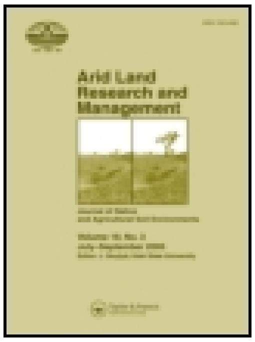

Arid Land Research and Management

ISSN: 1532-4982 (Print) 1532-4990 (Online) Journal homepage: http://www.tandfonline.com/loi/uasr20

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To cite this article: Mohammed Mouradi, Mohamed Farissi, Abdelaziz Bouizgaren, Bouchra Makoudi, Ablaa Kabbadj, Anne-Aliénor Very, Hervé Sentenac, Ahmed Qaddourya & Cherki Ghoulam (2015): Effects of water deficit on growth, nodulation and physiological and biochemical processes in Medicago sativa-rhizobia symbiotic association, Arid Land Research and Management, DOI: 10.1080/15324982.2015.1073194

To link to this article: http://dx.doi.org/10.1080/15324982.2015.1073194

ARID LAND RESEARCH AND MANAGEMENT
2016, VOL. 00, NO. 00, 1–16
http://dx.doi.org/10.1080/15324982.2015.1073194

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Effects of water deficit on growth, nodulation and physiological and biochemical processes in *Medicago sativa*-rhizobia symbiotic association

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ABSTRACT
The effects of water deficit on growth, nodulation, and several physiological and biochemical processes in six symbiotic combinations involving three Moroccan alfalfa (*Medicago sativa* L.) populations (Tafilalet1, Adis-Tata and Demnate2), an American Moapa variety and two rhizobial strains (RhL9 and RhL10) were studied. The experiment was conducted under greenhouse conditions. Seedlings were separately inoculated with the suspension of two rhizobial strains and grown under two irrigation regimes: 80% of field capacity (optimal irrigation) and 40% of field capacity (water deficit). The water stress was applied for five weeks and the agro-physiological and biochemical parameters related to water deficit tolerance were assessed. The results showed that the water deficit had significantly reduced the height of the plants, the dry biomass and nodulation. This constraint also negatively affected the relative water content of leaves, the membrane permeability, the stomatal conductance, the maximum quantum yield of photosystem II, the time to reach maximum fluorescence, the total chlorophyll content and the total nitrogen content. Comparison among the tested symbiotic combinations showed that their behaviors were significantly different. Under drought, oasis populations Ad and Ta maintained high PS II efficiency, membrane stability, relative water content, chlorophyll and nitrogen content in comparison to the mountain one Dm2 and the American Moapa variety. These parameters were maintained at adequate levels in the plants inoculated with the rhizobial strain RhL9 that showed a tolerance to water deficit conditions.

ARTICLE HISTORY
Received 22 February 2015
Accepted 13 July 2015

KEYWORDS
Alfalfa; chlorophyll fluorescence; nodulation; tolerance; water deficit

Introduction

Medicago sativa L. has a very favorable influence on soil fertility. It provides more nitrogen to the agricultural ecosystems then the total amount of nitrogen applied by fertilization (Rengel 2002). This species has the ability to make a specific symbiotic interaction with the soil bacteria known as rhizobia. On roots of alfalfa, rhizobia elicit the formation of nodules by the secretion of lipochito oligosaccharidic Nod factors (NFs). This organ contains differentiated forms of rhizobia called bacteroids, which reduce dinitrogen into

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NH_4^+. This then is assimilated by the host plant (Masson-Boivin et al. 2009), which provides carbon and energy sources to bacteroids in the form of succinate and malate (Lodwig and Poole 2003). Better N_2-fixing symbiosis requires greater capacity of alfalfa genotypes to establish interactions with tolerant rhizobia even under conditions of stress.

In Moroccan traditional agro-ecosystems, such as oasis and mountains, local populations of alfalfa contribute strongly to the socio-economic development of local families as a principal source for livestock nutrition (Bouizgaren, Kallida, and Al Faiz 2010). However, the environmental constraints such as water deficit and salinity, recorded in many world regions particularly arid and semi-arid areas, affect legume plants growth and the performance of their symbiosis with rhizobia (Terzi et al. 2010; Faghire et al. 2011; Latrach et al. 2014). Indeed, drought is one of the major abiotic stresses that limit plant growth and productivity of many *Medicago* species (Chebouti, Mefti, and Abdelguerfi 2001; Bouizgaren et al. 2013). This constraint causes many agro-physiological and biochemical changes that can be observed at the whole plant level as the plant dies and/or decreases in their productivity. Almost all of these changes are associated with activation of some physiological and biochemical processes allowing an adaptation to this constraint. Under water deficit conditions, several studies have shown that this constraint negatively affects legumes-rhizobia symbiosis by promoting nodules senescence and reducing their number (Ashraf and Iram 2005), reducing their leghemoglobin content and nitrogenase activity (Figueiredo et al. 2008). The stomatal closure, causing limitation of CO_2 assimilation and subsequently a strong perturbation of photosynthetic activity, and the decrease in relative water contents were reported under water deficit (Saruhan Güler et al. 2012; Bouizgaren et al. 2013; Amini, Arzani, and Karami 2014).

