

REACTION OF *MEDICAGO TRUNCATULA* TO *APHANOMYCES EUTEICHES* RACE 2

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Aphanomyces euteiches causes root rot disease of alfalfa, and two races of the pathogen have been identified based on the reaction of established check cultivars. A majority of alfalfa cultivars grown in regions where *A. euteiches* is prevalent have resistance to race 1 of the pathogen, but very few cultivars have resistance to race 2. The genetic basis of resistance in alfalfa to *A. euteiches* is unknown, in part due to characteristics of alfalfa that impede genetic analysis, including its tetraploid genome and susceptibility to inbreeding depression. The barrel medic, *Medicago truncatula*, has been suggested as a model organism for the study of legume genetics. Our objective was to evaluate plant introduction (PI) accessions of *M. truncatula* for resistance to *A. euteiches* race 2. In repeated experiments, employing standardized testing conditions used for evaluating resistance in alfalfa to *A. euteiches*, we identified seven *M. truncatula* accessions with levels of resistance similar to the resistant alfalfa check population WAPH-5. Two *M. truncatula* accessions were identified that were as susceptible as the susceptible alfalfa check cultivar Saranac. The utility of *M. truncatula* as a model for studying the genetics of resistance in alfalfa to *A. euteiches* race 2 is discussed.

Keywords: Alfalfa; *Aphanomyces euteiches*; Barrel medic; Disease resistance

INTRODUCTION

Aphanomyces euteiches Drechs. is the causal agent of Aphanomyces root rot of alfalfa (*Medicago sativa* L.), which is characterized by stunted seedlings, damping off and poor stand establishment (Grau, 1990). To minimize losses, the cultivation of resistant alfalfa cultivars is recommended, along with the avoidance of poorly drained and heavily infested fields (Grau, 1990). Several studies indicate that alfalfa cultivars with resistance to *A. euteiches* exhibit significantly better seedling health, yield, and persistence than varieties with low resistance when grown in naturally infested soils (Munkvold *et al.*, 2001; Vincelli *et al.*, 2000; Wiersma *et al.*, 1995, 1997).

Alfalfa cultivars must be evaluated for reaction to *A. euteiches* using a standardized test protocol (Fitzpatrick *et al.*, 1998) for the resistance rating to be accepted by the USA Alfalfa and Miscellaneous Legume Variety Review Board. The standard test uses a scale of 1 to 5 for evaluating resistance, where 1 = no visible symptoms, and 5 = dead

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plant. Plants scored as either a 1 or 2 (slight necrosis of roots and hypocotyls) are considered resistant. Cultivars are considered to express high resistance (HR) if greater than 50% of the plants in the cultivar are resistant, while cultivars with more than 30% resistant plants are considered to be resistant (R) (Fitzpatrick *et al.*, 1998). Within the alfalfa pathotype of *A. euteiches*, strains have been classified as either one of two races based on the reaction of established check cultivars under standardized testing conditions (Grau *et al.*, 1991). At the present, 71.4% (135/189) of the available certified alfalfa cultivars with a fall dormancy rating of 2 to 4 are rated as R or HR to race 1 of *A. euteiches* (Alfalfa Council, 2001). However, only 3.2% (6/189) of these varieties are rated as R or HR to race 2 of *A. euteiches* (Alfalfa Council, 2001). This suggests that although sufficient resistance to race 1 isolates of *A. euteiches* is available in alfalfa cultivars, there is a need for enhancing resistance in alfalfa cultivars to race 2 isolates of the pathogen. In even the most resistant alfalfa cultivars, the percentage of plants resistant to *A. euteiches* race 2 may not greatly exceed 40%. One report found that the average percentage of resistant plants was 38.5% for two commercial cultivars classified as HR to *A. euteiches* race 2 isolate NC-1 (Malvick and Grau, 2001).

Although the genetic basis of resistance to *A. euteiches* in alfalfa has not been determined, the observation that many cultivars are resistant to race 1 isolates but susceptible to race 2 isolates suggests that different genes are involved in resistance to each race. As an example, the population WAPH-1 (Grau, 1992) is the resistant check in standard tests for evaluating resistance to race 1 of *A. euteiches*, with approximately 50% resistant plants expected (Fitzpatrick *et al.*, 1998). However, in standard tests for evaluating resistance to race 2 of *A. euteiches*, WAPH-1 is a susceptible check, along with the cultivar Saranac, with approximately 2% resistant plants expected (Fitzpatrick *et al.*, 1998). Additionally, among the 135 certified alfalfa cultivars with a fall dormancy rating of 2 to 4 that are rated as R or HR to race 1 of *A. euteiches*, only 6 of these cultivars are rated as R or HR to *A. euteiches* race 2 (Alfalfa Council, 2001).

