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Nodule Senescence in *Medicago truncatula–Sinorhizobium* Symbiosis Under Abiotic Constraints: Biochemical and Structural Processes Involved in Maintaining Nitrogen-Fixing Capacity

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Abstract Nitrogen-fixing capacity (NFC) in nodules of four *Medicago truncatula* lines inoculated with four strains of *Sinorhizobium* was assessed, during the plant life cycle, in relation to parameters identified as indices of plant growth, photosynthetic capacity, nodule integrity, and functioning. Differences in duration of the NFC period were observed among symbiotic associations and were correlated with variability on plant biomass production. Senescence appearance and vigor varied in parallel with structural, physiological, and biochemical stability of nodules. Maintenance of a longer high-NFC period was correlated to a higher stimulation of antioxidant enzymes, mainly superoxide dismutase (SOD, EC 1.15.1.1) and guaiacol peroxidase (POX, EC 1.11.1.7), and a consequent longer maintenance of membrane integrity and nodule structure within the first stages of senescence. Salinity and drought stresses interfered with nodule functioning and triggered fast and global nodule senescence, albeit a superiority of nodules having a long high-NFC period. The protective role of POX activity on salt- and drought-stressed nodules was revealed. On the other hand, SOD stimulation was independent of stress application. Another strategy allowing the maintenance of longer NFC in salt-stressed nodules could be the accumulation of starch granules in the senescence-functioning interface of nodules. This finding is currently under investigation. Interestingly, the symbioses with different behaviors of nodule senescence identified in this work would be useful bases for biochemical, genomic, and proteomic studies dissecting nodule senescence.

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Introduction

The efficiency of legume–rhizobia symbiosis is partly dependent on the ability of this association to maintain functional nodules during the life cycle. Nodules attain their optimal activity around the flowering stage. After that, the nitrogen-fixing capacity (NFC) stabilizes and then decreases. The duration of the optimal nitrogen-fixing period is the determinant of symbiosis efficiency. Nodule senescence is defined as a rapid decline in biological nitrogen fixation and leghemoglobin content (Pfeifer and others 1983). It is an irreversible phenomenon that develops differentially between determinate and indeterminate nodules (van de Velde and others 2006). Evans and others (1999) defined senescence as a biochemical and physiological event observed at the final stage of maturation that requires the transcription of new genes and results in death of the nodule. The first symptoms of senescence are changes in color and loss of turgidity in the first-formed nodules (Swaraj and others 1995; Lequeño and others 2008), and the pink coloring due to the presence of leghemoglobin darkens or becomes green (Muller and others 2001). During senescence, membrane lipids, proteins, and nucleic acids degrade through peroxidation by reactive oxygen species (ROS), leading to degradation of the peribacteroid membrane (PBM) (Hernandez-Jimenez and others 2002) and decline in biological nitrogen fixation (Marino and others 2007). The density of bacteroids in nonsenescent cells is much greater than in senescent ones (Cermola and others 2000; Puppo and others 2005; Guerra and others 2010). Nodulins involved in

metal exchange between symbiotic partners were shown to be downregulated in senescent soybean nodules, suggesting a reduction of exchange during the senescence process (Alesandrini and others 2003). Nodule senescence is a highly organized process orchestrated in an age-dependent manner but also determined by environmental factors such as high temperature, darkness, salinity, drought, or pathogen infections (Matamoros and others 2003; Groten and others 2006; Guerra and others 2010). The morphological and metabolic damage that occurs during nodule senescence varies depending on stress conditions. Osmotic stresses such as drought and salinity cause rapid and excessive accumulation of ROS in plant cells leading to oxidative stress and an early senescence (Matamoros and others 2003; Wang and others 2003; Nandwal and others 2007; Garg and Manchand 2008). Increased activity of antioxidant enzymes in nodules protects biological nitrogen fixation under salinity and drought (Mhadhbi and others 2004, 2008, 2009, 2011), and consequently moderates the premature nodule senescence (Porcel and others 2003; Matamoros and others 2003). Retarding senescence is a determinant of enhanced nitrogen fixation and increased yields (van de Velde and others 2006).

Understanding how genes operate during senescence and identifying molecular markers is crucial for any subsequent senescence control (Webb and others 2008; Guerra and others 2010). As a model legume, *Medicago truncatula* (*Mtr*) is a suitable system to study the response to pathogenic agents and abiotic stresses (Rose 2008). Recently, Guerra and others (2010) published a study on *Mtr* nodule senescence where they reported different evolutions between natural and dark-induced senescence, mainly with respect to the expression of cysteine proteinase (CP) genes. The present study was performed on various *Mtr–Sinorhizobium* associations to identify contrasting behaviors and related mechanisms during natural and stress-induced nodule senescence by salinity and drought. Analyses were focused on nodule morphology, structure, integrity, and functioning, as well as on the antioxidant enzymes involved in the protection of the nodules against ROS, the major cause of nodule senescence.

