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Host status and reaction of *Medicago truncatula* accessions to infection by three major pathogens of pea (*Pisum sativum*) and alfalfa (*Medicago sativa*)

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Abstract

Ditylenchus dipsaci, the stem nematode of alfalfa (*Medicago sativa*), *Mycosphaerella pinodes,* cause of Ascochyta blight in pea (*Pisum sativum*) and *Aphanomyces euteiches,* cause of pea root rot, result in major yield losses in French alfalfa and pea crops. These diseases are difficult to control and the partial resistances currently available are not effective enough. *Medicago truncatula*, the barrel medic, is the legume model for genetic studies, which should lead to the identification and characterization of new resistance genes for pathogens. We evaluated a collection of 34 accessions of *M. truncatula* and nine accessions from three other species (two from *M. italica*, six from *M. littoralis* and one from *M. polymorpha*) for resistance to these three major diseases. We developed screening tests, including standard host references, for each pathogen. Most of the accessions tested were resistant to *D. dipsaci*, with only three accessions classified as susceptible. A very high level of resistance to *M. pinodes* was observed among the accessions, none of which was susceptible to this pathogen. Conversely, a high level of variation, from resistant to susceptible accessions, was identified in response to infection by *A. euteiches*.

Introduction

Pea (*Pisum sativum*) and alfalfa (*Medicago sativa*) are two of the principal legume crops in France and play an important part in the development of sustainable agriculture systems worldwide. However, these crops are affected by diseases, some of which may greatly reduce yield.

The stem and bulb nematode, *Ditylenchus dipsaci*, is an important pest of alfalfa, red and white clover, bean and pea (Plowright et al., 2002). This nematode invades the aerial parts of plants—stem, leaves, flowers and seeds—causing swelling of the tissues (Sturhan and Brzeski, 1991). Resistant cultivars are essential for nematode management because chemical control is generally uneconomic, seed treatment is not always effective and crop rotation is complicated by the wide host range of this nematode (Plowright et al., 2002). However, resistance is not available in all crop hosts and is genetically poorly understood in most species. Ascochyta blight, caused by *Mycosphaerella pinodes*, is one of the most important aerial diseases of pea worldwide (Bretag and Ramsey, 2001). In France, it has been reported to cause heavy losses in yield and seed quality (Tivoli et al.,

1996) and disease control is based principally on the use of fungicides. Pea cultivars currently in use are susceptible to *M. pinodes*. Increasing the level of resistance should help to stabilize yield and reduce fungicide applications. Pea root rot, caused by *Aphanomyces euteiches*, is the most damaging root disease of pea in most of the pea-producing areas of the world (Persson et al., 1997). In France, this fungus has caused severe losses since 1993 and is now considered the major limiting factor for cultivation of this crop; the only means of controlling the disease currently available is to avoid using infested fields for pea crops (Wicker et al., 2001a). Under field conditions, symptoms only occur on pea but some isolates of *A. euteiches* have extremely different host ranges (Wicker et al., 2001a; Grau et al., 1991). Genetics may provide other ways to control this disease. However, breeding for resistance to *A. euteiches* is difficult for two main reasons: only a few sources of partial resistance have been identified in pea germplasm, and the resistances identified are polygenic, with low heritability (Pilet-Nayel et al., 2002).

Medicago truncatula has attained the status of a model plant (Cook, 1999) because its characteristics make it suitable for both classical and molecular genetical studies (diploid, self-fertile, small genome size). It is also a useful tool for identifying and characterising new resistance genes for pathogens. This annual medic is closely related to economically important legumes species, including pea and alfalfa (Doyle et al., 1996) and conserved synteny has already been demonstrated (Gualtieri et al., 2002). There seems to be considerable genetic variation between *M. truncatula* ecotypes (Bonnin et al., 1996), but no systematic search for sources of disease resistance has yet explored this natural germplasm variability. The host status of *M. truncatula* with respect to *D. dipsaci* remains unclear (Whitehead, 1992) and the infection of *M. truncatula* by *M. pinodes* has never been reported. Resistance to *A. euteiches* race 2, the causal agent of alfalfa root rot, was recently evaluated for the first time (Vandemark and Grunwald, 2004) but no screening has yet been carried out for resistance to *A. euteiches*, the root rot pathogen of pea. Screening for resistance to these three major diseases of pea and alfalfa is required and could facilitate significant progress in breeding for resistance to these pathogens. We assessed host status and response to infection by *D. dipsaci*, *M. pinodes* and *A. euteiches* in a set of 43 *Medicago* accessions specially selected from the core collection conserved at INRA Montpellier, France (Prosperi et al., 2002), representing more than 80% of the genetic variability in this genus. Our principal aim was to identify potential sources of genetic resistance for breeding for resistance to these three major diseases of pea and alfalfa in France.

Materials and methods

Medicago *collection*

The French *Medicago* collection is conserved at INRA Montpellier and contains more than 3500 accessions, including 350 natural populations of *M. truncatula*. A core collection of 131 natural populations has been constituted and evaluated for morphologic and agronomic characteristics and for genetic diversity (Prosperi et al., 2002). Seeds of 34 accessions of *M. truncatula* and nine accessions of three other species of *Medicago* in the French core collection (two of *M. italica*, six of *M. littoralis* and one of *M. polymorpha*) were obtained from Dr J.M. Prosperi, INRA Montpellier, UMR DGPC, Mauguio–France (Table 1). Some accessions germinated poorly and were therefore not subjected to all the screening tests, due to a lack of seedlings.

