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Influence of Air SO_2 Pollution on Antioxidant Systems of Alfalfa Inoculated with Rhizobium

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Abstract
Air pollution is the most important environmental problem of last century that threatens the health of living organisms, especially plants. SO_2 is one of the main air pollutants that can cause to imbalance in growth and physiological function of plant in high concentrations. Symbiosis of *Rhizobium* bacteria with alfalfa can cause increasing plant growth and resistance to abiotic stresses. In order to study the effects of rhizobia inoculation on alfalfa antioxidant activity and capacity under air SO_2 pollution, 35 days plants (noninoculated and inoculated with native or standard *Rhizobium meliloti*) exposed to the different concentrations of SO_2 (0 as a control, 0.5, 1, 1.5 and 2 ppm) for 6 consecutive days (2 hours per day). Results showed inoculation had no significant effect on antioxidant activity and capacity of alfalfa plant. However different concentrations of SO_2 pollution had a significant effect on alfalfa antioxidant system. Increasing SO_2 stress increased antioxidant activities (I%) and decreased antioxidant capacities (IC_{50}) of alfalfa leaves significantly in comparison to the control plants (under 0 ppm) as well as increased superoxide dismutase, catalase and guaiacol peroxidase activity. Inoculation of alfalfa plant with *Rhizobium meliloti* reduced the negative effects of high concentrations of SO_2 on antioxidant activity and capacity significantly. In fact, the rate of this change of antioxidant system under SO_2 pollution in inoculated plants was lower than in the noninoculated plants. Therefore inoculation with *Rhizobium* strains could alleviate the effect of SO_2 pollution on antioxidant system.

Keywords: Alfalfa, Antioxidant activity and capacity, *Rhizobium*, SO_2 pollution

Introduction

Air pollution has become an extremely serious problem for the modern industrialized world. Air pollution may be defined as any atmospheric condition which certain substances are present in concentrations that may produce undesirable effects on human and ecosystem. These substances include gases (SO_2, nitrogen oxides, carbon monoxides, and hydrocarbons), particulate matters (smoke, dust, fumes, and aerosols) and radioactive materials (Gostin, 2009; Rai *et al*., 2011). Air pollution was earlier considered as a local problem around large point sources. But due to use of tall stacks and long range transport of pollutants, it has become a regional problem. Uncontrolled use of fossil fuels in industries and transport sectors has led to the increase in concentrations of gaseous pollutants such as SO_2, NO_x (Rai *et al*., 2011).

Sulfur dioxide (SO_2) is one of the most common and harmful air pollutants (Li and Yi, 2012) that its concentration is increasing in many metropolitan and industrial areas. It is a major atmospheric contaminant resulting primarily from the combustion of sulphur-rich fossil fuels such as coal and oil or naturally from forest fire and volcanic eruptions. In the atmosphere, when SO_2 combines with water, it forms sulphurous acid which is the main component of acid rain (Sha *et al.,* 2010). SO_2, normally 0.05–0.5 ppm in the urban areas and up to 2 ppm or more around sources of air pollution (Wali *et al*., 2007). SO_2 and acid rain be harmful to plants, turning leaves yellow and dry, bleaching and even killing foliage depending on the dosages (Lang *et al*., 2007). The 40% of SO_2 global emissions originating from Asia and it is growing (Smith *et al*., 2011). The phytotoxicity of SO_2 strongly depends on its concentration and exposure duration and is also influenced by the sulfur status of plants (Li and Yi, 2012).

In very low concentrations, SO_2 can cause positive effects on physiological and growth of plants, especially in sulfur-deficient soils (Swanepoel *et al.,* 2007), since sulfur is a

structural component of amino acids, proteins, vitamins and chlorophyll. Sulfur enhances the development of nodules and nitrogen fixation by legumes and also affects carbohydrate metabolism (Li and Yi, 2012). However, exposure to high doses of SO_2 causes toxicity and reduction of growth due to sulphite and sulphate accumulation within the plant (Swanepoel *et al.,* 2007). SO_2 can easily penetrate into chloroplasts and affect plant growth and development (Sha *et al.,* 2010). Even when stomata are closed, SO_2 can react with water to produce bisulfite and enter the leaf through the cuticle (Sha *et al.,* 2010). In the chloroplasts, SO_2 is mainly converted into sulfite, which causes a reduction of net CO_2 assimilation, inhibits photosynthetic enzymes, and decreases the photosynthetic electron transport rate (Sha *et al.,* 2010). SO_2-toxicity is mainly attributed to produce high reactive intermediates such as the sulphur trioxide radical (HSO_3^-), the superoxide radical and the hydroxyl radical, which are generated during the radical-initiated oxidation of SO_2. Illuminated chloroplasts can initiate oxidation of SO_2 and, hence, may be a primary site of radical production during SO_2 treatment. To counteract the toxicity of active oxygen species, a high efficient antioxidative defence system, composed of both non-enzymatic (e.g. glutathione, proline, α-tocopherols, carotenoids and flavonoids) and enzymatic (e.g. superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) constituents is present in all plant cells (Gill and Tuteja, 2010; Li and Yi, 2012).

