

## Oxidative Stress in Pea (*Pisum sativum* L.)-Rhizobia Symbiosis is Induced under Conditions of Salt Stress

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### ABSTRACT

After exposing Pea (*Pisum sativum* L.)-rhizobia symbiosis to two levels of NaCl (namely, 100 and 150 mM) in perlite culture, the salt-stressed plants were analyzed for nodulation, plant dry weight, total phenols, Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), Peroxidase (PO), PolyPhenol Oxidase (PPO), and Electrolyte Leakage (EL). In results, it was observed that the shoot dry weight of all examined *P. sativum*-rhizobia symbiosis had a statistically significant increase. It was also ascertained that the presence of salt increased the root length as well as the root dry weight. This increase ranged between 76 and 80%, respectively, under 100 and 150 mM NaCl. The same trend was detected for the nodule number and dry weight, which increased in response to salt stress in *P. sativum*-rhizobia symbiosis. Under salt stress (150 mM), shoot N content was three times more than in root. A relationship was revealed between nodulation and growth that was associated with N level in shoot and root. Generally, EL values were affected by salt in leaves with variations ranging between 22 and 37% under 150 and 100 mM NaCl supply, respectively. Concerning H<sub>2</sub>O<sub>2</sub> content in leaves, significant differences were noted in comparison to the control treatment that was stabilized after 30 days of inoculation. After 50 days of inoculation, H<sub>2</sub>O<sub>2</sub> content in leaves was almost six times higher than after 10 days. In general, salt stress did not affect PO activity. However, PPO activity increased over time, exceeding 10 µmol g<sup>-1</sup> FW.

**Keywords:** Pea (*Pisum sativum* L.), Rhizobia, Peroxidase, Phenols, Salinity.

### INTRODUCTION

Grown in many countries, Pea (*Pisum sativum* L.) is a vegetable crop that is used as dry pulses, edible-podded type, fresh peas, and even as fodder. As a legume, pea has an important characteristic that is its ability to fix atmospheric nitrogen in symbiosis with nodule-forming rhizobia (Beiranvand *et al.*, 2018). Rich in nutrients including proteins and carbohydrates (Hussein *et al.*, 2006), water-soluble fibers, thiamin (B<sub>1</sub>), and water-soluble antioxidants (Mukerji, 2004), peas are a winter crop. However, certain abiotic constraints like drought, heavy metals, cold, low nutrient

availability, and salinity can dramatically affect pea-rhizobia symbiosis (Abdi *et al.*, 2012; Clement *et al.*, 2008). Therefore, a major environmental threat is imposed on agriculture by salt stress since it limits pea growth, reduces N<sub>2</sub> fixation and related physiological parameters, and crop production (Mirtalebi and Banihashemi, 2019). It induces low osmotic potential, alterations in different metabolic activities of plants (Munns *et al.*, 2006), inhibition of enzymatic activities, ionic imbalance, and disturbances in solute accumulation, specific ion effects, or combination of all these factors (Munns *et al.*, 2006). Thus, several strategies have already

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been adopted and efforts are made to explore different mechanisms for salinity tolerance that would help in the alleviation of the harmful consequences of salinity stress (Logan, 2005). Increased enzymatic and physiologic reactions and phenol secretion are the most crucial of the various mechanisms to assume endurance to salt stress. Higher levels of antioxidant enzymes are generally seen in salt tolerant genotypes than in salt-sensitive genotypes (Logan, 2005). An increase in antioxidant enzymes/molecules was observed in these reports; the increase is at least a proportion of the mechanism of salt tolerance in pea. Reactive Oxygen Species (ROS) are endogenously generated during the normal plant metabolism (Foyer *et al.*, 1994), but their amounts shoot up and need to be scavenged under stressful conditions (Gueta-Dahan *et al.*, 1997; Chang *et al.*, 2009; Kamangar and Haddad, 2016). Oxidative stress is among the various influences caused by salt stress (Ashraf, 2009; Noreen *et al.*, 2009). It has been well established that to counter-act salt-induced oxidative stress plants generate different types of enzymatic and non-enzymatic antioxidants (Gupta *et al.*, 2005). In short, greater tolerance to environmental salinity (Lynch and Clair, 2004) and a rise in the activities of antioxidant enzymes (Demiral and Turkan, 2005; Bor *et al.*, 2003; Turkan *et al.*, 2005) have a stronger interrelation. Many activities like the concentration of hydrogen peroxide, the content of total phenols, leakage in electrolyte, and function of polyphenol oxidase and peroxidase marked a difference in the pea that fixes N<sub>2</sub> in plants for a longer time under salt stress. However, although a number of vegetable legumes species have shown a broad array of genetic adaptations to saline conditions, vegetable plants (including pea) have not yet been understood in the context of the fundamental mechanisms of oxidative stress tolerance. This study, thus, becomes among the first reports investigating N<sub>2</sub>-fixing pea under salt stress for morphological, physiological, and oxidative metabolism changes. Therefore, the major goals were to ascertain: (a) The extent of regulation of various antioxidants and metabolites, and (b)