Screening for resistance to D. dipsaci

We screened *M. truncatula* for resistance to *D. dipsaci*, using a slightly modified version of the method used to screen for resistance in *M. sativa* described by Bingeford and Eriksson (1968). Seeds were sown in pots containing steam-treated organic compost, two to six pots per accessions tested were used with five seedlings in each pot. Each seedling of a given accession constituted a replicate. Two alfalfa cultivars – Vertus (known to carry some resistance to stem nematode) and Europe (susceptible) – were included as controls (Whitehead, 1992). Three to four day-old seedlings with two cotyledons were inoculated with an aqueous suspension of nematodes containing 1% carboxymethyl cellulose as a gelling agent. Nematodes (50 nematodes/10 μl/plant) were administered in droplets applied to the first leaves close to

Table 1. Accessions from the *Medicago* core collection evaluated for resistance to *Ditylenchus dipsaci*, *Mycosphaerella pinodes* and *Aphanomyces euteiches*

Species and Accessions	Geographical origin	Screening tests			
		D.d[a]	Mp*(sd)*[b]	M.p*(dl)*[c]	A.e[d]
Medicago truncatula					
Borung	Australia	+		+	+
Caliph	Australia	+	+	+	+
Cyprus	Australia	+	+	+	+
Harbinger	Australia	+	+	+	+
Jemalong	Australia	+	+	+	+
Mogul	Australia	+		+	+
Parabinga	Australia	+	+	+	+
Paraggio	Australia	+	+	+	+
Salernes	France	+	+	+	+
Sephi	Australia	+	+	+	+
CRE.007	Crete	+	+	+	+
DZA.055	Algeria	+	+	+	+
DZA.105	Algeria	+	+	+	+
DZA.220	Algeria	+	+	+	+
DZA.233	Algeria	+	+	+	+
DZA.327	Algeria	+	+	+	+
DZA.045.5	Algeria				+
DZA.315.16	Algeria				+
ESP.039	Spain	+	+	+	+
ESP.105	Spain	+	+	+	+
ESP.158	Spain	+	+	+	+
ESP.159	Spain	+	+	+	+
ESP.165	Spain	+	+	+	+
ESP.171	Spain	+	+	+	+
F11.005	France	+	+	+	+
F11.013	France	+	+	+	+
F20.047	France	+	+	+	+
F20.061	France	+	+	+	+
F20.089	France	+	+	+	+
F83005.5	France		+	+	+
F83005.9	France				+
GRC.020	Greece	+	+	+	+
GRC.043	Greece	+	+	+	+
GRC.064	Greece	+	+	+	+
M. littoralis					
DZA.032A	Algeria	+	+	+	+
ESP.027	Spain	+	+	+	+
F20.026	France	+	+	+	+
F34.024A	France	+	+	+	+
GRC.036	Greece	+	+	+	+
PRT.180	Portugal	+	+		+
M. italica					
ESP.050	Spain	+	+	+	+
tornafield	Australia	+	+	+	+
M. polymorpha					
Circle valley	Australia	+			

[a]*D. dipsaci* ; [b]*M. pinodes* (seedling test) ; [c]*M. pinodes* (test on detached leaves) ; [d]*A. euteiches*.

the terminal meristem of the seedling. Inoculated seedlings were covered with a clear propagator hood (vents closed) and maintained in a controlled environment (15 °C and 16 h photoperiod). The first two to three days after inoculation were particularly critical for disease establishment. It was essential to maintain high relative humidity immediately after inoculation and to ensure that the plants grew slowly for one to two weeks, to allow the nematodes to establish an infection site. After one week, the seedlings were transferred to a controlled environment, with the temperature kept at 20 °C and no propagator hood. Plants were harvested one month after inoculation. The development of symptoms was visually assessed, and the relative nematode reproduction index (RI) for each plant was calculated by counting the number of nematodes per plant and dividing it by the number of nematodes inoculated. Plants with an RI < 1 were considered resistant. Assay A (10 plants tested per accession) was conducted using the Arbiotech population, classified as Lucerne race, and multiplied on callus from susceptible alfalfa, whereas assay B (30 plants tested per accession) was conducted using a *D. dipsaci* line obtained in laboratory conditions and less variable than the Arbiotech population.

Screening for resistance to M. pinodes

We used two tests to screen for resistance to *M. pinodes*. The seedling test was generally used to screen a large number of accessions. The test on detached leaves was used to describe disease progression.

Seedling test

This test was an adaptation of the pathogenicity test for pea developed by Onfroy et al. (1999). Seeds were allowed to germinate on moist filter paper for 48–72 h at 25 °C in the dark, and were then transferred to 500 ml plastic pots containing an unsterilized soil/compost mixture (1:1:1 soil–sand–peat). Four seeds were sown per pot, with four pots used per accession, each constituting a replicate. A fifth pot was included as an uninoculated control. Pots were arranged in trays in a completely randomized design and were placed in a controlled environment chamber under constant conditions (thermoperiod: 20/18 °C and 16 h photoperiod). Two lines of *Pisum sativum* – JI296 (John Innes Institute, UK) and DP (R. Cousin's collection, INRA, France), susceptible and resistant to *M. pinodes*, respectively – were used as controls (Onfroy et al., 1999).

When the plants reached the three- to four-leaf stage, they were inoculated with a suspension of pycnidiospores. The isolate used (*Mp* 91.31.12) was very aggressive on pea and representative of the French collection. The pycnoidispore suspension was prepared as previously described (Onfroy et al., 1999) and its concentration was adjusted to 10^6 spores ml^{-1}. One drop of Tween 20 was added as a wetting agent and the spore suspension was applied (0.5 ml per plant) with a hand-held garden sprayer. After inoculation, each tray was covered with a clear plastic cover to maintain 100% relative humidity and plants were regularly sprayed with distilled water. Disease severity was assessed on the first two trifoliolate leaves, 18 days after inoculation, using a 0–5 scale established by Tivoli et al. (1996) for the *P. sativum*/*M. pinodes* pathosystem and available for the *Medicago spp.*/*M. pinodes* pathosystem: 0 = no lesions; 1 = a few scattered flecks; 2 = numerous flecks; 3 = 10–15% of the leaf area necrotic and appearance of coalesced necrosis; 4 = 50% of the leaf area dehydrated or covered by lesions ; 5 = 75–100% of the leaf area dehydrated or necrotic.