The tetraploid nature ($2N = 4X = 32$) of alfalfa genetics and the susceptibility of alfalfa to inbreeding depression are factors contributing to difficulties in both population improvement and the analysis of genetic factors conditioning traits of interest in alfalfa (Busbice *et al.*, 1972). Considerable advances in understanding alfalfa genetics may be realized through the examination of a closely related plant species having characteristics making it more amenable to genetic analysis. Barrel medic (*Medicago truncatula* Gaertn.) is an annual pasture legume that has been chosen as a model species for genetic analysis based on several favourable characteristics. These include a small diploid genome ($2C = 2N = 16$), high efficiency of transformation, self-fertility and a short generation time (Bell *et al.*, 2001; Blondon *et al.*, 1994; Cook, 1999; Oldroyd and Geurts, 2001).

Several plant pathogens of *M. truncatula* have been identified due to its cultivation in dryland pasture systems in Australia, including *Pythium irregulare* (Barbetti, 1989), *Phytophthora medicaginis* (Mackie *et al.*, 1999), and which is a globally important causal agent of root rot of alfalfa (*M. sativa* L.) (Erwin, 1990). Additionally, a range of reactions to *Colletotrichum trifolii*, the causal agent of anthracnose disease of alfalfa, has been observed under greenhouse conditions among different accessions of *M. truncatula* (O'Neill and Bauchan, 2000). *A. euteiches* has not been isolated from *M. truncatula*. However, previous examples demonstrating the development of disease in *M. truncatula* due to infection by causal agents of disease in alfalfa have caused us to

consider if *A. euteiches* can cause disease in *M. truncatula* when the standardized test protocol for evaluating resistance in alfalfa to *A. euteiches* (Fitzpatrick *et al.*, 1998) is conducted.

The objective of this work was to screen a range of *M. truncatula* plant introduction (PI) accessions for reaction to *A. euteiches* race 2 type isolate NC-1 (Fitzpatrick *et al.*, 1998). The identification of accessions with different disease reactions will facilitate the development of populations that can be used to examine the genetic basis of resistance to *A. euteiches* race 2. The high degree of synteny observed between the genomes of *M. truncatula* and *M. sativa* (Bell *et al.*, 2001) suggests that advances in the understanding of the genetic basis of resistance in *M. truncatula* to *A. euteiches* may have applications for improving levels of resistance to the pathogen in alfalfa.

MATERIALS AND METHODS

Plant materials

Thirty *M. truncatula* plant introduction (PI) accessions were evaluated for resistance to *A. euteiches* race 2 isolate NC-1 (Table I). The PI accessions were chosen to sample the geographic diversity present among the accessions available from the National Plant Germplasm Service (NPGS) collection located at Pullman, WA, USA. The alfalfa population WAPH-5, the standard check for resistance to race 2 isolates of *A. euteiches*, and the population Saranac, a standard check for susceptibility to race 2, were also included as experimental controls (Fitzpatrick *et al.*, 1998) to enable comparison between resistance in *M. sativa* and *M. truncatula*.

Inoculations and evaluation of disease severity

A. euteiches NC-1, the type isolate of race 2 (Fitzpatrick *et al.*, 1998), was used for all inoculations. The isolate was maintained on potato dextrose agar (Difco Inc., Detroit, MI, USA) at room temperature. The standard test protocol for evaluating resistance in alfalfa to *A. euteiches* (Fitzpatrick *et al.*, 1998) was followed in this study. Briefly, seeds of *M. truncatula* PI accessions, the resistant alfalfa check population WAPH-5 and the susceptible alfalfa check cultivar Saranac were mixed separately in a commercial preparation of *Sinorhizobium meliloti* (LiphaTech Inc., Milwaukee, WI, USA), and planted in pots containing vermiculite. Seedlings were grown in the greenhouse with 16 h daylength at 20–24°C. Zoospores of *A. euteiches* NC-1 were produced in a mineral salt solution as described by Carman and Lockwood (1959). At 5 days post-germination, seedlings were thinned so that each pot contained six seedlings of a single accession or alfalfa check. The experiment consisted of three replicate pots for each population, and was repeated once.