Chlorophyll fluorescence has recently become one of the greatest tools to indicate stress and stress adaptation of plants. It may be detected before any physical or biochemical signs in tissues that might be manifested in plants under drought stress (Lichtenthaler et al. 2007). Additionally, one of the major consequences of this abiotic stress are observed in altering metabolic activities is the change of the redox homeostasis leading to oxidative stress by the liberation of reactive oxygen species (Baloğlu et al. 2012; Kavas et al. 2013). As a result, the induction of antioxidant enzyme activities is a general adaptation strategy that plants use to overcome oxidative stress (Mirzaee, Moieni, and Ghanati 2013).

Understanding legumes-rhizobia responses to drought is of great importance and a fundamental part of making symbiotic combinations stress-tolerant. In this context, the present work aims to assess the effects of water deficit on growth and nodulation in eight symbiotic associations involving three Moroccan alfalfa (*M. sativa* L.) populations, an American Moapa variety and two rhizobial strains isolated from the southeast region of Morocco. The study was focused on some agro-physiological and biochemical criteria with the hypothesis that the tolerance of alfalfa-rhizobia symbiosis to water deficit could be closely related to some determining parameters among growth and nodulation, relative water content, membrane permeability, stomatal conductance, maximum quantum yield of photosystem II (PS II), time to reach maximum fluorescence, and chlorophyll content.

Materials and methods

Plant culture and treatment

The experiments of this study were carried out under greenhouse conditions with an approximate temperature of 30/20°C (day/night), 50–80% of relative humidity and 16 h photoperiod (twenty-two Klux) at the Faculty of Sciences and Techniques, Marrakesh. Three Moroccan alfalfa (*M. sativa* L.) populations, Tafilalet1 (Ta), Adis-Tata (Ad), and Demnate2 (Dm2) and an American Moapa variety (Mo) were used as plant material. The first three populations originated from the southeast oasis, the southwest oasis and the High Atlas mountains of Morocco, where they have been cultivated for many centuries and are still widely used by farmers in their traditional agro-ecosystems (Bouizgaren et al. 2013). Seeds, supplied by the National Institute of Agronomic Research (INRA, Marrakesh), were surface-disinfected by immersion in ethanol 95% for 30 s and in 5% sodium hypochlorite for 5 min and then rinsed several times with sterile deionized water. Seeds were germinated in Petri dishes for 24 h and transferred to plastic pots measuring 12 cm tall and 16 cm diameter. Each pot contained 1300 g of sterile sand and peat with the proportion 4:1 respectively and the number of seedlings adjusted to ten per pot. Seven days after stage of true leaves, seedlings were separately cross inoculated with 10 mL of RhL9 and RhL10 bacterial suspensions. Each containing approximately 10^8 CFU/mL (CFU = Colony-forming unit). The rhizobial strains were isolated from nodules of alfalfa grown in soils from the southeast region of Morocco; each strain was previously subjected to an infectivity test under aseptic conditions and tested for its tolerance to different levels of water deficit induced by PEG 6000. Plants were watered alternately two times a week with distilled water and nitrogen free nutrient solution during the trial period (Bargaz et al. 2013). One week after inoculation, half of the plants (randomly selected) of each symbiotic combination were subjected to water deficit conditions (40% of field capacity), while the other half were kept under optimal irrigation conditions (80% of field capacity). To sustain field capacity during the experiment, the pots were weighed before irrigation. The stress was applied for five weeks and after harvest some agro-physiological and biochemical parameters related to water deficit tolerance were assessed. Three pots containing 10 plants each were used per symbiotic combination, per treatment and each pot was considered as a replicate.

Dry weight measurement

For the dry weight (DW) measurement, alfalfa plants were harvested, shoots and roots were separated, and nodules were root detached carefully. All plant parts were dried at 70°C for 48 h, then, the DW was determined on four plants for each treatment.

Plant height and leaf area

The plant's height was used to assess the effect of water deficit on the plants growth compared to the controls. It was measured on five plants per pot. Leaf area was determined at the end of the stress period in three plants from each treatment. This parameter was assessed by image analysis using Image J software available as a free download from National Institutes of Health (http://rsb.info.nih.gov/ij/index.html).

Relative water content (RWC)

RWC was estimated by recording the turgid weight (TW) of leaflet samples of 0.1 g fresh weight (FW) by keeping them in distilled water for 4 h as described by Ghoulam, Foursy, and Fares (2002), followed by drying in a hot air oven until constant weight was achieved (DW). RWC defined as follows:

$$RWC(\%) = ((FW - DW)/(TW - DW)) \times 100 \tag{1}$$

Three replicates per treatment per symbiotic combination were considered.

Membrane permeability (electrolyte leakage)

To determine the effect of water deficit on membrane stability, the electrolyte leakage (EL) was assessed as described by Ghoulam, Foursy, and Fares (2002) using young fresh healthy leaves. Firstly, samples were washed three times by deionized water to remove any surface-adhered electrolytes. Then placed in closed vials with 10 mL of deionized water and incubated at 25°C on a rotary shaker (100 t/min) for 24 h subsequently. Electrical conductivity of the solution (C_1) was determined using a (Hannah Instruments HI8820 N) conductivity meter. Samples were then autoclaved at 120°C for 20 min and the last electrical conductivity (C_2) was measured again after equilibration at 25°C. The electrolyte leakage was defined as follows:

$$\text{Electrolyte leakage}(\%) = (C_1/C_2) \times 100 \tag{2}$$

Six plants per treatment per symbiotic combination were considered and grouped as three replicates.