Test on detached leaves

This test was an adaptation of that described by Heath and Wood (1969), and adapted by Onfroy (UNIP-INRA, France, pers. comm.) for the *M. pinodes*/*Pisum sativum* pathosystem. Seedlings were grown in the conditions described for the seedling test, with the same controls. When the seedlings reached the four-leaf stage, the first and the second trifoliolate leaves were excised and maintained alive on a layer of water in a small clear plastic box divided into compartments.

A drop of pycnidiospore suspension (*Mp* 91.31.12, 10 μl, 2×10^5 spores ml^{-1}) was carefully applied to the centre of each leaf. Four replicates of two trifoliolate leaves were tested for each accession. Inoculated leaves were incubated in a controlled environment chamber (thermoperiod: 20/18 °C and 14 h photoperiod), in a completely randomized design. Disease progression was observed daily. The number of days after inoculation required to observe (1) the appearance of flecks,

(2) the coalescence of the flecks at the drop site and (3) the spread of necrosis beyond the drop site, was assessed. Both seedling tests and tests on detached leaves were repeated once.

Screening for resistance to A. euteiches

We used a modified version of the test developed for the evaluation of pea resistance to *A. euteiches* (Moussart et al., 2001). Seeds were allowed to germinate on moist filter paper for 48–72 h at 25 °C in the dark and were then transferred to 500 ml plastic pots containing unsterilized vermiculite (Vermex, M). Five seeds of a single accession were sown per pot, with each pot constituting a replicate. There were four replicates per accession. Pots were arranged in a completely randomized design, in a controlled environment chamber under constant conditions (thermoperiod: 25/23 °C and 16 h photoperiod). Two *Pisum sativum* genotypes – Baccara and PI180693 (USDA Plant Introduction Station, Pullman, USA), susceptible and resistant to *A. euteiches* (Wicker et al., 2003), respectively – were used as controls.

Seven days after sowing, seedlings were inoculated with a suspension of zoospores from a French strain of *A. euteiches* (RB84). This strain is very aggressive on pea and belongs to the main virulence group present in France (Wicker et al., 2001b). Zoospores were produced as previously described (Moussart et al., 2001), with the concentration of the suspension adjusted to 2,000 zoospores ml^{-1}. Seven day-old seedlings were inoculated by applying 25 ml of inoculum suspension per pot (10^4 zoospores per plant). Vermiculite was saturated with water after inoculation, to encourage disease development. Plants were removed 14 days after inoculation, and disease severity was scored on a 0–5 scale: 0 = no symptoms; 1 = traces of discolouration on the roots (< 25%); 2 = discolouration of 25–50% of the roots; 3 = discolouration of 50–75% of the roots; 4 = discolouration > 75% of the roots; 5 = dead plant . The screening test was repeated once.

Data analysis

We carried out an analysis of variance (ANOVA) for the screening results, and compared means, using a Newman–Keuls test ($P = 0.05$), in the General Linear Model procedure of SAS (SAS Institute, Cary, NC, USA). Relationships between scoring criteria were tested by Pearson correlation analysis (SAS, 1989).

Results

Screening for resistance to D. dipsaci

We assessed resistance to the stem nematode directly, by calculating the nematode reproduction index (RI) for each plant and the proportion of resistant plants (i.e. plants with RI < 1) for each accession tested. The susceptible alfalfa control accession, Europe, displayed severe swelling and a high percentage of susceptible plants (77–92%). *Ditylenchus dipsaci* infested the *Medicago* spp. studied but reproduced on only a few accessions of *M. truncatula* and *M. italica*. In the first assay (assay A) a significant genotype effect ($P < 0.05$) was observed and numerous sources of resistance were detected (Table 2). We repeated the screening test using 20 plants more per accession and a nematode line originating from a single cross, in an attempt to reduce variability. Unfortunately, similar levels of variability were observed in both assays, and the percentage of resistant plants differed between the two assays for several accessions. However, there was no significant difference in nematode reproduction levels in assays A and B and the classification of accessions as resistant and susceptible did not depend on the assay used. All but three of the accessions tested – Harbinger, Jemalong and Tornafield – were resistant to *D. dipsaci*. However, eight accessions – DZA.327, Caliph, DZA.055, ESP.159, F11.013, F20.061, GRC.020 and DZA.032A – had a relatively high proportion of susceptible plants (3–13%) with a high RI (RI ≥ 5). For three accessions – ESP.050, ESP.158 and F34.042 – 100% of the plants were resistant in both assays.

