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Behaviours of *Medicago truncatula–Sinorhizobium meliloti* Symbioses Under Osmotic Stress in Relation with the Symbiotic Partner Input: Effects on Nodule Functioning and Protection

H. Mhadhbi[1], V. Fotopoulos[2], N. Djebali[1], A. N. Polidoros[2] & M. E. Aouani[1,3]

1 Laboratoire Interactions Légumineuses Microorganismes (LILM), Centre de Biotechnologie, Technopôle Borj Cedria (CBBC), Hammam lif, Tunisia
2 Institute of Agrobiotechnology, CERTH, Thermi, Greece
3 NEPAD/North Africa Biosciences Network, National Research Center, El Buhouth St, Cairo, Egypt

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Correspondence
H. Mhadhbi
Laboratoire Interactions Légumineuses Microorganismes (LILM), Centre de Biotechnologie, Technopôle Borj Cedria (CBBC), BP 901, 2050 Hammam lif, Tunisia
Tel/Fax: +216 79 412 828
Email: mhadhbihay@yahoo.fr

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Abstract

Three genotypes of the model legume *Medicago truncatula* were assessed for symbiotic effectiveness in cross inoculation with two strains of *Sinorhizobium meliloti* under mannitol-mediated osmotic stress. Symbioses showed different tolerance levels revealed on plant growth, nitrogen-fixing capacity and indices of nodule functioning and protection. The variability of stress response was essentially correlated with performance at non-stressful conditions. Symbiosis attitude depended on bacterial partner, host-plant genotype and their interaction. Plant genotype manifested the highest contribution to symbiotic efficiency indices under osmotic stress, even for nodulation and nitrogen fixation where the bacterial strain effect is highly pronounced. Contrasting (tolerant/sensitive) associations were identified for tolerance behaviours, involving the same plant genotype with different rhizobial strains and vice versa. In nodules, osmotic stress leads to accumulation of oxidized lipids and decrease in total protein and leghaemoglobin contents. Antioxidant responses were manifested as induction of guaiacol peroxidase (POX, E.C. 1.11.1.7) and superoxide dismutase (E.C. 1.15.1.1). POX induction was higher in tolerant symbioses and both enzymes were suggested as contributors to the protection of nodule integrity and functioning under osmotic stress. In conclusion, symbiotic efficiency in *M. truncatula–S. meliloti* combinations under osmotic stress is determined by each symbiont's input as well as the plant–microbe genotype interaction, and POX induction could prove a sensitive marker of tolerant symbioses.

Introduction

Legumes in symbiosis with rhizobia have the ability to support production of protein-rich seeds and fodder by fixing atmospheric nitrogen, in addition to their beneficial effect on productivity of cereals and other crops in agricultural rotations (Graham and Vance 2003). However, this association is often compromised by severe environmental stresses, mainly salinity and drought (Zahran 1999). As much as 60 % of legume production in the developing world occurs under conditions of significant drought stress (Graham and Vance 2003, Zhang et al. 2007). It is therefore important to elucidate the drought tolerance mechanisms of these species in order to improve their agronomic performance. Nevertheless, the variability of legume responses to osmotic stress and the complexity of their genetic control make it difficult to find ways to alleviate water deficit-induced stress. Moreover, the interaction of plant with the micro-symbiont further convolutes the understanding of symbiosis tolerance mechanisms. The symbiosis is an associative mode-life between two partners where each one contributes to the energy charge and benefits from the produced nutrients. Plant behaviour is a determinant factor for the success of symbiosis under stressful conditions (Sadiki and Rabih 2001, Djilianov et al. 2003). The infection process is highly sensitive to stress, although rhizobia are tolerant even to stress conditions that inhibit plant growth (Zahran 1999), and have a

