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A highly osmotolerant rhizobial strain confers a better tolerance of nitrogen fixation and enhances protective activities to nodules of *Phaseolus vulgaris* under drought stress

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The effect of water deficiency on nodules of common bean (*Phaseolus vulgaris*) inoculated with three rhizobial strains differing in their osmotolerance, was investigated in two different experiments on sterile sand. In the first experiment, the control plants were maintained at 90% field capacity (FC) and water-deficient plants were grown at 35% FC. The nitrogen fixation and growth parameters drastically decreased under water deficiency, however the three rhizobial strains, *Rhizobium etli* A32 (sensitive), *Rhizobium tropici* CIAT899 (tolerant), and *Ensifer meliloti* 4H41 (highly tolerant), showed different symbiotic performances. *E. meliloti* 4H41 allowed the best acetylene reduction activity (ARA) and biomass production and the highest number of large-sized nodules, while no significant effect was observed on lipid peroxidation, protein and legheamoglobin contents. The effect on antioxidant activities was the lowest. In the second experiment, plants were maintained at 90% FC during 45 days and then watering was stopped. The results showed that, the response to water deficit was quite similar for the three analyzed symbioses until 35% FC, but below this value of FC, symbiosis involving strain *E. meliloti* 4H41 was the most tolerant. This tolerance was accompanied, by in both experiments, by a stability of metabolic indices and protective antioxidant activities. These results suggest that, the relative tolerance of the nodules induced by strain 4H41 could be due to a constructive adaptation involving specific cortex structure and stress-adapted metabolic activities acquired during nodule formation and growth, rather than to a timely inducible response due to the stimulation of antioxidant enzymes. This suggestion should be confirmed through microscopic structure analysis and supplemental key enzymes in nodule metabolism such as sucrose synthase and malate dehydrogenase.

Key words: Antioxidant activities, in pots experiment, leghemoglobin content, nodule, rhizobia, osmotolerance, symbiotic efficiency, water deficiency.

INTRODUCTION

Grain legumes are a major source of proteins in human and animal nutrition mainly in developing countries (Meuelenberg and Dakora, 2007). Drought represents, with soil salinity, major threats to crop yield worldwide. Under these osmotic stresses, the reduced water supply

of crops is the most critical growth factor (Zhu, 2001; Schleiff, 2008; Evelin et al., 2009). The osmotic constraint induced by both stresses reduces the amount of available water causing physiological drought. It leads the plant and the bacteria to decrease their internal water potential to avoid desiccation (Zahran, 1999; Tonon et al., 2004). For legume crops depending on nitrogen-fixation, drought affects plant growth and metabolisms (Ehsanpour and Amini, 2003; Aydi et al., 2008). Moreover, it affects several facets of nodule functioning including nitrogen

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fixation, metabolites synthesis (protein, malate and leghaemoglobin) and enzymatic activities (Marino et al., 2007; Mhadhbi et al., 2008, 2009a,b). Drought limits nodulation through its effects on persistence and survival of rhizobia in the soil, root-hair colonization and infection by rhizobia (Zahran, 1999; Vriezen et al., 2007). Rhizobia (*Rhizobiaceae*) include many species known to support severe salt and drought conditions; thanks to various adaptative strategies (Zahran, 1999; Vriezen et al., 2007; Mhadhbi et al., 2009b). However, species nodulating common bean (*Phaseolus vulgaris*), are considered sensitive to salt. The most tolerant strains have been reported to grow in salt concentrations up to 350 mM NaCl, but most of them are much less tolerant to salinity (Amarger et al., 1997; Mhamdi et al., 1999). The commonly used inoculant strain *Rhizobium tropici* CIAT899 is able to grow in YEM medium containing up to 300 mM (1.8%) NaCl (Mnasri et al., 2007b). Recently, a highly salt-tolerant strain (750 mM or 4.4% NaCl) was isolated from common bean nodules from a Tunisian oasis (Mnasri et al., 2007a). This strain (4H41) was shown to be more competitive and more effective in nitrogen fixation with common bean under water deficiency than the commonly used inoculant strain CIAT899 (Mnasri et al., 2007b). These results and others (Ben Rhomdhane et al., 2007; Meuelenberg and Dakora, 2007; Mhadhbi et al., 2008) suggested that, selecting stress-tolerant cultivars and stress-tolerant rhizobia could be a rational strategy to improve the yield of legumes in stressed environments.