Screening for resistance to M. pinodes

Seedling test

The tests were carried out twice and similar results were obtained (Table 3). Symptoms were observed on all the accessions inoculated with *M. pinodes*. However, these symptoms were different from

Table 2. Reaction of *Medicago* to the stem nematode *Ditylenchus dipsaci* (assays A and B)

Species	Accessions	Assay A				Assay B			
		Nb	Resistant Plants (%)	RI		Nb	Resistant Plants (%)	RI	
M. truncatula	Borung	33	64	1	R	–	–	–	–
M. truncatula	Caliph	29	45	2	R	–	–	–	–
M. truncatula	Cyprus	67	99	0	R	21	29	2	R/S
M. truncatula	Salernes	31	77	1	R	–	–	-	-
M. truncatula	Harbinger	43	14	14	S	17	0	6	S
M. truncatula	Jemalong	92	37	7	S	20	10	6	S
M. truncatula	Mogul	34	35	1	R	–	–	–	–
M. truncatula	Parabinga	24	88	0	R	20	85	1	R
M. truncatula	Paraggio	28	61	1	R	–	–	–	–
M. truncatula	Sephi	21	57	1	R	–	–	–	–
M. truncatula	CRE.007	12	92	1	R	17	88	1	R
M. truncatula	DZA. 055	10	80	1	R	17	35	2	R/S
M. truncatula	DZA.105	13	69	1	R	21	57	1	R
M. truncatula	DZA.220	11	82	1	R	12	92	1	R
M. truncatula	DZA.233	10	80	1	R	21	90	1	R
M. truncatula	DZA.327	12	50	1	R	18	28	2	R/S
M. truncatula	ESP.039	12	100	0	R	17	47	1	R
M. truncatula	ESP.105	8	88	0	R	21	86	1	R
M. truncatula	ESP.158	12	100	0	R	17	100	1	R
M. truncatula	ESP.159	12	100	0	R	16	19	2	R/S
M. truncatula	ESP.165	12	100	0	R	18	89	1	R
M. truncatula	ESP.171	12	100	0	R	21	71	1	R
M. truncatula	F11.005	9	100	0	R	22	91	1	R
M. truncatula	F11.013	9	100	0	R	19	58	1	R
M. truncatula	F34.042	11	100	0	R	18	100	0	R
M. truncatula	F20.047	13	77	1	R	19	76	1	R
M. truncatula	F20.061	12	92	1	R	19	53	2	R/S
M. truncatula	F20.089	12	100	0	R	21	37	1	R/S
M. truncatula	GRC.020	12	92	1	R	19	84	1	R
M. truncatula	GRC.043	13	85	1	R	17	94	0	R
M. truncatula	GRC.064	12	92	1	R	21	100	0	R
M. polymorpha	Circle Valley	29	86	1	R	17	100	0,4	R
M. littoralis	F20.026	12	83	1	R	20	60	1	R
M. littoralis	F34.024A	5	100	0	R	18	72	1	R
M. littoralis	ESP.027	13	92	0	R	18	94	0	R
M. littoralis	PRT.180	11	91	1	R	21	67	1	R
M. littoralis	DZA.032A	12	67	1	R	13	85	1	R
M. littoralis	GRC.036	11	100	0	R	20	100	1	R
M. italica	Tornafield	77	52	2	S	20	60	2	
M. italica	ESP.050	13	100	0	R	22	8	16	
M. sativa	Vertus	133	37	6					
M. sativa	Europe	102	23	9					

For each tested accession, the percentage of resistant plants and the average amount of nematode reproduction per plant (RI) are indicated. Nb = number of plants tested. *R* = accession classified as resistant ; *S* = accession classified as susceptible.

those usually observed on the host plant (*P. sativum*). Some flecks were present, but the lesions seemed to be restricted. A high level of resistance was detected, with little variability between accessions, mainly in experiment 2 (from 0.4 for Tornafield to 1.3 for F34.024A and GRC.043). Experimental conditions highly conducive to severe disease development resulted in severe symptoms and ratings on pea plants. The resistant (DP) and susceptible (JI296) references for the host plant (*P. sativum*) displayed complete necrosis and dehydration (respectively 4.4 and 5.0).

Test on detached leaves

Disease progression is described in Table 4. The first symptoms appeared two days after

Table 3. Assessment of *Medicago* accessions for resistance to *Mycosphaerella pinodes*, using the seedling test