At 1 week post-germination, the vermiculite was saturated with water and each seedling was inoculated with 1 ml of a 500 zoospore/ml suspension by pipetting the suspension to the base of the stem. The pots were placed in greenhouse flats without holes and were flooded for 5 days. For all *M. truncatula* accessions and alfalfa check populations, pots containing six uninoculated healthy plants were also placed in flats and similarly flooded. After the period of flooding, all pots were placed in greenhouse flats containing holes and allowed to drain. Seven days after inoculation, all pots were drenched with a nutrient solution (Miracle-Gro, The Scotts Co., Columbus, OH, USA).

TABLE I Reactions of *M. truncatula* Plant Introduction (PI) accessions and alfalfa (*M. sativa*) standard check populations WAPH-5 and Saranac to *Aphanomyces euteiches* isolate NC-1 race 2. Nonparametric analysis of variance conducted on ranks of DSI were significantly different for both experiments ($P < 0.0001$)

Population ^a	Origin	Mean DSI ^b		DW _i ^c
		Exp 1	Exp 2	
<i>M. sativa</i> WAPH-5	–	2.00 ab	2.11 a	0.882 (1)
PI 577643	Malta	1.72 a	2.44 a	0.838 (2)
PI 190082	Australia	2.33 abc	2.34 a	0.637 (17)
PI 493296	Portugal	2.33 abc	2.50 ab	0.817 (3)
PI 566890 (c)	Greece	1.92 ab	2.94 abcd	0.748 (6)
PI 384648 (c)	Morocco	2.50 abcd	2.83 abc	0.716 (9)
PI 284123	Cyprus	2.61 bcde	2.83 abc	0.679 (15)
PI 566888 (c)	Australia	1.67 ab	3.78 hijkl	0.807 (4)
PI 566887 (c)	Greece	2.54 abcde	2.83 abc	0.735 (7)
PI 239873	Algeria	3.11 efghi	2.67 abc	0.725 (8)
PI 577628	Spain	2.68 bcde	3.44 defgh	0.708 (10)
PI 577608	France	2.89 cdef	3.50 efghi	0.697 (12)
PI 534229	Algeria	3.00 cdefg	3.54 efghi	0.702 (11)
PI 442895	Australia	3.11 defgh	3.56 efghij	0.693 (13)
PI 577640	United States	3.50 hij	3.28 cdefg	0.383 (31)
PI 577610	United States	3.72 jkl	3.11 bcde	0.632 (18)
PI 577636	Spain	3.64 jk	3.50 fghij	0.749 (5)
PI 537168 (c)	Cyprus	3.67 jk	3.22 cdef	0.627 (19)
PI 517256	Ethiopia	3.30 fghij	3.61 efghij	0.64 (16)
PI 566889 (c)	Turkey	3.50 ghij	3.50 defghi	0.563 (27)
PI 577615	Italy	3.67 jk	3.33 cdefg	0.585 (23)
PI 535498	Tunisia	3.33 fghij	3.83 ikl	0.625 (20)
PI 197360	Malta	3.72 jkl	3.44 efghi	0.611 (22)
PI 292436 (c)	Israel	3.61 ijk	3.56 efghi	0.576 (24)
PI 577639	Sweden	3.56 hij	3.61 fghij	0.56 (28)
PI 535752	Morocco	3.50 ghij	3.72 hijk	0.567 (25)
PI 566886 (c)	Italy	3.56 hij	3.67 ghijk	0.558 (29)
PI 493295	Portugal	3.61 ij	3.78 hijkl	0.624 (21)
PI 577609	Sweden	3.61 ij	4.00 kl	0.688 (14)
PI 564941	Morocco	3.94 kl	4.00 jkl	0.493 (30)
PI 577614	Malta	4.28 l	4.22 l	0.565 (26)
<i>M. sativa</i> Saranac	–	4.44 l	4.39 l	0.383 (31)

^ac = accession is included in USA National Plant Germplasm Service (NPGS) core collection.