The aim of this work was to investigate the presumed added value of the newly isolated highly-osmotolerant strain 4H41, in comparison with the reference strain CIAT899 and a salt sensitive strain, towards nodule integrity and functioning under water deficiency.

MATERIALS AND METHODS

Biological material

Based on a preliminary work, three rhizobial strains were retained for subsequent analysis of their influence in the response of common bean nodules to water deficiency. These strains showed nearly similar symbiotic effectiveness, but different salt (NaCl) and drought (PEG) tolerance levels in liquid yeast extract mannitol medium (YEM): *R. tropici* CIAT899 (1.8% NaCl, 15% PEG), *Ensifer meliloti* 4H41 (4.4% NaCl, 30% PEG) and *Rhizobium etli* Ma1A32 (0.2% NaCl, 10% PEG).

The osmotolerant cultivar *P. vulgaris* cv. flamingo (Aydi et al., 2008) was used as the host plant.

Culture conditions and stress application

Nodulation, nitrogen fixation and NaCl tolerance tests were carried out as previously described (Mhamdi et al., 1999; Mnasri et al., 2007b). Seeds of *P. vulgaris* were surface sterilized by immersion for 10 min in 0.2% HgCl₂ and extensively washed with sterile distilled water. After germination, seeds were transferred into 500 ml plastic pots containing autoclave sterilized sand (one seed per

pot). The pots (12 pots for each treatment) were covered with lids to reduce contamination. After 3 days, the seedlings were inoculated with a suspension of each one of the three rhizobial strains at the rate of nearly 10⁶ cfu per strain and the cotyledons were kept out through a hole managed on the lid. Two modes of drought stress were applied: (1) A continuous stress where the plants were subdivided into two sets. The first set of control plants was kept to 90% of field capacity (FC) with a sterilized nitrogen-free solution (Mhadhbi et al., 2008). The second set of water-deficient plants was maintained at 35% FC. Regular weightings were performed every two days to restore the moisture levels by addition of an equivalent weight of nitrogen free solution; (2) a non permanent drought experiment where plants were maintained at 90% FC for 45 days and then watering was stopped and plants were monitored for their nodule activity each two days until complete shoot desiccation. Plants were harvested, nodules were counted and classed according to their sizes and then nodules were destined for biochemical assays or stored at -80°C for confirmative experiments. Aerial and root parts were dried for 72 h at 80°C and then shoot and root dry biomass was measured.

Acetylene reduction assay (ARA)

Nitrogenase (EC, 1.7.9.92) was assayed by acetylene reduction assay (ARA) by gas chromatography with Porapak-T column using pure ethylene as internal standard as detailed in Mhadhbi et al. (2008). Three plants were considered for each treatment with three replicated measures for each plant.

Leghaemoglobin content

Nodule leghaemoglobin content was determined according to the method described by Schiffman and Lobel (1970) with few modifications. 300 mg of fresh harvested nodules (mix 1/2/1/2 of small and big nodules) were homogenized in 5 ml Drabkin's solution and centrifuged at 5000 g for 15 min. The supernatant was added to 10 ml of Drabkin's solution (Schiffman and Lobel, 1970), homogenised and was centrifuged for 30 min at 15000 g. The leghaemoglobin content was determined using bovine haemoglobin as standard.

Lipid peroxidation assay

Lipid peroxidation in nodules was assayed using the thiobarbituric acid (TBARS) method modified according to Singh et al. (2007). 500 mg fresh harvested nodules (mix 1/2/1/2 of small and big nodules) were homogenized in 3 ml of 0.1% TCA solution. The homogenate was centrifuged at 10000 g for 20 min and 0.5 ml of the supernatant was added to 1 ml of 0.5% TBA in 20% TCA. The absorbance of the supernatant was determined at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of malondialdehyde (MDA) was calculated using the extinction coefficient $\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Enzymatic activities

Extracts were prepared at 4°C by homogenizing fresh nodules (500 mg) with 10% (w/w) polyvinylpyrrolidone in 50 mM phosphate buffer (pH 7.8) containing EDTA, 0.1% (v/v) triton X-100, 1 mM PMSF and was centrifuged for 20 min at 13000 g. Protein content was measured according to the method of Bradford (1976).

