



RESEARCH PAPER

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Effects of *Piriformospora indica* on biochemical parameters of *Oryza sativa* under salt stress

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Key words: Antioxidant enzyme, *Piriformospora indica*, Rice (*Oryza sativa*), Salt stress.

<http://dx.doi.org/10.12692/ijb/4.4.24-32>

Article published on February 20, 2014

Abstract

Salt stress is one of abiotic stress limiting growth and crop production worldwide. The endophytic fungus, *Piriformospora indica* is a recently discovered basidiomycete that increases plant resistance to environmental stresses. The aim of the present study was to measure biochemical changes in *Oryza sativa* L., colonized with *P.indica* and non-colonized (controls) under salt stress (0,100,200 and 300mM NaCl). We compared some biochemical parameters such as proline, the rate of lipid peroxidation level in terms of malondialdehyde, Na⁺ and K⁺ concentrations and antioxidant enzyme (Catalase (CAT), Peroxidase (POX), Superoxide dismutase(SOD) and Polyphenol oxidase (PPO)) activities of *O. sativa* colonized with *P.indica* and control plants under salt stress. The results demonstrated that proline concentration and antioxidant enzyme activities were found to be higher in shoot of colonized plants than controls in response to increasing NaCl concentrations. Increased activity of antioxidant enzymes reduces the chances of oxidative burst and therefore *P.indica* might be protected from the oxidative defense system during colonization. Malondialdehyde was more decreased in colonized plants than controls. In addition, colonized plants have lower Na⁺ and higher K⁺ concentration in shoots than controls under salt stress. The results of this study also indicated the effective role of *P.indica* to improve growth of *O. sativa* under salt stress conditions.

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Introduction

Environmental stress for example salt stress is one of the major abiotic stress limiting agricultural production and productivity in many areas of the world (Shannon, 1998; Silva *et al.*, 2008). Salinity affects all important processes, such as growth, photosynthesis, protein synthesis, and lipid and energy metabolism, resulting in all phases of plant life from germination to seed production (Maslenkova *et al.*, 1999; Naidoo and Naidoo, 2001). 10% of Iran's total arable land area is salt affected (Abtahi, 1992). Plants to cope with salt stress, utilize a series of morphological, biochemical and molecular mechanisms. Biochemical pathways that lead to products and process that are involved in the increased salt tolerance in plants (Iyengar and Reddy, 1996). Rice is the staple food for over half of the world. In Iran, rice is the staple food after wheat. *Piriformospora indica* is a new root- endophytic fungus that was isolated from the rhizosphere soil of *Prosopis juliflora* and *Zizyphus nummularia* in the Thar Desert of India (Varma *et al.*, 1998). The review that took place on ribosomal DNA sequences showed that *P.indica* belongs to the phylum of Basidiomycota and family of Sebacinacea (Waller *et al.*, 2005., Weiss *et al.*, 2004). *P.indica* can be cultivated easily on synthetic media and colonized cabbage and spinach of *Brassicaceae* family, in contrast of Arbuscular mycorrhizal fungi (AMF) (Kumari *et al.*, 2003). It has been proven the positive effects of endophytic fungus symbiosis between *P.indica* the survival and growth of host plants in arid and semiarid regions of the world (Rai and Varma., 2005., Rai *et al.*, 2001). Previous studies showed that *P.indica* stimulates plant growth and increased tolerance to salt and drought stress (Waller *et al.*, 2005). In this study, we studied the positive effect of *P.indica* on resistance of *O. sativa* under salt stress with an emphasis on biochemical parameters. In order to respond to the research question in this study, We compared some biochemical responses such as proline the rate of lipid peroxidation level in terms of malondialdehyde, Na⁺ and K⁺ concentrations and antioxidant enzyme (catalase, peroxidase, superoxide dismutase and *Polyphenol oxidase*) activities of *p.indica*-colonized

and non-colonized (control) *O.sativa* under levels of NaCl concentration.

Materials and methods

Fungal and Plant Material, Symbiotic and NaCl treatment

P. indica was provided by Vierheilig *et al* (1998) and was propagated on a rotary shaker at 18–22°C in liquid Aspergillus Complex Medium (CM) (Table. 1). Druege *et al.* (2007) described that fungal mycelium was prepared for root inoculation.