Species	Accessions	Disease severity [a]		
		Experiment I	Experiment II	Mean both experiments
P. sativum[b]	JI296	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
P. sativum[b]	PI180693	4.6 ± 0.3	4.2 ± 0.2	4.4 ± 0.3
M. littoralis	F34.024A	1.3 ± 0.5 ab	–	1.3 a
M. truncatula	GRC.043	1.6 ± 0.5 a	1.1 ± 0.5 a	1.3 ± 0.4 a
M. truncatula	GRC.020	1.3 ± 0.5 ab	1.0 ± 0.0 a	1.2 ± 0.2 a
M. truncatula	ESP.171	1.2 ± 0.1 abc	1.2 ± 0.3 a	1.2 ± 0.0 a
M. truncatula	ESP.165	1.2 ± 0.3 abc	–	1.2 a
M. truncatula	Parabinga	1.1 ± 0.1 abc	1.1 ± 0.5 a	1.1 ± 0.0 ab
M. truncatula	DZA.220	1.1 ± 0.2 abc	–	1.1 ab
M. littoralis	ESP.027	1.1 ± 0.7 abc	–	1.1 ab
M. truncatula	Borung	–	1.1 ± 0.3 a	1.1 ab
M. truncatula	ESP.039	1.1 ± 0.1 abc	1.0 ± 0.0 a	1.1 ± 0.1 ab
M. truncatula	Paraggio	1.1 ± 0.2 abc	–	1.1 ab
M. truncatula	DZA.055	1.1 ± 0.2 abc	1.0 ± 0.0 a	1.1 ± 0.1 ab
M. truncatula	ESP.159	1.0 ± 0.1 abc	–	1.0 abc
M. truncatula	DZA.327	1.0 ± 0.2 abc	1.1 ± 0.5 a	1.0 ± 0.1 abc
M. truncatula	ESP.105	1.0 ± 0.0 abc	–	1.0 abc
M. truncatula	Salernes	1.0 ± 0.1 abc	–	1.0 abc
M. truncatula	Harbinger	1.0 ± 0.3 abc	1.0 ± 0.3 a	1.0 ± 0.0 abc
M. littoralis	PRT.180	1.2 ± 1.0 abc	0.8 ± 0.3 a	1.0 ± 0.3 abc
M. truncatula	DZA.233	1.0 ± 0.1 abc	–	1.0 abc
M. truncatula	Jemalong	0.9 ± 0.1 abc	1.1 ± 0.5 a	1.0 ± 0.1 abc
M. truncatula	Cyprus	1.0 ± 0.2 abc	–	1.0 abc
M. truncatula	Sephi	1.1 ± 0.1 abc	0.8 ± 0.2 a	1.0 ± 0.2 abc
M. truncatula	GRC.064	0.9 ± 0.2 abc	1.0 ± 0.7 a	1.0 ± 0.1 abc
M. truncatula	F11.005	0.9 ± 0.5 abc	1.0 ± 0.0 a	1.0 ± 0.1 abc
M. truncatula	CRE.007	0.9 ± 0.1 abc	0.9 ± 0.5 a	0.9 ± 0.0 abc
M. truncatula	F20.047	0.9 ± 0.5 abc	–	0.9 abc
M. littoralis	DZA.032A	0.9 ± 0.2 abc	0.9 ± 0.6 a	0.9 ± 0.0 abc
M. truncatula	F20.089	0.9 ± 0.5 abc	–	0.9 abc
M. truncatula	ESP.158	1.1 ± 0.4 abc	0.7 ± 0.3 a	0.9 ± 0.3 abc
M. littoralis	GRC.036	1.0 ± 0.3 abc	0.6 ± 0.2 a	0.9 ± 0.3 abc
M. truncatula	Caliph	1.0 ± 0.2 abc	0.7 ± 0.3 a	0.9 ± 0.2 abc
M. truncatula	DZA.105	–	0.6 ± 0.1 a	0.8 abc
M. italica	ESP.050	1.0 ± 0.2 abc	0.6 ± 0.1 a	0.8 ± 0.3 abc
M. littoralis	F20.026	0.7 ± 0.5 abc	0.7 ± 0.4 a	0.7 ± 0.0 abc
M. truncatula	F11.013	0.5 ± 0.7 bc	0.7 ± 0.2 a	0.6 ± 0.1 abc
M. truncatula	Mogul	–	0.5 ± 0.2 a	0.6 abc
M. truncatula	F20.061	0.4 ± 0.4 c	0.5 ± 0.0 a	0.5 ± 0.1 bc
M. italica	Tornafield	0.6 ± 0.2 bc	0.2 ± 0.3 a	0.4 ± 0.2 c

[a]For each experiment, data are means and standard deviation of four replicates. Five plants were tested in each replicate. Plants were evaluated on a 0–5 scale. Mean values followed by the same letter are not significantly different (Newman–Keuls test $P = 0.05$).
[b]Standard reference cultivars of *P. sativum* for *Aphanomyces* root rot resistance.

inoculation on *Medicago* accessions and *P. sativum* cultivars. However, few flecks were present at the drop site on *Medicago* accessions whereas the drop site displayed complete necrosis on DP, the resistant pea cultivar. Necrosis had already spread beyond the drop site for JI296, the susceptible pea cultivar. Disease progression was slower for *Medicago* accessions and different reactions to the disease were identified. Six accessions (Borung, Harbinger, Mogul, Parabinga, F11.005, F20.026) showed several to many flecks at the drop site six days after inoculation. The drop site was completely necrotic two days after inoculation for one accession (GRC.036) and three days after

Table 4. Assessment of *Medicago* accessions for resistance to *Mycosphaerella pinodes*, using the test on detached leaves

Species	Accessions	Days after inoculation[a]				
		2	3	4	5	6
P. sativum[b]	JI296	+ + +	+ + +	+ + +	+ + +	+ + +
P. sativum[b]	DP	+ +	+ + +	+ + +	+ + +	+ + +
M. truncatula	Borung	+	+	+	+	+
M. truncatula	Caliph	+	+	+ +	+ +	+ +
M. truncatula	Cyprus	+	+	+ +	+ +	+ +
M. truncatula	Harbinger	+	+	+	+	+
M. truncatula	Jemalong	+	+	+ +	+ +	+ +
M. truncatula	Mogul	+	+	+	+	+
M. truncatula	Parabinga	+	+	+	+	+
M. truncatula	Paraggio	+	+ +	+ +	+ +	+ +
M. truncatula	Salernes	+	+ (+)*	+ +	+ +	+ +
M. truncatula	Sephi	+	+	+ +	+ +	+ +
M. truncatula	CRE.007	+	+	+ +	+ +	+ +
M. truncatula	DZA.055	+	+	+ +	+ +	+ +
M. truncatula	DZA.105	+	+	+ +	+ +	+ +
M. truncatula	DZA.220	+	+	+ +	+ +	+ +
M. truncatula	DZA.233	+	+ +	+ +	+ +	+ +
M. truncatula	DZA.327	+	+	+ +	+ +	+ +
M. truncatula	ESP.039	+	+	+ +	+ +	+ +
M. truncatula	ESP.105	+	+	+ +	+ +	+ +
M. truncatula	ESP.158	+	+	+ +	+ +	+ +
M. truncatula	ESP.159	+	+	+ +	+ +	+ +
M. truncatula	ESP.165	+	+ (+)*	+ +	+ +	+ +
M. truncatula	ESP.171	+	+	+ +	+ +	+ +
M. truncatula	F11.005	+	+	+	+	+
M. truncatula	F11.013	+	+	+ +	+ +	+ +
M. truncatula	F20.047	+	+	+ +	+ +	+ +
M. truncatula	F20.061	+	+	+ +	+ +	+ +
M. truncatula	F20.089	+	+	+ +	+ +	+ +
M. truncatula	GRC.020	+	+	+ +	+ +	+ +
M. truncatula	GRC.043	+	+ +	+ +	+ +	+ + +
M. truncatula	GRC.064	+	+	+ +	+ +	+ +
M. littoralis	DZA.032A	+	+	+ +	+ +	+ +
M. littoralis	ESP.027	+	+	+ +	+ +	+ +
M. littoralis	F20.026	+	+	+	+	+
M. littoralis	F34.024A	+	+	+ +	+ +	+ +
M. littoralis	GRC.036	+ +	+ +	+ +	+ +	+ +
M. italica	ESP.050	+	+	+ +	+ + (+)*	+ + +
M. italica	Tornafield	+	+ +	+ +	+ +	+ +