Superoxide dismutase (SOD) (EC, 1.15.1.1) activity was determined spectrophotometrically, based on the method of Beauchamp and Fridovich (1971) by monitoring the inhibition of

photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. The reaction mixture contained 50 mM K-phosphate (pH 7.8), 0.1 mM EDTA, 10 mM L-methionine, 2.7 μ M riboflavine and 75 μ M NBT.

One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25°C.

Peroxidase (POX) (EC, 1.11.1.7) was determined by monitoring the formation of tetraguaiacol from guaiacol (9 mM) at 470 nm during 1 min ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) in the presence of H_2O_2 (19 mM) added for starting the reaction.

Catalase (CAT) (EC, 1.11.1.6) was assayed by following the decline of absorbance at 240 nm caused by the decomposition of H_2O_2 (10 mM) during 3 min ($\epsilon = 36 \text{ M}^{-1} \text{ cm}^{-1}$).

Ascorbate Peroxidase (APX) (EC, 1.11.1.11) was measured by monitoring the disappearance of ascorbate (0.5 mM) at 290 nm during 1 min ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture contained 0.2 mM H_2O_2 in 50 mM K-phosphate buffer (pH 7).

Statistical analyses

The one-way ANOVA (STATISTICA program) was used for variance analysis and means were compared by the Tukey's honestly significant difference ($p \leq 0.05$). Nine replicates per treatment were considered for nodule number, dry weight and acetylene reduction activity.

For enzyme activities, protein, MDA and leghaemoglobin contents data were means of six values resulting from three plants with two replicates for each plant.

RESULTS

Effect of continuous water deficit on plant growth, nodulation and nodule activity

The symbioses involving the three rhizobial strains were submitted to water deficit imposed by maintaining field capacity (FC) at 35% during plant life cycle. However, control symbioses were grown under 90% FC. The results are summarized in Table 1. Plant biomass production and nodule number, mainly large nodules, were drastically reduced by drought; whereas root biomass was less affected. Symbiosis involving the highly osmotolerant strain *E. meliloti* 4H41 relatively maintained the highest shoot biomass production and large-size-nodule number, scoring 1.5 to 2 fold the values given by *R. tropici* CIAT899 and *R. etli* Ma1A32 (Table 1). *E. meliloti* 4H41 allowed also an important nitrogen-fixing capacity (ARA) averaging twice to three times the levels scored by the other strains (Table 2). The nodules induced by strain 4H41 under water deficiency were characterized by the stability of the level of lipid peroxidation estimated by MDA, amounts of total soluble proteins and leghaemoglobin contents. These nodules presented a moderate decrease of catalase (CAT) activity and the lowest increase in peroxidase antioxidant activities (POX and APX). Superoxide dismutase (SOD) activity was not significantly affected in nodules involving strain 4H41; however, it was decreased in nodules induced by *R. tropici* CIAT899 and *R. etli* Ma1A32 (Table 2).

Effect of intermittent water deficit on plant growth, nodulation and nodule activity

In the second part of this study, drought stress was induced on 45 day aged plants grown under 90% FC by stopping irrigation and monitoring nodule activity during the decrease of moisture level (Figure 1). The response of nodule activity to such brutal watering stopping was quite similar for symbioses implicating the three rhizobial strains. ARA decreased linearly from 90% FC to 35% FC during 8 days, then it stabilised at a low level in nodules involving all the strains with a slight increase for *E. meliloti* 4H41 (Figure 1). Protein level, leghaemoglobin content, APX activities and MDA content were not significantly changed mainly during the first 8 days (until 35% FC) of water deprivation. When FC dropped under 35%, the MDA content increased in nodules formed by strains CIAT899 and Ma1A32. However, it was stabilised in the nodules involving strain 4H41 until the last measure performed at 10% FC. POX was unchanged in nodules implicating strains CIAT899 and Ma1A32 during the water deficit treatment. However, in the nodules induced by strain 4H41, POX activity was increased until 60% FC, then it decreased to drop lower than the initial value at 35% FC (Figure 1). CAT decrease was prominent until 60% FC. Under this FC level, CAT activity was quite unchanged mainly in nodules involving strain 4H41 (Figure 1).