Stomatal conductance

Stomatal conductance is based on the measure of CO_2 entering rate, or water vapor exiting through the leaf stomata. This parameter was measured in mmol $H_2O/m^2 \cdot s$ on healthy leaves using a porometer (Leaf porometer, model SC1, DECAGON DEVICES, version 2012) under a temperature of 25 ± 1°C and a relative humidity of 55 ± 5%. Six plants per treatment per symbiotic combination were considered and grouped as three replicates.

Chlorophyll fluorescence: Maximum quantum yield of PS II and time to reach maximum fluorescence

The chlorophyll fluorescence was measured using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England) after 15 to 20 min of dark adaptation. Maximum quantum efficiency of Photosystem II (PSII) (Fv/Fm) was calculated as Fv/Fm = (Fm-Fo)/Fm, where Fo are maximal and minimal fluorescence of dark-adapted leaves respectively, and Fv is variable fluorescence (Jifon and Syvertsen 2003). It measures the fluorescence signal (Fo) received from dark adapted leaves using a low fluorescence intensity when all reaction centers are open, and the measure of maximum saturation (Fm) when all reaction centers are closed. The Photosystem II efficiency was also evaluated under stress by TFm (time to Fm), a parameter used to indicate the time at which the maximum fluorescence value was reached. Six plants per treatment per symbiotic combination were considered and grouped as three replicates.

Total chlorophyll content

Total chlorophyll concentration was measured by the method described by Arnon (1949). It was extracted with acetone in a mortar, using a proportion of 200 mg of fresh leaf tissue and 5 ml of acetone (80%, v/v). Chlorophyll concentration was measured after centrifugation (10 min at 5000 × g), the absorbance (OD) of the supernatant was measured at 663 and 645 nm. Total chlorophyll was determined using the following formula:

$$\text{Total Chlorophyll} = 8.02\,\text{OD}_{663} + 20.20\,\text{OD}_{645} \tag{3}$$

Three replicates per treatment per symbiotic combination were considered.

Total nitrogen content

Total nitrogen content was determined using the Kjeldahl method (Faghire et al. 2011; Bargaz et al. 2012). A dry leaf sample of 0.5 g was placed in Buchi tubes with 1 g of catalyst mixture (100 g of potassium sulfate, 20 g of copper sulfate and 2 g of selenium powder) and 10 mL of concentrated sulfuric acid. The digestion of the organic matter was held for 2 h at 400°C. After 5 min of distillation, the distillate was recuperated in H_3BO_3 solution at 1% and titrated by hydrochloric acid (N/100). The total nitrogen content was calculated and expressed in mg/plant. Three replicates per treatment per symbiotic combination were considered.

Statistical analysis

The statistical analysis was performed using SPSS (10.0) software. Two-way analysis of variance (ANOVA II) was performed using three replicates per combination per treatment for almost all studied parameters. The mean values and standard errors were calculated. Tukey’s test was applied for means comparison of the considered parameters.

Results

Effect of water deficit on growth

The total dry biomass was significantly affected by water deficit (Table 1). Irrigation by 40% FC reduced also, leaf area, plant height and shoot/root ratio in symbiotic interactions involving Ta, Ad, Dm2 and Mo alfalfa populations and two rhizobial strains RhL9 and RhL10 as represented in Table 2. For dry weights, the eight symbiotic combinations were affected differently by water deficit ($p < 0.001$, Table 1). Table 1 shows highly significant decreases ($p < 0.001$) in shoots, roots and nodules dry weight of stressed plants compared to their respective controls. We noted that Ad plants inoculated with rhizobial strain RhL9 were less affected, 14.3 and 12.2% reduction by this constraint in shoot and root parts respectively, while the most significant reductions of shoots was observed in Ta and Dm2 populations inoculated with RhL10 strain by 80.3 and 80.8% respectively. Symbiotic combinations involving rhizobial strain RhL9 have developed an intermediate behavior in terms of shoot and roots dry biomass (Table 1). For nodule dry weight, most of the symbiotic combinations associating RhL9 and alfalfa populations studied have shown weaker

Table 1. Effect of water deficit on: Shoot dry weight (SDW), root dry weight (RDW), and nodule dry weight (NDW) in four alfalfa populations (Ta, Ad, Dm2, and Mo) inoculated with rhizobial strains RhL9 and RhL10.

