativum*)	Lincoln	++	++	++	-
Lentil (*Lens culinaris*)	unknown	-	-	-	-
Common bean (*Phaseolus vulgaris*)	Coco Blanc	++	+++	+	-

(-) no symptoms, (+) low, (++) moderate, (+++) severe symptoms or pycnidia formation.

discrimination among isolates of *Phoma macdonaldii* infecting sunflower (Roustaee *et al.*, 2000) and *Cercospora medicaginis* infecting *M. truncatula* (Djébali *et al.*, 2012). In the present study, the most discriminating parameters between *P. medicaginis* isolates were the percentage of leaf necrosis they produced. In accordance, Djébali *et al.* (2012) showed that the percentage of leaf necrosis was the most discriminating parameter between *C. medicaginis* isolates 10 days post inoculation on F83005.5 leaves. On the basis of this parameter two aggressiveness groups were found in the present study to be independent from the site and host of isolate origins; a large group (11 isolates) that caused 50–67% necrosis and a smaller group (three isolates) that was less aggressive with 18–32% necrosis.

The inoculation of shoots of F83005.5 plants was more discriminating between the studied *P. medicaginis* isolates, in comparison to the inoculation on roots. This result was expected because the 14 *P. medicaginis* isolates were originally obtained from leaves of annual *Medicago* species. This result indicates that *P. medicaginis* may have adapted to infect host shoots (organ specialization). In addition, the parameters measured on leaves and roots were not correlated, indicating that the aggressiveness of *P. medicaginis* isolates was dependent on the inoculated organs. This probably indicates a difference in the genetic determination of resistance between shoots and roots. This was also demonstrated by Cazaux (2009), who showed that the locus involved in *M. truncatula* root resistance to *Colletotrichum trifolii* was not the same as that involved in foliar resistance.

The *P. medicaginis* Pm8 isolate, originally obtained from barrel medic leaves, was able to infect leaves of barrel medic, alfalfa, pea, common bean and chickpea (Figure 3), whereas no symptoms were noted on lentil leaves. *Phoma medicaginis* was reported to be pathogen on a wide range of legumes such as the genera *Melilotus*, *Trifolium*, *Pisum*, *Phaseolus* and *Cicer* (Edmunds and Hanson, 1960; Mead and Cormack, 1961; Haware and Nene, 1981; Leath, 1990). Nevertheless, in the present study *P. medicaginis* was more pathogenic on *M. truncatula* as a host of origin in comparison to the other legumes tested, suggesting that these species are likely to be secondary hosts for this pathogen. In agreement with this result, Boerema *et al.* (1965) indicated that black stem fungi isolated from alfalfa and red clover could attack both plant species, but the isolates were more pathogenic on the host from which they were isolated.

Inoculation of *P. medicaginis* Pm8 on roots showed that it was pathogenic to barrel medic, alfalfa, pea, chickpea and common bean, but not to lentil roots (Table 5). Boerema *et al.* (1965) showed that *P. medi-*

