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RESEARCH ARTICLE

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Antioxidant responses of alfalfa (*Medicago sativa* L.) nodules to water deficit stress

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ABSTRACT

The antioxidant responses of nodules to water deficit stress were studied in ten symbiotic combinations involving two Moroccan alfalfa populations (Tata and Dem) and five rhizobial strains (RHL2, RHL29, RHL48, RHL68 and RHL80). The experiment was conducted in a greenhouse at 32/22 °C d/n, 50-80% of relative humidity and a photoperiod of 16 h. The seedlings were separately inoculated with the suspensions of five rhizobial strains and grown under two water regime irrigations, 75% of Field capacity (optimal irrigation) and 25% of Field capacity (water deficit), in plastic pots filled with sterile sand and peat at 9/10 and 1/10 ratio, respectively. After 45 days of stress, the nodule biomass and some nodular antioxidant enzyme activities as peroxidase, polyphenol oxidase, catalase and superoxide dismutase were evaluated. The results indicated that the water deficit caused a significant reduction in nodule biomass with the significant differences between the tested symbiotic combinations. The reductions, comparatively to optimal irrigation conditions, varied from 18.63 to 30.14 %. The lowest reductions were marked with the symbiotic combination Tata-RHL80 and Tata-RHL2 (18.63 and 18.98% respectively). However, the highest reductions were observed when the seedlings were inoculated with the rhizobial strain RHL4 (30.14 and 28.54% for Dem-RHL4 and Tata-RHL4, respectively).The activities of the antioxidant enzymes analyzed were found increased in nodules of all combinations under water deficit and the high nodule biomass marked in some combinations was associated with the highest antioxidant activities suggesting that the enhancing of the antioxidant activities of these enzymes could play a critical role in water deficit tolerance.

Key words: alfalfa, water deficit, nodule, antioxidant enzymes

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a crop that has a very favorable influence on soil fertility by contributing to the incorporation of nitrogen in pastoral ecosystems with beneficial economic impact, helping to reduce or limit the use of chemical fertilizers by nitrogen-fixing symbiosis involving rhizobial strains [1]; [2]; [3]. In Morocco, alfalfa constitutes the first forage crop and occupies over 22% of the total area devoted to forage crops and over 80% of forage area in oasis agro-ecosystems. Local populations of this species are widely used in the Moroccan traditional agro-ecosystems, oasis and mountain, and strongly contributes to socio-economic development of local families [4]; [5]; [6]; [7]. However, the environmental constraints recorded in the arid and semi-arid ecosystems constitute the limiting factors for plant growth and productivity and affected the symbiotic nitrogen fixation [8]; [9]. In fact, water deficit recorded in many world regions is the major environmental factor limiting plant growth and productivity and constitutes an important constraint to alfalfa (*Medicago sativa* L.) production in Morocco and in many parts of the world [10]; [1].

Several studies have shown that the water deficit negatively affects legumes-rhizobia symbiosis by promoting nodules senescence and reducing their number [11], and reduce the leghemoglobin content in nodules and nitrogenase activity [12]. Baloğlu et al. (2012) noted that the one of the major consequences of this constraint is the alteration of the antioxidative metabolism leading to an oxidative stress induced by an overproduction of reactive oxygen species (ROS) [13].

To solve water deficit problem and benefit from nitrogen-fixing symbiosis, many symbiotic combinations tolerating to water deficit conditions must be selected and the understanding of the mechanisms involved in tolerance to this environmental factors will be helpful to enhance the productivity in the areas affected by this constraint. In this context, the present work aims to study the effect of water deficit conditions on symbiotic interaction associating two Moroccan alfalfa populations to five rhizobial stains isolate five from different areas of Morocco. The nodule biomass and the role of some nodular antioxidant responses in alfalfa-tolerance were analyzed and discussed.

MATERIALS AND METHODS

Plant materials and growth conditions

The Experiment was conducted under greenhouse conditions with an approximate temperature of 30/20°C (day/night) and 16 h photoperiod at the Faculty of Sciences and Techniques of Marrakesh, Morocco. Ten symbiotic combinations involving two Moroccan alfalfa populations, *Tata* and *Dem*, selected by their tolerance to water deficit conditions [1], and five rhizobial strains isolated from nodules of *Medicago sativa* L. grown in soils from different Moroccan areas [3]. These local strains have been previously subjected to infectivity test under aseptic conditions and evaluated for their tolerance to many environmental constraints [3]. The seeds were surface-sterilized with sodium hypochlorite (5%) for 5 min, rinsed four times with sterile deionized water and germinated in plastic pots filled with sterile sand and peat at 9/10 and 1/10 ratios, respectively. After germination, the pots were separately inoculated with the suspensions of five rhizobial strains, *RHL2*, *RHL29*, *RHL48*, *RHL68* and *RHL80* (10^8 cells. ml^{-1}). Six day after inoculation, the culture was submitted to two water regimes of irrigation: 75% of Field capacity, FC (optimal irrigation) and 25% FC (water deficit). After 45 days of stress, the plants were harvested. Analysis in different symbiotic combinations were focused on the nodule biomass as well as some nodular antioxidant enzymes activities as peroxidase (POD), polyphenol oxydase (PPO), catalase (CAT) and superoxide dismutase (SOD).