Symbiotic combinations	Total Biomass (g/plant)			Shoots dry weight (g/plant)			Roots dry weight (g/plant)			Nodule dry weight (g/6 plants)		
	Irrigation treatments		Reduction %	Irrigation treatments		Reduction %	Irrigation treatments		Reduction %	Irrigation treatments		Reduction %
	80% FC	40% FC		80% FC	40% FC		80% FC	40% FC		80% FC(1)	40% FC(2)	
Ta-RhL9	0.670 ± 0.022^{b}	0.308 ± 0.009^{d}	54	0.437 ± 0.012^{ab}	0.128 ± 0.009^{efg}	70.7	0.233 ± 0.010^{def}	0.180 ± 0.003^{fg}	22.7	20.75 ± 0.51^{ab}	14.15 ± 0.84^{cd}	31.8
Ta-RhL10	0.670 ± 0.024^{b}	0.182 ± 0.024^{ef}	72.7	0.352 ± 0.013^{cd}	0.069 ± 0.021^{g}	80.3	0.317 ± 0.022^{b}	0.113 ± 0.012^{hi}	64.3	23.22 ± 1.2^{a}	9.72 ± 0.81^{d}e	58.1
Ad-RhL9	0.662 ± 0.012^{b}	0.562 ± 0.009^{c}	15	0.363 ± 0.014^{cd}	0.311 ± 0.008^{d}	14.3	0.287 ± 0.004^{bcd}	0.252 ± 0.002^{cde}	12.2	23.46 ± 1.2^{a}	17.52 ± 0.74^{bc}	25.3
Ad-RhL10	0.617 ± 0.011^{bc}	0.232 ± 0.011^{def}	62.3	0.330 ± 0.014^{d}	0.089 ± 0.009^{fg}	73	0.299 ± 0.003^{bc}	0.144 ± 0.01^{gh}	51.8	20.25 ± 1.2^{ab}	7.52 ± 1.01^{e}	62.8
Dm2-RhL9	0.608 ± 0.018^{bc}	0.270 ± 0.018^{de}	55.6	0.406 ± 0.002^{abc}	0.150 ± 0.009^{ef}	63	0.202 ± 0.017^{ef}	0.120 ± 0.011^{hi}	40.6	24.27 ± 1.04^{a}	11.52 ± 0.85^{de}	52.5
Dm2-RhL10	0.682 ± 0.011^{b}	0.158 ± 0.006^{f}	76.7	0.417 ± 0.005^{abc}	0.080 ± 0.005^{fg}	80.8	0.265 ± 0.008^{bcd}	0.078 ± 0.003^{i}	70.5	19.8 ± 0.68^{ab}	7.95 ± 1.19^{e}	59.8
Mo-RhL9	0.874 ± 0.018^{a}	0.296 ± 0.03^{d}	66.1	0.463 ± 0.018^{a}	0.159 ± 0.015^{e}	65.6	0.410 ± 0.006^{a}	0.136 ± 0.019^{gh}	66.8	20.97 ± 0.8^{ab}	8 ± 0.14^{e}	61.8
Mo-RhL10	0.683 ± 0.03^{b}	0.227 ± 0.001^{def}	66.8	0.377 ± 0.034^{bcd}	0.092 ± 0.002^{efg}	75.6	0.305 ± 0.004^{bc}	0.134 ± 0.002^{gh}	56	22.1 ± 0.5^{a}	7.5 ± 0.5^{e}	66
	dF	F		dF	F		dF	F		dF	F	
Symbiosis	3	17.37**–*		3	8.45***–*		3	13.07***–*		3	12.45***–*	
Water deficit	1	2044.96***–*		1	1409.43***–*		1	742.31***–*		1	665.65***–*	
Interactions	3	23.49***–*		3	13.33***–*		3	27.96***–*		3	4.84**–*	

Note: Values are means of four replicates ± standard errors.

*: Significance at 0.05 probability level; **: Significance at 0.01 probability level; ***: Significance at 0.001 probability level; NS: Not significant at 0.05.

Table 2. Effect of water deficit on plant height, leaf area, and shoot/root ratio in four alfalfa populations (Ta, Ad, Dm2, and Mo) inoculated with rhizobial strains RhL9 and RhL10.

	Plant height (cm)			Leaf area (cm^2)			Shoot/root ratio (four plants)	
	Irrigation treatments			Irrigation treatments			Irrigation treatments	
Symbiotic combinations	80% FC	40% FC	Reduction %	80% FC	40% FC	Reduction %	80% FC	40% FC
Ta-RhL9	32 ± 1.2^{ab}	17.6 ± 1.5^{defg}	44.8	302.8 ± 6.4^{bc}	113.7 ± 5.8^{f}	62.4	1.87 ± 0.048^{a}	0.7 ± 0.056^{de}
Ta-RhL10	28 ± 1.5^{abc}	15 ± 0.6^{fgh}	46.4	284 ± 8^{bcd}	99.6 ± 5.9^{f}	64.9	1.13 ± 0.095^{bcd}	0.62 ± 0.183^{e}
Ad-RhL9	33.3 ± 1.8^{a}	21.6 ± 0.9^{cdef}	35	361.8 ± 9^{a}	167.2 ± 8.4^{e}	53.7	1.21 ± 0.05^{bc}	1.23 ± 0.029^{bc}
Ad-RhL10	30.3 ± 1.5^{ab}	18.6 ± 0.9^{defg}	38.4	317.2 ± 6.1^{b}	115.9 ± 5.8^{f}	63.4	1.15 ± 0.06^{bcd}	0.63 ± 0.08^{e}
Dm2-RhL9	27.6 ± 0.9^{abc}	17 ± 1^{efgh}	38.5	257.5 ± 4.7^{d}	100.4 ± 5.8^{f}	61	2.05 ± 0.16^{a}	1.26 ± 0.086^{bc}
Dm2-RhL10	25.3 ± 1.2^{bcd}	12 ± 2.1^{gh}	52.6	270.7 ± 8.9^{cd}	93.5 ± 4.9^{f}	65.4	1.57 ± 0.042^{ab}	1.01 ± 0.048^{cde}
Mo-RhL9	27.2 ± 1.7^{abc}	16.1 ± 2.1^{fgh}	40.6	286.3 ± 7.1^{bcd}	91.9 ± 6.7^{f}	67.8	1.13 ± 0.053^{bcd}	1.21 ± 0.15^{bc}
Mo-RhL10	24.4 ± 1.9^{bcd}	9.9 ± 2.3^{h}	59.4	281.2 ± 11^{bcd}	83.1 ± 6.3^{f}	70.4	1.23 ± 0.19^{bc}	0.68 ± 0.024^{de}
	dF	F		dF	F		dF	F
Symbiosis	3	0.192NS		3	9.64***–*		3	0.83NS
Water deficit	1	274.16***–*		1	2768.83***–*		1	112.95***–*
Interactions	3	0.54NS		3	0.53NS		3	11.02***–*

Note: Values are means of three replicates ± standard errors.