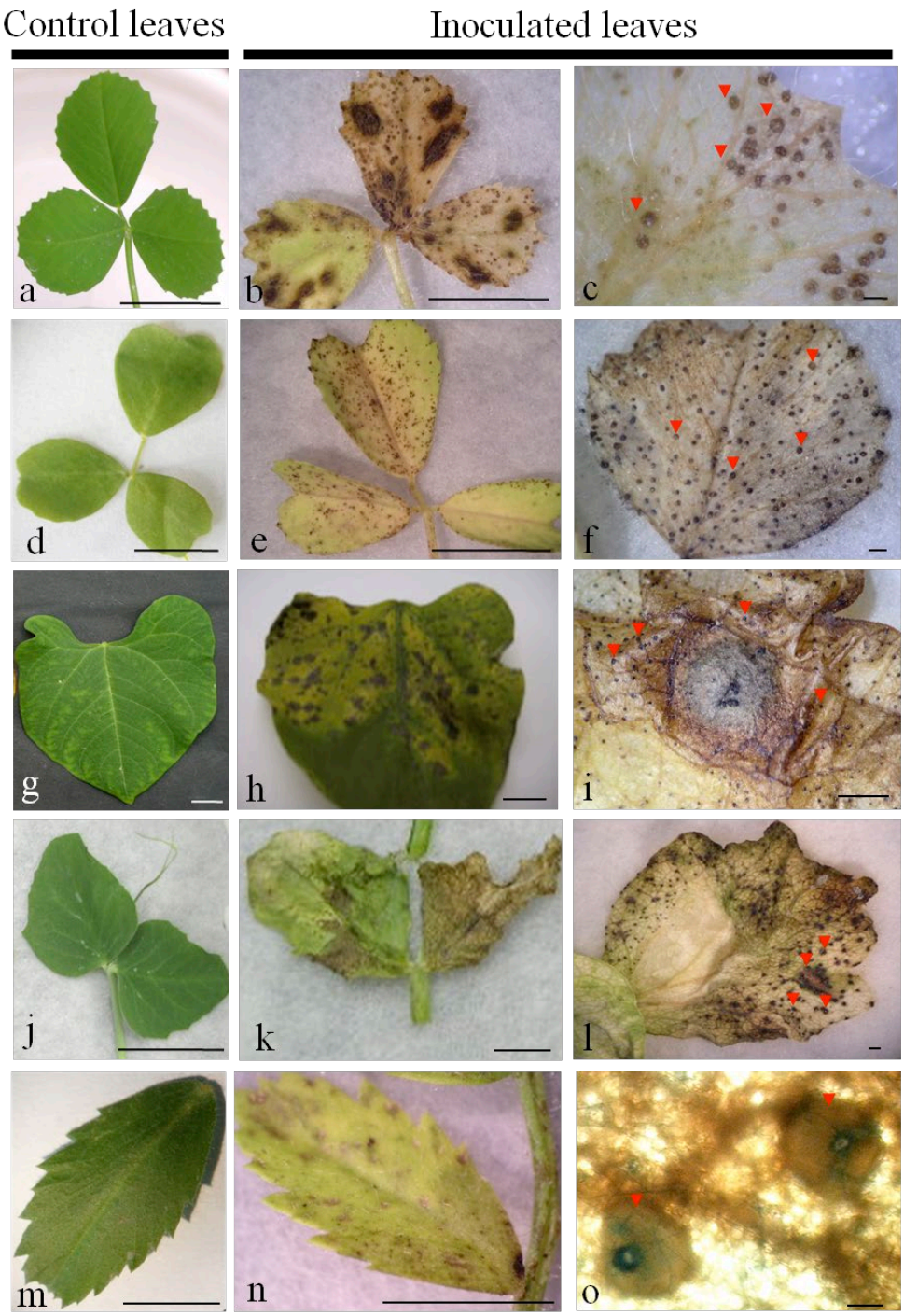

Figure 3. Macroscopic symptoms on control (a, d, g, j and m) and *Phoma medicaginis* inoculated leaves (b, e, h, k and n) of legumes and microscopic observations of pycnidium (red arrows) production of this pathogen on inoculated leaves (c, f, i, l and o) at 30 days post inoculation. a–c: *Medicago truncatula* (line F83005.5) leaves; d–f: *Medicago sativa* (cv. Gabes) leaves; g–i: *Phaseolus vulgaris* (cv. Coco blanc) leaves; j–l: *Pisum sativum* (cv. Lincoln) leaves; m–n: *Cicer arietinum* (cv. Chetoui) leaves; o: *P. medicaginis* pycnidia morphology on *M. truncatula* inoculated leaf. Bars correspond to 1 cm (a, b, d, e, g, h, j, k, m and n), 1 mm (c, f, i and l) and 100 μm (o).

caginis isolated from alfalfa leaves was able to infect alfalfa, pea and red clover roots causing yellow lower leaves and brownish to black stems and roots. In addition, Rodriguez *et al.* (1990) and Rodriguez and Leath (1992) showed that *P. medicaginis* is a primary pathogen of roots and crowns of alfalfa. The present study is the first report of infection of chickpea and common bean roots by *P. medicaginis*. Furthermore, it has been demonstrated that *P. medicaginis* can infect entire roots of *M. truncatula* by colonizing root cotices and steels (Figure 2) damaging the vascular tissues which disturbs water and mineral nutrition of the infected plants. It has also been shown that the pathogen can produce fertile pycnidia that release conidia on *M. truncatula* infected roots (Figure 2), demonstrating the possibility of reproduction of *P. medicaginis* via root infection. *Phoma medicaginis* when inoculated on *M. truncatula* roots was able to grow into the the stem bases with the production of pycnidia. This indicates that infected roots in the field may constitute primary sources of inoculum for the development of infection on *Medicago* plants and crops. Lentil was not a host for *P. medicaginis* (Table 5), so this legume could be used in rotation with alfalfa and annual *Medicago* crops to reduce the damage of this pathogen by interrupting its life cycle in the field.

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