[a] + : few flecks on the drop site ; + + : drop site completely necrosed ; + + + : lesion extension beyond the drop site.
[b] Standart reference cultivars of *P. sativum* for *M.pinodes* resistance.
*Stage of infection which only occurred in one experiment.

inoculation for five accessions (Paraggio, Salernes, DZA.233, GRC.043 and Tornafield). For most accessions, the drop site was completely necrotic four days after inoculation. No lesion was observed beyond the drop site for *Medicago* accessions, with the exception of GRC.043 and ESP.050: progressive lesions with fruiting bodies were observed six days after inoculation. However these lesions progressed much more slowly than those observed on pea leaves and rapidly stopped progressing. Symptoms on *Medicago* spp. did not progress after six days.

Screening for resistance to A. euteiches

A highly positive correlation was found between the two repeated tests ($R^2 = 0.82$; $P = 0.0001$). Typical disease symptoms, with honey brown root discolourations, were observed in *Medicago* spp.

Table 5. Assessment of *Medicago* accessions for resistance to *Aphanomyces euteiches*

Species	Accessions	Disease severity [a]		
		Experiment I	Experiment II	Mean both experiments
P. sativum[b]	Baccara	4.8 ± 0.4 a	5.0 ± 0.0 a	4.9 ± 0.2 a
P. sativum[b]	PI180693	4.3 ± 0.5 ab	3.9 ± 0.1 b	4.1 ± 0.2 b
M. italica	ESP.050	4.0 ± 0.0 abc	3.6 ± 0.2 bcd	3.8 ± 0.3 bc
M. truncatula	GRC.064	3.6 ± 0.5 bcde	–	3.6 bcd
M. truncatula	Paraggio	4.0 ± 0.0 abc	3.2 ± 0.5 bcde	3.6 ± 0.6 bcd
M. truncatula	Borung	3.4 ± 0.5 bcdefg	3.6 ± 0.4 bc	3.5 ± 0.1 bcde
M. truncatula	Caliph	3.8 ± 0.4 abcd	3.3 ± 0.5 bcde	3.5 ± 0.4 bcde
M. truncatula	ESP.105	3.7 ± 0.6 bcde	3.2 ± 0.3 bcde	3.4 ± 0.3 cdef
M. truncatula	Parabinga	3.8 ± 0.4 abcd	3.1 ± 0.6 bcde	3.3 ± 0.5 cdefg
M. truncatula	F20.061	3.0 ± 0.0 cdefghij	3.4 ± 0.4 bcde	3.3 ± 0.3 cdefgh
M. truncatula	ESP.158	3.3 ± 0.7 bcdefgh	–	3.3 cdefgh
M. littoralis	PRT.180	3.6 ± 0.5 bcdef	3.0 ± 0.0 bcdef	3.2 ± 0.4 cdefgh
M. truncatula	F83005.5	2.9 ± 0.8 cdefghij	3.4 ± 0.1 bcde	3.2 ± 0.3 cdefgh
M. truncatula	GRC.043	3.2 ± 0.6 bcdefghi	2.9 ± 0.2 cdef	3.1 ± 0.2 cdefghi
M. truncatula	Cyprus	2.9 ± 0.9 cdefghij	3.3 ± 0.6 bcde	3.1 ± 0.3 cdefghi
M. truncatula	Harbinger	3.0 ± 0.0 cdefghij	3.0 ± 1.0 bcdef	3.0 ± 0.0 defghij
M. truncatula	DZA.233	2.9 ± 0.3 cdefghij	3.2 ± 0.3 bcde	3.0 ± 0.2 defghij
M. truncatula	DZA.327	2.8 ± 0.6 cdefghijk	3.1 ± 1.2 bcde	2.9 ± 0.2 defghij
M. truncatula	ESP.171	2.8 ± 1.0 cdefghijk	3.1 ± 0.4 bcde	2.9 ± 0.2 defghij
M. truncatula	ESP.039	2.8 ± 1.1 cdefghijk	3.0 ± 0.4 bcdef	2.9 ± 0.1 defghij
M. truncatula	DZA.055	2.7 ± 0.6 cdefghijk	3.1 ± 0.1 bcde	2.9 ± 0.3 defghij
M. littoralis	DZA.032A	2.7 ± 0.4 cdefghijk	2.9 ± 0.2 cdef	2.8 ± 0.1 defghij
M. truncatula	Sephi	2.5 ± 0.8 defghijk	3.0 ± 0.3 bcdef	2.8 ± 0.3 efghij
M. truncatula	F11.005	3.0 ± 0.5 cdefghij	2.4 ± 0.5 efgh	2.7 ± 0.4 fghijk
M. littoralis	GRC.036	2.2 ± 0.9 fghijkl	3.0 ± 0.0 bcdef	2.6 ± 0.5 ghijk
M. truncatula	Mogul	2.6 ± 1.0 defghijk	2.6 ± 0.3 defgh	2.6 ± 0.0 ghijk
M. truncatula	GRC.020	3.0 ± 0.0 cdefghij	2.5 ± 0.4 efgh	2.6 ± 0.3 ghijk
M. truncatula	ESP.159	2.3 ± 0.7 efghijk	2.6 ± 0.6 cdefgh	2.6 ± 0.2 ghijk
M. italica	Tornafield	2.8 ± 1.6 cdefghijk	2.4 ± 0.8 efgh	2.5 ± 0.3 hijkl
M. littoralis	F34.024A	2.5 ± 0.5 defghijk	2.5 ± 0.4 efgh	2.5 ± 0.0 hijkl
M. truncatula	F20.089	2.2 ± 0.9 ghijklm	2.8 ± 0.2 cdefg	2.3 ± 0.4 ijkl
M. truncatula	Jemalong	2.2 ± 0.9 ghijklm	2.5 ± 0.7 efgh	2.3 ± 0.2 jklm
M. truncatula	Salernes	1.9 ± 0.8 ijklm	2.6 ± 1.2 cdefgh	2.2 ± 0.5 jklm
M. truncatula	CRE.007	2.3 ± 0.6 efghijk	2.0 ± 0.6 fghi	2.2 ± 0.2 jklm
M. littoralis	F20.026	1.9 ± 1.0 hijklm	2.4 ± 0.2 efgh	2.2 ± 0.3 jklm
M. truncatula	F20.047	1.9 ± 0.9 hijklm	2.0 ± 0.8 fghi	1.9 ± 0.1 klmn
M. truncatula	DZA.315.16	1.8 ± 0.6 jklm	2.0 ± 0.2 fghi	1.9 ± 0.1 klmn
M. littoralis	ESP.027	2.3 ± 0.9 efghijk	1.8 ± 0.2 hi	1.9 ± 0.4 klmn
M. truncatula	F11013	1.8 ± 0.7 jklm	2.0 ± 0.0 fghi	1.9 ± 0.1 klmn
M. truncatula	DZA.220	1.5 ± 0.7 klm	2.0 ± 0.4 fghi	1.8 ± 0.3 lmno
M. truncatula	DZA.045.5	1.7 ± 0.5 jklm	1.8 ± 0.4 ghi	1.8 ± 0.1 lmno
M. truncatula	F83005.9	1.7 ± 0.5 jklm	1.4 ± 0.3 i	1.5 ± 0.2 mno
M. truncatula	ESP.165	1.0 ± 0.4 m	1.4 ± 0.4 i	1.3 ± 0.3 no
M. truncatula	DZA.105	1.1 ± 0.6 lm	1.4 ± 0.4 i	1.2 ± 0.2 o

