Medicago truncatula in Interaction with *Fusarium* and *Rhizoctonia* Phytopathogenic Fungi: Fungal Aggressiveness, Plant Response Biodiversity and Character Heritability Indices

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(Received on February 4, 2021; Revised on May 6, 2021; Accepted on May 20, 2021)

Fusarium and *Rhizoctonia* genera are important pathogens of many field crops worldwide. They are constantly evolving and expanding their host range. Selecting resistant cultivars is an effective strategy to break their infection cycles. To this end, we screened a collection of *Medicago truncatula* accessions against *Fusarium oxysporum*, *Fusarium solani*, and *Rhizoctonia solani* strains isolated from different plant species. Despite the small collection, a biodiversity in the disease response of *M. truncatula* accessions ranging from resistant phenotypes to highly susceptible ones was observed. A17 showed relative resistance to all fungal strains with the lowest disease incidence and ratings while TN1.11 was among the susceptible accessions. As an initiation of the characterization of resistance mechanisms, the antioxidant enzymes' activities, at the early stages of

infections, were compared between these contrasting accessions. Our results showed an increment of the antioxidant activities within A17 plants in leaves and roots. We also analyzed the responses of a population of recombinant inbred lines derived from the crossing of A17 and TN1.11 to the infection with the same fungal strains. The broad-sense heritability of measured traits ranged from 0.87 to 0.95, from 0.72 to 0.96, and from 0.14 to 0.85 under control, *F. oxysporum*, and *R. solani* conditions, respectively. This high estimated heritability underlines the importance of further molecular analysis of the observed resistance to identify selection markers that could be incorporated into a breeding program and thus improving soil-borne pathogens resistance in crops.

Keywords : biodiversity, *Fusarium oxysporum*, *Medicago truncatula*, recombinant inbred lines, *Rhizoctonia solani*

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Handling Editor : Inhwa Yeam

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Fungal diseases are becoming a serious problem to the majority of plants, and associated with severe losses in their production around the world (Farooq et al., 2009). Breeding plants employing the approach of vertical resistance have been used as strategy to reduce these diseases' impacts such as with the rust fungi (Park, 2008). But no strong resistance was identified for many necrotrophic fungi, and this class of pathogens remain a sustainable issue for agriculture in many parts of the world (Anderson

et al., 2010). *Fusarium* and *Rhizoctonia* genera are such pathogens that are difficult to control and require the implication of several approaches to limit their impacts (Anderson et al., 2013; Landa et al., 2004). These pathogens are found globally and mentioned as ubiquitous soil inhabitant that cause severe diseases, such as vascular wilt, *Fusarium* crown and root rot in host plants which could be responsible of up to 100% yield loss in susceptible cultivars (Belete et al., 2013; Ramírez-Suero et al., 2010). *Fusarium* spp. can persist in affected soil as dormant chlamydospores, as microconidia on host plant's surface, and as mycelium or spores on dead tissues for a long period of time (Di Pietro et al., 2003). And *R. solani* exists in nature in the form of vegetative hyphae and sclerotia because of the scarcity of sexual spores and conidia (Tuncer and Eken, 2013).

For these pathogens, alternative approaches such as crop rotation and crop association have been employed as strategies to control their related diseases (Marburger et al., 2015; Peters et al., 2004). Legume crops are constantly used in rotation or association in agricultural systems due to their nutritional importance and protein richness and to their ability to make the soil more fertile by fixing atmospheric nitrogen into the soil (Graham and Vance, 2003; Stagnari et al., 2017; Trabelsi et al., 2012). Moreover, legumes help to control pathogens by breaking their life cycles. But this strategy have met with only little success because of the emerging of new variants that could infect new plant species (Anderson et al., 2013; Djébali et al., 2014).

This emergence of new variants warranted detailed investigations and better understanding of the mechanisms underlying quantitative or partial resistance, and the manipulation of these mechanisms in the attempt to provide enhanced resistance in crop plants and new molecular targets that may be used in crop efficient and durable breeding programs. The legume model plant, *Medicago truncatula*, is suited for comparative studies of pathogenic interactions (Ramírez-Suero et al., 2010; Rispail et al., 2010). It is a valuable resource to gain insight on important agronomic traits and to understand the mechanisms of resistance to diseases within grain legumes since it is a host plant for several pathogenic fungi that may cause yield losses in legumes fields (Cook, 1999; Rispail and Rubiales, 2014). As such, *M. truncatula* has been already used to decipher mechanisms of resistance towards biotrophic, hemibiotrophic, and necrotrophic pathogens (Djébali et al., 2009; Prats et al., 2007; Yang et al., 2008).

In Tunisia, soil-borne pathogens are very destructive to legumes and to other diverse crops (Boughalleb and Mahjoub, 2006; Jedidi et al., 2018; Samet et al., 2018). Among

the primary methods to controlling these diseases is the selection of resistant cultivars which can be used to dissect the mechanisms and markers of resistance. Utilizing a model host for broad-host-range fungal pathogens will facilitate this dissection. This study was designed and implemented to (1) assess the disease incidence (DI) of *Fusarium oxysporum* and *Fusarium solani*, *Vicia faba*-infecting isolates (Haddoudi et al., 2021), and of *Rhizoctonia solani* AG3 isolate, originally isolated from *Solanum tuberosum* and causes disease on *V. faba* (Djébali et al., 2014), on a collection of *M. truncatula* accessions and its relevant collection of recombinant inbred lines (RILs), and to (2) estimate the broad-sense heritability of some measured traits which will initiate the elucidation of resistance markers for breeding programs.