Materials and Methods

Growth Conditions, Rhizobia Inoculation, and Abiotic Stress Application

Medicago truncatula line Jemalong 6 and the local lines TN8.20, TN6.18, and TN1.11 were scarified and germinated as previously described (Mhadhbi and others 2005). *Sinorhizobium meliloti* strains RCR2011, TII7, and E4 and *S. medicae* strain SII4 were grown on YEM liquid medium for 24 h at 28°C and used to inoculate 48-h old seedlings (approximately 10^9 cfu). Seedlings were grown in sterile-sand pots in a glass house at 25/22°C day/night temperatures, 60–70% relative humidity, and 16/8-h photoperiod. Plants were regularly watered with a free-nitrogen nutritive solution for inoculated plants and a nitrogen-rich solution for controls (Mhadhbi and others 2005) to maintain field capacity at 85–90%. Stress application was performed on 30-day-old nodulated plants. For salt treatment, 75 mM NaCl was added to the watering solution. For drought treatment, field capacity was maintained at 35%.

Acetylene Reduction Assay (ARA)

Nitrogenase (EC 1.7.9.92) was assayed by acetylene reduction activity (ARA) by gas chromatography with a Porapak-T column using pure acetylene and ethylene as internal standards, as detailed by Mhadhbi and others (2009). ARA was monitored during the nodule life cycle using three plants for each treatment.

Chlorophyll and Leghemoglobin Contents

Chlorophyll content in leaves was spectrophotometrically determined after extraction in 80% acetone according to Lichtenthaler (1987). Nodule leghemoglobin content was determined according to the method described by Schiffman and Löbel (1970) with few modifications. Fresh nodules (100 mg) were homogenized in 3 ml Drabkin's solution. Leghemoglobin content was determined using bovine hemoglobin as standard. Three plants were considered for each treatment.

Lipid Peroxidation Assay

Lipid peroxidation in nodules was assayed using the thiobarbituric acid (TBARS) method modified according to Singh and others (2007). Nodules (300 mg) were homogenized in 3 ml of 0.1% TCA solution. The homogenate was centrifuged at $10{,}000 \times g$ 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 nonspecific absorption at 600 nm was subtracted. The amount of MDA was calculated using the extinction coefficient $\varepsilon = 155\ \mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$. Three plants were considered for each treatment.

Enzyme Activity Assays

Superoxide dismutase (SOD; EC 1.15.1.1) was determined spectrophotometrically by monitoring the inhibition of the 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 determined as previously described (Mhadhbi and others 2005). These were performed by monitoring the formation of tetraguaiacol from guaiacol at 470 nm for guaiacol peroxidase (POX; EC 1.11.1.7) ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$), the decomposition of H_2O_2 at 240 nm for catalase (CAT; EC 1.11.1.6) ($\varepsilon = 36\ M^{-1}\ cm^{-1}$), and the disappearance of ascorbate at 290 nm for ascorbate peroxidase (APX; EC, 1.11.1.11) ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). Three plants were considered for each treatment.

Microscopic Analysis

Nodules were fixed in formaldehyde, washed with distilled water, dehydrated, and embedded in paraffin. Longitudinal sections of nodules (15 μm) were made using the KD-400 vibration microtome (Kedee, Korea) and mounted on slides, deparaffined with toluene, rehydrated in distilled water, and stained with toluidine blue/malachite green. The specific localization of starch granules was performed by lugol staining (0.3 g I_2 and 1.5 g KI in 100 ml distilled water). Photographs were taken with a light microscope equipped with a camera (Olympus BX41, Tokyo, Japan).

Results

Development of the Nitrogen-Fixing Capacity During Nodule Life Cycle

Based on previous unpublished results conducted on 25 different associations where ARA was monitored from the onset of nodule formation until late in the plant life cycle (90 days after sowing [das]), the present experiment was designed to monitor the evolution of the NFC *in vivo* by monitoring ARA from 63 to 82 das. Analyses were performed on four lines of *M. truncatula* (Jemalong 6, TN8.20, TN6.18, and TN1.11) inoculated with three strains of *S. meliloti* (RCR2011, TII7, and E4) and one strain of *S. medicae* (SII4). The maximum ARA activity was observed at 68 das, corresponding to the main flowering stage. Then the nitrogenase activity dropped and was severely affected following the end of the nodule life cycle corresponding to the late senescence phase of nodules (82 das) (Fig. 1). Differences in the rapidity of ARA decrease were revealed. These differences depended on plant genotype and bacterial strain. Symbiosis involving strain TII7 and line TN8.20 maintained a longer period of high NFC (ARA) in nodules (68–74 das). However, for nodules of Jemalong 6–TII7 symbiosis, ARA dropped immediately after reaching the maximum at 68 das. The TN6.18 line produced nodules with different senescence behaviors when inoculated with TII7 or RCR2011. Nodules

