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High diversity of responses among *Medicago truncatula* lines to *Phoma medicaginis* infection.

Maiza Nourhene[1,2], Jabri Cheima[1], Zaidi Nawres[1,2], Rafik Khawla[1], Khiari Bilel[3], Djébali Naceur[3], Sánchez-Ballesta Maria Teresa[4] & Badri Mounawer[1]*

[1]*Laboratory of Extremophile Plants, Centre of Biotechnology of Borj Cedria, B.P. 901, Hammam-Lif 2050, Tunisia.*

[2]*University of Tunis El Manar, Faculty of Mathematical, Physical and Natural Sciences of Tunis, Campus Universitaire El-Manar, 2092 El Manar Tunis.*

[3]*Laboratory of Bioactive Substances, Centre of Biotechnology of Borj Cedria, B.P. 901, Hammam-Lif 2050, Tunisia.*

[4]*Laboratory of Biotechnology and Postharvest Quality. Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), Jose Antonio Novais, 10, 28040 Madrid, Spain.*

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*** Corresponding author**
mounawer.badri@cbbc.rnrt.tn

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Abstract

Medicago truncatula is an omni-Mediterranean species grown as an annual forage legume. In addition to its small genome size and simple genetics, *M. truncatula* harbors several attributes, which make it an attractive model forage plant. In this study, we investigated the variation of responses in ten parental lines of *M. truncatula* to *Phoma medicaginis* infection. Plants were cultivated in the growth chamber under controlled conditions and were inoculated after two months with *P. medicaginis*. At harvest, 13 quantitative traits of growth and pathogenicity were measured. Results from ANOVA showed that the variation of analyzed parameters was explained by the effect of line. All measured parameters, except the root fresh weight, showed significant difference among the 10 studied lines. Most tolerant lines are those with the lowest ratios of the number of infected and dead leaves. Studied lines were clustered into three groups according to their responses to *P. medicaginis* infection. The most resistant TN6.18 line and most sensitive F83005.5 to *P. medicaginis* are useful for the exploration of physiological mechanisms and genetic determinants for *M. truncatula* tolerance to this constraint.

1. INTRODUCTION

In their natural habitat, plants are constantly subjected to several biotic stresses that seriously reduce their productivity. Among these constraints, fungal attacks, especially spring black stem and leaf spot, caused by *Phoma medicaginis*, represent the main factors limiting the productivity of legume plants in the Mediterranean basin. A better understanding of the genetic bases and physiological mechanisms for tolerance to this constraint in forage legume plants is a prerequisite for any program aimed at improving their yields. *Medicago truncatula* has been proposed as a model legume due to the many assets it has (Young and Udvardi, 2009). Indeed, it is annual, herbaceous, diploid (2n = 16), self-pollinated, with a small genome (500-550 Mbp), with a short life cycle (3 to 4 generations per year), with a high level of biodiversity and having a sufficient number of lines available (Badri et al., 2016). Moreover, the genomes of approximately 330 inbred *Medicago truncatula* lines are resequenced at least 5X coverage (http://www.medicagohapmap.org/). This provides a foundation for discovering single

nucleotide polymorphisms (SNPs) which allows the identification of the genetic determinants of the agronomic characters of interest in this species by using a genome wide association study (GWAS) approach.

Phoma medicaginis Malbr. & Roum. the causal agent of spring black stem and leaf spot on perennial Lucerne (*Medicago sativa* L.) (Gray et al., 1990) and annual *Medicago* species (Barbetti 1995) and a model pathosystem for *M. truncatula*, occurs under a wide range of environmental conditions. This disease is common on *Medicago* species in North America, Europe, Australia (Tivoli et al., 2006) and North Africa, particularly in Tunisia (Djébali, 2013). *Phoma medicaginis* is a major cause of yield loss and stand decline in M. sativa. The fungus also causes losses in forage quality and it is more destructive in irrigated fields of this species than dry land crops. Black lesions are frequently observed on leaves and stems of M. sativa.

The *P. medicaginis* Pm8 isolate (BrMt0404Ph8), originally obtained from barrel medic leaves, was able to infect leaves of barrel medic, alfalfa, pea, common bean and chickpea (Djébali, 2013). The same authors reported that Pm8 and Pm10 strains of *P. medicaginis* caused about 50 to 70% plants death of *M. truncatula*.