Materials and Methods

Plant material and growth conditions

Parental accessions and RILs. The studied accessions of *M. truncatula* are derived from natural populations from Tunisia (TN1.11, TN6.18, TN8.20, TN7.4, TN14.1, TN12.2, TN6.5, TN4.22, TN4.16, and TN2.12) collected from different eco-geographical sites (Arraouadi et al., 2009, 2011; Lazrek et al., 2009) with two reference accessions Jemalong A17 from the Australian collection and A20 from Moroccan collection (An et al., 2002). The RILs collection was consisted of nine lines (H1, H2, H4, H5, H6, H7, H8, H9, and H10), they were created by manually crossing A17, as the male parent, and TN1.11, as the female parent, to produce the F1 generation, which was later selfed. F2 generation was used in self-fertilization and single-seed descent for five generations at the Center of Biotechnology of Borj Cedria (CBBC) in Tunisia. Seeds of RILs are F6-derived.

Germination and growth conditions. Seeds of *M. truncatula* were surface scarified with sulfuric acid solution for 5 min and rinsed six times using sterile distilled water. After 2 h of imbibition in sterile distilled water, seed were transferred into petri dishes containing sterile wet filter paper and germinated in the dark for 3 days at 4°C and for 24 h at 25°C, always in the dark. The germinated seed were transplanted on medium M (Bécard and Fortin, 1988) in square petri dishes (12 cm × 12 cm). These dishes were closed with parafilm and placed in a growth chamber at 24°C/20°C at a photoperiod of 16 h light/8 h dark. In all experiments, each petri dish contained five seedlings of the same accession, with three replicate.

Experimental design. The experimental unit consisted of one square petri dish containing five seedlings of the same accession. The screen test of *M. truncatula* and RILs collections consisted of three replicate of the experimental unit for control (non-infected) plants, and for infected plants (with *F. oxysporum*, *F. solani*, and *R. solani*), arranged in randomized complete blocks. The data presented are from two independent experiments.

Fungal material, inoculum preparation and disease evaluation

Fusarium spp. *F. oxysporum* (KLR13) (GenBank reference no. MK615110) and *F. solani* (KLF13) (GenBank reference no. MK615111) initially isolated from *V. faba* (Haddoudi et al., 2021) were used in this study. The fungal strains were routinely grown on potato dextrose agar (PDA) at 25°C with a photoperiod of 16 h light and 8 h obscurity. To prepare the spore suspensions for both strains, six fungal agar-discs (6 mm in Ø) from 21-day-old culture were sub-cultured in 100 ml liquid potato dextrose broth (PDB) medium at 27°C with shaking at 160 rpm. The obtained suspensions were filtered and adjusted to 10^6 conidia/ml. To assess the disease on *M. truncatula* accessions, germinated seeds were placed on medium M, at rate of five seedlings per box (12 cm × 12 cm). Four days later, roots of seedlings were submerged in the spore suspension (10^6 conidia/ml) for 15 min, or in PDB solution for control, and then transferred again on medium M. 21 days post-inoculation (dpi) shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), and root dry weight (RDW) were measured. To closely evaluate the DI, symptoms related to the leaf level and the whole plant level were scored every 4 days from 5 dpi to 21 dpi and to estimate the disease symptom rate (DR) at the leaf and the whole plant level and to calculate the area under the disease progression curve (AUDPC) as described by Bani et al. (2012). Another parameter was calculated as the proportion of dead plants for each accession, the DI.

Rhizoctonia solani AG3. *R. solani* strain, RS1.2, initially collected from *S. tuberosum* L., and infecting *V. faba* (Djébali et al., 2014) was routinely left to culture on PDA medium at 25°C under 16 h of florescent light and 8 h of obscurity and stored on PDA slants at 4°C in the dark. The *R. solani* inoculum was prepared as described by Anderson et al. (2013). Briefly seven agar-discs (9 mm in Ø) from 7-day-old culture were sub-cultured in 100 ml PDB medium for 7 days at 25°C with shaking. The obtained mycelium, after draining broth, was crushed and homogenized in sterile water, then used for seedlings inoculation at a

concentration of 2.2 mg/ml. To inoculate seedlings with *R. solani*, 50 µl of the obtained mycelium, as described above, was deposited on the roots. Different parameters relating to different features of the disease were measured 21 dpi, mentioning SFW, SDW, RFW, and RDW. The number of surviving plants was determined for each square dish which was initially planted with five plantlets, to evaluate the seedlings damping off. These surviving plants were the support to score the number of healthy leaves (NHL), without any symptoms (chlorosis or necrosis), and to determine the RFW which can indicate the direct effect of the fungus.