Whether there exists a positive association of antioxidant metabolites with the degree of salt tolerance in *P. sativum*-rhizobia symbiosis.

## MATERIALS AND METHODS

Salt-tolerant strain of Rhizobia nodulating pea is used to protect seeds of *Pisum sativum* that are cultivated widely in Tunisia. This efficient strain named “Ar.13” was selected based on the results of nodulation test and its salt tolerance to inoculate pea seedlings. Experiments were conducted in glasshouse conditions under natural light with day/night temperatures of 28/20°C, relative humidity above 78%, and a 16-h photoperiod at the National Institute for Agricultural Research of Tunis (INRAT), Tunisia. Special greenhouse conditions and plant growing pots were set for growing the seedlings. Seeds were primarily sterilized in 2% calcium hypochlorite after washing with sterile distilled water for 5 times and germinated in soft agar with 100 mL of Bergersen solution at 28°C (Vincent, 1970). Rhizobial inoculant was prepared with salt-tolerant rhizobia strains and preserved at 4°C in tubes in Yeast Extract Mannitol (YEM). Liquid YEM solution was used to grow Rhizobia into an Erlenmeyer with agitation. This was accomplished at a temperature of 28°C for 2 days, in complete darkness. Four-day-old seedlings were soaked for 30 minutes as part of inoculation; approximately 10<sup>8</sup> cells mL<sup>-1</sup> were contained in 100 mL of inoculants. Sterile plastic pots of 1 kg sterilized perlite were used to transplant these seedlings. These were then separated into 2 groups (4 plants per treatment) and irrigated with a N-free nutrient solution composed of KH<sub>2</sub>PO<sub>4</sub> (50 µM), MgSO<sub>4</sub> (100 µM), K<sub>2</sub>SO<sub>4</sub> (700 µM), CaCl<sub>2</sub> (1650 µM), Fe-EDTA (16 µM), MnSO<sub>4</sub> (4 µM), H<sub>3</sub>BO<sub>3</sub> (22 µM), ZnSO<sub>4</sub> (0,4 µM), NaMoO<sub>4</sub> (0,05 µM) and CuSO<sub>4</sub> (1,6 µM) (Bargaz *et al.*, 2011). This solution was supplemented with NaCl quantities: 0 (control), 100, and 150 mM. At flowering stage, plants were harvested i.e. it was segregated into its various components of shoots, nodules, and root. The dry matter

was determined after the plants were dried at 70°C for 48 hours to constant weight.

### Determination of N Contents in Shoots and Roots

For N determination, 0.5 g of shoot and root sub-samples were used and examined by the Kjeldahl method (Abdi *et al.*, 2015; Hemissiet *et al.*, 2015).

### Electrolyte Leakage (EL)

According to Ghoulam *et al.* (2002), stability of the leaves membrane is ascertained with *EL*, which is correlated with *EC* (Electrical Conductivity). The formulae for *EL* calculation is as follows:

$$EL (\%) = (EC_1/EC_2) \times 100$$

Where,  $EC_1$  indicates the value of electrolyte leakage (EL) of incubated leaves at 25°C for 24 hours, and  $EC_2$  refers to value of electrolyte leakage (EL) of autoclaved leaves at 120 °C for 20 minutes.