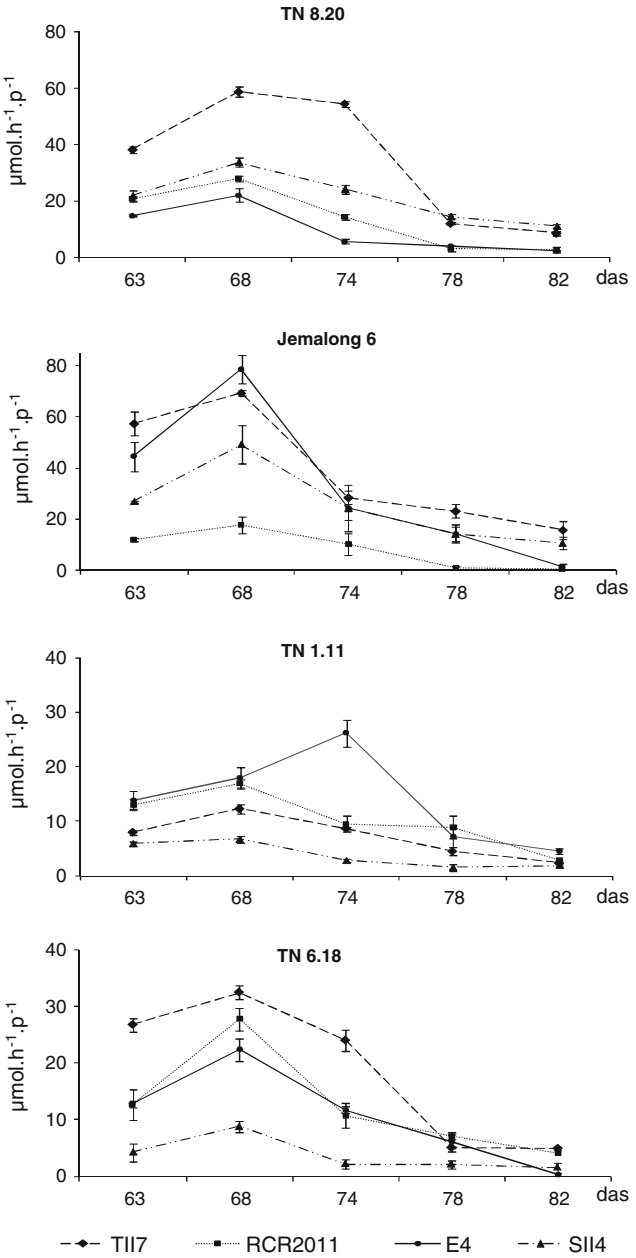

Fig. 1 Age-dependent (days after sowing, das) development of nitrogen-fixing capacity, estimated in vivo by ARA (μmol $C_2H_4\ h^{-1}\ plant^{-1}$) in nodules of four *Medicago truncatula* lines (TN1.11, Jemalong 6, TN8.20, TN6.18) inoculated with four *Sinorhizobium* strains (RCR2011, TII7, E4, SII4). ARA was monitored from the beginning of the flowering stage (63 das) to the end of the pods' maturation stage (82 das). Data are means ± SD

of TN1.11–E4 reached the maximum of ARA at 74 das, whereas activities of symbioses involving TN1.11 and all other strains dropped at 68 das. Four contrasting symbioses were maintained for subsequent analyses, with TN8.20–TII7 and TN6.18–TII7 exhibiting longer high-ARA periods and Jemalong 6–TII7 and TN6.18–RCR2011 showing short high-ARA periods.

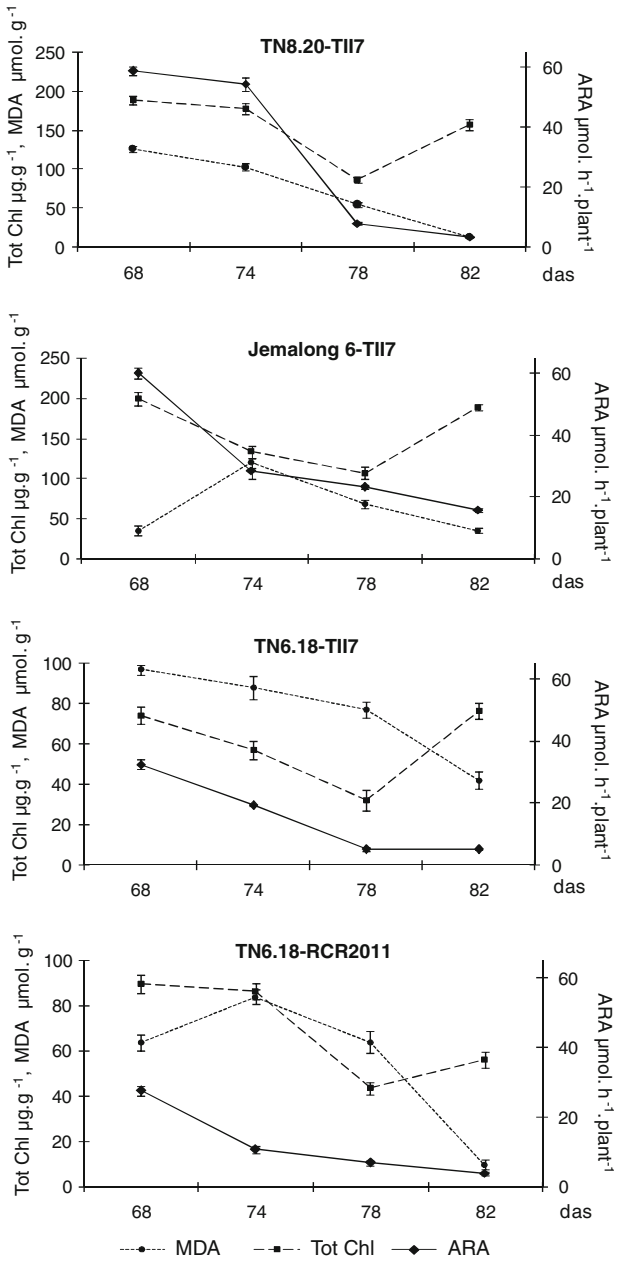

Fig. 2 Coevolution of total chlorophyll content (Tot Chl µg g^{-1} leaf fresh weight, Y_1 axis) and lipid peroxidation in nodules (MDA µmol g^{-1} nodule fresh weight, Y_1 axis) with the nitrogen-fixing capacity (ARA µmol C_2H_4 h^{-1} $plant^{-1}$, Y_2 axis) during the senescence phase (68–82 das) of *Mtr–Sinorhizobium* symbioses TN8.20–TII7 and TN6.18–TII7 and Jemalong 6–TII7 and TN6.18–RCR2011. Data are means ± SD