Antioxidant enzymes assays. After the infection with *F. oxysporum* and *R. solani*, leaves and roots of A17, TN1.11, H7, and H6 were sampled at two time points 1 dpi and 3 dpi. Five hundred mg of frozen leaves and roots of control and infected seedlings were grinded to a fine powder in a mortar with liquid nitrogen. To extract soluble proteins, the powder was re-suspended, in 2 ml of extraction buffer containing: 50 mM potassium phosphate buffer (pH 7.8) 0.1 mM EDTA, 0.1 mM PMSF, and 10 mM DTT. The homogenate was then aliquoted in new tubes after centrifugation at 13,000 ×g for 20 min. Extracts were used immediately for enzymatic and protein assays or stored at -80°C. Protein concentration was measured using the Bradford (1976) method using a protein assay kit (Bio-Rad, Munched, Germany) and bovine serum albumin as a standard (Sigma, St. Louis, MO, USA). Guaiacol peroxidase (GPOX) activity was determined at 470 nm during 1 min ($\epsilon = 26.6/\text{mM}/\text{cm}$) by its ability to produce 1 µmol/min of guaiacol oxidized in the presence of H_2O_2 according to the method described by Anderson et al. (1995). Superoxide dismutase (SOD) activity was assayed according to the method of Yu and Rengel (1999) in measuring the ability of the enzyme to inhibit the photochemical reduction of NBT (Sigma). The reaction was initiated by the addition of riboflavin, and the absorbance (A_{560}), was measured after 10 min of incubation under the light. Catalase (CAT) activity is determined by following the decomposition of H_2O_2 at 240 nm ($\epsilon = 36/\text{M}/\text{cm}$) (Anderson et al., 1995). Four replicates for proteins content and enzymes activities were used.

Statistical analysis. All statistical analysis was performed with PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). To analyze the significance of differences between the treatments, one way analysis of variance (ANOVA) was performed and Tukey's *post hoc* test ($P \leq 0.05$) was applied. To get a clearer idea about the variability of the responses of *M. truncatula* accessions and RILs, we per-

Fig. 1. Disease symptoms induced by *Fusarium oxysporum* (KLR13), *F. solani* (KLf13), and *Rhizoctonia solani* (RS1.2) on different accessions of *Medicago truncatula*, 21 days post-inoculation (dpi). (A) A17. (B) TN1.11. (C) TN6.18. (D) A20. (E-K) *M. truncatula* in interaction with RS1.2: (E) non-infected, (F) A17 showing some root rot, (G) A20 showing root rot and necrosis on cotyledons, (H) TN6.18 showing root rot, dead cotyledons, and chlorosis on first real leaves, (I) TN1.11 showing root rot and crown and beginning of seedlings damping off, (J) TN8.20 showing seedlings damping off, and (K) TN4.22 showing dead plants. Three replicates were analyzed each with five seedlings.

formed a principal component analysis (PCA). To calculate the broad-sense heritability (H^2) for all measured parameters in RILs experiment, the variance components σ_g^2 (genotypic variance) and σ_e^2 (error variance) were estimated. Genetic variance was calculated considering the line effect as a random effect, while error variance was performed considering the treatment and line as fixed effects.

$H^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2/K)$ Where K is the number of replicates per line.

Results

Disease development

Fusarium oxysporum. *In vitro* culture was performed to assess the DI of 12 *M. truncatula* accessions towards *F. oxysporum* (KLR13). Seedlings were inoculated with *F.*

oxysporum and parameters were analyzed at 21 dpi or were recorded periodically. Variability in responses to the infection was observed among the studied accessions. *F. oxysporum* caused severe disease symptoms on the majority of accessions and reduced SDW and RDW (Supplementary Table 1) and no total resistant behavior was revealed. Accessions such as A17 were the least affected with intermediate symptoms on cotyledons and dark-brown discoloration on the roots (Fig. 1A). TN1.11 showed more susceptibility with dead cotyledons, chlorotic first leaves and development of fungal mycelium on the root surface and root growth was reduced (Fig. 1B). Accessions such as TN6.18 and A20 were highly susceptible and they showed early symptoms development on leaves and roots and at 21 dpi all seedlings were dead with massive development of fungal mycelium on all the plantlet (Fig. 1C and D). The

Table 1. Disease ratings of differential lines of *Medicago truncatula* to *Fusarium oxysporum* (KLR13) and to *F. solani* (KLf13)