The N-free nutrient solution [14] was added once a week and the experimental layout was a completely randomized design with five pots (five plants) and each one was considered as one replicate with five pots per treatment per combination. All results were subjected to two-way analysis of variance (ANOVA II) using SPSS (10.0) software. The means were compared with Student-Newman-Keuls test.

Nodule dry weight determination

The plants were removed from the pots and the roots were thoroughly rinsed with water. For dry weight determination, Roots and nodules were carefully separated and the nodules were oven-dried for 48h at 70 °C and their dry weights were determined [14]. For standardizing data, the results were expressed as the relative reduction of nodular dry weight comparatively to the nodules formed under optimal irrigation (75% of FC), using the following formula:

Relative reduction (%) = [(1 - (stressed/optimal irrigation)] × 100 [15]

Nodular antioxidant enzymes analysis

The nodules (100 mg) were homogenized in 1 mL of phosphate buffer (20 mM, pH 7). The homogenate was centrifuged at 15000 g for 20 min at 4 °C. The supernatant obtained was used for the determination of the enzymatic activities of POD (EC 1.11.1.7) and PPO (EC 1.14.18.1) according to the technique previously described by Hori *et al.* (1997) [16].

The CAT (EC 1.11.1.6) activity was determined according to the method described by Gong *et al.* (2001). 100 mg of nodules was homogenized in 1.5 mL of Tris-HCl buffer (pH 8.5) including 2 mm EDTA and 10% (w/v) PVPP) [17]. The homogenate was centrifuged at 16 000 rpm for 14 min at 4 °C. Supernatant was used for the activity measurement.

The activity of superoxide dismutase (EC 1.15.1.1) was determined as described by Chagas *et al*. (2008) [18].

For all enzymes, the results were expressed in relative percentage of specific activity compared to the antioxidant activities recorded in nodules formed under optimal irrigation (75% FC).

RESULTS AND DISCUSSION

Effect on nodular dry biomass

The effect of water deficit on nodule dry weight (NDW) was indicated in the table. Results showed that under this environmental constraint the NDW was significantly reduced (P<0.001) in all symbiotic combinations tested. The post hoc test of Student-Newman-Keuls showed that the behavior of the ten combinations studied was significantly different (P<0.05). Generally, the nodules formed by inoculation with the *RHL80* and *RHL2* strains have developed the lowest reductions. Indeed, in *Tata-RHL2*, *Tata-RHL80*, *Dem-RHL80* and *Dem-RHL2* symbiotic combinations, the reductions were 18.98, 18.63, 20.23 and 19.23% respectively. The highest reductions were noted in *Tata-RHL4*, *Dem-RHL3* and *Dem-RHL4* while the intermediate reductions were showed by the remaining combinations. Under water deficit conditions, Sinclair et *al*. (1988) observed a significant decrease in dry weight of soybean nodule relatively to the well-watered treatment [19]. Similar observation was reported in nodules of *Phaseolus vulgaris* L. and *Sesbania aculeata* L. [11]. Reducing nodular biomass under water deficit can be explained primarily by the decrease in the number and diameter of root hairs or inhibiting the emergence and elongation of these bodies [20] and second, the limited growth of rhizobia and thus reducing the initiation and development of nodules [21]; [22].