*: Significance at 0.05 probability level; **: Significance at 0.01 probability level; ***: Significance at 0.001 probability level; NS: Not significant at 0.05.

reductions under water deficit comparative to other symbiosis, especially in symbiotic combination Ad-RhL9 (25.3%). We observed that this abiotic constraint has negatively affected plant height and leaf area ($p < 0.001$, Table 2) in all tested symbiotic combinations with significant differences in their behaviors. Under water deficit conditions, the symbiotic combination Ad-RhL9 showed the least reduction percentage in terms of leaf area and plant height (35 and 53.7% respectively, Table 2) in comparison with other combinations. The highest values were recorded with this symbiotic combination under optimal irrigation (80% FC). For shoot/root ratio, this parameter was significantly reduced ($p < 0.001$, Table 2) under 40% FC in tested alfalfa genotypes. Associations Ad-RhL9 and Mo-RhL9 seem not to be affected by water deficit in terms of this ratio (Table 2).

Effect on relative water content

The growth of alfalfa-rhizobia symbiosis under water restriction conditions reduced significantly ($p < 0.001$, Table 3). The leaves RWC in all of the studied symbiotic combinations, with the exception of the combination Ad-RhL9 for which the reduction did not exceed 2.05% (Figure 1). For other combinations the reduction percentage ranged from 18.5% in Dm2-RhL10 to 5.61% in Ta-RhL9, respectively. When the culture was well watered, the highest RWC were noted in seedlings inoculated with RhL10 (Figure 1).

Effect on membrane permeability

Figure 2 shows that water deficit caused highly significant increases of leaves electrolyte leakage in almost all plants grown under this constraint ($p < 0.001$, Table 3). The comparison between the different combinations studied showed that the lowest percentages of electrolyte leakage were observed in the symbiotic combinations involving Ad population and the two-rhizobial strains tested (19.64 and 18.54% for Ad-RhL9 and Ad-RhL10, respectively). In contrast, the highest values in terms of this parameter have been observed in other combinations, particularly symbiotic combinations Ta-RhL9 (30.51%) and Mo-RhL10 (27.86%).

Effect on stomatal conductance

The restriction of irrigation by 40% of field capacity significantly reduced the stomatal conductance in all of the studied symbiotic combinations ($p < 0.001$, Table 3, Figure 3).

Table 3. Results of two-way analysis of variance (ANOVA II) of water deficit and symbiotic combination effects and their interactions for the considered parameters.

	Independent variables					
	Symbiotic combination		Water deficit		Interactions	
Dependent variables	dF	F	dF	F	dF	F
Relative water content	3	17.18***–*	1	133.75***–*	3	1.75NS
Electrolyte leakage	3	4.20*	1	238.83***–*	3	0.13NS
Stomatal conductance	3	8.84***–*	1	823.16***–*	3	2.43NS
Fv/Fm	3	7.10**–*	1	645.43***–*	3	8.58***–*
TFm	3	2.10NS	1	772.54***–*	3	11.32***–*
Chlorophyll content	3	9.58**–*	1	655.56***–*	3	19.07***–*
Nitrogen content	3	5.73**–*	1	1838.94***–*	3	0.32NS

*: Significance at 0.05 probability level; **: Significance at 0.01 probability level; ***: Significance at 0.001 probability level; NS: Not significant at 0.05.

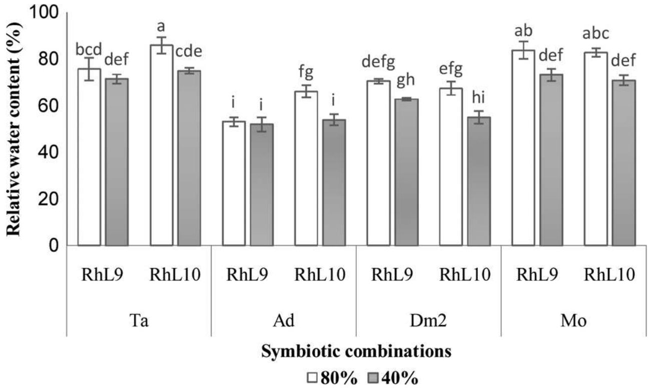

Figure 1. Effect of water deficit on relative water content in four Moroccan alfalfa genotypes (Ta, Ad, Dm2, and Mo) inoculated with rhizobial strains RhL9 and RhL10. Values are means of three replicates and bars represent standard errors.