Line	Strain	DI (%)	Disease ratings ^a			
			Plant mean disease index		Leaves showing symptoms (%)	
			VI	AUDPC	PS	AUDPC
TN1.11	KLR13	33.3	3.8 ± 0.44 b	39.9 ± 4.71 b	56.2 ± 6.03 b	583.3 ± 63.29 b
A17	KLR13	0	1.0 ± 0.00 d	10.5 ± 0.00 d	8.75 ± 2.29 c	91.8 ± 24.09 c
TN6.18	KLR13	100	4.8 ± 0.06 a	50.7 ± 0.63 a	100.0 ± 0.00 a	1,050 ± 0.00 a
TN8.20	KLR13	100	5.0 ± 0.00 a	52.5 ± 0.00 a	100.0 ± 0.00 a	1,050 ± 0.00 a
A20	KLR13	100	5.0 ± 0.00 a	52.5 ± 0.00 a	100.0 ± 0.00 a	1,050 ± 0.00 a
TN2.12	KLR13	0	2.2 ± 0.09 b	22.6 ± 1.01 c	38.4 ± 9.63 b	404.2 ± 101.12 b
TN4.16	KLR13	100	5.0 ± 0.00 a	52.5 ± 0.00 a	100.0 ± 0.00 a	1,050 ± 0.00 a
TN4.22	KLR13	0	2.7 ± 0.17 b	28.7 ± 1.84 c	54.7 ± 4.76 b	575 ± 50.00 b
TN6.5	KLR13	100	5.0 ± 0.00 a	52.5 ± 0.00 a	100.0 ± 0.00 a	1,050 ± 0.00 a
TN12.2	KLR13	100	5.0 ± 0.00 a	52.5 ± 0.00 a	100.0 ± 0.00 a	1,050 ± 0.00 a
TN14.1	KLR13	100	5.0 ± 0.00 a	52.5 ± 0.00 a	100.0 ± 0.00 a	1,050 ± 0.00 a
TN7.4	KLR13	100	5.0 ± 0.00 a	52.5 ± 0.00 a	100.0 ± 0.00 a	1,050 ± 0.00 a
TN1.11	KLf13	55.5	3.7 ± 0.17 b	39.9 ± 1.87 b	68.5 ± 4.45 bc	719.6 ± 46.73 bc
A17	KLf13	0	1.0 ± 0.00 g	10.5 ± 0.00 g	8.9 ± 2.22 f	93.3 ± 23.83 f
TN6.18	KLf13	44.4	3.3 ± 0.27 bc	34.7 ± 2.88 bc	56.7 ± 7.5 bcd	595.9 ± 78.81 bcd
TN8.20	KLf13	0	1.5 ± 0.19 fg	15.9 ± 2.02 fg	51.1 ± 5.77 bcd	536.6 ± 60.66 bcd
A20	KLf13	100	5.0 ± 0.00 a	52.5 ± 0.00 a	100.0 ± 0.00 a	1,050.0 ± 0.00 a
TN2.12	KLf13 KLf13	41.6	2.7 ± 0.11 cd	29.3 ± 1.2 cd	53.1 ± 4.81 bcd	557.1 ± 50.60 bcd
TN4.16	KLf13	0	2.2 ± 0.3 def	23.1 ± 3.22 def	68.5 ± 5.89 bc	719.2 ± 61.89 bc
TN4.22	KLf13	100	5.0 ± 0.00 a	52.5 ± 0.00 a	100.0 ± 0.00 a	1,050.0 ± 0.00 a
TN6.5	KLf13	0	2.6 ± 0.00 cde	27.3 ± 0.00 cde	40.0 ± 0.00 de	420.0 ± 0.00 de
TN12.2	KLf13	0	1.0 ± 0.00 g	10.5 ± 0.00 g	20.0 ± 0.00 ef	210.0 ± 0.00 ef
TN14.1	KLf13	0	2.4 ± 0.08 de	25.2 ± 0.85 de	47.9 ± 2.83 cd	503.1 ± 29.77 cd
TN7.4	KLf13	41.6	2.9 ± 0.16 bcd	31.1 ± 1.70 bcd	71.5 ± 3.61 b	750.6 ± 37.98 b

DI, disease incidence; VI, mean value of visual index of all leaves on an individual plant; AUDPC, area under the disease progression curve; PS, percentage of leaves having symptoms for each individual plants.

^aData are means of three replicates, each with five seedlings; different letters means significant difference between values according to Tukey's HSD range test at $\alpha = 5\%$. Each strain and each parameter was analyzed separately.

Fig. 2. Principal component analysis (PCA) (A) on five variables measured for the 12 accessions of *Medicago truncatula* infected with *Fusarium oxysporum* according mainly to PC1 (97.88% of the total variance and defined by VI, PS, and their AUDPC); (B) on five variables measured in interaction with *F. solani* according to PC1 (90.718% of the total variance and defined by VI, its AUDPC, and DI) and to PC2 (7.091% of the total variance and defined by PS and its AUDPC); and (C) on four variables measured in interaction with *R. solani* according to PC1 (48.66% of the total variance and defined by RDW, RFW, and SP) and to PC2 (47.42% of the total variance and defined by NHL). (D) PCA on four variables measured for the 9 RILs in interaction with *F. oxysporum* according mainly to PC1 (91.998% of the total variance and defined by VI, PS, and their AUDPC). (E) PCA on four variables measured in interaction with *F. solani* according to PC1 (84.827% of the total variance and defined by VI, its AUDPC, and DI) and to PC2 (13.081% of the total variance and defined by PS and its AUDPC). (F) PCA on six variables measured in the interaction with *R. solani* according to PC1 (46.306% of the total variance and defined by SDW, RDW, SFW, RFW, HL, and SP) and to PC2 (31.576% of the total variance and defined by SDW, SFW, and SP). Three replicates were analyzed each with five seedlings. VI, mean value of visual index of all leaves on an individual plant; PS, percentage of leaves having symptoms for each individual plants; AUDPC, area under the disease progression curve; DI, disease incidence; RDW, root dry weight; RFW, root fresh weight; SP, number of plants surviving seedling damping off; NHL, number of healthy leaves; RIL, recombinant inbred line; SDW, shoot dry weight; SFW, shoot fresh weight. .

DI ranged from 0 to 100%, and DR varied from 1.0/8.75% to 5/100% according to mean value of visual index of all leaves on an individual plant (VI) and to percentage of leaves having symptoms for each individual plant (PS) parameters, and from 10.5/91.8 to 52.5/1,050 for their respective AUDPC values (Table 1). To closely segregate contrasting behaviors of lines we performed a PCA taking into account five variables (Fig. 2A). The presentation of the first two components (PC1 = 97.88%, PC2 = 1.503%) showed three groups, the first one is positively correlated with PC1 and PC2 showing thereby the highest DI and DR values, the second group is negatively correlated with PC2 and presents lower DI and high DR values, and the last one is negatively correlated with both components and this group contains only A17 with the lowest DI and DR values.