### H<sub>2</sub>O<sub>2</sub> Content of the Leaves and Nodules

Following Velikova *et al.* (2000), 100 mg of leaf sample aliquot was centrifuged at 15,000×g at 4°C for 15 minutes after grinding it in 2 mL of TCA (20 %). The supernatants of these extracts were collected to determine their H<sub>2</sub>O<sub>2</sub> content. To 0.5 mL of the extract, 0.5 mL of potassium phosphate buffer (10 mM, pH 7) was added. One mL of potassium iodine (1M) at 390 nm was found after incubating this for an hour in the dark. H<sub>2</sub>O<sub>2</sub> content was expressed in μmol per g of Fresh Weight (FW). H<sub>2</sub>O<sub>2</sub> content was determined using a standard curve established under the same conditions with known concentrations of H<sub>2</sub>O<sub>2</sub> range.

### Enzyme Extractions and Assays

To prepare the enzyme extract of PPO (PolyPhenol Oxidase) and PO (Peroxidase),

100 mg of leaves samples was homogenized at 0-4°C with a mix of 1 mL of 0.1M phosphate buffer (pH 7.0) and 10% (w/w) polyvinyl polypyrrolidone (Bargaz *et al.*, 2013). After this, for 20 minutes at 4°C, the homogenate was centrifuged at 13,000 rpm. The supernatant issued to check the actions of these enzymes (Tejera *et al.*, 2004). The PO activity was determined according to Diani *et al.* (2009).

The reaction mixture consisted of 200 μL of H<sub>2</sub>O<sub>2</sub> at 0.3%, 300 μL of guaiacol at 20 mM, 2 mL of phosphate buffer (0.1M, pH 6), 1 mL of distilled water and 50 μL of enzymatic extract. PO activity was determined by following the decomposition of H<sub>2</sub>O<sub>2</sub> at 470 nm. For PPO activity assay (Hori *et al.*, 1997), the reaction mixture consisted of 500 μL catechol at 1.6 % in phosphate buffer (0.1M, pH 6), 250 μL of distilled water, 200 μL of phosphate buffer (0.1M, pH 6) and 100 μL of enzymatic extract. Thereafter, the absorbance was recorded at 410 nm. For both enzymes, reading of the optical density was checked once every 60 seconds during 3 min of incubation against a control where enzymatic extract was replaced by distilled water. Enzyme activities were expressed as the amount of protein decomposing 1 μmol of H<sub>2</sub>O<sub>2</sub> per g FW.

### Total Phenols Content

A pestle and a pre-cooled mortar were used to turn frozen roots to fine powder through different treatments. At 4°C with 80% methanol, these were extracted three times with continuous stirring. The supernatants were observed by a spectrophotometer and the homogenate was centrifuged for 3 minutes. Total phenols contents were estimated based on Feline-Ciocalteu method adapted from Dicko *et al.* (2002). The absorption was read at 760 nm. A calibration curve was constructed from freshly prepared solutions of (+)-catechin. According to Bargaz *et al.* (2013), the results were



calculated as  $\pm$ mg of catechin per g of fresh weight (FW)

### Statistical Analysis

The experiments were performed in a completely randomized design. All data were subjected to three-way Analysis Of Variance (ANOVA) and the means were compared by LSD test at 1% probability. Results are means of five replicates for both growth and nodulation and four replicates for all the remaining tested parameters. Data followed by the same letter are not significantly different at the 1% probability level.

## RESULTS

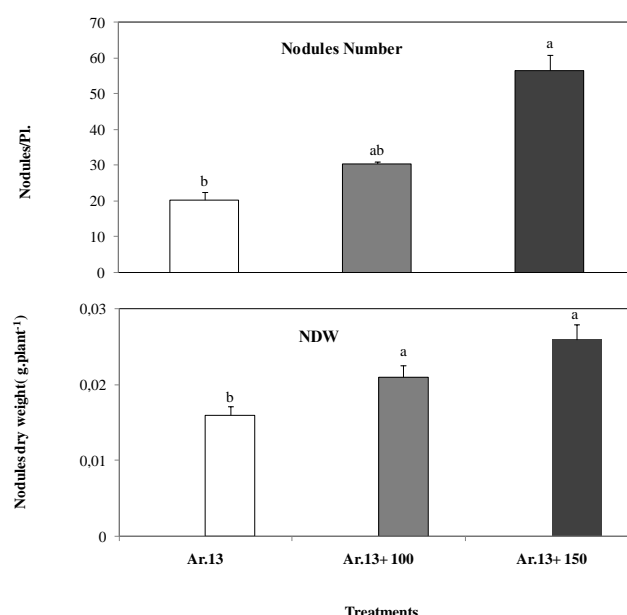
### Growth and Nodulation of Plant

Impact caused by salt stress overgrowth of plant and nodulation was analyzed in *Pisum sativum*-rhizobia symbiosis. The results