Seeds of *O. sativa* L., cultivars Tarrom were obtained from the University of Agricultural and Natural Resources (Iran, Sari). Seeds were surface-sterilized for 10 minutes in 25% sodium hypochlorite and finally washed six times with the sterile water. Seeds germinated at 28°C for 4 days in the dark on sheets of Whatman No. 1 filter paper in Petri dishes.

After 4 days, one part of the germinating seeds (four seeds per pot) was transferred to pots (15 cm height by 12cm diameter) and grown in a mixture of sand/vermiculite (1:1) in green house at 25: 18 °C at day: night cycle, 70% relative humidity and a photoperiod of 16 h light: 8 h dark and fertilized daily with Yoshida solution (Yoshida *et al.*, 1976).

The other part of the seeds was inoculated with *P. indica*: developing roots of 4-d-old germinating seeds were immersed in *P. indica* homogenate (The spore concentration was adjusted to 500 000 spores/ml) for 12 h before transferring to pots and grown under the same conditions. Three weeks after incubation, colonization was observed under a light microscope after staining root fragments described by Vierheilig *et al* (1998). After 4 weeks, *P. indica*-infected and control plants were exposed to different concentrations of NaCl (0, 100, 200 and 300mM) for one week. During one week, plants were grown in Yoshida solution treated with different of NaCl concentration. After NaCl treatment, plants for each group were harvested and then stored at -70°C for biochemical analyses.

Sodium and Potassium assay

Na⁺ and K⁺ were measured by flame photometer (Jenway PFP7; ELE instrument Co. Ltd.).

Proline content Assay

Free proline content was extracted and determined according to the method described by Bates *et al.* (1973).

Fresh of shoots (0.5 g) was homogenized in 10 ml of 3% (w/v) aqueous sulfosalicylic acid. This aqueous solution was filtered through Whatman's No. 1 filter paper and 2 ml of filtrated solution mixed with 2 ml Acid-ninhydrin and 2 ml of Glacial acetic acid in a test tube. The mixture was heated at 100°C for 1 h in water bath, and the reaction was stopped by using ice bath. The reaction mixture was extracted with 4 ml Toluene, after which the Chromophore-containing toluene was aspirated and subjected to absorbance measurement at 520 nm. Proline concentration was determined through using a calibration curve and expressed as $\mu\text{mol proline g}^{-1}$ FW.

Lipid peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content according to the method of Heath and Packer (1968). Fresh shoots (1 g) was homogenized in 10 ml of 0.25% thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA). The mixture was heated at 95°C for 30 min and then cooled quickly on an ice bath. Afterwards, the mixture was centrifuged for 10 min at $10,000 \times g$ and the absorbance of the supernatant was measured at 532 nm and at 600 nm for correction of nonspecific turbidity. The MDA content was calculated according to its extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as $\mu\text{mol g}^{-1}$ FW.

Enzyme extraction

For determining of enzyme activity, homogenate of shoots were extracted with ice-cold 0.5 M Tris-HCl (pH 6.8) buffer. The extracts were centrifuged at $16,000 \times g$ for 30 minutes at 4 °C and resulting supernatants were frozen in liquid nitrogen, kept in -70 °C and used for protein determination and

enzyme assays. A high-speed centrifuge (*J2-21M*, Beckman, Palo Alto, USA) and UV-visible spectrophotometer (*UV-160*, Shimadzu, Tokyo, Japan) with 10 mm matched quartz cells were used for centrifugation of the extracts and determination of the absorbance, respectively.

Antioxidant enzyme activity

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined according to the protocol of Beauchamp and Fridovich (1971), measuring inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm.

The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8) with 0.1 mM ethylenediaminetetraacetic acid (EDTA), 75 μM NBT, 13 mM methionine, 2 μM riboflavin and 20 μl of protein extract.

Reactions were carried out for 10 minutes at a light intensity of $300 \mu\text{mol}^{-1} \text{ m}^{-2}\text{s}^{-1}$. The non-irradiated reaction mixture served as control and was deducted from absorption at 560 nm. One unit of SOD activity was defined as the amount of enzyme, which causes 50% inhibition of NBT reduction under the assay condition, and the results were expressed as U mg^{-1} protein.