^bDSI = disease severity index. Differing letters following means indicate significant differences among accessions for DSI following a nonparametric mean comparison using Fisher's protected LSD based on ranks of DSI. Each experiment included six plants per replication and three replications for each accession. Plants were individually scored using a DSI scale of 1–5. Ratings: 1 = no necrosis of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyls, slight chlorosis of cotyledons, and moderate stunting of stem; 4 = extensive necrosis of roots, hypocotyls and cotyledons, and severe stunting of stem; and 5 = dead plant.

^cDW_i reflects a reduction in biomass yield due to disease calculated as the ratio of overall mean dry weight of shoots plus stems of inoculated plants divided by dry weight of uninoculated control plants. Numbers in parenthesis are rankings of ratio among the 32 different populations.

Fourteen days after inoculation, seedlings were removed and disease severity index (DSI) ratings were taken on seedlings using an integer scale from 1–5 as follows: 1 = no necrosis of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyls, slight chlorosis of cotyledons, and moderate stunting of stem; 4 = extensive necrosis of roots, hypocotyls and cotyledons, and severe stunting of stem; and 5 = dead seedling (Fitzpatrick *et al.*, 1998).

The effect of disease on plant growth was evaluated by determining shoot dry weight and calculating the percentage of shoot dry weight in inoculated relative to

uninoculated treatment. Dry weight was determined after removing and drying stems and leaves of each plant in a forced hot air drying facility for 2 days.

Data analysis

The two independent greenhouse experiments were arranged in a completely randomized design with three replications for each *M. truncatula* accession and both alfalfa check populations. Mean DSI and mean dry shoot weight were calculated per replication as an average over six seedlings planted per pot. As a measure of yield loss due to disease, a dry weight index (DW_i) was calculated for each *M. truncatula* accession and both alfalfa check populations as the ratio of dry shoot weight in inoculated divided by noninoculated tissue. DW_i consisted of only one observation per accession per experiment based on an overall mean, because observations in inoculated and uninoculated treatments were not paired. Because DSI is a categorical variable the assumptions for parametric analyses of variance were violated. Accordingly, a nonparametric analysis of variance based on ranks was conducted (Conover and Iman, 1981; Eskridge, 1995): ranks were first assigned to all observations using PROC RANK (SAS User's Guide, 2000) and ranks were then subjected to analysis of variance using PROC ANOVA. The same procedure was used to assess whether rankings of accessions for DSI were significantly different between the two experiments. The Wilcoxon–Mann-Whitney test (Walpole and Myers, 1978) was employed to test the hypothesis that the frequency distributions of DSI values were not significantly different between experiments 1 and 2. Exact distributions of the test statistic were performed using PROC NPAR1WAY (SAS User's Guide, 2000). For each experiment, the Spearman rank correlation (Ostle, 1954) between DSI rating and dry shoot and leaf weight was calculated. Additionally, for each experiment the Spearman rank correlation between mean dry shoot and leaf weight in inoculated and uninoculated plants was calculated both including and excluding the two alfalfa populations WAPH-1 and Saranac.

RESULTS

Accessions of *M. truncatula* showed considerable variation for resistance to race 2 of *A. euteiches* ranging from a mean DSI rating of 1.67 to 4.28 (Table I). Nonparametric ANOVA indicated that significant differences were observed between *M. truncatula* PI accessions inoculated with *A. euteiches* NC-1 race 2 for DSI ratings in both experiments ($P < 0.0001$). The two independent experiments yielded consistent results. Rankings between the two experiments for DSI did not significantly change ($P = 0.92$) and frequency distributions of DSI between the two experiments were not significantly different following a Wilcoxon–Mann-Whitney exact test ($P = 0.5657$) (Figure 1). The Spearman rank correlation between DSI rating and dry shoot and leaf weight was -0.72 for experiment 1 and also was -0.72 for experiment 2, with both correlations being significant ($P < 0.0001$).

Results for comparisons of means between PI accessions and the resistant and susceptible alfalfa check populations, WAPH-5 and Saranac, respectively, are presented for both experiments (Table I). The population with the lowest mean DSI rating (2.06) when averaged over both experiments was the resistant alfalfa check WAPH-5, while

