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Variation of *Medicago sativa* varieties tolerance to *Phoma medicaginis* infection.

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Abstract

Due to its very important agronomic value and nutritional quality, *Medicago sativa* L. is considered as the queen of fodder and the first cultivated forage crop in the world. In field conditions, *M. sativa* is exposed to several biotic and/or abiotic constraints that affect its quality. In this regard, research is still underway to improve *M. sativa* resistance to many biotic stresses and, in this context, we analyzed the responses of a core collection of 10 varieties of *M. sativa* to *Phoma medicaginis* infection. Results from ANOVA showed that most growth parameters exhibited significant differences between the studied varieties. Nevertheless, only the number of healthy leaves among infection parameters varied significantly between the varieties. The local variety Gabès2355 exhibited the highest biomass. Positive correlations were found between the measured parameters. PCA based on the traits showing significant differences among the studied lines showed that the Gabès variety formed a separate group. Cluster analysis revealed that the studied varieties are classified into three major groups. The first group is formed by Gabès2353, the second group is composed of the Californian and El Hamma varieties, and the third group is constituted of the seven remaining varieties. Gabès2355 was the most tolerant to the Pm8 strain of *P. medicaginis* while Magna601 variety was the most susceptible. These two varieties will be useful to analyze the physiological and genetic determinants for *M. sativa* tolerance to *P. medicaginis* infection.

1. INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a perennial autotetraploid (2n = 4x = 32) forage legume and a cross-pollinated species. It is called the "queen of forage" and is the main fodder crop worldwide. Thanks to its great agronomical value, it is highly cultivated in more than 80 countries (Tlahig et al., 2017), with over 31 million hectares as a global total area planted (Benabderrahim et al., 2009), representing up to 2.5% of all crops cultivated in the world. *M. sativa* has many benefits, such as its low production cost, high yield, high nutritional value and protein quantity, nitrogen fixing

capability, and its ability to improve soil fertility and soil structure by reducing erosion. *M. sativa* has a wide distribution in irrigated semi-arid and arid regions (Chen et al., 2012). Populations of *M. sativa* are phenotypically and genetically very heterogeneous at inter and intra-genotypic levels. Under field conditions, *M. sativa* is subjected to a wide range of diseases affecting its persistence and its yield. Among these, *Phoma medicaginis* is the main causative pathogenic agent of yield loss and fodder quality reduction for *M. sativa* (Samac et al., 2003).

In Tunisia, *M. sativa* is the most widely cultivated fodder legume, with more than 12400 hectares (Ministry of the Environment and Sustainable Development, 2007). It is cultivated as a second crop under palm trees in Southern Tunisia on 75% (9,720 ha) of the surface of the oases (Tlahig et al., 2017). This study aimed to analyze the morpho-physiological responses to *Phoma medicaginis* infection in a core collection of *Medicago sativa* varieties.

2. MATERIALS AND METHODS

2.1 Plant material and growth conditions

A core collection of 10 varieties of *M. sativa* were used. They include the two Tunisian local varieties Gabès 2355 and El Hamma, the Californian and Magna-601 varieties from United States, the Moroccan variety Erfoud1, the Italian Mamuntanas and Agsalfa varieties, the Super Aurora, Sardi10 and ML99 Multileaf Australian varieties. Seeds were germinated in Petri dishes with double-layer filter paper soaked in distilled water. Seedlings were transferred into 2-liter pots (diameter of 16.5 cm and deep of 13 cm) 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 tap water. Fourteen replicates per variety were used, giving 140 plants. Plants were cultivated in the growth chamber into a split plot design with randomized blocks.

2.2. Measured characteristics

At harvest, fourteen parameters were measured. Among them, nine were related to growth, including the number of internodes (NIN), number of main axes (NMA), number of total leaves (NTL), length of stems (LS), shoot dry weight (SDW), shoot fresh weight (SFW), root length (RL), root dry weight (RDW), and root fresh weight (RFW). The other five parameters were related to infection, including the number of dead leaves (NDL), number of healthy leaves (NHL) number of infected petioles (NIP), number of infected stems (NIS) and number of infected leaves (NIL), which were taken at 15dpi. Previous studies (Djébali et al., 2013; Badri et al., 2016a,b) have shown that these traits are good descriptors of the variability of responses to biotic and abiotic constraints in lines of M. truncatula.