Catalase (CAT; EC 1.11.1.6) activity was measured according to the method of Aebi (1974), which measures the decline of the extinction of H₂O₂ at the maximum absorption at 240 nm.

The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 15 mM H₂O₂ and 20 μl protein extract. The decrease of H₂O₂ level was monitored and quantified by its molar extinction coefficient ($36 \text{ M}^{-1} \text{ cm}^{-1}$), and the results were expressed as $\mu\text{mol H}_2\text{O}_2\text{min}^{-1} \text{ mg}^{-1}$ protein.

Peroxidase (POX; E.C. 1.11.1.7) activity was measured according to the method of Abeles and Biles (1991).

The assay mixture consisted of 4 cm^3 of 0.2 M acetate buffer (pH 4.8), 0.4 cm^3 H₂O₂ (3 %), 0.2 cm^3 20 mM

benzidine and 0.05 cm³ enzyme extract. The absorbance was recorded at 530 nm.

Polyphenol oxidase (PPO; E.C. 1.10.3.1) activity was estimated following the method of Raymond *et al.* (1993) by measuring the increase in absorbance at 430 nm.

The reaction mixture contained 2.5 cm³ 200 mM sodium phosphate buffer (pH 6.8), 0.2 cm³ pyrogallol 20 mM and 0.1 cm³ enzyme extract. The temperature of the reaction mixture was 40 °C.

Statistical analysis

At least two independent experiments were carried out in each case. Each data was pointed the mean of three replicates of all plants. Mean values and standard deviations (S.D.) were calculated for *P. indica*-inoculated and control cuttings of all plants. Analysis of variance was conducted through using one-way ANOVA test using SPSS version 14 for

Microsoft Windows. Means were compared by Duncan's test at the 0.05 level of confidence.

Result and discussion

P. indica is a root endophytic fungus

Cytological analysis of rice roots in contact with *P. indica* revealed the fungal mycelium penetrate in epidermis, cortical cells and never reaches the endodermis layer and Vascular tissue. Pear-shaped chlamydospores were localized intercellularly in the root cortical tissue (fig. 1).

We observed the difference morphological changes in shoots of *O. sativa* colonized with *P. indica* compared with control plants in four levels of NaCl. The old leaves of rice plants grown in saline condition showed typical symptoms as leaf yellowing, stunted growth, wilting, chlorosis and subsequent necrosis (Fig. 2). However, these symptoms are increased control plants than *O. sativa* colonized with *P. indica*.

Table 1. *Aspergillus* complex medium for produce of *P. indica*.

MDA ($\mu\text{molg}^{-1}\text{FW}$)	Proline ($\mu\text{molg}^{-1}\text{FW}$)		NaCl (mM)
53±7.11c	150±9.09c	- <i>p. indica</i>	0
50±9.6cd	163±9.16d	+ <i>P. indica</i>	
77±15.76c	180±37bc	- <i>p. indica</i>	100
55±7.8bc	271±38c	+ <i>P. indica</i>	
140±8.6b	220±8.1abc	- <i>p. indica</i>	200
70±7.11bc	349±3.3b	+ <i>P. indica</i>	
190±4.08a	300±57ab	- <i>p. indica</i>	300
90±8.1a	490±45a	+ <i>P. indica</i>	

P. indica increase proline in shoots of salt-treated rice

Proline is an osmoprotectant shown to accumulate in plants in response to salt and drought stress. In concurrence with this, our results (Table. 2) showed that rice colonized with *P. indica* was higher levels of

proline than control plants under salt stress. For instance, when exposed to 300mM NaCl, Shoot prolines of rice colonized increased 3-fold but in uncolonized rice were 2-fold relatively to the omM NaCl.

Table 2. The effect of *P. indica* on proline and lipid peroxidation under NaCl concentration.