significant input into the tolerance level (Mhadhbi et al. 2004, 2008, Zacarias et al. 2004, Mnasri et al. 2007). In nodules, one of the earlier results of osmotic constraint is the over-production of reactive oxygen species (ROS) (Figueiredo et al. 2007, Naya et al. 2007, Zhang and Nan 2007). ROS lead to damage of tissue integrity and nodule function through oxidative attack to lipids, proteins and nucleic acids (Becana et al. 2000, Matamoros et al. 2003, Porcel et al. 2003, Naya et al. 2007). Symbiosis response to oxidative stress includes morphological modifications such as the change of nodule cortex structure (Matamoros et al. 1999), and biochemical adaptations such as the modulation of antioxidant enzyme expression in nodules. Several reports underline the intimate relationship between enhanced or constitutive antioxidant enzyme activities and increased symbiosis resistance to environmental stress (Mhadhbi et al. 2004, Tejera et al. 2004, Marino et al. 2007, Zhang and Nan 2007). After *Medicago truncatula* was chosen as a suitable plant model for studying legume–rhizobia interactions, numerous studies of molecular, genetic, proteomic and physiological aspects have focused on its symbiosis with *Sinorhizobium meliloti* (Barker et al. 1990, Rolfe et al. 2003, Badri et al. 2007, Lopez et al. 2008, Rose 2008). Therefore, understanding the physiological and biochemical mechanisms conferring drought tolerance to this symbiosis is very important for the development of selection and breeding strategies.

In the present work, we examined the effect of mannitol-mediated osmotic stress on the behaviour of different *M. truncatula–S. meliloti* associations. The analyses were performed in order to investigate the interaction of plant–bacteria under stressful conditions, and to subsequently identify symbioses with contrasting (tolerant/sensitive) response to osmotic stress. Antioxidant enzyme activities in the nodules of the contrasting symbioses were estimated in order to identify potential biochemical markers of specific responses to osmotic stress.

Material and Methods

Biological material

This study was performed with three *M. truncatula* genotypes: a reference one (Jemalong J6) and two local genotypes (TN8.20 and TN6.18) from different Tunisian regions (Mhadhbi et al. 2005). Plants were inoculated with two *S. meliloti* strains: one reference (RCR2011) and one native (TII7) strain (Zribi et al. 2004).

Glasshouse trial

Medicago truncatula seeds were sterilized in concentrated sulphuric acid for 6 min, rinsed several times with sterile distilled water and then germinated on 0.9 % agar in Petri plates at 4 °C for 4 days. Germinated seeds were transferred onto sterile perlite, and then inoculated (approximately 10^9 cfu). One week later, each seedling was transferred into 0.5 l syrup bottles containing nutrient solution (Mhadhbi et al. 2005). The seedlings were then re-inoculated (10^9 cfu). The nutrient solution was aerated with airflow of 400 ml min^{-1}. The experiment was conducted in a glasshouse at 25 °C and 16/8 h (day/night) photoperiods. Osmotic stress was induced with the addition of 75 mM mannitol to the nutrient solution (−215 kPa). Plants were harvested at the flowering stage.

Nitrogen-fixing assay

Nitrogenase (E.C. 1.7.9.92) was assayed by acetylene reduction activity (ARA; Hardy et al. 1968) using a gas chromatograph (HP 4890A; Hewlett Packard, Palo Alto, CA, USA) with a Porapak-T column. Nodule-bearing roots were incubated in 10 % C_2H_2 atmosphere. After 60 min, three replicates of 0.5 ml samples were withdrawn from the root atmosphere of each plant (three plants/association) and ethylene production was measured by gas chromatography. Pure acetylene and ethylene were used as internal standards (Mhadhbi et al. 2005).

Leghaemoglobin content

The content of leghaemoglobin in nodules was determined by the extraction of the pigment in Drabkin's solution using the method described by Shiffmann and Lobel (1970) with few modifications. Fresh nodules (100 mg) were homogenized in 3 ml Drabkin's solution. The homogenate was centrifuged at 5000 ***g*** for 15 min. The supernatant was added to 10 ml of Drabkin's solution, homogenized and centrifuged for 30 min at 15 000 ***g***. The supernatant was then collected and absorbance was determined at 540 nm. The content of leghaemoglobin was estimated referring to a standard curve determined from samples of bovine haemoglobin monitored at the same conditions.

Lipid peroxidation assay

Lipid peroxidation in nodules was assayed using the thiobarbituric acid (TBARS) method modified according to Singh et al. (2007). This test determines malondialdehyde (MDA) as an end product of the TBARS reaction. Nodules (300 mg) were homogenized in 3 ml of 0.1 % TCA solution. The homogenate was centrifuged at 10 000 ***g*** for 20 min and 0.5 ml of the supernatant was added to 1 ml of 0.5 % TBA in 20 % TCA. The mixture was incubated in boiling water for 30 min, and the reaction stopped by

placing the reaction tubes in an ice bath. The samples were then centrifuged at 10 000 ***g*** for 5 min, and the absorbance of the supernatant was read at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The amount of MDA was calculated using the extinction coefficient $\varepsilon = 155\ \text{mM}^{-1}\ \text{cm}^{-1}$.