[a]For each experiment, data are means and standard deviation of four replicates. Five plants were tested in each replicate. Plants were evaluated on a 0–5 scale. Mean values followed by the same letter are not significantly different (Newman–Keuls test $P = 0.05$).
[b]Standard reference cultivars of *P. sativum* for *Aphanomyces* root rot resistance.

after inoculation with *A. euteiches*, and oospores were visible in the root cortex. Disease severity varied considerably between accessions (Table 5), from 1.2 for the most resistant accession (DZA.105) to 3.8 for the most susceptible ones (ESP.050). *Medicago* species showed much higher resistance than the best pea line (PI18693) currently available. Most of the plants of the

susceptible reference accession (*P. sativum*, cv. Baccara) died (score = 4.9). The roots of plants of the resistant reference accession (*P. sativum*, PI180693) were completely discoloured and the leaves were chlorotic (score = 4.1), due to the high concentration of inoculum used in the test and the very late scoring. *Medicago truncatula*, *M. littoralis* and *M. italica* accessions displayed moderate to high levels of resistance.

Discussion

Until now, the host status of *M. truncatula* with respect to *D. dipsaci* and *M. pinodes* was unclear. We clearly demonstrate here, for the first time, that annual *Medicago* spp. are hosts for both pathogens. This is also the first report of screening for resistance to three major pathogens of pea and alfalfa – *D. dipsaci*, *M. pinodes and A. euteiches* – in accessions from a collection of annual *Medicago* spp.

The tests performed for each pathosystem were adapted from tests developed for the host plant. However, particular observations were made when screening for resistance in annual *Medicago* spp. Resistance to nematodes was interpreted as the ability of a plant to reduce nematode reproduction and multiplication (Plowright et al., 2002). The symptoms expressed by plants in response to the invasion and/or multiplication of *D. dipsaci* are good indicators of susceptibility or resistance in alfalfa and faba bean (Caubel and Leclercq, 1989; Leclercq and Caubel, 1991). However, we found no clear correlation between symptoms such as hypertrophy at the infection site or vegetative meristems and the observed RIs, suggesting that symptoms in seedlings of annual *Medicago* spp. are not reliable indicators for the assessment of susceptibility or resistance status. Considerable variability in nematode multiplication rate was also observed between plants of a given accession. This variability was not reduced in assays using a nematode line originating from a single cross, suggesting that the heterogeneity of the nematode population used cannot account for this result. Such high levels of variability have already been observed in other screenings for resistance to nematodes and numerous reasons for this variability have been proposed (Plowright et al., 2002).

Ditylenchus dipsaci development is strongly influenced by environmental conditions (particularly relative humidity and temperature) and actively growing plants may outgrow the infection. Mercer and Grant (1995) estimated that more than 75% of the *Ditylenchus* inoculum may be lost during host invasion. The problem of plants escaping infection for reasons unrelated to resistance can be minimized but rarely eliminated, so it is also important to establish the resistance status in *M. truncatula* by determining the percentage of plants with an RI < 1, as in certified alfalfa cultivars. In some cases, we observed variability between plants of a given accession when screening for resistance to *M. pinodes* or *A. euteiches*. It is difficult to identify the source of the variation. Some of the variation could be due to the techniques employed, but some of it may come from genetic variation. Although *M. truncatula* is generally self-pollinated, it displays high levels of genetic diversity at the population level (Bonnin et al., 1996, 2001), which may contribute to the variability observed in the three tests. For the *M. pinodes*/*Medicago* spp. pathosystem, we carried out two tests.