***F. solani*.** *F. solani* (KLf13) was less aggressive compared to *F. oxysporum*. A17 showed always a resistant phenotype with symptoms on cotyledons and some root rot (Fig. 1A). Its DI was 0%, DR scores were 1.0/8.9% for VI and PS parameters, the respective AUDPC values were 10.5/93.3

(Table 1). TN1.11 and TN6.18 remained among the susceptible accessions with affected cotyledons and later-formed leaves which in some individual plants were dried and dead, and appearance of root rot for all seedlings (Fig. 1B and C). DI for the majority of lines ranged from 0 to 41.6% and DR from 1/20% to 2.9/71.5% for VI and PS parameters and from 10.5/210 to 31.1/750.6 for their respective AUDPC values (Table 1). PCA presented two components, PC1 with 90.718% of total variance and defined by DI, VI and its AUDPC and PC2 with 7.091% of total variance defined by PS and its AUDPC (Fig. 2B). A17 was in the group negatively correlated with PC1 and PC2 with the lowest DI and DR, in the contrary to TN1.11 which showed higher DI and DR.

***M. truncatula* as a host for *R. solani*.** At 21 dpi, moderate pathogenicity of *R. solani* was observed. RS1.2 caused the typical disease symptoms including crown rot and seedlings damping off (Fig. 1G, H, and K). The seedlings damping off which was observed only for some accessions such as TN1.11, TN8.20, and A20, occurred until the full expansion of the first true leaf associated with root rot and reduc-

Table 2. Disease ratings of differential inbred lines to *Fusarium oxysporum* (KLR13) and to *F. solani* (KLf13)

Line	Strain	DI (%)	Disease ratings ^a			
			Plant mean disease index		Leaves showing symptoms (%)	
			VI	AUDPC	PS	AUDPC
H1	KLR13	77.7	4.8 ± 0.85 a	50.5 ± 8.99 a	93.3 ± 6.66 a	980.0 ± 70.00 a
H2	KLR13	0	3.7 ± 0.62 ab	39.4 ± 6.60 ab	61.7 ± 7.26 bc	647.5 ± 76.28 bc
H4	KLR13	88.8	4.5 ± 0.38 a	47.4 ± 4.60 a	88.8 ± 7.34 ab	933.3 ± 77.16 ab
H5	KLR13	0	1.7 ± 0.13 bc	18.5 ± 1.37 bc	52.6 ± 5.09 cd	552.8 ± 53.50 cd
H6	KLR13	77.7	5.0 ± 0.00 a	52.5 ± 0.00 a	90.5 ± 9.52 ab	950.0 ± 100.00 ab
H7	KLR13	0	1.4 ± 0.05 c	14.6 ± 0.58 c	25.8 ± 2.98 d	270.4 ± 31.33 d
H8	KLR13	0	3.3 ± 0.35 abc	35.0 ± 3.70 abc	62.7 ± 2.01 bc	658.7 ± 36.65 bc
H9	KLR13	44.4	4.2 ± 0.46 a	43.7 ± 4.81 a	83.3 ± 4.16 ab	875.0 ± 43.75 ab
H10	KLR13	50	4.2 ± 0.12 a	44.5 ± 1.33 a	66.3 ± 4.33 abc	696.6 ± 45.52 abc
H1	KLf13	100	5.0 ± 0.00 a	52.5 ± 0.00 a	100.0 ± 0.00 a	1,050 ± 0.00 a
H2	KLf13	0	1.0 ± 0.00 e	10.5 ± 0.00 e	40.0 ± 0.00 cd	420 ± 0.00 cd
H4	KLf13	30.55	3.5 ± 0.39 bc	36.3 ± 4.10 bc	59.9 ± 6.73 bc	628.6 ± 70.69 bc
H5	KLf13	0	2.6 ± 0.05 cd	27.3 ± 0.60 cd	42.6 ± 2.70 cd	447.7 ± 28.29 cd
H6	KLf13	0	1.7 ± 0.04 de	17.5 ± 0.44 de	83.3 ± 16.66 ab	875 ± 175.00 ab
H7	KLf13	0	1.0 ± 0.00 e	10.5 ± 0.00 e	20.0 ± 0.00 d	210 ± 0.00 d
H8	KLf13	66.66	4.6 ± 0.19 ab	48.45 ± 2.02 ab	97.2 ± 2.77 a	1,020.8 ± 29.16 a
H9	KLf13	41.66	3.6 ± 0.66 bc	38.5 ± 7.00 bc	40.0 ± 10.00 cd	420 ± 105.00 cd
H10	KLf13	0	1.0 ± 0.00 e	10.5 ± 0.00 e	40.0 ± 0.00 cd	420 ± 0.00 cd

DI, disease incidence; VI, mean value of visual index of all leaves on an individual plant; AUDPC, area under the disease progression curve; PS, percentage of leaves having symptoms for each individual plants.