This study aims to explore the variation of tolerance among ten parental lines of *M. truncatula* to Pm8 strain infection and to determine the most discriminant parameters of pathogenicity.

2. MATERIALS AND METHODS

2.1 Plant material and growth conditions

Ten lines of *M. truncatula* were used in this study. They include TN1.11, TN1.21, TN8.3, TN8.20, and TN6.18 from Tunisia, the lines DZA315.16 and DZA45.5 from Algeria, the line A20 from Morocco, the line JemalongA17 (JA17) from the Australian collection and the French line F83005.5. The later line was used as a reference line because it was previously described by Djébali et al. (2007) as highly susceptible to *P. medicaginis* attack. Scarified seeds of *M. truncatula* using sand paper were immersed in sodium hypochlorite for 1 min, and then washed five times with distilled water. Imbibed seeds were transferred to Petri dishes on a filter paper and incubated at 25°C in phytotron until radical emergency.

Seedling were sown on pots of two liters (diameter of 16.5 cm and deep of 13cm) filled with a mixture of sand and compost (1:2) in a growth chamber at 25°C with 16 h daily photoperiod, and were irrigated every two days with a nutritive solution (Vadez et al., 1996). Each genotype was replicated twenty one times, giving 210 plants.

2.2. Measured traits

After 3 months, we measured for each individual plant, 13 quantitative traits of aerial and root growth and of infection. They include the length of stems (LS, cm), length of roots (LR, cm), number of internodes (NI), number of healthy leaves (NHL), number of dead leaves (NDL), number of infected leaves (NIL), number of infected petioles (NIP), number of infected axes (NIA), number of total petioles (NTP), aerial fresh weight (AFW, g), shoot dry weight (SDW, g), root fresh weight (RFW, g), and root dry weight (RDW, g). We also measured the frequency of healthy leaves (FHL), frequency of total leaves (FTL), frequency of dead leaves (FDL) and frequency of infected Leaves (FIL) as follows: number of FHL or FTL or FDL or FIL * 100 / total number of leaves.

2.3. Inoculum production and inoculation tests

P. medicaginis Pm8 strain was grown on PDA medium in the phytotron at 25°C and 16h daily photoperiod. 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}. Aggressiveness tests on leaves 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 10 days to maintain high humidity to stimulate infection.

2.4. Statistical analysis

The data collected were subjected to an analysis of variance of one factor using the GLM procedure. Comparison of means of measured traits for studied lines was performed using Duncan's multiple range test at 5%. Estimated correlations between measured characters was made using the CORR procedure. Principal component analysis (PCA) and cluster analysis were performed using the means of analyzed parameters showing significant differences

between the lines. All these analyses were performed using SAS 2000 software (Statistical Analysis System).

3. RESULTS

3.1 Analysis of variance

Results from ANOVA showed that all morphological parameters, except root fresh weight, displayed significant differences between studied lines (Table 1). Furthermore, all the five traits of infection revealed significant variation among analyzed lines. Our results suggest that the traits measured are good descriptors of the high degree of variation found between studied lines to *P. medicaginis* infection.

Table 1. Effect of line on the variation of measured traits and synthetic parameters for studied lines of *M. truncatula* under *P. medicaginis* infection.

Parameters	Mean	F value	Pr > F
LS	5.11	7.72	<0.0001
NIN	10.10	8.17	<0.0001
NTP	13.97	6.17	<0.0001
LR	12.93	2.62	0.0102
AFW	380.23	4.82	<0.0001
ADW	113.11	3.85	0.0004
RFW	136.96	1.59	0.1326
RDW	27.68	2.43	0.0169
NIA	1.04	2.08	0.0395
NIP	9.18	7.60	<0.0001
NHL	3.62	4.31	0.0002
NIL	5.50	8.51	<0.0001
NDL	6.67	2.81	0.0055

F: coefficient of Snedecor-Fisher, significant ($P \leq 0.05$). Length of stems (LS, cm), number of internodes (NIN), number of total petioles (NTP), length of roots (LR, cm), aerial fresh weight (AFW, g), aerial dry weight (ADW, g), root fresh weight (RFW, g), and root dry weight (RDW, g), number of infected axes (NIA), number of infected petioles (NIP), number of healthy leaves (NHL), number of infected leaves (NIL), number of dead leaves (NDL).