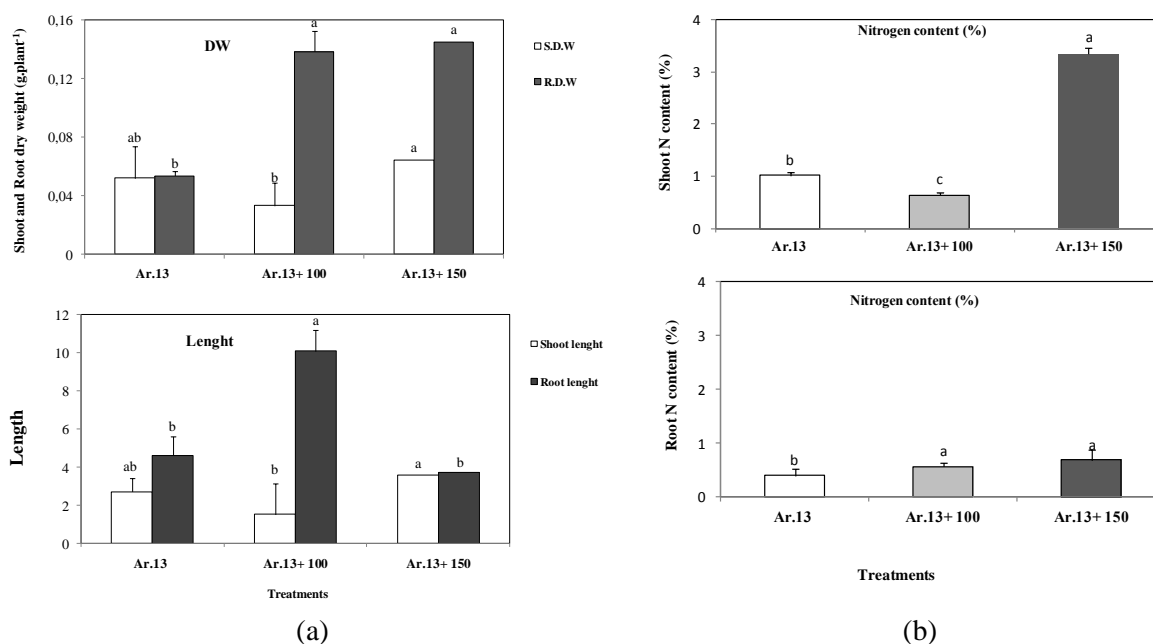
exhibited a statistically significant increase in the dry shoot weight (g per plant) of every investigated *P. sativum*-rhizobia symbiosis (Figure 1). Largely, root dried weight (g per plant) and salt affected root length (cm per plant), the increase was within the range of 76 and 80% pertaining to 100 and 150 mM NaCl, respectively. In the case of nodule number (nodules per plant), a similar trend was detected. A statistically significant increase of growth parameters (dry weight and length of shoot and root) was noticed at 150 mM NaCl (Figure 2-a). Under salt stress (150 mM), nodulation was in the order of 60 nodules plant<sup>-1</sup> and it showed the highest nodule biomass (0.025 g). Considering the entire tested treatments, nodule biomass was increased under salt stress and appeared highly significant under 150 mM compared with the control inoculation, wherever differences were not significant between the two doses supply.

### N Content of Shoot and Root

Interestingly, shoot N content increased



**Figure 1.** Effect of different salinity levels (100 and 150 mM NaCl) on nodules number and Nodule Dry Weight (NDW) of pea-rhizobia symbiosis grown under greenhouse conditions. Values are means of five replicates harvested at flowering stage and significant differences between means, as determined by Duncan test ( $P < 0.05$ ), are indicated by different letters.