^aData are means of three replicates, each with five seedlings; different letters means significant difference between values according to Tukey's HSD range test at $\alpha = 5\%$.

tion in the root biomass. All the plantlets of A17 survived seedlings damping off and developed less severe symptoms with limited cankers on the root and brown lesions on cotyledons (Fig. 1F). As a direct effect of the fungus, it reduced significantly RFW and NHL for the majority of

the collection (Fig. 3A and B). To discriminate between different groups in the collection based on their responses, we performed a PCA taking into account four variables (Fig. 2C). The obtained graphic showed different groups based on PC1 = 48.66% of total variance and PC2 = 47.42% of

Fig. 3. Performance of *Medicago truncatula* accessions and recombinant inbred lines (RILs) following infection with *Rhizoctonia solani*. (A) Root fresh weight (RFW) of *M. truncatula* accessions. (B) Number of healthy leaves of *M. truncatula* accessions (NHL). (C) RFW of RILs. (D) NHL of RILs. (□) correspond to non-infected (C, control) plants. (■) correspond to *R. solani*-infected plants. All measurements were taken at 21 days post-inoculation. Different letters means significant difference between values according to Tukey' HSD range test at $P = 0.05$. Data are means of three replicates each with five seedlings.

total variance. The first one represents the least affected accessions (A17) with the least affected RFW, RDW and surviving plants. The group gathering (TN1.11, TN4.22, TN12.2, and TN6.5) negatively correlated with PC1, thus

representing the lowest RFW and RDW. A20, TN8.20, and TN2.12 are the most susceptible accessions with low RFW, RDW, NHL, and surviving plants.

Fig. 4. Effect of the inoculation with *Fusarium oxysporum* (KLR13) and *Rhizoctonia solani* (RS1.2) on the activity of guaiacol peroxidase (GPOX), superoxide dismutase (SOD), and catalase (CAT) in leaves and roots of A17, TN1.11, H6, and H7 at two sampling dates 1 day post-inoculation (dpi) and 3 dpi. (A) Activity of GPOX in leaves of A17 and TN1.11. (B) Activity of GPOX in roots. (C) Activity of CAT in leaves. (D) Activity of CAT in roots. (E) Activity of SOD in leaves. (F) Activity of SOD in roots. (G) Activity of GPOX in leaves of H6 and H7. (H) Activity of GPOX in roots. (I) Activity of SOD in leaves. (J) Activity of SOD. (□) corresponds to non-infected plants (C). (■) corresponds to KLR13-infected plants. (▒) corresponds to RS1.2-infected plants. *Means significant differences between control and its respective fungal infection at $P = 0.05$. Data are means $\pm$ SD of four replicates.

Disease assessment of RILs population

***F. oxysporum*.** A population of 9 RILs at F6 was tested against *F. oxysporum*. Twenty-one dpi the same parameters, previously recorded with the natural collection of *M. truncatula*, were taken. *F. oxysporum* developed symptoms on all RILs with DI ranging from 0% to 88.8%, DR from 1.4/25.8% to 4.8/93.3% according to VI and PS parameters and from 14.6/270.4 to 50.5/980 to their respective AUDPC values (Table 2). The PCA (Fig. 2D) confirmed the distribution given by the DR scores: H7 was in the group negatively correlated with (PC1 = 91.998%) with the lowest values of DI and DR. while the group positively correlated with PC1 (H1, H6, H8, and H4) presented the least resistant inbred lines.

***F. solani*.** Infection with *F. solani* developed disease symptoms on all RILs with variability in responses. DI ranged from 0% to 100%, DR from 1/20% to 5/100% for VI and PS parameters, and from 10.5/210 to 52.5/1,050 for their respective AUDPC values (Table 2). H7 and other lines revealed more resistance to this fungus with the lowest DI and DR (Table 2). While H6 for example showed more susceptibility with high PS and AUDPC. This distribution of RILs was highlighted by the PCA analysis (Fig. 2E) that segregated lines based on two factors: PC1 with 84.82% of total variance and defined by VI and its AUDPC and DI, PC2 with 13.08% of total variance defined by PS and its AUDPC.

Susceptibility of RILs population to *R. solani*. SFW, SDW, and RDW results (Supplementary Table 2) showed that *R. solani* showed moderate severity on the inbred collection generally. RS1.2 decreased significantly RFW of all RILs (Fig. 3C). However, no significant effect was observed

on the NHL except for H6 (Fig. 3D). To have clearer idea about the distribution of the population, we proceeded to a PCA analysis on six parameters (Fig. 2F). The graphic presented two principal components, PC1 = 46.306% and PC2 = 31.576%, the first component gathered mainly the NHL, while SFW, SDW and number of surviving plants were positively correlated with PC1 and PC2. As a consequence, the group situated in the positive side of both components, representing H7, can be considered as the most resistant inbred line to *R. solani*. While the group negatively correlated with both components represents the least resistant lines mentioning H6, H1, and H10.

Investigation of oxidative stress response during plant fungi interaction.

These analyses were performed on the already selected couples and under infection with *F. oxysporum* and *R. solani*, at two different time points. At 1 dpi (Fig. 4A and B), a significant increase, compared to control conditions, of GPOX activity by 51.69% and 58.97% in leaves and roots of A17-RS1.2 interaction was observed respectively, while within TN1.11 a significant decrease in both tissues and under both fungi was observed. Three dpi, GPOX activity increased significantly in leaves and roots of A17-KLR13 and of TN1.11-RS1.2 interactions. CAT activity, at 1 dpi, increased in roots of A17 infected with *R. solani* by 88.94% when compared to control plants. This enhancement was sustained at 3 dpi, and accompanied by an increase of the activity in leaves by 38.56%. Also at 3 dpi, CAT activity increased in leaves and roots of A17 infected with *F. oxysporum* increased by 74.27% and 80.10%, respectively. For TN1.11, in interaction with both fungi, CAT activity marked a decrease (Fig. 4C and D). SOD activity, at 1 dpi, significantly increased in roots of A17-*F. oxysporum* interaction, by 84.55%. At 3 dpi, an en-