We also estimated the percentage of infected leaves and the percentage of dead leaves as follows: (number of infected, dead, or healthy leaves) * 100 / total number of leaves.

2.3 Inoculum production and inoculation tests

The strain Pm8 of *Phoma medicaginis* was grown on potato dextrose agar (PDA) medium in a phytotron. Conidium suspensions were prepared by gently scraping the plates with autoclaved distilled water. The conidium suspensions used for the inoculations were prepared from one month old cultures, with a concentration of 1.107 conidia/mL. Three-month-old plants were spray inoculated by the prepared inoculum with the conidium suspension until run-off. Plants were covered with transparent plastic bags during 10 days in order to keep high humidity to stimulate infection. Plants were harvested 10 days post-inoculation

2.4 Statistical analysis

Obtained data were subjected to analysis of variance for one factor (variety) using the GLM procedure. Comparison of means was performed using the Duncan multiple range test at 5% significance level. Correlations were carried out between measured traits using the correlation procedure (PROC CORR). Multivariate analyses including principal component analysis (PCA) and cluster analysis were performed using the means of the measured parameters that showed significant differences among studied lines. All these analyses were performed using the SAS software (SAS, 2000).

3. RESULTS

Results from ANOVA showed that the variation in seven of the nine growth parameters was explained by the variety effect (Table 1). However, the variation of parameters of infection, except NHL, was not dependent on the variety factor.

Table 1. Effect of variety on parameters measured in varieties of *Medicago sativa* infected by *Phoma medicaginis*.

Parameters	Mean	Ms	F	*P*
NMS	2.40	0.56	7.36	<0.0001
NIN	9.46	9.77	2.20	0.0275
LS	14.61	4.52	0.79	0.6233
SFW	545.78	230.60	3.27	0.0307
SDW	152.17	83.93	2.20	0.0015
RL	13.68	4.50	2.16	<0.0001
RFW	237.87	85.08	7.60	<0.0001
RDW	85.93	51.40	2.80	0.0056
NTL	28.97	9.77	0.87	0.5574
NIP	14.83	5.14	0.92	0.5076
NDL	11.55	5.01	0.95	0.4815
NIL	8.41	4.32	1.64	0.1125
NHL	9.22	59.00	2.01	0.0452

Ms: mean square, F: coefficient of Snedecor-Fisher, significant ($P \leq 0.05$). Number of internodes (NI), number of main sheets (NMS), number of total leaves (NTL), length of stems (LS), shoot dry weight (SDW), shoot fresh weight (SFW), root length (RL), root dry weight (RDW), root fresh weight (RFW), number of dead leaves (NDL), number of healthy leaves (NHL), number of infected petioles (NIP), number of infected stems (NISA) and number of infected leaves (NIL).

3.1. Comparison of means

The highest number of main axes (NMA) was found in the Gabès variety while the lowest value was noted for ML99 variety (Table 2). Moreover, the highest number of internodes (NIN) was recorded for the El Hamma variety while the lowest value was registered for the Californian variety. The largest values of shoot and root fresh weights were observed for the Gabès variety whereas the lowest values were noted for Mamuntanas and SupA varieties, respectively. On the other hand, the highest number of healthy leaves (NHL) was found for Gabès variety while the lowest value was registered for Magna-601. Overall, Gabès variety exhibited the highest plant vigor.

The four varieties namely Agsalfa, Super Aurora, El Hamma and Magna-601 showed much more infected leaves than healthy leaves (Fig. 1.), with the highest value registered for Magna-601.

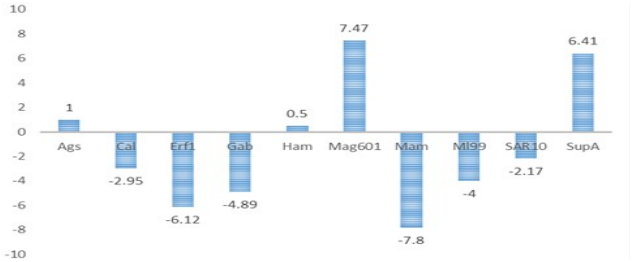

Fig. 1. Differences between the percentage of infected leaves and healthy leaves. Investigated cultivars were Gabès2355 (Gab), El Hamma (Ham), Californian (Cal), Magna-601 (Mag601), Erfoud1 (Erf1), Mamuntanas (Mam), Agsalfa (Ags), Super Aurora (SupA), Sardi10 (Sar10) and ML99 (ML99).