Preparation of enzyme extracts

All operations were performed at 4 °C to maintain enzyme activity. Extracts were prepared by homogenizing 0.2 g of nodules in a mortar with 10 % (w/w) polyvinyl-polypyrrolidone and 1 ml of 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1 % (v/v) Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF) as an inhibitor of proteases, added with 5 mM ascorbate for APX activity. Extracts were centrifuged at 13 000 ***g*** for 20 min and the supernatant was used to determine enzyme activities. Protein rate was measured according to the method of Bradford (1976) using bovine serum albumin as a standard.

Enzyme assays

Superoxide dismutase (SOD) activity was determined spectrophotometrically according to Beauchamp and Fridovich (1971) by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50 % at 25 °C.

Spectrophotometric assays of the other antioxidant enzymes were performed by monitoring the formation of tetraguaiacol from guaiacol at 470 nm for POX ($\varepsilon = 26.6\ \text{mM}^{-1}\ \text{cm}^{-1}$), the decomposition of H_2O_2 at 240 nm for catalase (CAT; $\varepsilon = 36\ \text{M}^{-1}\ \text{cm}^{-1}$) and the disappearance of ascorbate at 290 nm for ascorbate peroxidase (APX; $\varepsilon = 2.8\ \text{mM}^{-1}\ \text{cm}^{-1}$) as described previously (Mhadhbi et al. 2005).

Statistical analyses

For number of nodules, dry weight and nitrogen fixation, nine to ten replicates were used per treatment. Six replicates were used for enzyme activity and protein content. Data were subjected to analysis of variance (two-way ANOVA) and comparison of means by Duncan's multiple-range test ($P \leq 0.05$). Correlation between effectiveness indices and antioxidant activities was performed for the different associations.

Results

Effect of osmotic stress on symbiotic performance and plant–microbe interaction

Osmotic stress induced by the application of 75 mM mannitol decreased all parameters used to estimate plant growth (Table 1). Nitrogen-fixing capacity (ARA) was the most affected parameter under stress conditions in all symbiotic associations (Table 2). Nevertheless, we noted variability in the magnitude of the effect between symbioses according to the inoculated rhizobial strain and the genotype of host-plant. Indeed, symbioses implicating Jemalong J6 with the local *S. meliloti* (TII7) strain, and those associating TN8.20 genotype with both microbial strains manifested the highest level of tolerance to osmotic stress. These symbioses maintained high levels of aerial biomass production and nitrogen-fixing capacity (Tables 1 and 2). Symbioses implicating TN6.18 genotype were the most affected by mannitol application with inhibition rate reaching 60 % on shoot biomass and ARA

Table 1 Effect of mannitol-mediated osmotic stress (75 mm) on shoot dry biomass production (SDW, g/plant), root dry biomass (RDW, mg/plant), number of nodules (NN) and nodule fresh biomass (NFW, mg/plant) of *Medicago truncatula* genotypes Jemalong J6, TN8.20 and TN6.18 inoculated by RCR2011 and TII7 *Sinorhizobium meliloti* strains

	Jemalong J6				TN 8.20				TN 6.18			
	RCR2011		TII7		RCR2011		TII7		RCR2011		TII7	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
SDW	1.85^{c}	0.87^{f}	1.97^{b}	1.59^{d}	2.30^{a}	1.66^{d}	1.95^{b}	1.53^{d}	1.17^{e}	0.39^{g}	1.06^{e}	0.39^{g}
% Decrease	52.97		19.29		27.83		21.86		66.67		63.21	
RDW	414.70^{a}	336.50^{b}	360.53^{b}	303.30^{b}	378.22^{a}	248.90^{c}	206.80^{c}	263.80^{c}	$289.25b^{c}$	120.00^{e}	158.60^{d}	121.90^{e}
% Decrease	18.86		15.58		34.19		27.56		58.51		23.14	
NN	109^{a}	75^{b}	86^{b}	67^{c}	78^{b}	52^{d}	74^{b}	59^{c}	72^{b}	41^{e}	53^{d}	30^{f}
% Decrease	31.19		22.09		33.33		20.27		43.06		43.40	
NFW	183.12^{f}	95.45^{i}	215.7^{e}	174.32^{f}	438.1^{a}	370.14^{b}	312.5^{c}	281.77^{d}	183.85^{f}	101.9^{h}	147.2^{g}	93.78^{i}
% Decrease	47.88		19.18		15.51		9.83		44.57		36.29	

Values are means of 10 replicates. Means denoted with different superscript letters differ significantly at $P \leq 0.05$ based on Duncan's multiple-range test.