Relationship Between Nitrogen Fixation, Biomass Production, Plant Photosynthetic Apparatus, and Nodule Integrity Indices in the Contrasting Symbioses

ARA was assessed during the senescence phase of nodules (between 68 and 82 das) for the chosen contrasting symbioses (TN8.20–TII7/Jemalong 6–TII7 and TN6.18–TII7/TN6.18–RCR2011) concomitantly with the concentration of chlorophyll pigments, MDA, total protein, and leghemoglobin contents. The evolution of chlorophyll content was similar to that of NFC until 78 das, and then it increased for all analyzed symbioses, whereas ARA continued to drop (Fig. 2). On the other hand, nodule protein and leghemoglobin contents did not show important variations during senescence (data not shown). Lipid peroxidation estimated by MDA accumulation showed similar behavior to that of nitrogenase activity (of continuous decrease) in the nodules of TN8.20–TII7 and TN6.18–TII7 symbioses. However, MDA content of Jemalong 6–TII7 and TN6.18–RCR2011 symbioses increased during the first senescence phase (68–74 das), and then dropped similarly to ARA (Fig. 2). At 82 das, biomass measurement showed that symbioses with nodules maintaining a long high-ARA period produced shoot biomasses 1.5–2-fold higher than those given by the symbioses characterized by an ARA that rapidly drops at 68 das. Nevertheless, for root biomass no significant difference was found between the two groups of symbioses (Table 1).

Fig. 3 Change in nodule structure of TN8.20–TII7 symbiosis during developmental and stress-induced senescence localized by toluidine blue/malachite green staining. **A** Expansion of the senescence zone upon aging identified on longitudinal section of nodules at 68 das (*a*, *a*′), 74 das (*b*, *b*′), and 78 das (*c*, *c*′). **B** Salt-related plasmolysis of cells and destruction of nodule symbiosomes. *a* apparition of nonregular cell walls and plasmolyzed cells, *b* destruction of cell walls and dispersion of symbiosome structure, *c* expansion of deserted area and degeneration of nodule structures. **C** Specific lugol staining showing accumulation of starch granules in the approximate functional zone (bacteroid mass) of salt-stressed senescent nodules. *m* meristem, *i* infection zone, *f* fixation zone, *sc* senescence zone, *sy* symbiosome, *st g* starch granules

Changes in Nodule Structure During Senescence

Light microscope observation of longitudinal sections of nodules stained with toluidine blue/malachite green was performed at different senescence phases (Fig. 3). Mature nodules (68 das) presented the common developmental zones of indeterminate nodules, cortex, apical meristem, infection zone, and fixation zone (Fig. 3A, a and a′). Senescent nodules showed the establishment of a senescence zone proximal to the fixation zone (Fig. 3A, b). Upon aging, this zone gradually expanded until reaching the apical part (Fig. 3A, c) and invading the entire nodule surface (82 das). The senescence zone was characterized by the disintegration of the peribacteroid membrane leading to the loss of the integrity of the fixation zone and the degeneration of the microsymbiont (Fig. 3A, b′ and c′). Expansion of this senescence zone was more moderate within nodules maintaining a long high-ARA period.