Table 2. Means of measured traits for the studied varieties of *Medicago sativa* infected by *Phoma medicaginis.*

	NMA	NIN	NHL	SFW	SDW	RL	RFW	RDW
Ags	2.36b	9.57ab	8.57abc	618.86ab	158.57abc	14.96ab	243.42bc	70.07cde
Cal	2.35b	8.29b	8bc	440.44bc	179.24ab	13.82abc	225.29bc	104.47abc
Erf1	2.47b	8.71b	10.71ab	534.06bc	102.65c	11.77b	196.76c	87.29abde
Gab	3.5a	8.67b	12.67a	768.56a	153.28abc	13.79abc	348.72a	97.76abcd
Ham	2.28b	10.72a	8.28abc	593.5bc	149.67abc	13.01abc	225.06bc	58.22de
Mag601	2.18b	9.59ba	6.18c	505.13bc	126.76bc	13.19abc	269.59b	120.65a
Mam	2.15b	9.65ba	11.05ab	425.55c	208.85a	13.85abc	237cb	110.53ab
Ml99	2.12b	10.12ab	8.47abc	549.47bc	156.94abc	15.294a	226.88bc	55.13e
SAR10	2.28b	9.11ab	9.22abc	520.28bc	137.78bc	14.317ab	217.44bc	73.78bcde
SupA	2.4b	10.1ab	8.5abc	516.53bc	134.16bc	14.317abc	190.63c	75.69bcde

Means followed by the same letter(s) or common letters are not significantly different according to the Duncan test at 5%. Parameters measured were number of main axes (NMA), number of internodes (NIN), number of total leaves (NTL), length of stems (LS), shoot dry weight (SDW), shoot fresh weight (SFW), root length (RL), root dry weight (RDW), root fresh weight (RFW), number of dead leaves (NDL), number of healthy leaves (NHL), number of infected petioles (NIP), number of infected stems (NIS) and number of infected leaves (NIL). Varieties investigated were Gabès2355 (Gab), El Hamma (Ham), Californian (Cal), Magna-601 (Mag601), Erfoud1 (Erf1), Mamuntanas (Mam) and Agsalfa (Ags), Super Aurora (SupA), Sardi10 (Sar10), and ML99 (ML99).

On the other hand, only the Gabès variety among the studied varieties revealed a higher number of healthy leaves than dead leaves (Fig. 2.). Nevertheless, Magna-601 variety showed more dead leaves than healthy leaves.

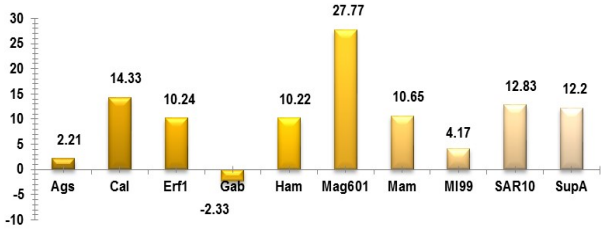

Fig. 2. Differences between the percentage of dead leaves and healthy leaves. Varieties investigated were Gabès2355 (Gab), El Hamma (Ham), Californian (Cal), Magna-601 (Mag601), Erfoud1 (Erf1), Mamuntanas (Mam) and Agsalfa (Ags), Super Aurora (SupA), Sardi10 (Sar10) and ML99 (ML99).

Accordingly, Djébali (2013) reported that the symptoms of *Phoma medicaginis* on leaves of *M. truncatula* are the main discriminative criterion for tolerance among *Medicago* lines.
Among the 28 possible correlations between measured parameters, 14 of them were significant. Significant positive correlations were found between growth traits ($r \geq 0.30$; $P \leq 0.001$) (Table 3). The number of main stems was positively correlated with NHL, SFW and RFW. Furthermore, the number of healthy leaves was positively correlated with SFW and RFW.

Table 3. Estimated correlations between the measured parameters for studied varieties of *Medicago sativa* infected by *Phoma medicaginis*.