Table 2 Osmotic stress effect on nitrogen-fixing capacity (ARA, μmol h^{-1}/plant), nodule protein content (mg g^{-1} nfw), lipid peroxidation [malondialdehyde (MDA), μmol g^{-1} nfw] and leghaemoglobin content (lghb, mg g^{-1} nfw) of *Medicago truncatula* genotypes Jemalong J6, TN8.20 and TN6.18 inoculated with RCR2011 and TII7 *Sinorhizobium meliloti* strains

	Jemalong J6				TN 8.20				TN 6.18			
	RCR2011		TII7		RCR2011		TII7		RCR2011		TII7	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
ARA	11.18^{b}	5.88^{e}	12.78^{a}	7.76^{d}	12.65^{a}	6.96^{d}	11.86^{ab}	7.22^{d}	9.73^{c}	2.95^{f}	10.50^{c}	3.26^{f}
% Decrease	47.41		39.28		44.98		39.12		70.74		68.95	
Proteins	15.36^{b}	12.69^{c}	19.48^{a}	17.58^{ab}	13.58^{c}	11.62^{d}	13.69^{c}	11.45^{d}	15.75^{b}	10.30^{e}	19.05^{a}	11.56^{d}
% Decrease	17.38		9.75		14.38		15.73		34.60		39.32	
MDA	8.6^{d}	12.16^{b}	12.86^{b}	16.36^{a}	8.88^{d}	11.16^{c}	9.86^{d}	12.19^{b}	8.86^{d}	13^{b}	11.2^{c}	15^{a}
% Increase	41.39		27.21		25.67		23.63		46.72		33.92	
Lghb	3.75^{c}	2.6^{e}	4.25^{b}	3.25^{cd}	4.75^{b}	3.75^{c}	5.65^{a}	4.72^{b}	3.07^{d}	2.15^{e}	3.22^{cd}	2.25^{e}
% Decrease	30.67		23.53		21.05		16.46		31.60		30.12	

Values are means of nine replicates for ARA and six for MDA and lghb. Means denoted with different superscript letters differ significantly at $P \leq 0.05$ based on Duncan's multiple-range test.

activity. Plants of Jemalong J6 genotype inoculated with RCR2011 reference strain also showed high sensitivity to mannitol-mediated osmotic stress.

We performed a two-way ANOVA to estimate the impact of each partner on symbiosis behaviour. The variance of symbiotic effectiveness indices was dependent on the genotype of plant and rhizobia, as well as the interaction between the two partners. For biomass production, the contribution of plant genotype to the total variance was high (Fig. 1), with an average of 82 % for SDW and RDW, whereas it reached 66 % for NFW. A similar figure was observed in non-stressed plants. However, for the number of nodules and mainly ARA, the bacterial partner – that was the essential factor controlling the total variance of these parameters at non-stressful conditions – remained highly significant (30 %) even under osmotic stress. The interaction effect was slightly pronounced

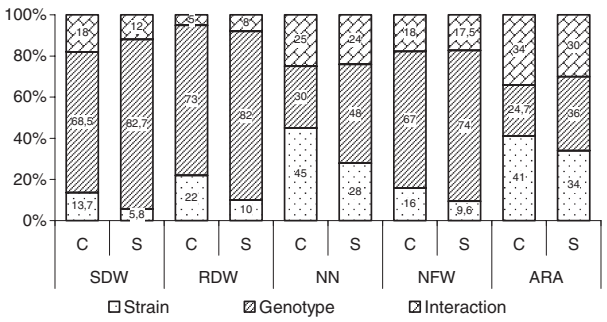

Fig. 1 Contribution of each partner of the symbiosis and the interaction between the two partners in the total variance of shoot dry weight (SDW), root dry weight (RDW), nodule fresh weight (NFW), nodule number (NN) and nitrogen fixation (ARA) of *Medicago truncatula–Sinorhizobium meliloti* symbiosis at control (unstressed) conditions (C) and under osmotic stress induced by 75 mm mannitol (S). Percentages given in the data are calculated from two-way ANOVA, $P \leq 0.05$.

under stress conditions except for nitrogen-fixing activity (ARA) where it averaged 30 %.