Amount	Materials	Amount	Materials	Amount	Materials
120g NaNO ₃ ,10.4gKCL,10.4g MgSO ₄ +7H ₂ O,30.4g KH ₂ PO ₄	20× salt Solution	15g	Agar	20g	Glucose
6g MnCL ₂ +4H ₂ O,1.5g H ₃ BO ₃ ,2.65g ZnSO ₄ +7 H ₂ O,2.4mg NaMO ₄ + 2H ₂ O, 750mg KI,130mg CuSO ₄ +5H ₂ O	Microelement	1mL	Water	2g	Pepton
		1g	Casamin acids	1g	Yeast extract

Zarea and *et al* (2012) reported that proline more increases more in wheat colonized with *P. indica* than control plants under salinity. This increase (Proline) may be due to amino acid production and/or from increased stress-induced protein breakdown (Widodo Patterson *et al.*, 2009).

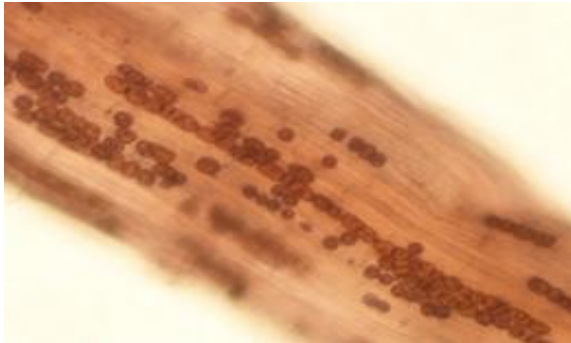


Fig. 1. The fungus *P. indica* into *O. sativa* root cells.

P. indica reduces lipid peroxidation in salt-treated rice

Changes in lipid peroxidation serves as an indicator of the extent of oxidative damage under stress (Borsani *et al.*, 2001). To determine if NaCl causes oxidative damage in rice, we monitored changes in lipid peroxidation by measuring thiobarbituric acid reactive substances in shoots of inoculated rice and control plants. Lipid peroxidation levels in shoots of rice inoculated with *P. indica* and controls, measured as the content of MDA, are given in Table. 2. Increased lipid peroxidation was observed with increasing NaCl concentrations in both groups. This increase was significantly higher in control compared to colonized rice with *P. indica* at all NaCl concentrations. For instance, when exposed to 300mM NaCl, shoot MDA of rice colonized increased 1.8-fold but in uncolonized rice were 3.6 relatively to the 0mM NaCl. These observations suggest that the rate of lipid peroxidation can also be used to characterize how effectively *P. indica*-treated plants cope with salt stress. Previous studies have demonstrated a salt-induced increase in lipid peroxidation (Hernandez *et al.*, 1995; Yang *et al.*, 2004). Our results are consistent with the results of Baltruschat *et al* (2008) that showed *P. indica* reduces lipid peroxidation in leaves of salt-treated barley.

Increasing K/Na in salt-treated rice under salt stress

Our results showed that shoot Na⁺ concentration increased with increasing NaCl levels in both plant groups. The measurements showed that the Na⁺ concentration in all treatments were generally lesser in rice inoculated with *P. indica* than control plants (Fig.3).

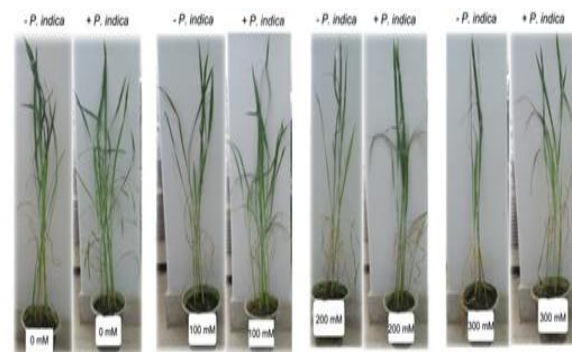


Fig. 2. Effects of *P. indica* on shoot morphology of rice plant under salt stress.

K⁺ concentration in all treatments were generally higher in rice inoculated with *P. indica* than control plants (Fig.3).

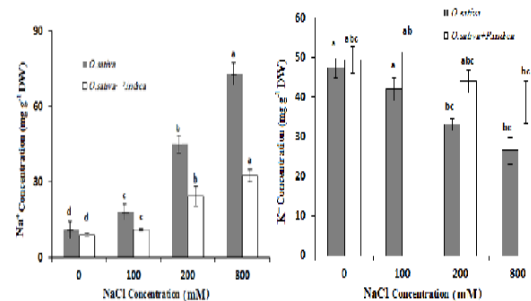


Fig. 3. The effect of *P. indica* on K⁺ and Na⁺ concentration under NaCl concentration. Vertical bars indicate mean±SE of three replicates. Means followed by the same letter were not significantly different at P<0.05.