3.2 Comparison of means

The largest aerial and root fresh weight were found for TN6.18 and TN1.11 lines while the bblowest values were noted for F83 line (Table 2). Furthermore, the greatest values of ADW and RDW were registered for TN6.18 and DZA315 whereas the lowest values were noted for F83 and JA17. Overall, the TN6.18 line exhibited the highest vigor by showing the largest values for NHL AFW, ADW and NTP.

For the lines studied, the frequency of infected leaves is higher than the frequency of dead leaves for studied lines, except for TN6.18, which showed the lowest frequency of dead leaves and the highest frequency of infected leaves (Fig. 1.). On the other hand, F83005.5 showed the lowest frequency of infected leaves and the highest frequency of dead leaves.

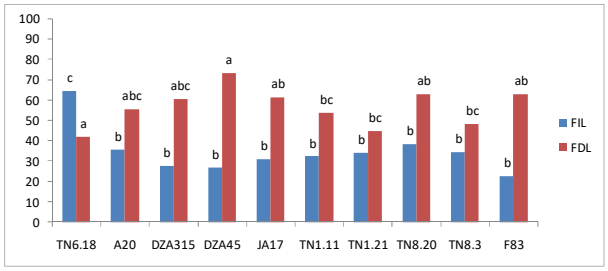

Fig. 1. Comparison of means of the frequency of dead leaves (FDL) and the frequency of infected leaves (FIL) for studied lines of *M. truncatula* under *P. medicaginis* infection. DZA315.16 (DZA315), DZA45.5 (DZA45), F83005.5 (F83), Jemalong A17 (JA17).

Means followed by the same letter(s) or common letters are not significantly different among the lines for each according to Duncan test at 5%.

Table 2. Means of measured traits for studied lines of *M. truncatula* under *P. medicaginis* infection

Lines/ parameters	LS	NIN	NTP	RL	AFW	ADW
A20	4.05cb	8,5294bc	10.78cde	14.19ab	314.44d	107.94bcd
DZA315	3.85cb	9,1875b	16.5b	14.4a	359cd	124.25abc
DZA45	4.89b	9,4286b	9.55ed	13.13abcd	288.21d	99.43cd
F83	2.79cb	8,6429bc	8.21e	11.25d	241.21d	114abcd
JA17	4.3b	11,1538a	13.78bcd	11.92cd	297.85d	79.07d
TN1.11	9a	11,9231a	14.2bcd	13.55abc	526.38abc	140.77ab
TN1.21	5.61b	11,25a	15.8bc	12.41abcd	539.6ab	131.2abc
TN6.18	1.16c	7,625c	24.84a	13.75abc	582.9a	147.6a
TN8.20	10.8a	11,2308a	14.78bcd	13.07abcd	392.54bcd	107.31bcd
TN8.3	3.65cb	10,95a	13.5bcd	11.9bcd	308.05d	92.15cd
Lines/ parameters	**RFW**	**RDW**	**NIP**	**NHL**	**NIL**	**NDL**
A20	145.72abc	30.5ab	7.55cd	2.18cd	3.706bc	5.73d
DZA315	147.25ab	32.125a	11.29b	4.91ab	5bc	8.47ab
DZA45	145.36abc	29.25ab	7.42cd	2.5bcd	2.818bc	6.31cd
F83	96.21c	18.429c	4.93d	2.41d	1.833c	4.37cd
JA17	133.46abc	28.077ab	10.42bc	2.81abc	5.364b	7.35bcd
TN1.11	170.77a	29.462ab	8.2bc	4.5bcd	5.41b	6.267bc
TN1.21	144.65abc	29.45ab	10.65bc	3.3abcd	5.6b	6.9bcd
TN6.18	107.6bc	27.333ab	16.46a	6.8a	12.74a	9.15a
TN8.20	111.31bc	22.846bc	7.76cd	4.8abcd	5.385b	6.73ab
TN8.3	147.5ab	27.25ab	8.95bc	3.33bcd	4.947bc	6.10bcd

Length of stems (LS, cm), number of internodes (NIN), number of total petioles (NTP), length of roots (LR, cm), aerial fresh weight (AFW, g), aerial dry weight (ADW, g), root fresh weight (RFW, g), and root dry weight (RDW, g), number of infected petioles (NIP), number of healthy leaves (NHL), number of infected leaves (NIL), number of dead leaves (NDL). DZA315.16 (DZA315), DZA45.5 (DZA45), F83005.5 (F83), JemalongA17 (JA17). Means followed by the same letter(s) or common letters are not significantly different among the lines for each trait according to Duncan test at 5%.