**Figure 2.** Effect of different salinity levels (100 and 150 mM NaCl) on (a) shoot and root Dry Weight (DW) and length of pea-rhizobia symbiosis grown under greenhouse conditions (b) on nitrogen content in shoot and root of pea-rhizobia symbiosis grown under greenhouse conditions. Values are means of five replicates harvested at flowering stage and significant differences between means, as determined by Duncan test ( $P < 0.05$ ), are indicated by different letters.

significantly for all tested treatment (Figure 2-b). Shoot N content (%) increase of almost 71.4% was pronounced less in 150 mM as compared to the 100 mM treatment, of which shoot N remained significantly the lowest. In contrast, root N content did not vary. It was in order of 0.7% for all tested treatments (Figure 2-b). However, 150 mM NaCl treatment accumulated statistically significant N in root of *P-sativum* inoculated with Ar.13. Cordovilla *et al.* (1996) mentioned that plants dependent on nitrogen fixation are not always more sensitive to salinity. Under salt stress (150 mM), shoot N content was three times more than in root (Figure 2-b).

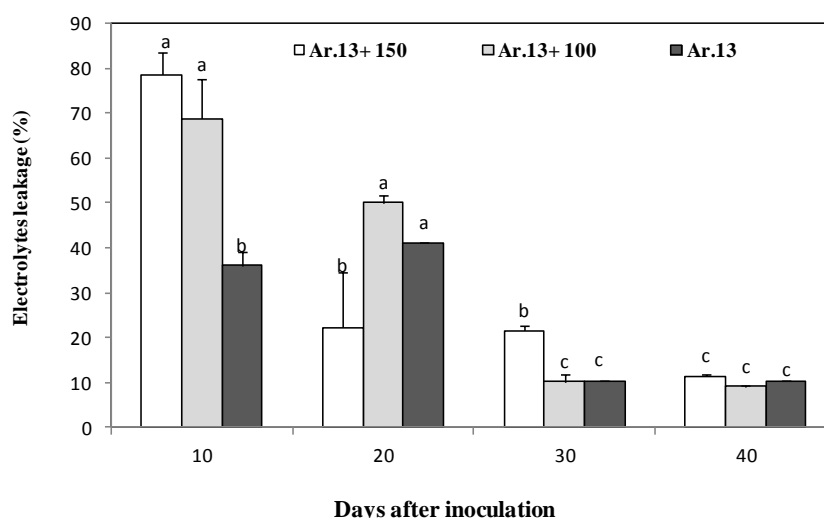
#### Leakage of Electrolyte

Results presented in Figure 3 indicated that salt stress caused a significant variation in *EL* of *P. sativum* symbioses, and it

decreased significantly with time. Notably, the highest leaves *EL* under salt stress was observed after 10 days of inoculation by Ar.13 strain and it declined significantly after 20 days. Results also showed that after 40 days, *EL* significantly decreased in leaves of all treatments (Figure 3). Generally, *EL* values were affected by salt in leaves with variations ranging between 22 and 37% under 150 and 100 mM NaCl treatments, respectively. In contrast, considering the means of *EL* for all the tested treatments, a high increase in leaves (62.5%) was observed under 100 mM NaCl against (57.14%) in leaves after 10 days of inoculation.

#### H<sub>2</sub>O<sub>2</sub> Content (μmol g<sup>-1</sup> FM)

The highest level of H<sub>2</sub>O<sub>2</sub> content was detected after 50 days of inoculation and was in the order of 30 μmol g<sup>-1</sup> FM in the



**Figure 3.** Effect of different salinity levels (100 and 150 mM NaCl) on electrolytes leakage in leaves of pea-rhizobia symbiosis grown under greenhouse conditions. Values are means of five replicates harvested at flowering stage and significant differences between means, as determined by Duncan test ( $P < 0.05$ ), are indicated by different letters.

symbiotic combination under 100 and 150 Mm NaCl. Concerning the  $H_2O_2$  content in leaves, significant differences were noted compared to the control treatment that was stabilized after 30 days of inoculation, symbiotic combinations and due to their interaction effect (Figure 4). After 50 days of inoculation,  $H_2O_2$  content in leaves was almost six times higher than after 10 days.