We noted that plants inoculated with RhL9 showed higher reductions of this parameter compared to their relative controls. With this constraint, the parameter varied between 84.13 and 190.16 mmol of $H_2O/m^2.s$ in Ta-RhL9 and Ad-RhL10, respectively. Compared to their respective controls, percentages of reductions were 73.96, 70.14, and 49.6% in symbiotic combinations Ad-RhL9, Ta-RhL9, and Ad-RhL10, respectively.

Effect on maximum quantum yield of PS II and time to reach maximum fluorescence (tfm)

Figure 4 illustrates the effect of water deficit on quantum yield of PS II in symbiotic interaction involving four alfalfa populations and two rhizobial strains, RhL9 and RhL10. Results showed that water deficit caused a decrease of PS II activity, evaluated by Fv/Fm ratio, in all eight tested symbiotic combinations ($p < 0.001$, Table 3). We noted that when the inoculation was done with strain RhL9 and under drought conditions, the plants developed the highest values in terms of this parameter comparative to those inoculated with RhL10. The values recorded in Ta-RhL9 and Ad-RhL9 were respectively 0.77 and

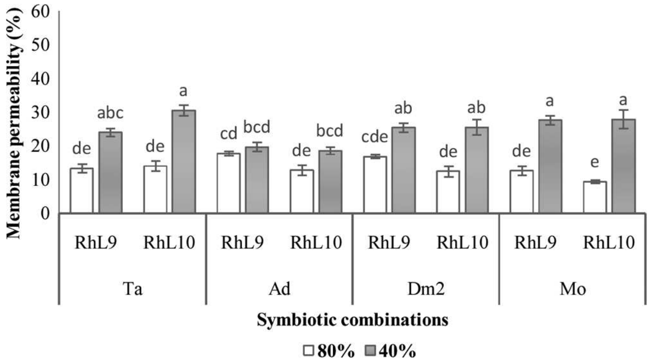

Figure 2. Effect of water deficit on electrolyte leakage in four Moroccan alfalfa genotypes (Ta, Ad, Dm2, and Mo) inoculated with rhizobial strains RhL9 and RhL10. Values are means of three replicates and bars represent standard errors.

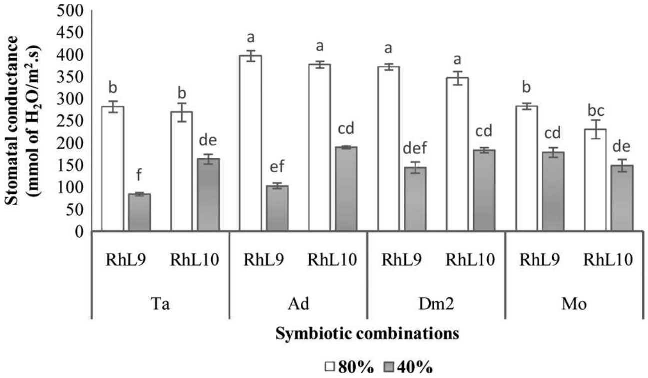

Figure 3. Effect of water deficit on stomatal conductance in four Moroccan alfalfa genotypes (Ta, Ad, Dm2, and Mo) inoculated with rhizobial strains RhL9 and RhL10. Values are means of three replicates and bars represent standard errors.

0.78. However, the values did not exceed 0.68 in other symbiotic combinations. The time to reach maximum fluorescence (Fm) under water restriction is shown in Figure 5. These results indicate that all symbiotic combinations took less time to reach the Fm with water deficit, Ad and Ta populations showed the least reductions with water deficit in this parameter when the symbiosis includes RhL9 strain (193 and 170 ms respectively). The other symbiotic combinations developed the same behavior, and values varied from 102 to 145 ms under drought constraint (Figure 5).

Effect on total chlorophyll content

Figure 6 shows the effect of water deficit on total chlorophyll contents. The results showed that total chlorophyll contents in all of the studied symbiotic combinations were significantly reduced by water stress ($p < 0.001$, Table 3) with significant differences between these combinations ($p < 0.001$, Table 3). The least reductions were observed in Ad-RhL9 and Ta-RhL9 with reduction of 5.93 and 7.53% respectively in comparison to controls. However, the reductions reached 69.76% in Ad-RhL10 combination, 85.06% and 88.34% for both symbiosis Mo-RhL9 and Mo-RhL10, respectively (Figure 6).

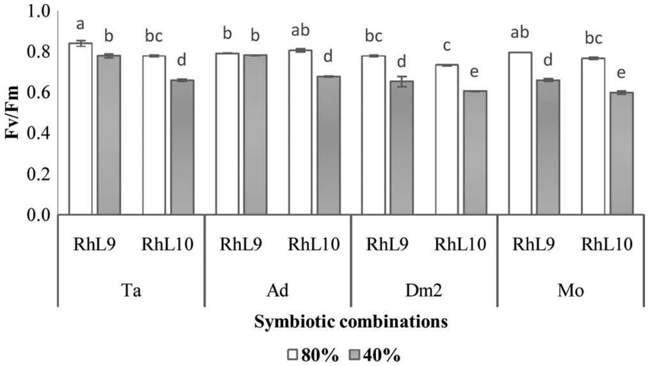

Figure 4. Effect of water deficit on Chlorophyll fluorescence in four Moroccan alfalfa genotypes (Ta, Ad, Dm2, and Mo) inoculated with rhizobial strains RhL9 and RhL10. Values are means of three replicates and bars represent standard errors.