Table 3. Means $\pm$ SD, genotypic variance σ_g^2 , environmental variance σ_e^2 , and heritability (H^2) for six traits measured within RILs of *Medicago truncatula* cultivated under control and *Fusarium oxysporum* (KLR13) inoculation

	SFW	SDW	RFW	RDW	AUDPC (VI)	AUDPC (PS)
Control						
Means	133.66 $\pm$ 32.22	8.53 $\pm$ 2.50	91.99 $\pm$ 13.71	2.99 $\pm$ 0.71	0.00	0.00
σ_g^2	188.326	0.69	133.117	0.149	-	-
σ_e^2	22.629	0.03	10.207	0.022	-	-
H^2	0.89	0.95	0.93	0.87	-	-
KLR13						
Means	80.55 $\pm$ 44.55	6.50 $\pm$ 2.74	64.80 $\pm$ 28.56	2.74 $\pm$ 0.67	38.47 $\pm$ 14.54	729.39 $\pm$ 239.88
σ_g^2	198.607	0.712	58.169	0.085	45.646	25,443.58
σ_e^2	8.080	0.069	3.576	0.012	156.154	53,728.36
H^2	0.96	0.91	0.94	0.87	0.72	0.81

RIL, recombinant inbred lines; SFW, shoot fresh weight; SDW, shoot dry weight; RFW, root fresh weight; RDW, root dry weight; AUDPC (VI), area under the disease progression curve using VI values; AUDPC (PS), area under the disease progression curve using PS values.

hancement of this activity by 62.06% and 85.46% in leaves and roots was scored. Within TN1.11, inoculation with *R. solani* enhanced SOD by 63.43% in leaves and by 85.47% in roots (Fig. 4E and F).

The inbred lines showed a little difference in their antioxidant enzymes activities compared to parental accessions. At 1 dpi, roots of H7 recorded an increase in GPOX activity, compared to control, by 81.38% under *F. oxysporum* infection and by 61.35% under *R. solani* (Fig. 4G). While in H6, this activity increased in leaves by 26.90% compared to control under *R. solani* infection. Three dpi, GPOX activity remained high in roots of H7 in interaction with RS1.2, with concomitant increase in leaves activity as well (Fig. 4G and H). For H6, GPOX in leaves increased under infection with KLR13. SOD activity significantly increased within H7 leaves, at 1 dpi and after infection with RS1.2, values increased from 92.64 unit/mg protein in control to 147.68 unit/mg protein (Fig. 4I). For roots, SOD activity was 17.14 unit/mg protein and 105.70 unit/mg protein for control plants of H6 and H7, respectively. When infected with *F. oxysporum*, values increased by 70.33% and 14.12% in H6 and H7 respectively (Fig. 4J).

Heritability. On average all RILs showed similar phenotypes as parental accessions, either the relatively resistant (A17) or the susceptible (TN1.11), under *F. oxysporum* and *R. solani* infections. To evaluate the importance of scored parameters in the illustration of the noticed phenotypic variability among studied RILs, an estimation of broad-sense heritability (H^2) of these parameters was done. All traits showed high heritability values (≥ 0.87) for control conditions and under *F. oxysporum* inoculation (Table 3). The same high levels in heritability were observed in *R. solani* treatments and its control conditions (≥ 0.76) except

for SDW (Table 4).

Discussion

A collection of *M. truncatula* accessions and a population of RILs were screened against *F. oxysporum*, originally isolated from *V. faba*, in a controlled environment using methods previously developed by Bani et al. (2012). Many pathosystems involving *M. truncatula* and root-infecting pathogens were studied to identify the degree of resistance and susceptibility within this plant model (Ben et al., 2013; Djébal et al., 2009; Vaillau et al., 2007). In all these pathosystems, explanatory set of parameters was commonly used to estimate the variability of resistance within *M. truncatula* accessions. This set was based on scoring symptoms such as chlorosis/necrosis on leaves, restriction of the root growth, and the concomitant effect on the whole plant. Our results revealed variability in responses of the studied accessions from relative resistance to high susceptibility based on DI and DR related parameters. As expected from previous studies such as Rispaill et al. (2015), our results confirmed that the inoculation with *F. oxysporum* caused typical wilt symptoms, leaf chlorosis and necrosis, complete wilting and death of the plants of the susceptible and the moderately susceptible accessions. Ramírez-Suero et al. (2010) used a *M. truncatula*–*F. oxysporum* f. sp. *medicagenis* pathosystem based on a small hydroponic culture, and after using a visual scale 0–5 based on the appearance of chlorosis or necrosis on leaves, they identified A17 as the susceptible one. In our study we used a similar pathosystem but with a solid medium instead, after calculating DI and scoring DR with more detailed parameters, we found A17 as the most resistant accession. The rest of the collection showed moderate and high susceptibility with a

Table 4. Means $\pm$ SD, genotypic variance σ_g^2 , environmental variance σ_e^2 , and heritability (H^2) for six traits measured within RILs of *Medicago truncatula* cultivated under control and *Rhizoctonia solani* inoculation