3.3. Estimated correlations between measured traits

Among the 78 possible correlations, 64 are significant and positive (Table 3). The number of

Table 3. Correlations between measured traits for the ten lines of *M. truncatula* under *P. medicaginis* infection.

	LS	NIN	NTP	LR	AFW	ADW	RFW	RDW	NIA	NIP	NHL	NIL	NDL
LS	1.00												
NIN	0.35*	1.00											
NTP	0.18*	0.24*	1.00										
LR	0.02ns	0.01ns	0.24*	1.00									
AFW	0.34*	0.26*	0.75*	0.22*	1.00								
ADW	0.32*	0.23*	0.68*	0.25*	0.84*	1.00							
RFW	0.34*	0.20*	0.33*	0.38*	0.54*	0.49*	1.00						
RDW	0.17*	0.07ns	0.32*	0.27*	0.39*	0.39*	0.64*	1.00					
NIA	0.10171ns	0.004ns	0.24*	0.12ns	0.098ns	0.17*	0.16*	0.27*	1.00				
NIP	0.007ns	0.24*	0.83*	0.19*	0.53*	0.51*	0.16*	0.24*	0.19*	1.00			
NHL	0.18*	0.12ns	0.80*	0.13ns	0.67*	0.54*	0.36*	0.27*	0.21*	0.43*	1.00		
NIL	0.09ns	0.097ns	0.81*	0.12ns	0.66*	0.58*	0.16*	0.19*	0.08ns	0.70*	0.63*	1.00	
NDL	0.04ns	0.22*	0.50*	0.19*	0.25*	0.32*	0.11ns	0.20*	0.20*	0.66*	0.18*	0.19*	1.00

*significant ($P \leq 005$), non significant (ns) ($P > 0.05$). Length of stems (LS, cm), number of total petioles (NTP), length of roots (LR, cm), aerial fresh weight (AFW, g), aerial dry weight (ADW, g), root fresh weight (RFW, g), and root dry weight (RDW, g), number of infected axes (NIA), number of infected petioles (NIP), number of healthy leaves (NHL), number of infected leaves (NIL), number of dead leaves (NDL).

healthy leaves (NHL) was strongly correlated with NIL, NDL, AFW and RFW while the number of dead leaves was correlated with NIP (r=0.66). In addition, the AFW was correlated with RFW (r=0.54) and ADW was correlated with RDW (r=0.39). Length of stems (LS) was correlated with ADW, RFW and NIN while length of roots (LR) was correlated with RFW and ADW.

3.4. Principal Components Analysis (PCA) and clustering analysis

Our results showed that the first three axes explained 75% of the total variation found between studied lines. The first axis was defined on the positive side by the parameters of infection such as NIL, NHL, NDL and NIP while the second axis was formed by the traits of growth as LS, LR, AFW, ADW, RFW, and RDW (Fig. 2.).

Studied lines formed three groups according to the factorial plan (1-2). A first group is composed of TN6.18, a second group is formed of F83, and a third group is constituted of the remaining lines (Fig. 3.).

The lines showed different behavior in responses to Pm8 infection. We found that TN6.18 line replaces infected leaves with new ones and it increases its biomass. Furthermore, TN8.20 tended to increase the length of its axes in order to avoid the harmful effect of the pathogen.

On the other hand, A20, DZA315 and TN618 have developed their roots in order to maximize the surface contact with the soil.

Studied lines of *M. truncatula* were clustered into three different major groups (Fig. 4). The first group is composed of TN6.18, TN1.11 and

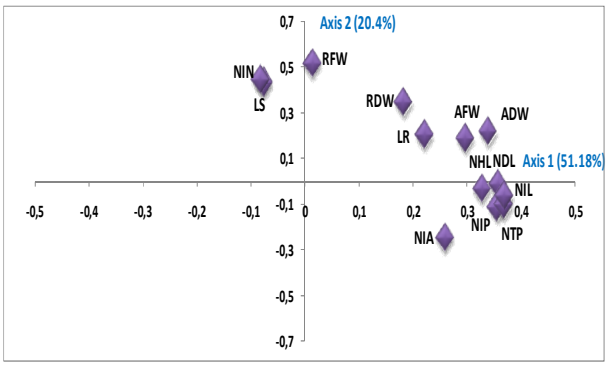

Fig. 2. Plot (1-2) of the principal component analysis applied to analyzed parameters for studied lines of *Medicago truncatula*. Length of stems (LS, cm), number of internodes (NIN), number of total petioles (NTP), length of roots (LR, cm), aerial fresh weight (AFW, g), aerial dry weight (ADW, g), root fresh weight (RFW, g), and root dry weight (RDW, g), number of infected axes (NIA), number of infected petioles (NIP), number of healthy leaves (NHL), number of infected leaves (NIL), number of dead leaves (NDL).