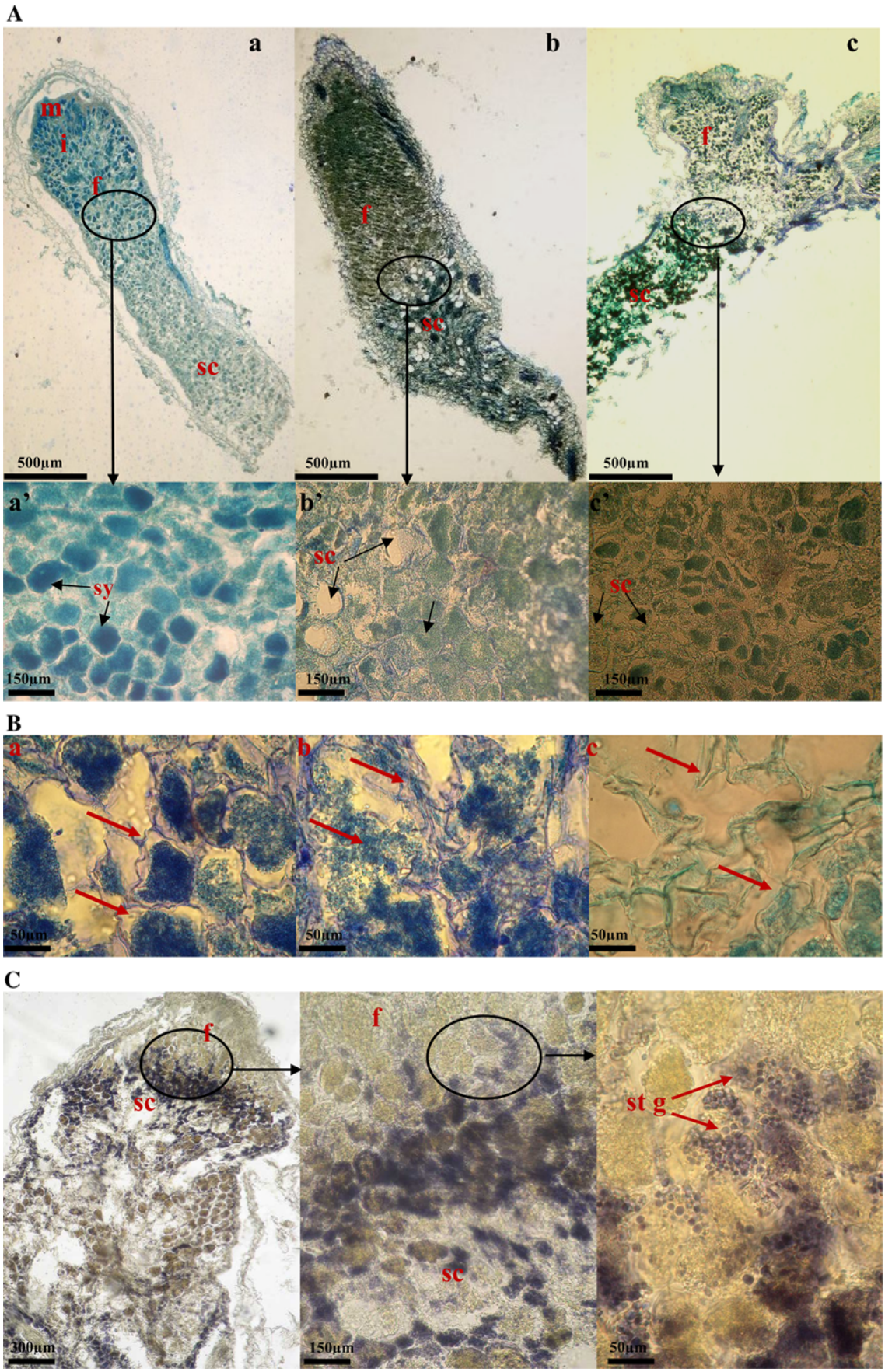
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Table 1 Shoot dry weight (SDW) and root dry weight (RDW) biomass production of *Medicago truncatula* lines TN8.20, Jemalong 6, and TN6.18 irrigated with nitrogen-rich solution (N) or nitrogen-free solution and inoculated with *Sinorhizobium meliloti* TII7 and RCR2011 strains

Symbioses	SDW (g plant^{-1})	RDW (g plant^{-1})
TN8.20–TII7	1.93 ± 0.28[a]	0.44 ± 0.09[a]
Jemalong 6–TII7	0.86 ± 0.07[c]	0.34 ± 0.07[a]
TN6.18–TII7	1.19 ± 0.06[b]	0.21 ± 0.05[b]
TN6.18–RCR2011	0.80 ± 0.1[c]	0.23 ± 0.05[b]
TN8.20 N	2.01 ± 0.16[a]	0.32 ± 0.02[a]
TN6.18 N	1.87 ± 0.19[a]	0.21 ± 0.01[b]
Jemalong 6 N	2.10 ± 0.09[a]	0.36 ± 0.03[a]

Plants were harvested 82 days after sowing. Data denoted with different letters are significantly different according to Tukey test at $P \leq 0.05$

Nevertheless, there was no specific nodule structure characterizing this senescence ability.

Antioxidant Enzyme Activities Within Senescent Nodules

Monitoring of SOD, APX, CAT, and POX activities in senescent nodules along with the drop in ARA revealed different behaviors of enzyme expression between nodules of symbioses maintaining longer high-ARA periods (TN8.20–TII7 and TN6.18–TII7) and symbioses with rapidly dropping ARA (Jemalong 6–TII7 and TN6.18–RCR2011). Between 68 and 74 das, CAT activity showed a general decrease in nodules of all analyzed symbioses (Fig. 4). However, POX, APX, and SOD activities were increased. POX and SOD showed higher increased levels in nodules maintaining longer high-ARA periods. This difference between the two enzymes was still obvious until 78 das. The kinetics of APX activity was generally similar for all nodules. Beyond 78 das, POX and SOD were moderately decreased in nodules maintaining a long high-ARA period but remained elevated in nodules with a short high-ARA period.