DISCUSSION

The use of tolerant rhizobia to alleviate salt and drought stresses on legumes showed promising results (Zahran, 1999; Mhadhbi et al., 2008; Mnasri et al., 2007b; Ben Rhomdhane et al., 2007; Meuelenberg and Dakora, 2007). Nevertheless, rhizobia nodulating common bean are known as sensitive to osmotic stresses (Amarger et al., 1997; Mhamdi et al., 1999). Recently, highly osmotolerant *E. meliloti* strains isolated from common bean nodules from South Tunisia were found to be more effective and more competitive than the commonly used inoculant strain CIAT899 under water deficiency (Mnasri et al., 2007a, b); however, their effect on nodule functioning remains unclear. This study was focused on investigating the implication of highly osmotolerant strains in enhancing nodule functioning under water deficiency in comparison with less tolerant strains.

We showed that nodule number was relatively maintained for symbiosis involving osmotolerant strains under water deficiency. The highly tolerant *E. meliloti* strain 4H41 enabled more large-sized nodules ($\geq 1 \text{ mm}$) under 35% FC than those formed by symbiosis involving the reference and sensitive strains. Under water deficiency, the ARA of the nodules induced by osmotolerant strain 4H41 was two or three fold less affected than the nodules induced by sensitive strains (Table 2). This relative higher efficiency of strain 4H41

Table 1. Effect of continuous water deficiency (35% FC) on growth indices of *P. vulgaris* cv. flamingo inoculated with three rhizobial strains differing in their osmotolerance (NaCl and PEG): *R. etli* Ma1A32 (sensitive), *R. tropici* CIAT899 (tolerant) and *E. meliloti* 4H41 (very tolerant).

Strain	Treatment*	Shoot dry weight (g plant ⁻¹)	Root dry weight (g plant ⁻¹)	Nodule number		
				Total	Ø ≥ 1 mm	Ø < 1 mm
Ma1A32	Control	1.85 ^b	1.62 ^b	196 ^a	93 ^a	103 ^a
	Water-deficient	0.65 ^d	0.94 ^d	56 ^d	14 ^d	42 ^b
CIAT899	Control	2.13 ^b	2.63 ^a	153 ^b	55 ^b	98 ^a
	Water-deficient	0.47 ^d	1.38 ^c	35 ^d	10 ^d	25 ^c
4H41	Control	2.65 ^a	2.13 ^b	161 ^b	52 ^b	109 ^a
	Water-deficient	0.96 ^c	1.86 ^b	71 ^c	22 ^c	49 ^b

*Control plants were maintained at 90% field capacity (FC) and water-deficient plants were maintained at 35% FC. In each column, data denoted with different letters are significantly different according to the HSD Tukey's test ($p < 0.05$).