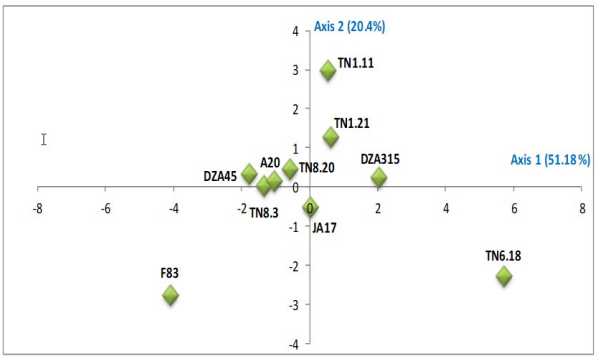

Fig. 3. Plot (1-2) of the principal component analysis (PCA) of the core collection of *Medicago truncatula* applied to measured traits. DZA315.16 (DZA315), DZA45.5 (DZA45), F83005.5 (F83), JemalongA17 (JA17).

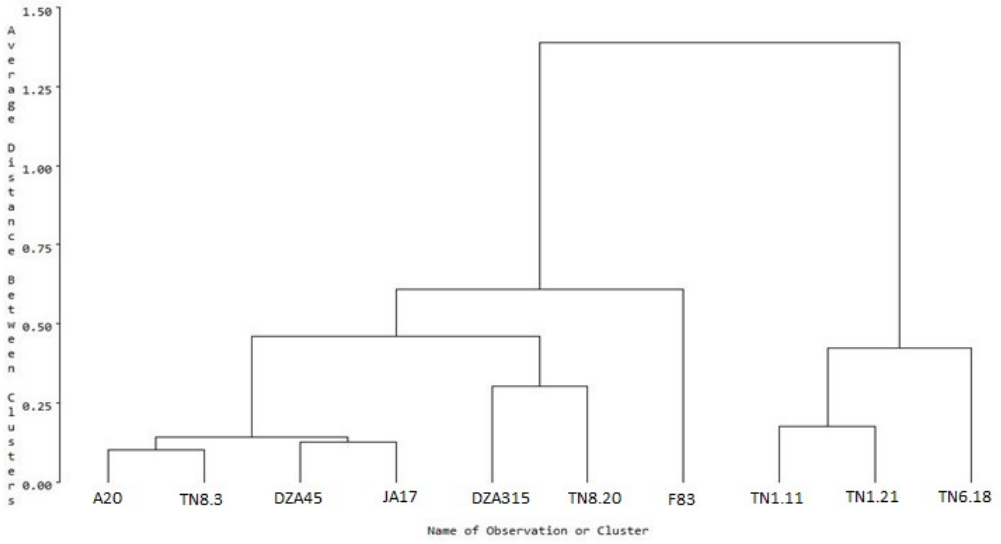

Fig. 4. Cluster analysis of *Medicago truncatula* lines infected by *P. medicaginis.* Group 1 (G1), group 2 (G2), and group 3 (G3).

TN1.21 characterized by the highest values of growth parameters (NHL, AFW, ADW and NTP). The second group is formed by F83 which is the most sensitive to Pm8 infection. The third one was formed by the remaining lines of intermediate behavior. In accordance with Tlahig et al. (2016), lines from the first and second group could be useful in a future breeding program.

3. CONCLUSION

A high level of diversity was found among studied lines to *P. medicaginis*. The lines were clustered into three groups including one tolerant line, eight moderately tolerant, and one susceptible line. Tolerant lines exhibited the lowest frequency of dead leaves and having the highest frequency of infected leaves compared to healthy leaves. A further study is needed to analyze the physiological and genetic determinants of *M. truncatula* tolerance to *P. medicaginis* infection by using the two contrasting lines TN6.18 and F83005.5.

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