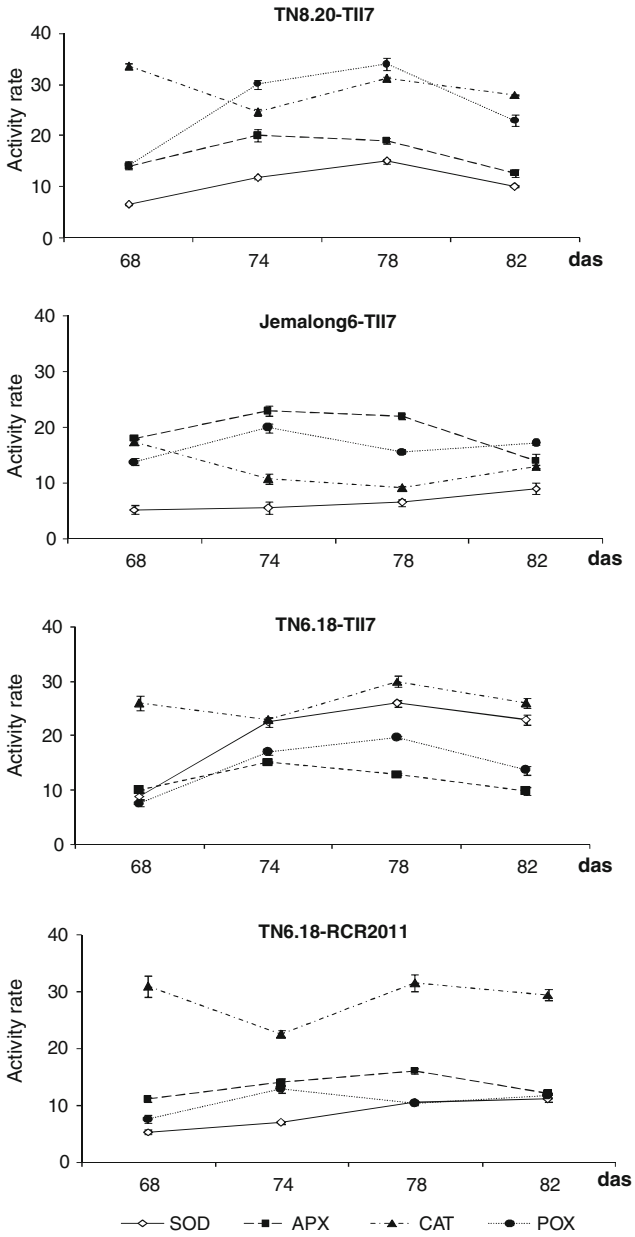

Fig. 4 Modulation of the antioxidant enzyme activities (SOD, U mg^{-1} proteins; POX, CAT, and APX, μmol H_2O_2 min^{-1} mg^{-1} proteins) during nodule senescence in nodules of symbioses TN8.20–TII7 and Jemalong 6–TII7. Data are means ± SD

Effect of Abiotic Stresses on Nodule Senescence

The evolution of nodule senescence under salinity and water deficit was assessed. Analyses were focused on the contrasting couple of symbioses, TN8.20–TII7 (longer high-ARA period) and Jemalong 6–TII7 (shorter high-ARA period). The inhibiting effects of salinity and drought on ARA activity were drastic (Fig. 5). Symbioses grown under salinity and water deficit showed a lower NFC during the nodule life cycle. Moreover, there was an earlier drop in ARA compared to those of control symbioses. The effect of salinity and drought on nodule functioning was also observed on protein, chlorophyll, and leghemoglobin contents (data not shown) and on lipid peroxidation (Fig. 5), where MDA levels, already higher than those of controls, increased in Jemalong 6–TII7 mainly during the period of 56–74 das. However, in nodules of TN8.20–TII7, the MDA level did not significantly change in the same period.

Senescent nodules under drought were not different from those in natural senescence at the structural level (data not shown). However, in salt-stressed nodules, there

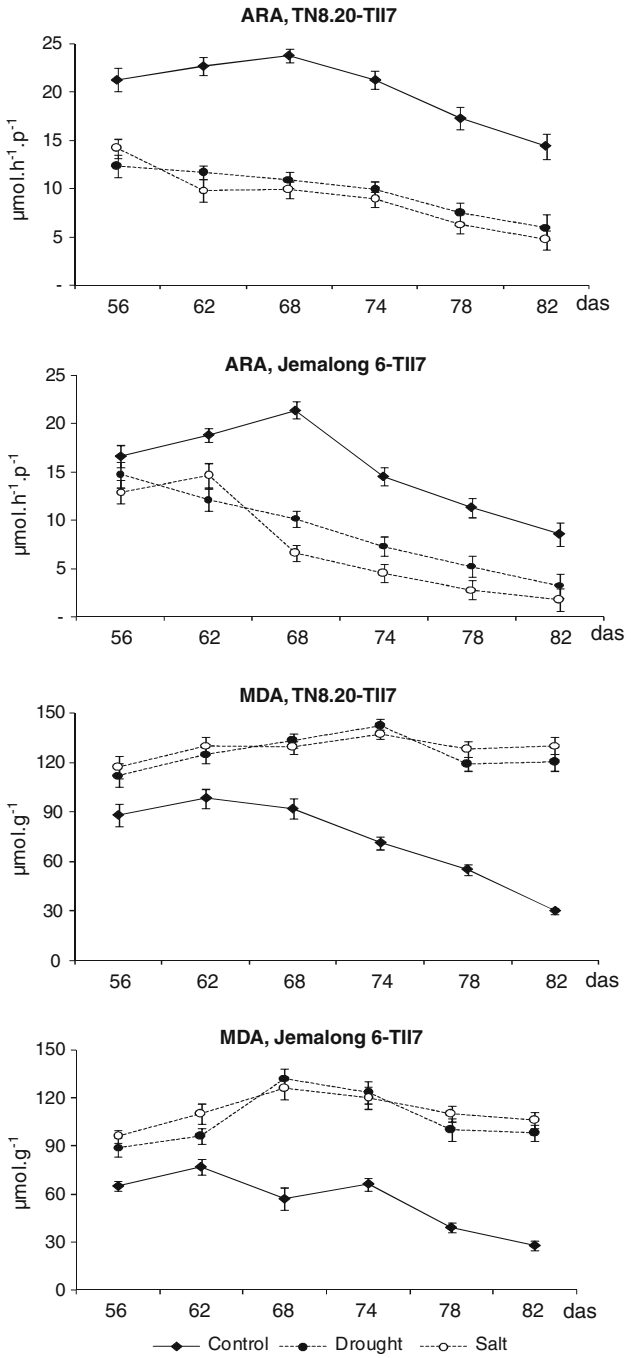

Fig. 5 Effects of salinity (75 mM NaCl) and water deficiency (35% field capacity) on the evolution of the nitrogen fixing capacity (ARA, µmol C_2H_4 h^{-1} $plant^{-1}$) and lipid peroxidation (MDA µmol mg^{-1} nodule fresh weight) of nodule from TN8.20–TII7 and Jemalong 6–TII7 symbioses. Data are means ± SD