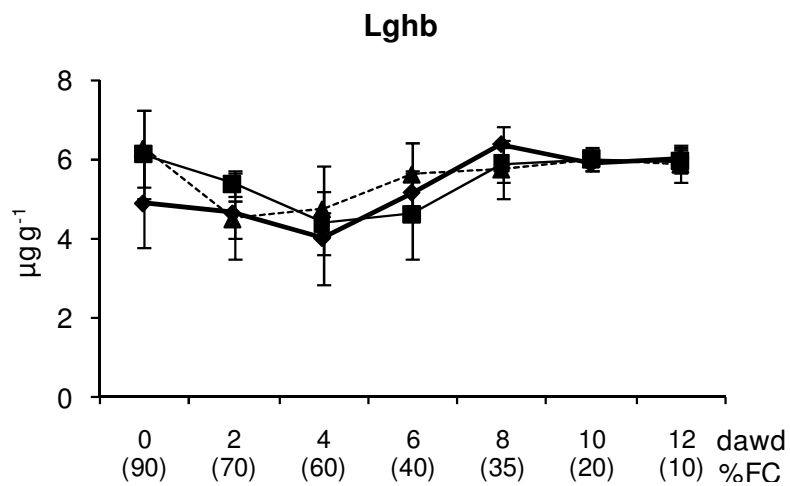
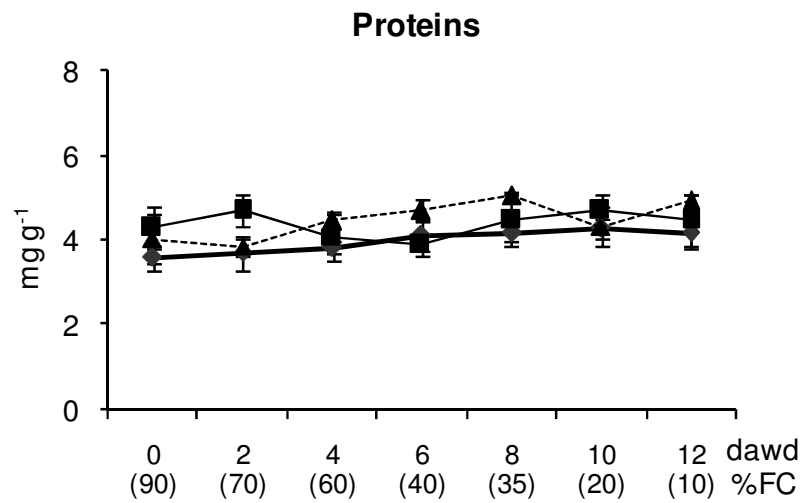
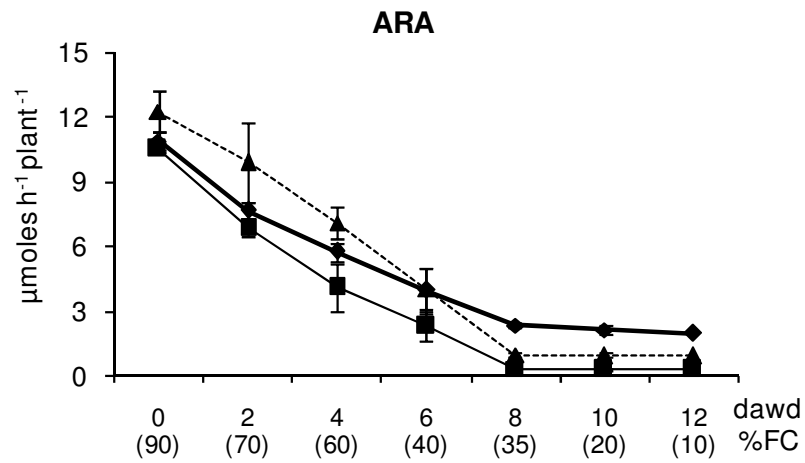
Table 2. Effect of continuous water deficiency (35% FC) on nodule functioning indices and antioxidant enzymes of *P. vulgaris* cv. Flamingo inoculated with three rhizobial strains differing in their osmotolerance (NaCl and PEG): *R. etli* Ma1A32 (sensitive), *R. tropici* CIAT899 (tolerant) and *E. meliloti* 4H41 (very tolerant). In each column data denoted with different letters are significantly different according to the HSD Tukey's test ($p < 0.05$).