The severity of reactions observed in the bioassay on detached leaves was similar to that observed in the seedling test. This method has the advantage of providing a quantitative means of identifying and characterizing the resistance present in *Medicago* spp. It also made it easier to distinguish between accessions. The screening test used for the *A. euteiches*/*Medicago* spp. pathosystem appeared to be well adapted and discriminated between the accessions well. It could be used to screen large numbers of accessions rapidly. We can specify that the inoculum level used (10,000 zoospores/plant) for the *A. euteiches* screenings is much more similar to inoculum levels used for peas, whereas only 100–1,000 zoospores are required for evaluating alfalfa for resistance to alfalfa pathovars of *A. euteiches* (Vandemark and Grundwald, 2004).

We observed little variation among *Medicago* accessions in terms of the reaction to infestation by *D. dipsaci*. Screening was carried out in conditions optimal for infestation and high levels of resistance were found, especially in accessions ESP.050, ESP.158 and F34.042, for which 100% of the plants were resistant in both assays. Only three accessions (Harbinger, Jemalong and Tornafield) were classified as susceptible to the Lucerne race of the stem nematode *D. dipsaci*, like the perennial

M. sativa cv. Europe. Conversely, considerable variation in resistance to *A. euteiches* was observed, with disease severity scores ranging from 1.0 for ESP.165–4.0 for Paraggio and ESP.050. Such variation was previously reported for *M. truncatula* accessions evaluated for resistance to *A. euteiches* race 2 (Vandemark and Grunwald, 2004). Screening for resistance to major necrotrophic fungal pathogens of legumes (*Ascochyta rabiei*, *Botrytis fabae*, *Colletotrichum trifolii*, *Phoma medicaginis*) has also been initiated and differential responses have been obtained from the accessions tested (Ellwood et al., 2001; O'Neill and Bauchan, 2000, 2003). For the *M. pinodes*/*Medicago* spp. pathosystem, a very high level of resistance to *M. pinodes* was observed in all the accessions tested, with very little variability in disease reactions. However, none of the accessions was found to be susceptible. A comparison of the results obtained in the three screening tests did not lead to the identification of accessions with the same reaction to infection by all three pathogens. This suggested that different genes were involved in resistance to the three diseases. Similarly, accessions within a species had markedly different reactions to the three diseases.

Phytoalexins have been reported to function as resistance factors against nematode invasion and multiplication (Baldridge et al., 1998). Edwards et al. (1995) reported systemic induction of isoflavonoids conjugate accumulation in alfafa roots when shoots were inoculated with the stem nematode, *D. dipsaci*. The increase in these antifungal compounds occurred in response to a signal from infected shoots and was correlated with resistance. Partial resistance to *M. pinodes* in *P. sativum* has been attributed to pisatin, a phytoalexin of the isoflavonoid family (Cruickshank and Perrin, 1965; Shiraishi et al., 1978). Ingham (1979) identified several different phytoalexins in the genus *Medicago*. The much higher level of resistance to *M. pinodes* observed in *M. truncatula* than in *P. sativum* may be due to the production of phytoalexins by *M. truncatula* that are different from those produced by the host plants. Only small necrotic reactions were observed on the seedlings of *Medicago* spp. inoculated with *M. pinodes*, indicating that fungal development was limited to the cell initially infected. Three different reactions during the interaction between *Medicago* spp. and *M. pinodes* were identified with the other test. The first reaction was an absence of lesions, possibly due to failed spore penetration: six accessions (Borung, Harbinger, Mogul, Parabinga, F11.005, F20.026) had only flecks at the drop site. The second reaction, observed for most of the accessions, consisted of fungal development restricted to the drop site. This reaction occurred between days 2 and 4 for the various accessions. The third reaction, observed for two accessions (GRC.043 and ESP.050) was the slowing of lesion progression, followed by a halting of lesion progression. O'Neill and Bauchan (2000) also demonstrated that fungal development was restricted in resistant tissues during pathogenic interaction between annual *Medicago* spp. and *C. trifolii*. They suggested a hypersensitive response, possibly involving phytoalexins, as in the *M. sativa*-*C. trifolii* pathosysten (O'Neill, 1996). However, many systems may be involved and the pathogenic interactions should be investigated by biological and molecular approaches. The wide range of plant reactions observed in response to infection by *A. euteiches* suggested that the *Medicago* spp. harboured several mechanisms of resistance to the fungus. Recently initiated studies for investigation of the interaction between *M. truncatula* and the fungus should increase our understanding of the infection process or of the components of partial resistance to *A. euteiches* in pea (Nyamsuren et al., 2003; Colditz et al., 2004).

The variability between *M. truncatula* accessions in response to infection by *D. dipsaci* and *A. euteiches* should facilitate broad biological and genetic investigations of interactions between *Medicago* and these two pathogens. Moreover, cross hybridizations between susceptible and resistant accessions may facilitate rapid determination of the inheritance of resistance in the progeny and the potential number of resistance genes involved. For the *M. pinodes*/*Medicago* spp. pathosystem, we need to identify suitable ecotypes with markedly different phenotypes, for genetic dissection of the basis of *M. pinodes* resistance and analysis of resistance gene expression. We therefore intend to enlarge our screening to include all the annual *Medicago* spp. in the French core collection or to other core collections.

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