#### Peroxidase and Polyphenol Oxidase Activities

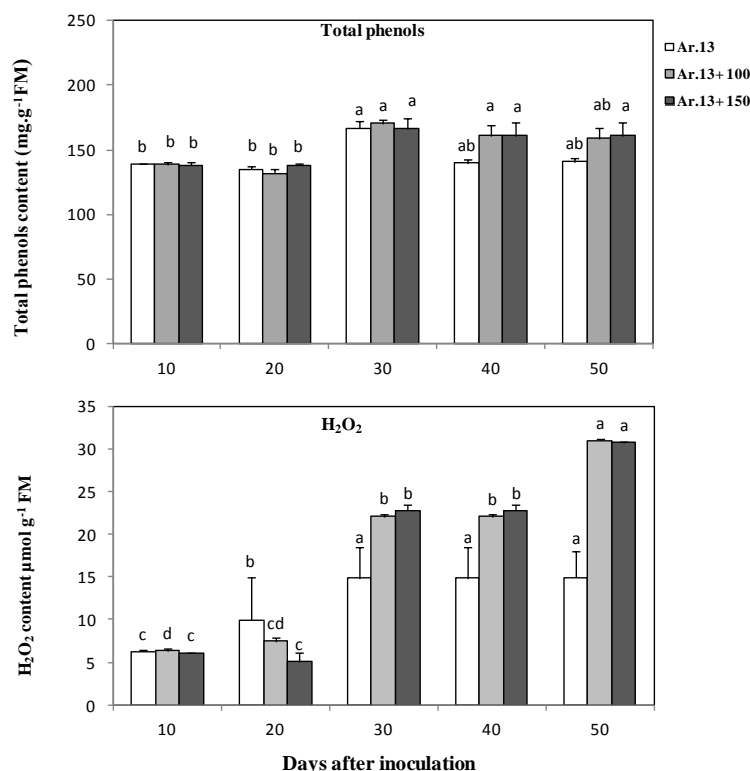
Concerning the activity of PO in leaves, results showed a non-significant increase over time. PO was in the order of  $3 \mu\text{mol g}^{-1}$  FM after 10, 30, and 40 days of inoculation with all treatments. However, this activity declined at 20 and 50 days of inoculation. In general, salt stress did not affect PO activity (Figure 5-a). However, PPO activity was increased over time and exceeded  $10 \mu\text{mol g}^{-1}$  FM, except at 30 days of inoculation under 150 mM of NaCl when PPO activity was in the order of  $2 \mu\text{mol g}^{-1}$  FM (Figure 5-b).

#### Overall Content of Phenol

The overall phenol content of salt stressed roots of *P-sativum* symbioses escalated considerably (Figure 4). The range of increase was recorded after thirty days of inoculation (12.5%) in the case of every treatment and showed the maximum phenol content ( $160 \text{ mg g}^{-1}$  FM). After twenty inoculation days, every combination exhibited most fragile roots' phenol composition that dwindled within the range of 120 and  $130 \text{ mg g}^{-1}$  FM. Similarly, the phenol composition was further substantially reduced and was stable in the roots after *P-sativum* inoculation of forty days and under the effect of 150 mM NaCl (Figure 4).

#### DISCUSSION

The results have shown a statistically validated increase in shoot dried weight (g per plant) of every *P. sativum*-rhizobia



**Figure 4.** Effect of different salinity levels on total phenols content in roots and H<sub>2</sub>O<sub>2</sub> content in leaves of pea inoculated with the rhizobia strain Ar.13 grown under salt stress (100 and 150 mM NaCl) in greenhouse conditions. Values are means of five replicates harvested at different stages of culture (10, 20, 30, 40 and 50 days) after inoculation. Statistical analysis was done separately for each stage of culture. Significant differences between means, as determined by Duncan test ( $P < 0.05$ ), are indicated by different letters.

symbiosis. Overall, salinity enhanced root elongation (cm per plant) and dry root weight (g per plant); similar results were obtained for number of nodules (per plant) and dried weigh (g per plants), which increased while responding to the salt stress entailed in *P. sativum*-rhizobia symbiosis. Under salt stress (150 mM), shoot N content was three times more than in root. It is certain from the current study that higher NaCl supply increases pea variety growth as well. Moreover, plant growth stimulation caused by higher salinity level is integrated with certain biochemical and physiological processes affecting plant growth. Salt tolerance in the pea variety observed in the present study might have been due to variation in photosynthesis, nutrient

imbalance, accumulation of compatible solutes, enzyme activities, etc., which in turn can affect crop growth (Noreen and Ashraf, 2009; Akram *et al.*, 2009). A significant interrelation has been evidenced between growth and nodulation (Figures 1 and 2-a) which were in conjunction with N content in root and shoot (Figure 2-b). Plants exposure to low salinity level stimulates multifaceted phenomenon causing an improvement in plant stress endurance. A similar trait has been observed for other plant species like rice, sorghum, and soybean (Umezawa *et al.*, 2000).