	SFW	SDW	RFW	RDW	NHL	SP
Control						
Means	133.66 $\pm$ 32.22	8.53 $\pm$ 2.50	91.99 $\pm$ 13.71	2.99 $\pm$ 0.71	5.56 $\pm$ 0.69	5 $\pm$ 0.00
σ_g^2	188.326	0.69	133.117	0.149	4.539	5.252
σ_e^2	22.629	0.03	10.207	0.022	0.499	0.686
H^2	0.89	0.95	0.93	0.87	0.90	0.88
RS1.2						
Means	121.36 $\pm$ 28.34	6.90 $\pm$ 2.77	87.92 $\pm$ 15.22	3.11 $\pm$ 0.64	2.39 $\pm$ 1.54	2.44 $\pm$ 0.84
σ_g^2	123.430	0.027	168.527	0.101	242.152	4.482
σ_e^2	41.828	0.157	28.272	0.021	76.119	0.950
H^2	0.74	0.14	0.85	0.82	0.76	0.82

RIL, recombinant inbred lines; SFW, shoot fresh weight; SDW, shoot dry weight; RFW, root fresh weight; RDW, root dry weight; NHL, number of healthy leaves; SP, number of plants surviving seedling damping off.

DI up to 100%. The resistance observed within A17 was concomitant to a significant increase in SOD, CAT, and GPOX activities. This early activation (1 dpi and 3 dpi) can be due to the involvement of the apoplastic reactive oxygen species (ROS) in producing an oxidative burst to impede the progression of the fungus towards conducting vessels (Camejo et al., 2016; García-Limones et al., 2002; Gay and Tuzun, 2000). This underlies the importance of further analyzing the ROS contribution to the observed resistant phenotype.

With *F. solani*, which is a major factor of root rot and *Fusarium* crown, the tested collection showed variability in their responses. DR parameters showed low pathogenicity of *F. solani* on *M. truncatula* accessions which may be explained by its specific host range since it showed a high pathogenicity against *V. faba*, its host plant, as presented in the study of Belete et al. (2013). Despite the limited number of accessions used in this study, this *M. truncatula* collection contained biodiversity in its response, from highly resistant (such as A17) to moderately resistant (such as TN8.20 and TN6.5) to susceptible lines (such as TN1.11 and A20), and this can provide new genetic variation that underlines this wide range of responses to *F. solani*.

R. solani is a widespread soil born pathogen, it causes big losses on wide range of crops such as legumes crops (Anderson et al., 2016). In our study we used strain of *R. solani* from AG3 originally isolated from potato and known to infect Faba bean (Djébali et al., 2014) to inoculate our collection of *M. truncatula*. To assess the effect of the infection with *R. solani*, we evaluated two characteristics of the disease phenotype: first, scoring the number of the plants surviving the seedlings damping off, and secondly, estimating the direct effect of the fungus by measuring the RFW and calculating the NHL on each surviving plants. RS1.2 developed the typical symptoms of disease, root rot and crown symptoms, but the accessions responded differently to the infection. Some such as TN1.11, TN8.20, TN4.22, and A20 were found among the most susceptible accessions, while A17 was amongst the least susceptible ones. In a previous study (Anderson et al., 2013), it was reported that *M. truncatula* is a potential host for AG8 strains of *R. solani*, though AG8 is known for its pathogenicity against cereals, with a biodiversity in responses of different accessions. *R. solani* AG3 is a major pathogen of the potato crop, thus resistance in legume crops would have benefits with respect to effective crop rotation strategies. Our findings showed that *M. truncatula* represents a potential host that can be used to determine the mechanisms acting in these resistant accessions at the cellular and molecular levels.

Previous studies (Ameline-Torregrosa et al., 2008; Ben

et al., 2013; Djébali et al., 2009; Vailleau et al., 2007; Yang et al., 2008) used RILs populations derived from the cross between *M. truncatula* accessions for QTL analysis to facilitate the advancement of genetic studies once biodiversity in responses was observed. We assessed the disease caused by *F. oxysporum*, *F. solani*, and *R. solani* to differentiate between the inbred lines based on their resistance level. The scored parameters revealed similar phenotypes (resistance and susceptible) of RILs compared to the parental lines A17 or TN1.11 and the broad-sense heritability (H^2) analyses of some measured parameters showed high values in control conditions and under fungal stress. These findings highlight the importance of a QTL mapping to identify loci contributing to moderate resistance to *F. oxysporum* and *R. solani*. Such analysis can provide new insights into how the resistance mechanisms may be employed in crops to enhance resistance to these and other similar intractable pathogens.

In this study two genera of fungi, *Fusarium* and *Rhizoctonia*, were used to screen a collection of *M. truncatula* accessions and RILs population. Our results showed a high susceptibility within *M. truncatula* towards *F. oxysporum*, and variability in responses to *R. solani*. This screening allowed us to identify contrasting couple, TN1.11 as susceptible and A17 as relative resistant accession. This resistance was concomitant to an increasing in the activity of the protective antioxidant enzymatic system, peroxidase at an early stage of infection and catalase and superoxide dismutase at another stage of the infection. The contrasting behavior of A17 and TN1.11 towards these fungi was observed within their RILs. The high estimated broad-sense heritability of some measured traits underlines the importance of pursuing to the identification of genomic regions (QTLs) which contributed to this resistance, and thus understanding the mechanisms of basal resistance and trying to identify selection markers of resistant cultivars to root pathogens.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

The author acknowledge Jamila Hammemi and Fathi Barhoumi, technicians at Laboratory of Legumes, CBBC, for their technical assistance in the procedure of the screening test.

This work was supported by the [Tunisian Ministry of

Higher Education and Scientific Research] in the frame of the work program of L₂AD-CBBC, 2019-2022.

Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (<http://www.ppjonline.org/>).

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