	NMS	NHL	NIN	RL	SFW	RFW	SDW	RDW
NMS	1.00							
NHL	0.35*	1.00						
NIN	-0.13	0.19*	1.00					
RL	0.02	0.23*	0.09	1.00				
SFW	0.31*	0.42*	0.20*	0.17*	1.00			
RFW	0.32*	0.34*	0.00	0.38*	0.48*	1.00		
SDW	0.04	0.09	-0.03	0.01	0.22*	0.21*	1.00	
RDW	0.02	-0.01	-0.06	0.11	0.09	0.11	0.36*	1.00

*significant ($P \leq 0.05$). Number of main stems (NMS), number of internodes (NIN), shoot dry weight (SDW), shoot fresh weight (SFW), root length (RL), root dry weight (RDW), root fresh weight (RFW), number of healthy leaves (NHL)

3.2 Principal component analysis (PCA) and cluster analysis

The first three factors of PCA justified 80% of the total variation among the studied varieties. Factor 1 was formed by NMA, NHL, SFW and RFW, while the factor 2 was defined by RL, PFA, and NIN (Fig. 3.).

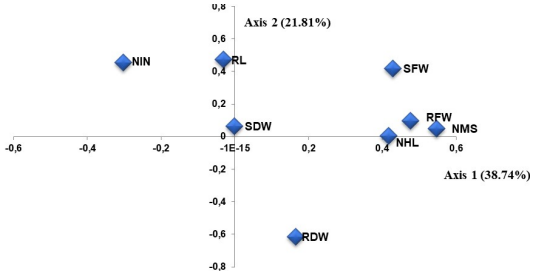

Fig. 3. Plot (1-2) of the principal component analysis (PCA) applied to the measured traits in the studied populations of *M. sativa*. The traits were number of main axes (NMA), number of internodes (NIN), number of total leaves (NTL), length of stems (LS), shoot dry weight (SDW), shoot fresh weight (SFW), root length (RL), root dry weight (RDW), root fresh weight (RFW), number of dead leaves (NDL), number of healthy leaves (NHL), number of infected petioles (NIP), number of infected stems (NIS) and number of infected leaves (NIL).

The distribution of the varieties according to the factorial plan (1-2) showed that they formed three groups (Fig. 4.). A first group was formed by Gabès 2355 with the highest values of NHL, SFW, RFW and NMA. A second group was formed by ML99, Hamma, Agsalfa, Super Aurora and Sardi10 with highest values of RL, NIN and SDW. A third group composed of the remaining varieties (Erfoud1, Mamuntans and Californian) with intermediate behavior.

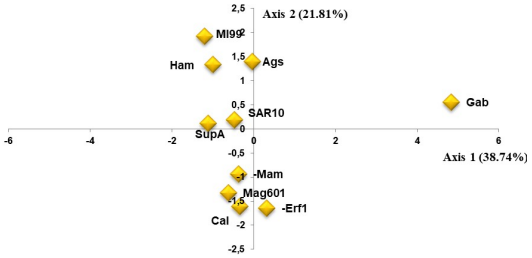

Fig. 4. Plot (1-2) of the principal component analysis (PCA) of the core collection of *M. sativa* applied to the measured traits. The varieties were Gabès2355 (Gab), El Hamma (Ham), Californian (Cal), Magna601 (Mag601), Erfoud1 (Erf1), Mamuntanas (Mam), Agsalfa (Ags), Super Aurora (SupA), Sardi10 (Sar10) and ML99 (ML99).

Our results showed that the studied varieties possess different mechanisms against *Phoma medicaginis* infection. The Gabès variety seems to replace its infected leaves with new ones and

it develops its biomass normally to survive, as found by Djébali (2013). The varieties ML99, Hamma, Agsalfa, Super Aurora and Sardi10 reorients their mechanisms to elongate in order to escape infection. However their biomass was strongly affected, as described by Djébali (2013) that *Phoma medicaginis* reduces shoot fresh weight in *M. truncatula* plants. Furthermore, Mamuntanas, Magna601, Erfoud1 and Californian enhanced their root growth to maximize the surface contact with the soil, which is a similar observation as found by Djébali (2013) who associated this observation with survival.