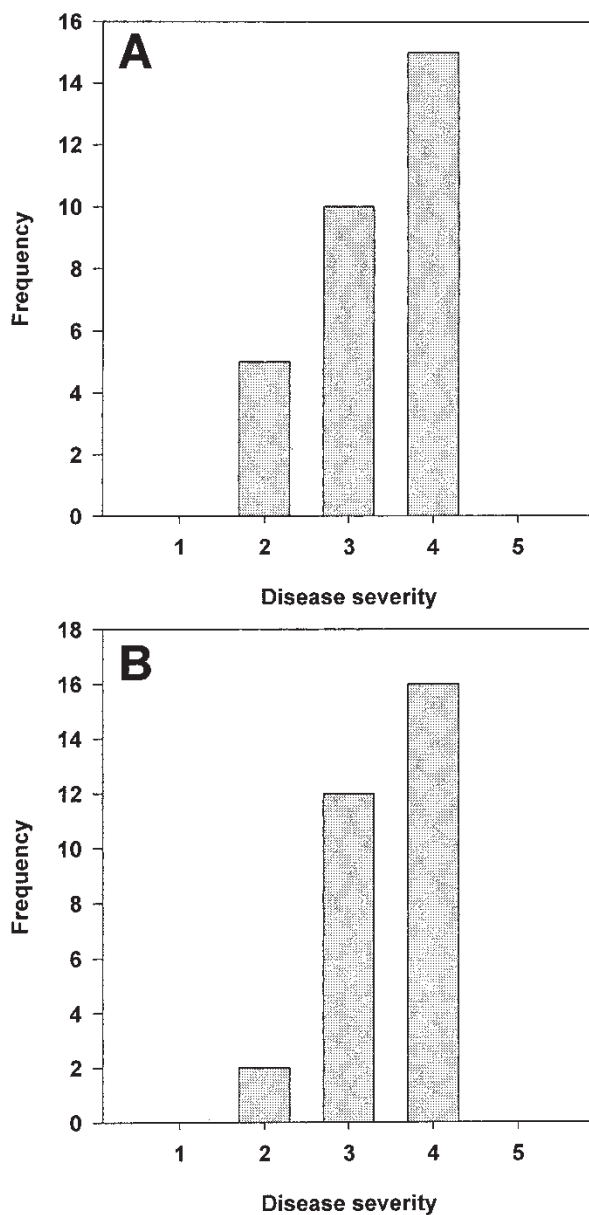


FIGURE 1 Histogram of number of *M. truncatula* accessions within each class of disease severity in **A**, first and **B**, second experiment ($n = 30$). Disease severity was rated visually on a scale of 1–5 on uprooted and washed roots. Ratings: 1 = no necrosis of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyls, slight chlorosis of cotyledons, and moderate stunting of stem; 4 = extensive necrosis of roots, hypocotyls and cotyledons, and severe stunting of stem; and 5 = dead plant. The frequency distributions between the two experiments were not significantly different following a Wilcoxon-Mann-Whitney exact test ($P = 0.5657$).

the highest mean DSI rating (4.42) was for the susceptible alfalfa check Saranac. Seven *M. truncatula* PI accessions, 577643, 190082, 493296, 566890, 384648, 284123, and 566887 had DSI ratings in both experiments that were not significantly different than

the resistant alfalfa check population WAPH-5. Conversely, two *M. truncatula* PI accessions, 564941 and 577614 had DSI ratings in both experiments that were not significantly different than the susceptible alfalfa check population Saranac. Considerable variation was observed for DSI ratings (Table I) among the eight *M. truncatula* accessions that are included in the NPGS annual *Medicago* core germplasm collection (Diwan *et al.*, 1994). Additional evaluations should be conducted on PI 566888, as its disease reaction was not consistent over experiments. When the mean DSI rating was averaged over both experiments, the most resistant of these accessions was PI 566890 (Greece; mean DSI = 2.43), and the most susceptible was PI 566886 (Italy; mean DSI = 3.62).

Reduction in shoot and leaf dry weight due to disease (DW_i) was estimated as the ratio between the mean dry weight of plants infected with *A. euteiches* NC-1 and mean dry weight of uninoculated plants for each accession (Table I). As observations for inoculated and uninoculated treatments were not paired, DW_i could only be calculated for means and accordingly, analysis of variance could not be conducted. The highest ratio (0.88) was observed for the resistant alfalfa check WAPH-5, while the lowest ratio (0.38) was observed for the susceptible alfalfa check Saranac. The highest ratio among *M. truncatula* PI accessions was 0.84, for 577643 (Malta), while the lowest ratio was 0.49, for 564941 (Morocco). The highest ratio among the eight PI accessions included in the NPGS annual *Medicago* core germplasm collection (Diwan *et al.*, 1994) was 0.81 for PI 566888 (Australia), while the lowest ratio was 0.56 for PI 566886 (Italy).