In *O. sativa* inoculate with *P. indica*, K⁺ concentration increased with increasing NaCl to 100mM and then decreased at 200 to 300mM NaCl but in control plants K⁺ concentration decreased with increasing NaCl levels. Potassium is one of the major ions that affect the water balance, cell development, nutrient transfer in xylem, maintaining cytoplasmic pH and osmotic potential of vacuoles (Marschner, 2012). Increasing of sodium concentration can affect many

enzymatic processes in the cytoplasm (Giri *et al.*, 2007; Giri and Mukerji, 2004). The ratio K/Na can prevent degradation of metabolic process and protein synthesis under salt stress (Giri and Mukerji, 2004). Previous studies showed that ratio of K / Na is a higher level in plants inoculated with arbuscular mycorrhiza than the control plants under salt stress (Giri *et al.*, 2007; Giri and Mukerji, 2004).

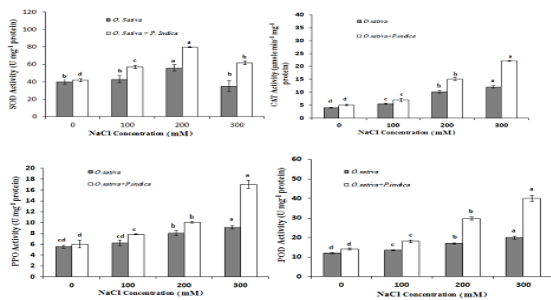


Fig. 4. Positive effect of *P.indica* on antioxidant enzyme activity. Vertical bars indicate mean±SE of three replicates. Means followed by the same letter were not significantly different at $P < 0.05$.

P. indica increases antioxidant enzyme activities induced by salt treatment in rice

Statistical analysis revealed significant ($P < 0.05$) effects of *P.indica* on the activities of antioxidant enzymes in several of NaCl concentration (Fig. 4). NaCl affected Enzyme activities in shoots of rice. We assayed SOD, POX, CAT and PPO enzyme activity.

CAT, PPO and POX activities increased in both plant groups with increasing NaCl levels, but superoxide dismutase (SOD) activity increased with increasing NaCl to 200mM and then decreased at 300mM NaCl in both controls and *p.indica* –inoculated rice.

There are many reports of increased activity of antioxidant enzymes such as CAT, POX, PPO and SOD in roots and shoots of plants colonized with endophytic or AM fungi in compared with control plants under environmental stress (He *et al.*, 2007; Hajiboland *et al.*, 2010; Pacovsky *et al.*, 1991). The studies were performed by waller and *et al* (2005) showed that barley coloized with *P.indica* CAT and SOD activities, respectively, 48.23 times compared to the control plants under salt stress.

Vertical bars indicate mean±SE of three replicates. Means followed by the same letter were not significantly different at $P < 0.05$.

Conclusion

Salinity is a major abiotic stresses that limit plant growth and crop production in agriculture worldwide. Several possible symbiotic mechanisms proposed for salt tolerance (Koyro *et al.*, 2008). Many studies show that symbiosis of plants with endophytic fungi could be a good solution for reducing the effects of salinity. *P. indica* is a root endophytic fungus interacts with many plant species (Waller *et al.*, 2005). Our results showed that salt stress has indicated the negative effect on all of biochemical parameters on *P.indica* inoculated *O.sativa* and control plants, but these negative effects were more reduced on rice when colonized with *P. indica* than control plants. Our results indicate that rice colonized with *P.indica* has higher activity of antioxidant enzymes, ratio of K/Na, proline content and lower lipid peroxidation than control plants under four level of NaCl. We suggest the consideration of this root endophyte as a tool for tolerable agriculture.

Acknowledgements

The authors are grateful to the Seed and Plant Improvement Institute, Rice Research Institute of Agricultural Sciences and Natural Resources University, Sari for providing seed and inoculum necessary for this study. We would like to thank Laboratory of Tehran University and laboratory of Islamic Azad University, Science and Research Branch technicians for cooperation in various stages of research.

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