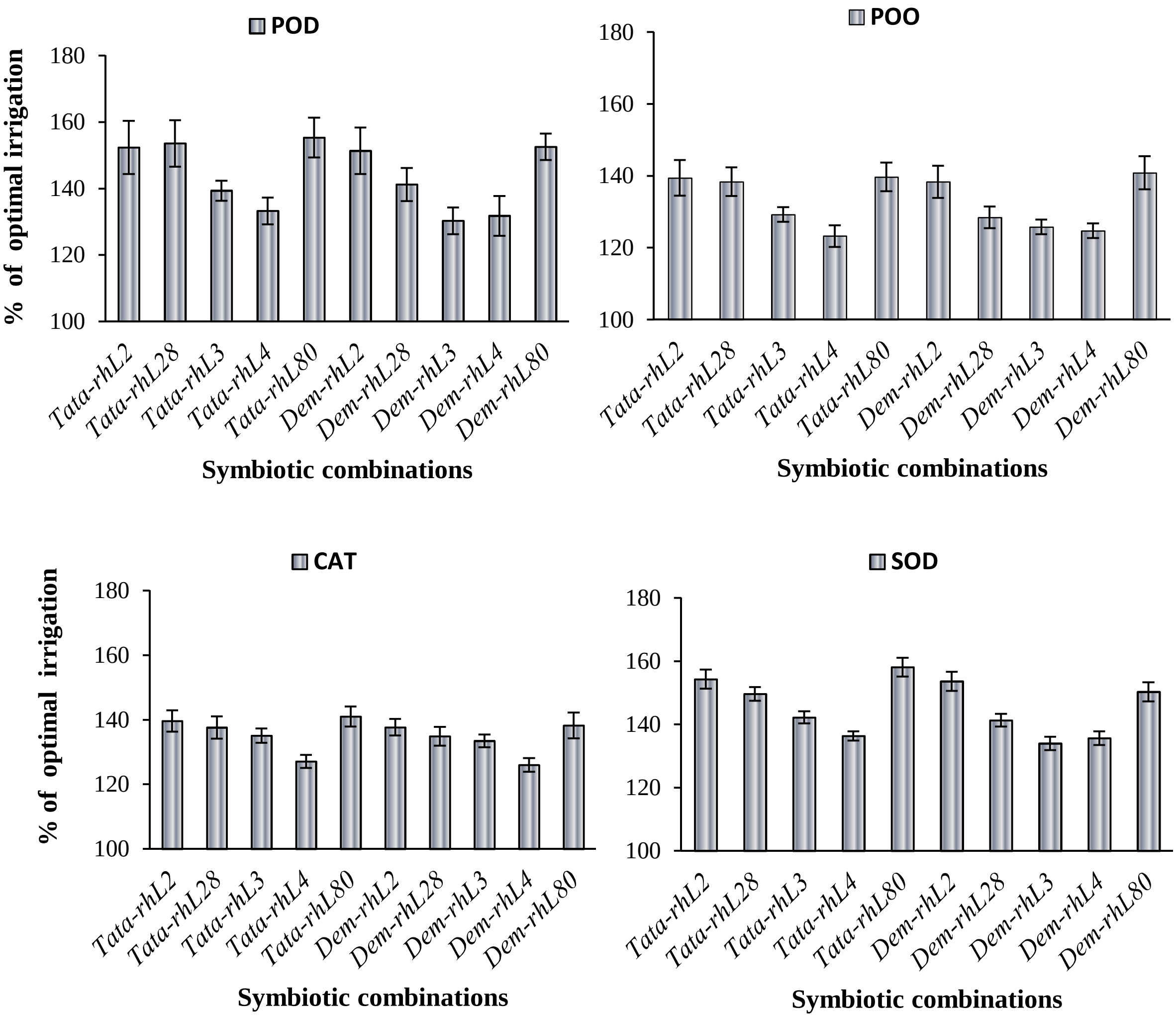

Figure . Effect of water deficit on nodular antioxidant enzymes, POD, PPO, CAT and SOD, in ten symbiotic combinations involving two Moroccan alfalfa populations (*Tata* and *Dem*) and five rhizobial strains (*RHL2, RHL29, RHL48, RHL68* and *RHL80*). The results were expressed in percentage of specific activity compared to the nodules formed under optimal irrigation (75% FC).Values are means of five replicate sand bars are standards errors.

Table . Effect of water deficit on nodular dry weight in ten symbiotic combinations involving two Moroccan alfalfa populations (*Tata* and *Dem*) and five rhizobial strains (*RHL2, RHL29, RHL48, RHL68* and *RHL80*). Results are expressed as reduction percentage of optimal irrigation (75% FC). Values are means of five replicates.

Symbiotic combination	% of reduction from optimal irrigation
	Nodular dry weight
Tata-RHL2	18,98 e
Tata-RHL28	22,44 c
Tata-RHL3	27,14 b
Tata-RHL4	28,54 a
Tata-RHL80	18,63 e
Dem-RHL2	20,23 de
Dem-RHL28	26,32 b
Dem-RHL3	28,46 a
Dem-RHL4	30,14 a
Dem-RHL80	19,23 e

Means followed by the same letter are not significantly different (P<0.05) using Student-Newman-Keuls test.

Effect on nodular antioxidant potential

Under water deficit conditions, the enzymatic activities of nodular antioxidant enzymes significantly increased (P<0.001) depending on the symbiotic combinations tested (Figure). Except for CAT activity, the highest and significant (P<0.001) antioxidant activities were noted in nodules formed by inoculation with the *RHL80* and *RHL2* strains. However, the CAT activity increased in almost all the symbiotic combinations (P>0.05). The water deficit is inevitably associated with increased oxidative stress due to enhanced accumulation of ROS, particularly superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in chloroplasts, mitochondria, and peroxisomes [23]. As a result, the induction of antioxidant enzyme activities is a general adaptation strategy that higher plants use to overcome the oxidative stress induced by environmental stress conditions [24]; [1]. SOD is considered to be the first defense against ROS, being responsible for the dismutation of superoxide to hydrogen peroxide and molecular oxygen [1]. CAT and POD are enzymes that catalyze the conversion of hydrogen peroxide to water and molecular oxygen [25].

CONCLUSION

We conclude that the water deficit caused a significant reduction in nodule biomass with the significant differences between the tested symbiotic combinations. The activities of the antioxidant enzymes analyzed increased in nodules of all combinations under water deficit and the high nodule biomass observed in some combinations was associated with highest antioxidant activities, suggesting that the enhancing of the antioxidative capacity could play a critical role in the water deficit tolerance.

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