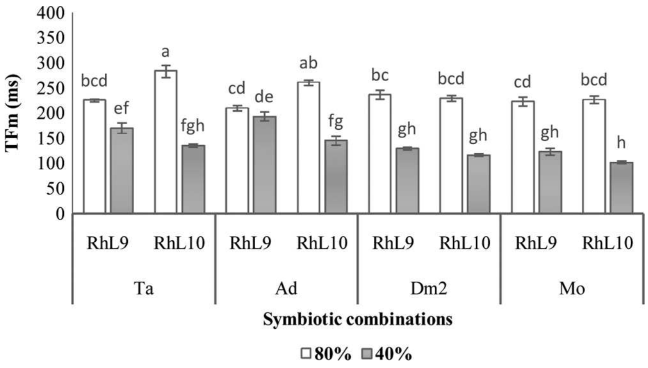

Figure 5. Effect of water deficit on TFm (time at maximum fluorescence) in four alfalfa genotypes (Ta, Ad, Dm2, and Mo) inoculated with rhizobial strains RhL9 and RhL10. Values are means of three replicates and bars represent standard errors.

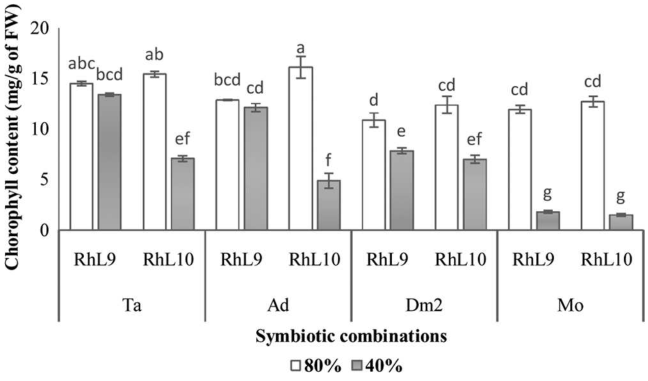

Figure 6. Effect of water deficit on chlorophyll content in four alfalfa genotypes (Ta, Ad, Dm2, and Mo) inoculated with rhizobial strains RhL9 and RhL10. Values are means of three replicates and bars represent standard errors.

Effect on nitrogen content

Figure 7 shows that drought stress induced significant ($p < 0.001$, Table 3) decrease of the total nitrogen content in plants in all of the studied symbiotic associations. The associations

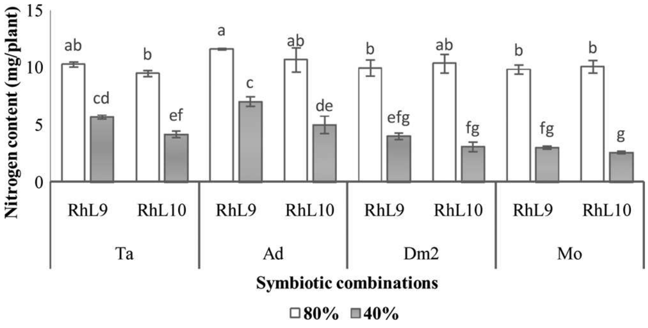

Figure 7. Effect of water deficit on total nitrogen content in four alfalfa genotypes (Ta, Ad, Dm2, and Mo) inoculated with rhizobial strains RhL9 and RhL10. Values are means of three replicates and bars represent standard errors.

involved RhL9 strain presented the least percentage reduction, especially with Ad and Ta populations, which presented reductions of 39.4 and 44.6%, respectively. The associations Mo-RhL10 and Dm2-RhL10 were the most affected with reductions of 74.1 and 70.09%, respectively.

Discussion

The effects of water deficit on symbiotic combinations involving three Moroccan alfalfa (*Medicago sativa* L.) populations, an American Moapa variety and two rhizobial strains isolated from the southeast region of Morocco were assessed. The restriction of water irrigation by 40% of field capacity caused a significant reduction in shoot, root and nodule dry weights, leaf area, plants height, and shoot/root ratio compared to the control conditions (irrigation at 80% of field capacity). Results obtained in this study indicated that the behavior of tested symbiotic combinations was significantly different for almost all of the analyzed parameters. The plants inoculated with the RhL10 strain appeared to be the most sensitive to this constraint, while biomass reductions were lower in plants inoculated with the strain RhL9. The effect of water deficit on plants growth has been documented by Farissi et al. (2013), Benabderrahim et al. (2013) and Erice et al. (2010) on some Moroccan populations of *Medicago sativa* L., such as Tafilalet1, Tata, and Demnate. The decrease in shoot and root dry biomass in stressed plants could be the result of a reduction of leaf expansion and the stomata number that limit water loss through transpiration. Thereby, it limits CO_2 assimilation, disrupts the photosynthetic activity resulting in the decrease of shoot biomasses, as well as the inhibition of photosynthates transport to nodules (Bekki, Trinchant, and Rigaud 1987; Antolín, Muro, and Sánchez-Díaz 2010).