Under salt stress conditions, plants have evolved complex mechanisms allowing for adaptation to osmotic and ionic stress caused by high salinity levels (Ghoulam *et al.*,

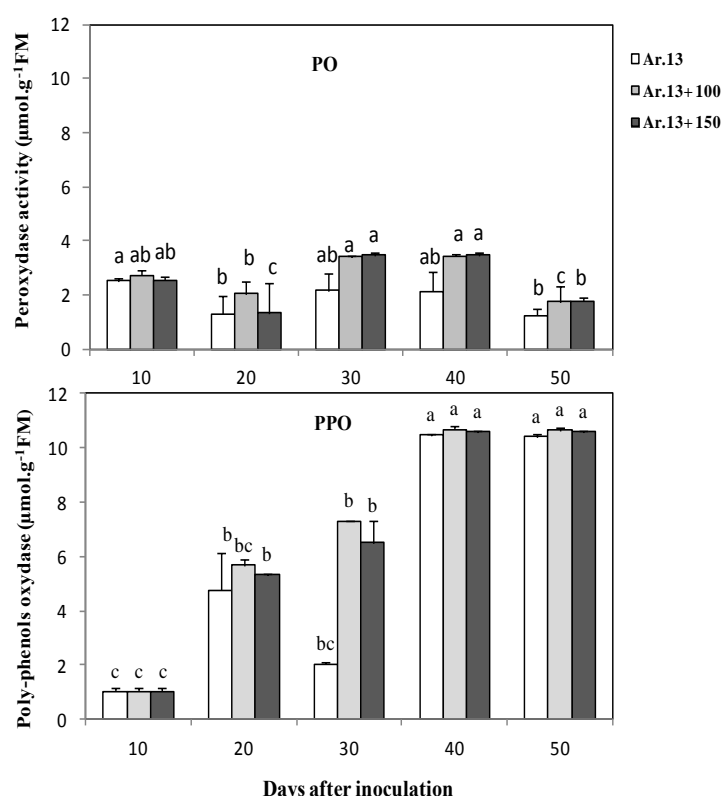


2002). However, regulation of these ROS depends on their rates of generation. For example, leaves  $H_2O_2$  content significantly increased over time in the combinations of *P-sativum* and rhizobia strain (Figure 4). It is generally known that salt stress enhances the production of singlet oxygen, superoxide anion,  $H_2O_2$  and hydroxyl radical in plants (Mateo *et al.*, 2004).

In general, salt stress did not affect PO activity. However, PPO activity was increased with time and exceeded  $10 \mu\text{mol g}^{-1}\text{FM}$ . Compared to PO, P-deficiency slightly stimulated PPO activity in leaves (Figures 5-a and -b). A significant increase in PPO activity (80%) was detected only in leaves of *P-sativum*-Ar. 13 under salt stress.

This statement agrees with previous findings on polyphenols that are triggered in plants in response to biotic and abiotic

stresses such as in soybean aluminum tolerance (Dechassa *et al.*, 2010). Similarly, Mandhania *et al.* (2006) reported disturbance in cell membrane stability as reflected by the increase over time in PPO and total phenols, particularly in the leaves and root, under salt stress. It was reported in leaves where high *EL* was generally noticed at 10 days after inoculation accompanied with a significant variation of PO as an evidence of oxidative damage. From a number of studies, it is evident that high levels of antioxidants are associated with plant salt tolerance (Bor *et al.*, 2003). Also, it has been reported that plant containing high level of polyphenols and PO are likely to be more tolerant to high concentrations of metals (Lavid *et al.*, 2001; Michalak, 2006), and accumulation of phenolic compounds have important antioxidant properties in



**Figure 5.** Effect of different salinity levels (100 and 150 mM NaCl) on Peroxidase (PO) and PolyPhenol Oxidase (PPO) activities in leaves of pea-rhizobia symbiosis grown under salt stress in greenhouse conditions. Values are means of five replicates harvested at different stages of culture (10, 20, 30, 40 and 50 days) after inoculation. Statistical analysis was done separately for each stage of culture. Significant differences between means, as determined by Duncan test ( $P < 0.05$ ), are indicated by different letters.



protecting membranes by neutralizing lipid radicals (Moran *et al.*, 1997; Takahama and Oniki, 2000).