Nodule metabolism modulation by osmotic stress

Osmotic stress effect was assessed on nodules, the central organs of symbiosis. In addition to assessment of nitrogenase activity – the key enzyme present in nodules – by ARA analysis, we analysed some indices of structure stability (lipid peroxidation) and function (leghaemoglobin). The total protein rate was decreased for all symbioses (Table 2). The ARA and the leghaemoglobin content also declined, and decrease in the former was positively correlated with decrease in the latter ($r = 0.87$, $P \leq 0.001$). In stressed nodules there was an accumulation of MDA as final product of lipid peroxidation, mainly in sensitive symbioses (Table 2).

Analysis of enzymatic antioxidant defence, the main protective system in nodules (Becana et al. 2000), showed that osmotic stress differently affected the examined enzymes (Table 3). CAT activity was slightly lower but the decrease was nonsignificant in many cases. SOD and POX activities were stimulated by mannitol application. The maximum level of SOD increase (64 %) was found in nodules of the sensitive *M. truncatula* genotype TN6.18. However, POX activity was mainly induced in those involving the tolerant genotype TN8.20 (up to 65 %). APX activity was slightly but non-significantly increased by the osmotic stress in nodules of the overall analysed symbioses.

Discussion

Modulation of symbiotic performances by osmotic stress

Mannitol application affected all parameters used as indices to estimate symbiosis effectiveness. Nevertheless, ARA

Table 3 Modulation of the expression of antioxidant activities, superoxide dismutase (SOD, unit mg^{-1} protein), catalase (CAT, μmol H_2O_2 mg^{-1} protein), guaiacol peroxidase (POX, μmol H_2O_2 mg^{-1} protein) and ascorbate peroxidase (APX, μmol H_2O_2 mg^{-1} protein) in the nodules of *Medicago truncatula* genotypes Jemalong J6, TN8.20 and TN6.18 inoculated with RCR2011 and TII7 *Sinorhizobium meliloti* strains

	Jemalong J6				TN 8.20				TN 6.18			
	RCR2011		TII7		RCR2011		TII7		RCR2011		TII7	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
SOD	17.42^{d}	23.40^{b}	17.75^{d}	19.86^{c}	19.17^{c}	23.86^{b}	22.44^{b}	27.74^{a}	16.63^{d}	27.37^{a}	20.31^{bc}	30.04^{a}
% Activation	34.31		11.89		24.47		23.62		64.58		47.91	
CAT	69.61^{d}	56.79^{e}	97.48^{a}	84.77^{b}	96.18^{a}	84.64^{b}	102.60^{a}	94.60^{ab}	97.09^{a}	78.06^{c}	88.98^{b}	71.76^{d}
% Decrease	18.42		13.04		12.00		7.80		19.60		19.35	
POX	55.33^{b}	72.56^{a}	44.25^{c}	70.10^{a}	41.62^{c}	67.14^{a}	35.85^{d}	58.89^{b}	$38.70c^{d}$	53.75^{b}	29.57^{e}	40.13^{c}
% Activation	31.14		58.19		61.32		64.27		38.89		35.71	
APX	28.53^{c}	30.84^{b}	36.97^{a}	39.48^{a}	22.19^{e}	24.26^{d}	30.08^{b}	32.55^{b}	26.73^{c}	29.18^{bc}	30.13^{b}	32.80^{b}
% Activation	8.10		6.79		9.33		8.21		9.17		8.86	

Values are means of six replicates from three different experiments. Data denoted with different superscript letters differ significantly at $P \leq 0.05$ based on Duncan's multiple-range test.

was the most affected parameter. This sensitivity of nitrogenase activity compared to plant growth was reported previously (Verdoy et al. 2004, Lopez et al. 2008) and is explained by the complexity of interaction and the need for energy required by the nodule. Efficient symbioses at standard conditions remained the best performing ones under osmotic stress. Such results suggest that high biomass production and nitrogen-fixing capacity under osmotic stress is a consequence of high potential biomass production under non-stressful conditions (Mhadhbi et al. 2007, Pimratch et al. 2008).