In this study, we observed a significant variation in the behaviors of studied symbiotic associations under water deficit. It has been suggested that a combination between stress-tolerant rhizobia and some stress-tolerant forage cultivars may enhance the plants ability to grow and survive under drought conditions (Zahran 1999: Mhadhbi et al. 2009). Ben Rhomdhan et al. (2009) reported that, in general, rhizobial strains seem to be more tolerant to drought then host plants, and the formers present a variation between them for growth and surviving under this constraint. The reduction of nodule biomass under water deficit could be explained primarily by the decrease in the number and diameter of root hairs, or the inhibition of the emergence and elongation of these bodies (Zahran and Sprent 1986); secondly, by the reduction of rhizobia growth and the initiation and development of nodules (Saadallah et al. 2001; Antolín, Muro, and Sánchez-Díaz 2010). The water deficit induced a significant decrease in relative water content in the majority of studied symbiotic combinations. The combination Ad-RhL9 was able to maintain an adequate level in terms of this parameter. Significant reductions of RWC in *Medicago* submitted to this constraint has been reported by Nunes et al. (2008) and Maghsoodi and Razmjoo (2015). However, Aranjuelo et al. (2011) showed that no significant difference was detected between the stressed and unstressed control plants in alfalfa.

Maintaining an adequate level of RWC is probably related to a good osmotic adjustment capacity to preserve the structural and functional integrity of tissues (Farissi, Ghoulam, and Bouizgaren 2013). As well as RWC, the stomatal conductance was found to be reduced in all symbiotic combinations. This confirms the results found by Aranjuelo et al. (2011) and Leung et al. (2012). The fact that these associations exhibited a lower stomatal conductance when they presented high forage yield even under water-limited conditions, supports the hypothesis that the tolerant cultivars should develop mechanisms for better use of fixed CO_2. These mechanisms are related to differential day/night control (Rosales et al. 2012). In this study, no significant correlation was noted between leaf area and RWC and between stomatal conductance and RWC ($p > 0.05$), while leaf area was found positively correlated ($r^2 = 0.674$; $p < 0.01$) to stomatal conductance. It could be considered that it might have indirect positive effects on RWC by controlling the behavior of stomata under drought, maintaining leaf area and facilitating water absorption (Thameur, Lachiheb, and Ferchichi 2012). The rate of electrolyte leakage is considered a good physiological index that reflects the degree of cell membranes stability in the plants under stressful conditions (Ghoulam, Foursy, and Fares 2002; Farissi et al. 2013). An increase in electrolyte leakage indicates that the membrane integrity is affected. The results obtained in this study have shown a significant increase in this parameter under water deficit for all studied symbiotic combinations. The same has been reported by Slama et al. (2011) and Farissi et al. (2013). Chlorophyll fluorescence measurements could be used as a valid tool for the rapid screening of drought-tolerant alfalfa plants.

In the present study, we focused on the Fv/Fm ratio and TFm. The first parameter is commonly known as maximum quantum yield of primary photochemistry or maximal relative electron transport rate of PS II (Waldhoff, Furch, and Junk 2002) which is an indicator of the functional integrity of photosystem II (PSII) in dark-adapted leaves. The TFm is a parameter used to indicate the time at which the maximum fluorescence value (Fm) was reached. This parameter is used to indicate stress in plants, which causes the Fm to be reached much earlier than expected. For both parameters, the significant reductions were found after 35 days of drought. This decline could reflect the eventual disorder in PS II and photosynthetic apparatus damage in drought sensitive cultivars. In this study, we have noted a close relationship between the leaf water status and PS II efficiency. In fact, the symbiotic combinations, maintaining high level in RWC under stress, sustain a high Fv/Fm ratio that would be related to the lower injury of PS II performance. Based on our results, the decrease of chlorophyll content under drought stress was associated to the decrease in PS II performance this might be related to chlorophyll degradation that might cause photosynthetic inactivation (Blackburn 2007). The concentration of total chlorophyll decreased generally under water deficit due to its slow synthesis or fast degradation (Majumdar et al. 1991). Under water deficit, osmotic stress suppresses enzymes of metabolic pathways involved in N_2-fixation. These effects decrease nodule functioning and accelerate there early senescence (Mhadhbi et al. 2009). Several reports underlined the positive relationship between the increase of antioxidant defense enzymes expressions in nodules and the enhancement of symbiosis tolerance to stress (Naya et al. 2007). Nitrogen content was reduced in all associations under drought stress, Ad and Ta oasis populations showed important shoot nitrogen when inoculated by RhL9 strain. This decline is possibly due to nitrogen metabolism decrease (Antolín, Yoller, and Sinchez-Diaz 1995). In our work, the studied parameters Fv/Fm, TFm, and stomatal conductance reflected the differences between the most tolerant and less tolerant alfalfa genotypes in their responses to water deficit. Thus, selection and breeding for tolerance to water deficit could be based on these reliable parameters.

In summary, oasis-originated alfalfas inoculated by RhL9 strains showed better RWC, membrane integrity, PS II efficiency, chlorophyll and nitrogen content under water deficit.

Hence maintaining better growth under drought stress compared to those inoculated by RhL10. We concluded that water deficit negatively affected the assessed growth and nodulation parameters in all of the tested alfalfa-rhizobia combinations with differences between them. Indeed, combinations involving RhL9 rhizobial strain expressed more tolerance levels than the other combinations. Some of the studied parameters such as chlorophyll fluorescence and stomatal conductance reflected the differences between the most tolerant alfalfa genotype (Ad) and the less tolerant one (Dm2). Therefore, these parameters could be used as reliable criteria for selection and breeding for tolerance to water deficit in alfalfa.

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