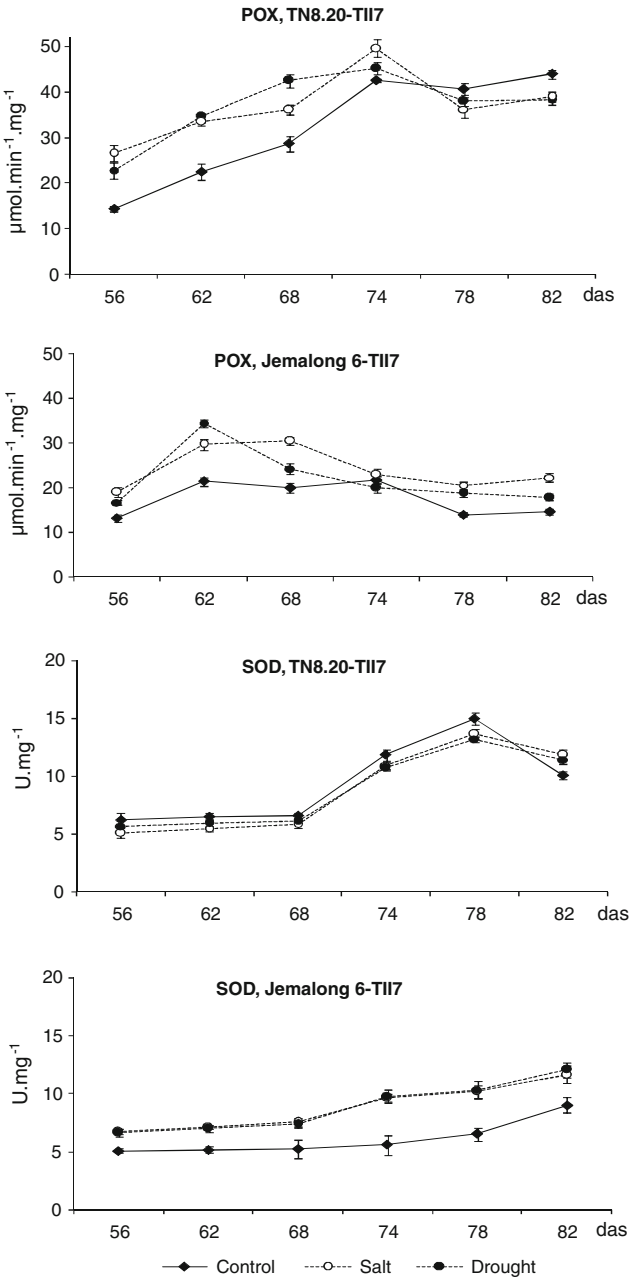

Fig. 6 Effects of salinity (75 mM NaCl) and water deficiency (35% field capacity) on the activities of guaiacol peroxidase (POX, µmol H_2O_2 min^{-1} mg^{-1} proteins) and superoxide dismutase (SOD, U mg^{-1} proteins) in nodules of TN8.20–TII7 and Jemalong 6–TII7 symbioses. Data are means ± SD

were plasmolyzed cells with nonregular cell walls (Fig. 3B) that gradually degenerated leading to loss of nodule structure. Moreover, specific lugol staining showed an accumulation of starch granules on salt-stressed senescent nodules at the interface between the fixation and the senescence zones (Fig. 3C). This accumulation was adjacent to the bacteroids (symbiosomes) and was more abundant in the nodules of TN8.20–TII7 symbiosis characterized by a long high-ARA period.

Nodule Antioxidant Defense Under Abiotic Stresses

Based on modulation of antioxidant enzymes during natural senescence (Fig. 4), we focused on the evolution of POX and SOD enzymes under abiotic stresses.

Under all conditions, POX activity in TN8.20–TII7 nodules increased up to 74 das and decreased only in stressed conditions thereafter. In contrast, POX activity in Jemalong 6–TII7 nodules showed an initial increase only and returned to control levels around 74 das. From 78 das, the evolution of POX activity in stressed nodules dropped below the control level in the case of TN8.20–TII7 nodules. However, it remained above the control level for nodules of Jemalong 6-TII7 symbiosis. Salt and drought treatments induced a significant increase (1.5–2-fold) on SOD activity in nodules of Jemalong 6–TII7 symbiosis. The main difference between both types of symbioses was the decrease in SOD activity at 78 das in nodules of TN8.20–TII7 symbiosis, maintaining a long high-ARA period (Fig. 6).