DISCUSSION

Although a majority of alfalfa cultivars have resistance to *A. euteiches* race 1, resistance to *A. euteiches* race 2 is lacking in these same cultivars. At the present in the United States, only six of 189 certified alfalfa cultivars with a fall dormancy rating of 2 to 4 are rated as resistant (R) or highly resistant (HR) to *A. euteiches* race 2 (Alfalfa Council, 2001). Results presented in this investigation suggest that the standardized test for evaluating resistance in alfalfa to *A. euteiches* race 2 can be used to evaluate resistance in *M. truncatula*. In this study seven *M. truncatula* PI accessions were identified that were not significantly different than WAPH-5, the resistant alfalfa check population, for DSI rating in both experiments (Table I). Although the most susceptible population identified in this study was the susceptible alfalfa check Saranac, two *M. truncatula* PI accessions were identified that were not significantly different than Saranac for DSI rating in both experiments (Table I).

Significant differences were detected between *M. truncatula* PI accessions for DSI ratings in response to infection by *A. euteiches* isolate NC-1 (race 2). Previously, significant differences have been observed among *M. truncatula* populations for resistance to root rot caused by *P. medicaginis* (Mackie *et al.*, 1999), powdery mildew caused by *Erysiphe pisi* (Yaeger and Stuteville, 2002), and anthracnose caused by *Colletotrichum trifolii* (O'Neill and Bauchan, 2000). Seven of eight *M. truncatula* PI accessions included in the NPGS annual *Medicago* core germplasm collection have now been evaluated for resistance to *A. euteiches* (this study), *E. pisi* (Yaeger and Stuteville, 2002) and *C. trifolii* (O'Neill and Bauchan, 2000). A comparison of results obtained with the different pathogens indicates that PI 566886 and PI 566889 are susceptible to all three pathogens, while PI 566887 is resistant to all three pathogens.

The Spearman rank correlation between DSI rating and dry shoot and leaf weight was negative and significant ($P < 0.0001$) in both experiments, suggesting that infection of *M. truncatula* by *A. euteiches* results in decreased shoot weight of seedlings. Additionally, Table I also demonstrates a trend in which the more resistant populations, based on DSI, generally exhibited less decrease in shoot weight associated with infection by *A. euteiches* than did the more susceptible populations, as evidenced by higher estimates of DW_i in the more resistant populations. Larsen *et al.* (2000) also observed that dry shoot weight was reduced in peas inoculated with *A. euteiches*. However, it could be possible that the relationship between DSI and dry weight is the result of susceptible plants having inherently less yield potential than more resistant plants. Our data cannot reject this hypothesis since we also observed significant, positive Spearman rank correlations between the mean shoot and leaf dry weight of a population for inoculated and uninoculated plants. For experiment 1, the correlation was 0.78 ($P < 0.0001$) for data that both included and excluded the two alfalfa populations WAPH-1 and Saranac, while for experiment 2 the correlations were 0.76 ($P < 0.0001$) and 0.80 ($P < 0.0001$), respectively, for data both including and excluding the two alfalfa populations WAPH-1 and Saranac.

It is difficult to conduct genetic studies on alfalfa due to polyploidy and inbreeding depression (Busbice *et al.*, 1972). Conversely, *M. truncatula* has a diploid nuclear genome and is self-fertile, two characteristics facilitating genetic analysis (Blondon *et al.*, 1994). *M. truncatula* cv. Jemalong (PI 442895) has been widely adopted as the cultivar of choice for the development of *M. truncatula* as a model species for legume genomics (Bell *et al.*, 2001; Blondon *et al.*, 1994; Cook, 1999; Oldroyd and Geurts, 2001). However, results presented in this study indicate that Jemalong has an intermediate level of resistance (Table I) to *A. euteiches* relative to the 30 PI accessions examined. This suggests that other PI accessions may have more utility as parents for producing segregating populations for use in examining the genetic control of resistance to *A. euteiches* in *M. truncatula*. At the present, we are producing inbred lines from a cross of PI 566890 (resistant) \times PI 566886 (susceptible) (Table I). These lines will be useful for numerous areas of investigation, including analysis of the inheritance of resistance to *A. euteiches*, linkage analysis using molecular markers, and the identification and characterization of genes involved in host resistance.

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