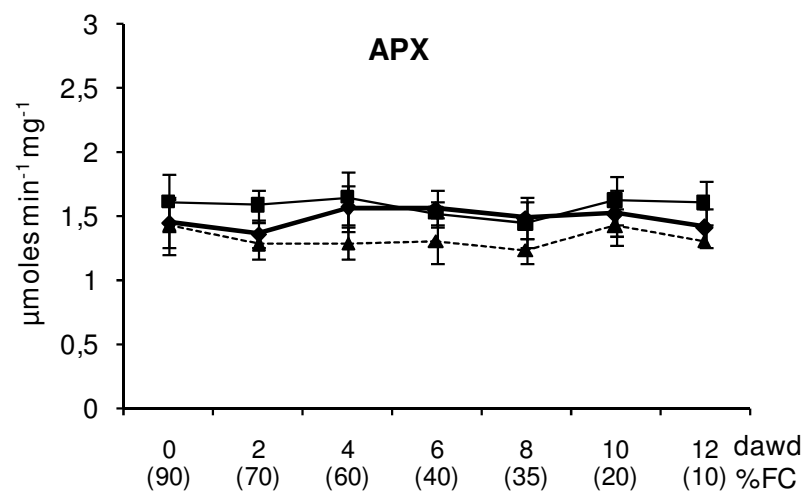
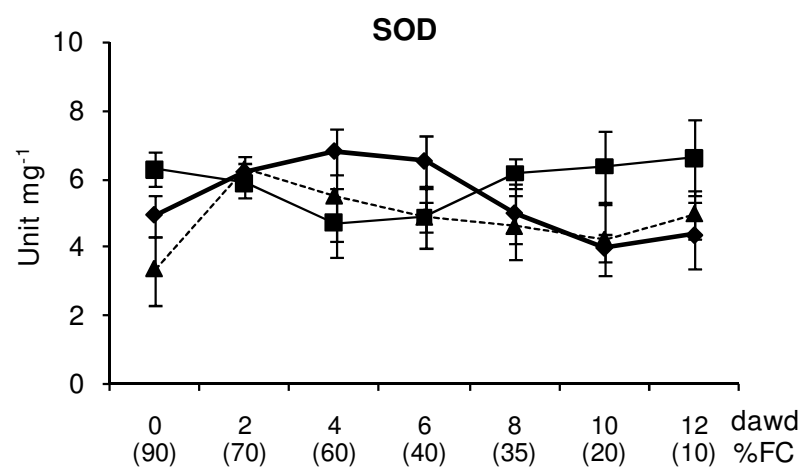
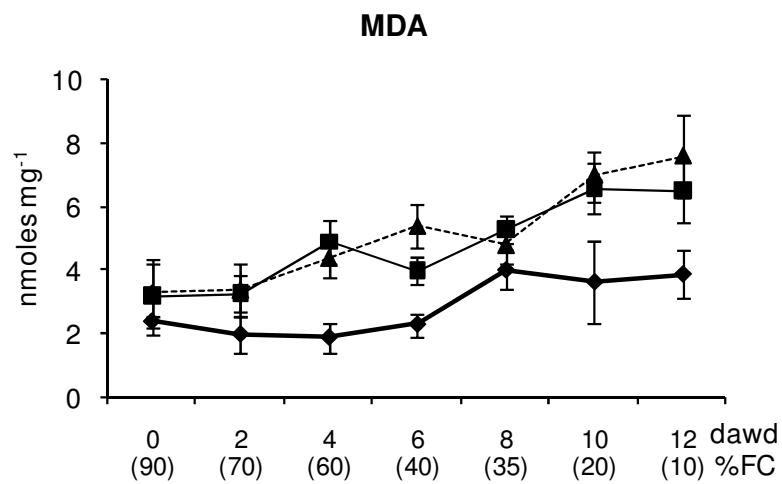
Strain*	Treatment [§]	ARA [¥] (µmoles h ⁻¹ plant ⁻¹)	Soluble proteins (mg g ⁻¹)	MDA (nmols mg ⁻¹)	Lghb [#] (µg g ⁻¹)	CAT (µmoles min ⁻¹ mg ⁻¹)	APX (µmoles min ⁻¹ mg ⁻¹)	SOD (Unit mg ⁻¹)	POX (µmoles min ⁻¹ mg ⁻¹)
Ma1A32	Control	12.8 ^a	10.86 ^a	1.29 ^d	6.02 ^a	38.02 ^c	0.66 ^d	6.1 ^b	9.5 ^e
	Water-deficient	2.22 ^c	6.57 ^b	1.72 ^c	4.84 ^c	25.62 ^d	0.79 ^c	2.4 ^d	15.9 ^c
CIAT899	Control	12 ^a	6.20 ^b	2.12 ^b	6.69 ^a	87.93 ^a	1.04 ^b	7.2 ^a	13.2 ^d
	Water-deficient	1.39 ^d	3.66 ^c	3.18 ^a	6.37 ^a	46.4 ^c	1.32 ^a	5.5 ^b	28.3 ^a
4H41	Control	11.86 ^a	5.51 ^b	1.81 ^{bc}	5.12 ^b	63.68 ^b	0.99 ^b	2.7 ^d	20.4 ^b
	Water-deficient	3.96 ^b	5.36 ^b	2.05 ^b	5.88 ^{ab}	42.72 ^c	1.23 ^a	2.8 ^d	21.7 ^b

*§Control plants were maintained at 90% Field capacity (FC) and water-deficient plants were maintained at 35% FC. ¥ARA, acetylene reduction activity; # Lghb, leghaemoglobin content.

could be attributed to the maintenance of the nodule structure and key metabolic indices (proteins, MDA and Lghb). This stability was potentially due to an intrinsic constitutive aspect of adaptation to water deficiency (structure and metabolism modulation); since, in these nodules the antioxidant protective enzymes (POX, APX and SOD) were slightly or not significantly affected. These observations did not support the other reports suggesting that nodule protection under drought and salt is mainly assured by antioxidant enzymes (Tejera et al., 2004; Jebara et al., 2005, 2010; Mhadhbi et al., 2008,