Roots of salt stressed symbiont *P-sativum* increased significantly their total phenol content (Figure 4). Similarly, many studies have found higher phenols concentrations in stress tolerant than in stress sensitive plants (Ashraf and Harris, 2004). Also, Abdi *et al* (2012) reported that root phenols content was a sign of resistance of plants to abiotic stress. Of various secondary metabolites, phenolics are considered the principal metabolites of tolerance to abiotic stress due to their structural properties (Ruiz and Romero, 2001). For example, enhanced synthesis of soluble phenolics has been directly correlated with salt and heat tolerance of sugarcane (Wahid and Ghazanfar, 2006).

### CONCLUSIONS

In conclusion, the results of this study indicate that pea inoculated with Ar.13 demonstrated a strong growth tolerance at high NaCl concentration (150 mM NaCl). Of various antioxidant enzymes and metabolites activity such as PPO and H<sub>2</sub>O<sub>2</sub> as well as the amounts of total antioxidants were found to be associated with the differential response of pea-rhizobia symbiosis to salt stress. Moreover, despite the fact that the leaves are the organ of antioxidant activity analysis, the enzymatic reaction varies differently against salt stress. Significant variation exists between the redox states after inoculation in the leaves, which can help prevent the toxic accumulation of variability in the plant host under conditions of contrast over time.

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## القای تنش اکسیداتیو در همزیستی نخود (*Pisum sativum* L.)- ریزوبیوم در شرایط تنش شوری

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### چکیده

بعد از قرار دادن همزیستی نخود (*Pisum sativum* L.)-ریزوبیوم در معرض دو سطح شوری (شامل ۱۰۰ و ۱۵۰ میلی مول) در محیط پرلیت، گیاهانی که تحت این تنش شوری بودند برای موارد زیر تجزیه و تحلیل شدند: گره بندی، وزن خشک گیاه، فنل کل، پراکسید هیدروژن ( $H_2O_2$ )، پراکسیداز (PO)، پلی فنل اکسیداز (PPO)، و نشت الکترولیت (EL). در نتایج مشاهده شد که افزایش وزن خشک شاخساره همه موارد همزیستی نخود (*Pisum sativum* L.)-ریزوبیوم از نظر آماری معنادار بود. همچنین مشخص شد که حضور نمک منجر به افزایش طول ریشه و وزن خشک ریشه شد. مقدار این افزایش در شوری های ۱۰۰ و ۱۵۰ میلی مول NaCl به ترتیب ۷۶٪ و ۸۰٪ بود. همین روند در مورد تعداد گره ها و وزن خشک آنها مشاهده شد که در واکنش به تنش شوری در همه موارد همزیستی نخود (*Pisum sativum* L.)-ریزوبیوم افزایش یافتند. در شرایط تنش شوری (۱۵۰ میلی مول) نیتروژن موجود در شاخساره سه برابر بیشتر از ریشه بود. نیز، رابطه ای میان تشکیل گره ها و رشد گیاه پیدا شد که با مقدار نیتروژن در شاخساره و ریشه همراه بود. به طور کلی، مقدار EL تحت تاثیر مقدار نمک در برگ بود و برای تیمارهای ۱۵۰ و ۱۰۰ میلی مول NaCl به ترتیب در محدوده ۲۲٪ و ۳۷٪ تغییر میکرد. در مورد  $H_2O_2$  در برگ، تفاوت های معناداری در مقایسه با تیمار شاهد وجود داشت که ۳۰ روز بعد از تلقیح پایدار شد. پنجاه روز بعد از تلقیح، مقدار  $H_2O_2$  در برگ تقریباً ۶ برابر بیشتر از مقدار آن در ده روز بعد از تلقیح بود. به طور کلی، تنش شوری اثری روی فعالیت PO نداشت. اما، فعالیت PPO با زمان افزایش یافت و از  $10 \mu\text{mol g}^{-1} \text{FM}$  بیشتر شد.