Cluster analysis showed that the studied varieties were classified into three groups (Fig. 5.). The first group is composed of the Gabès2355 variety characterized by the highest values of growth parameters (NHL, SFW, RFW and NMS). The second group is formed by Mamuntanas and Californian varieties with highest values of RDW. The third group is constituted of the remaining varieties with an intermediate behavior. According to Djébali (2013) the percentage of healthy leaves (NHL) is the most discriminating parameter between lines of *M. truncatula* in terms of responses to *P. medicaginis*. As mentioned by Tlahig et al. (2017), the varieties with extreme values from group 1 and group 3 will be useful for breeding programs.

3. CONCLUSION

Overall, a high level of diversity occurred in responses of the studied varieties to *Phoma medicaginis* infection. The varieties were classified into three groups according to their response to *P. medicaginis* infection, with one tolerant variety, five which are moderately tolerant, and four which are susceptible. The two contrasting varieties Gabès and Magna-601 could be useful for the identification and characterization of physiological and genetic determinants for *M. sativa* tolerance to *P. medicaginis* infection.

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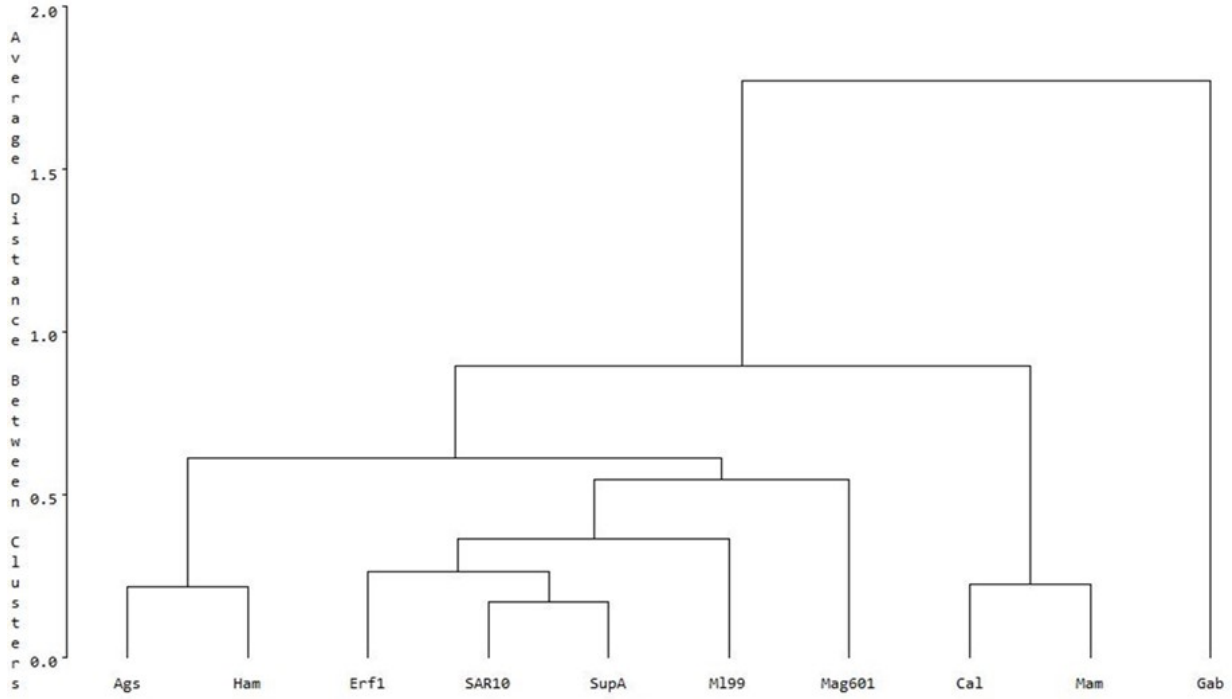

Fig. 5. Cluster analysis of the studied varieties of *Medicago sativa* infected by *Phoma medicaginis.* Group 1 (G1), group 2 (G2), group 3 (G3), Gabès2355 (Gab), El Hamma (Ham), Californian (Cal), Magna-601 (Mag601), Erfoud1 (Erf1), Mamuntanas (Mam), Agsalfa (Ags), Super Aurora (SupA), Sardi10 (Sar10), and ML99 (ML99)

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