2009a). Therefore, it could be suggested that, under the experimental conditions of our study, nodules underwent structural and metabolic changes, leading to the adaptation of nodule metabolism to water deficiency. Such adapted metabolism avoids ROS over-production and oxidative stress generation. This speculation is supported by the stability of MDA (as a final product of lipid peroxidation) and the leghaemoglobin contents, since these two parameters are known as highly sensitive to oxidative stress in nodules and overall plant tissues (Redondo et al., 2008; Mhadhbi et al., 2009b, Aydi et al.,





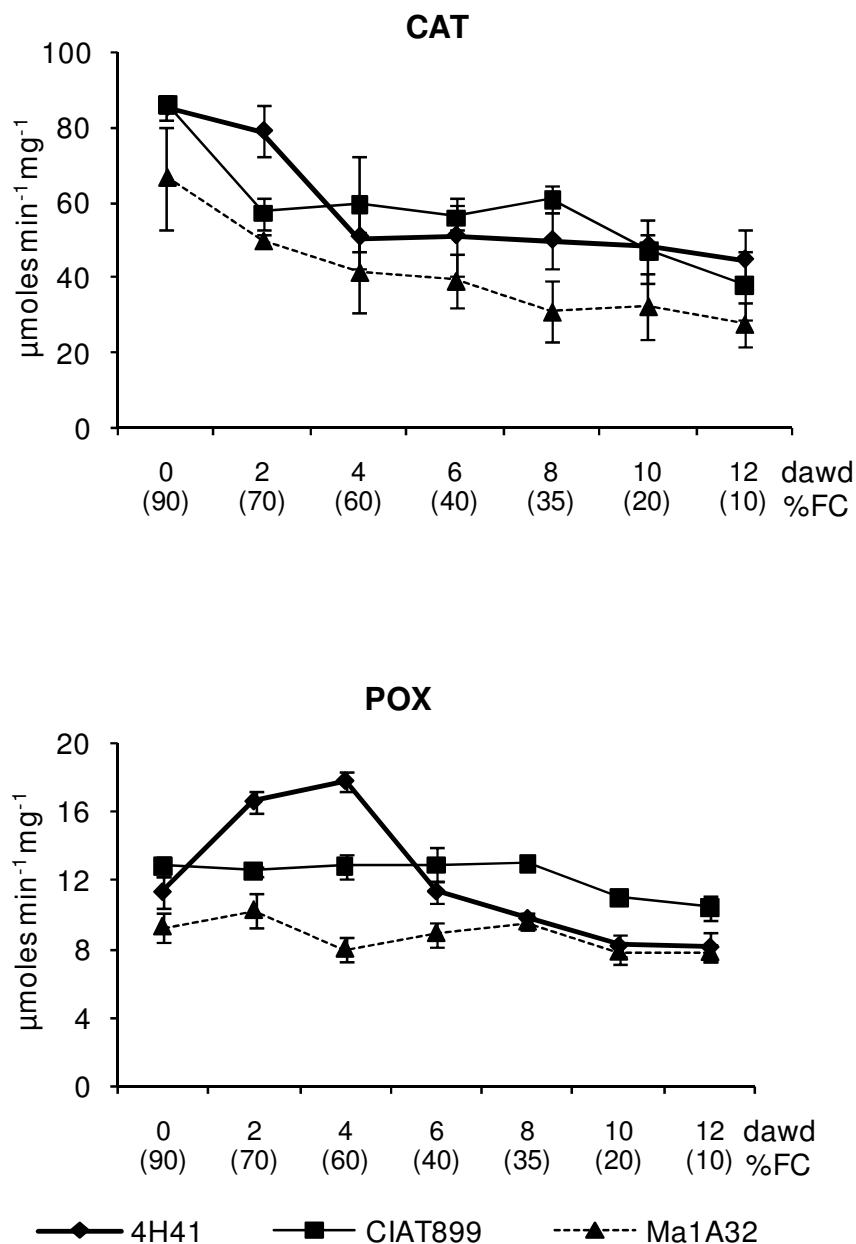


Figure 1. Evolution of nitrogenase activity (ARA), leghaemoglobin content (Lghb), protein content, lipid peroxidation (MDA) and some antioxidant enzymes (APX, SOD, POX, CAT) in nodules of *P. vulgaris* cv. flamingo inoculated with three rhizobial strains (*R. tropici* CIAT 899, *E. meliloti* 4H41 and *R. etli* Ma1A32) after water deprivation of 45 day-old plants. The field capacity (% FC) corresponding to each measure is given between parenthesis. Data are means of nine (ARA) or six (Lghb, APX, SOD, POX, CAT) replicates \pm standard error – dwad (days after water deprivation).