Discussion

Development of Integrity, Functioning, and Protection Parameters During Nodule Natural Senescence in Contrasting Symbioses

Decline in nitrogenase activity, indicating development of nodule senescence, could be measured using the ARA (Webb and others 2008; Stanchev and others 2010). In this study, ARA monitoring led to the identification of symbioses, with contrasting behaviors toward natural senescence, having either long or short high-ARA periods. The differences in the ARA evolution between the two groups of symbioses were supported by concomitant differences revealed on biomass production (Table 1). This suggests that even if the difference in senescence delay is not so important (6 days, 68–74 das), this period of difference in ARA significantly influenced plant growth. Contrasting symbioses involving the same rhizobial strain but different plant genotypes were identified. The involvement of plant genotype in nodule senescence was reported by Lequeño and others (2008) for common bean (*Phaseolus vulgaris*) and by Espinosa-Victoria and others (2000) for soybean (*Glycine max*). Moreover, we identified contrasting symbioses involving the same *Mtr* genotype and different rhizobial strains. These contrasting symbioses would be useful bases for biochemical, genomic, and proteomic studies dissecting nodule senescence. Selected symbioses were analyzed with respect to ARA and additional markers such as photosynthetic capacity, nodule integrity, and functioning. For nodules maintaining a long high-ARA period, the concomitant drop in MDA and ARA could be explained by a general slowing down of nodule activity and consequently the decrease of ROS production (the main cause of lipid peroxidation) in nodules as coproducts with metabolic processes. In this group of nodules, membrane integrity was protected essentially by an increase of SOD and POX antioxidant activities that control the development of nodule metabolism during senescence. The increase of MDA during the first phase of nodule senescence (68–74 das) for nodules showing a rapid drop of ARA, which indicates membrane disintegration, could be attributed to an insufficiency of antioxidant enzymes that were weakly stimulated in this group of nodules. In the second phase (74–78 das), CAT was induced only in the first group of nodules, forming a supplemental means of antioxidant defense. The concomitant evolution of ARA and chlorophyll content described here was also reported for common bean and pea (Verdoy and others 2004; Groten and others 2006). For both kinds of nodules, the last phase of senescence was characterized by a general decrease of all analyzed functioning indices. Decline of protein and leghemoglobin contents at the end of the nodule life cycle could be related to the activation of proteolytic activities (van de Velde and others 2006) and the accumulation of ROS, causing oxidative stress (Hernandez-Jimenez and others 2002; Wang and others 2003; Marino and others 2007). Hernandez-Jimenez and others (2002) and Lequeño and others (2008) reported that during senescence, membrane lipids, proteins, and nucleic acids are degraded by generated free radicals because ROS are involved in the degenerative process associated with senescence and cell death (Becana and others 2000; Pauly and others 2006).

Variability of Nodule Response to Stress-induced Senescence

In this experiment we aimed to investigate the effect of salinity and drought on nodule senescence. Salinity and drought induced a drastic effect on ARA. These two osmotic stresses are known to cause rapid and excessive accumulation of ROS, leading to oxidative stress (Wang and others 2003; Clement and others 2008). The environmental stress-induced nodule senescence develops much more rapidly than developmental senescence (Guerra and others 2010) and presents features of oxidative stress (Puppo and others 2005; Pauly and others 2006; Garg and Manchand 2008). This led us to investigate the role of the antioxidant system in the control of nodule senescence. SOD and POX were correlated to the evolution of senescence between contrasting nodules. Indeed, between 68 and 78 das, stimulation of SOD and POX activities paralleled the maintenance of high-ARA periods in TN8.20–TII7 nodules. SOD and POX were consequently monitored under abiotic constraints. In comparison to controls, SOD was stimulated only in nodules with a short ARA period. This suggests that SOD implication in senescence modulation is independent of stress application. Indeed, nodule SOD is not stimulated in salt- and drought-tolerant nodules but in

sensitive ones (Mhadhbi and others 2004, 2009). In nodules maintaining a longer high-ARA period, the POX was stimulated by stress application until 74 das. During this period, nodules showed a relatively constant ARA activity and a moderate increase of lipid peroxidation. In the second group of nodules, the stimulation of POX declined starting at 62 das, similar to ARA. The differential evolution of POX activity under abiotic stresses between the two groups of nodules concomitantly with NFC could be related to the protective role played by this enzyme. Another strategy that allows nodules to maintain longer and high nitrogen-fixing activity under salt stress could be the accumulation of starch granules in the senescence-functioning interface revealed in nodules that maintain a longer high-ARA period. Starch granules could be involved as a homeostasis keeper through Na^+ binding (Kanai and others 2007) and as an energetic source for bacteroid functioning.

Conclusion

Different nodule senescence developments were revealed in symbioses involving the same rhizobial strain with different plant genotypes, but also in nodules of the same *M. truncatula* genotype inoculated with different rhizobial strains. This study allowed the identification of two behaviors toward nodule senescence: nodules that maintain a long high-ARA period and others that show a rapid drop in ARA. These contrasting symbioses would be useful bases for biochemical, genomic, and proteomic studies dissecting nodule senescence. Comparative analysis of the development of nodule integrity, functioning, and protection indices between the two groups of nodules under standard conditions and abiotic constraints showed that the difference in senescence velocity is related to the subsequent difference in maintaining nodule integrity and metabolism. The delay of nodule senescence was correlated to the efficiency of the protective antioxidant enzymatic system, mainly to the increased guaiacol peroxidase activity during the different senescence phases. Under salt stress, accumulation of starch granules could be an additional tool of homeostasis maintenance as well as an energy source for nitrogen fixation.

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