The host-plant genotype was the major contributor to the total variance of the parameters used to estimate symbiotic effectiveness (shoot and root dry weight, nodule number and dry weight and nitrogen-fixing capacity) as was observed at non-stressful conditions in our previous study (Mhadhbi et al. 2005). This agrees with Fesenko et al. (1994), Robinson et al. (2000) and Pimratch et al. (2008) who reported the dependence of shoot biomass production on plant genotype, both at standard conditions and under water deficit. Otherwise, the contribution of inoculated rhizobial strain into ARA and nodulation observed in our results was reported earlier (Fesenko et al. 1994, Robinson et al. 2000, Kantar et al. 2003, Mhadhbi et al. 2007). ARA is known to strongly depend on the interaction between the two symbiotic partners (Mhadhbi et al. 2005, 2007) and the effect of environmental constraints (Serraj et al. 1999). Consequently, it could be concluded that each partner contribute to the variability of the symbiosis behaviour. Indeed, in this work we identified contrasting symbioses related to plant genotype – tolerant associations with TN8.20 and sensitive ones involving TN6.18, as well as contrasting behaviours of symbioses involving the same plant genotype (Jemalong J6) but different bacterial partner RCR2011 (sensitive) or TII7 (tolerant).

Stress effects and antioxidant system in nodules

Osmotic stress suppresses enzymes of metabolic pathways involved in nitrogen fixation such as sucrose synthase, phosphoenolpyruvate carboxylase, glutamate synthase, etc. (Figueiredo et al. 2007, Naya et al. 2007). This effect appears in the form of general decrease in ARA, nodule protein rate and leghaemoglobin content and an increase in lipid peroxidation. The correlation between the level of decrease in ARA and leghaemoglobin is explained by the key role of the latter –as oxygen transporter – in the process of bacteroids and cell respiration and, therefore, the adequate functioning of nitrogenase. A similar result was found in nodules submitted to salt stress (Nandwal et al. 2007, Lopez et al. 2008). The limitation in metabolic capacity of bacteroids and oxidative damage of cellular components are contributing factors to the inhibition of nitrogenase activity in nodules (Naya et al. 2007). One of the indices of cellular damage is the accumulation of MDA (Singh et al. 2007, Zhang et al. 2007) that was increased in stressed nodules. Lipid peroxidation was differentially affected depending on symbiosis tolerance levels. Taken together, the above results suggest that the level of nitrogen-fixing activity under stressful conditions is related to stability of membrane lipids and maintenance of sufficient amount of key metabolic proteins such as leghaemoglobin.

Catalase activity decreased under osmotic stress in nodules as reported previously (Mhadhbi et al. 2004, 2007, Nandwal et al. 2007). CAT is not regarded as the forefront of antioxidant defence under osmotic stress because of the low affinity to its substrate (Matamoros et al. 2003, Tejera et al. 2004), although this enzyme plays an important role in the nodulation process and nodule functioning (Jamet et al. 2003). SOD also plays a crucial role in symbiosis functioning (Santos et al. 2000), but it was not modulated by

moderate stress (Rubio et al. 2002). Present results showed an increase in SOD under stressful conditions, mainly in nodules of the most affected symbioses. Consequently, the protective role of SOD, for nitrogenase functioning, is essentially solicited when the physiological or even the structural state of nodules is greatly damaged by stress. POX is another important enzyme in the antioxidant defence of nodules under osmotic stress (Mhadhbi et al. 2004, 2007). The induction and protective role of peroxidases under salt and water osmotic stresses, as well as mineral toxicity, were widely reported in legume-rhizobia nodules (Mhadhbi et al. 2004, Jebara et al. 2005, Khan et al. 2007, Nandwal et al. 2007), other plant tissues (Türkun et al. 2005, Zhang and Nan 2007) and free-living rhizobia (Barloy-Hubler et al. 2004). Our data indicate that the increase in POX activity is significant in all stressed symbioses. Thus, POX induction could provide a sensitive marker for distinguishing tolerant symbioses. The high level of POX induction in tolerant nodules could protect nodule integrity via lignification, supporting the relative observed SOD increase.

In conclusion, symbiotic responses to osmotic stress are dependent on both symbiotic partners. Contrasting tolerant and sensitive symbiotic responses were identified for each host-plant genotype in association with different bacterial strains, as well as for each bacterial strain inoculated in different plant genotypes. Tolerance of the symbiotic association is primarily determined by the degree of host-plant tolerance. The bacterial partner has an input related to its potential efficiency under stress conditions. The forefront of antioxidant defence under osmotic stress in nodules is primarily an increase in POX activity while SOD involvement is more evident in nodules showing high levels of decrease in nitrogen-fixing capacity.

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