2008; Hafsi et al., 2010). Thus, the stability of MDA and leghaemoglobin indicates a healthy state of cell membranes in nodules and the conservation of oxygen balance through these organs (Matamoros et al., 2003). It seems that under stressful conditions, tolerant rhizobia induce the formation of nodules that have more strong structure and more efficient metabolism able to fix

nitrogen and to avoid metabolic deviations allowing a best growth performance.

To investigate the involvement of the rhizobial partner in the modulation of the nodule metabolism under stressful conditions, we applied water deficiency on 45 day-old plants to focus on the stress effect on nodule activity and discard interference with infectivity and

nodule growth parameters. The similar decrease of Ascorbate peroxidase (ARA) observed in all nodules (involving the three rhizobial strains) indicated that, under non permanent stress application, the symbiosis response to watering deprivation was independent of the rhizobial partner, unlike the response to a continuous water deficiency where tolerant rhizobia enhance the symbiosis tolerance. The response of symbiosis to watering interruption is therefore, primarily dependent on the plant reaction. Indeed, during the first 8 days after water was stopped (until 35% FC), there were no significant changes in leghaemoglobin and MDA contents, which suggests that ARA decrease could be caused by the insufficiency of photosynthetic products delivered to the nodules not to structural or biochemical alterations of nodules. This suggestion is supported by the stability in SOD and APX activities known as sensitive enzymes to osmotic stress mediated nodule damages (Mhadhbi et al., 2008, 2009a; Jebara et al., 2010). POX activity was stimulated in nodules involving strain 4H41 during the first 4 days when water was stopped (until 40% FC). This was potentially due to the localisation of POX in the peripheral tissues of the nodules (Matamoros et al., 2003), which make it the first enzyme involved in stress response. POX stimulation was faster manifested in the nodules of “tolerant symbiosis” as a rapid response to stress perception in these organs. Indeed, this enzyme is a marker of drought and salt tolerance (Mhadhbi et al., 2009b), which is involved in the cell wall enforcing to cope with stress expansion (Matamoros et al., 2003). After this adaptative phase, the POX activity dropped indicating that nodules “reached” the stressful conditions and modulated their metabolism to maintain an equilibrated activity under stressful conditions. This observation is supported by the relative stability of lipid peroxidation in nodules of the tolerant strain 4H41 compared with those involving the other strains.

It could be summarized that under drought stress, the success of *P. vulgaris*-*E. meliloti* 4H41 symbiosis to maintain efficient nitrogen fixation seemed to be the outcome of a constructive adaptation acquired during nodule formation and growth not to a timely inducible physiological or biochemical response. The osmotolerant *E. meliloti* strain induced the formation of nodules that had more strong structure and more efficient metabolism to fix nitrogen and to avoid metabolic deviations such as overproduction of ROS and allowing a better growth performance. Such hypothesis should be confirmed through microscopic structure analysis and supplemental key enzymes in nodule metabolism such as sucrose synthase and malate dehydrogenase.

Despite their difference in osmotolerance as free living bacteria, *R. tropici* CIAT899 and *R. etli* Ma1A32 showed quite similar behaviours when inoculated to common bean under water deficit. This suggests that, the difference in stress tolerance is reflected on the symbiotic mode of life only when free living rhizobia reach high

stress levels. More rhizobial strains should be assessed in this context before drawing final conclusions in this regard.

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Abbreviations

APX, Ascorbate peroxidase; **ARA**, acetylene reduction activity; **BSA**, bovine serum albumin; **CAT**, catalase; **MDA**, malondialdehyde; **NBT**, nitroblue tetrazolium; **PMSF**, phenylmethylsulphonyl fluoride; **POX**, guaiacol peroxidase; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase.

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