Rhizobium is one of the most prominent Plant growth promoting rhizobacteria (PGPR) members that activate plant root nodulation in leguminous plants (Gentili and Jumpponen, 2006). PGPR can promote plant growth by several mechanisms, directly and/or indirectly, include (i) the ability to produce or change the concentration of plant growth regulators like indoleacetic acid, gibberellic acid, cytokinins and ethylene, (ii) N_2 fixation, (iii) production of siderophores, antibiotics and cyanide (iv) solubilization of mineral phosphates and other nutrients (Aeini *et al.,* 2012; Martínez-Viveros *et al*., 2010; Saharan and Nehra, 2011). PGPR can prevent the deleterious effects of environmental stressors (Han and Lee, 2005; Tank and Saraf, 2010). The use of PGPR offers an attractive way to replace chemical fertilizer, pesticides and supplements. Some PGPR have been produced commercially as inoculants for agriculture to improve plant growth through supply of plant nutrients and may help to sustain environmental health and soil productivity (Estefan *et al*., 2013; Tank and Saraf, 2010).

Alfalfa (*Medicago sativa* L.) is a very used forage legume with over 32 million hectares in the world (Benabderrahim *et al.,* 2009) and it´s the most important forage crop for the arid and semi-arid areas (Salehi *et al*., 2008). The objective of this study was to determine the effects of rhizobium inoculation on antioxidant activity and total antioxidant of alfalfa under different concentrations of SO_2 (0, 0.5, 1, 1.5 and 2 ppm).

Materials and Methods

Bacterial culture and inoculant preparation

Alfalfa plants were collected from Arak farm lands (a city of Iran that high levels of SO_2 has been reported (Moini *et al*., 2011) and native strain of *Rhizobium meliloti* was extracted from roots of this plants. For this purpose, alfalfa roots were sterilized with 70% ethanol and were washed with sterile distilled water (Swift and Bignell, 2001). Then the pink nodules (containing active bacteria) isolated from roots, crushed in distilled water and cultured in solid medium of YMA (Yeast Manitol Agar) (Molla *et al.,* 2001). These cultures were transferred to incubator at 25°C. After incubation, gram reaction and morphology of bacteria were studied under the microscope. Formation of convex prominent semi-transparent slimy and mucilage colonies and gram-negative reaction were considered a sign of successful isolation of rhizobium (Swift and Bignell, 2001).

Standard strain of rhizobium (*Rhizobium meliloti* PTCC 1684) were obtained from the Persian type culture collection (PTCC, Iran). For activation these bacteria, 1 ml of liquid medium of YMA under sterile conditions added to powdered of bacteria. For proliferation of bacteria, one inoculation loop of these bacteria dissolved in 100 ml of liquid YMA and incubated on an orbital shaker at 200 rpm for 24 h. Optimum amount of rhizobium to stimulate alfalfa growth was reported 10^5 cells/mL (Caetano-Anolles *et al.*, 1988), for this purpose two strains of *Rhizobium meliloti* (native and standard) were cultured separately in liquid medium of YMA (Molla *et al.*, 2001) and incubated on an orbital shaker at 200 rpm for 24 h at 25°C (Sadovinkova *et al.*,

2003). Then these cultures were centrifuged at 1000g for 10 min and were resuspended with phosphate buffer. The optical density (OD_{620}) of this solution was 0.1 it means 10^8 cells/mL (Bai *et al.*, 2003). For preparation optimum amount of inoculum (10^5 cells/mL), this solution was diluted by phosphate buffer.

Seed preparation and its inoculation

The seeds of alfalfa (Medicago sativa cv. Hamedani) were prepared from Arak Agriculture Research Center. They were sterilized by 70% ethanol for 2 min and 1% sodium hypochlorite for 5 min, then they washed with distilled water 5 times (Wang and Oyaizu, 2009). After washing, the seeds were divided into three groups. First group of seeds inoculated with native *R. meliloti* inoculum, second group of seeds inoculated with standard *R. meliloti* inoculum and third group soaked in sterile phosphate buffer. All of the groups were placed under vacuum and ambient temperature for 2 h (Bashan *et al*., 1989).

Hydroponic cultivation of seed

Inoculated and non-inoculated seeds were placed to plates system containing nutrient solution (without nitrogen) in the dark for 24 h. The germinated seeds were transferred to sterile microtubes in plastic container containing 2 L of nutrient solution without nitrogen (Millner and Kitt, 1992). Containers were oxygenated by the air compressor. Each container was considered as an experimental group. These containers were maintained under 12 h photoperiod, at 25°C during day and 20°C during night (Bashan *et al*., 1989).

SO_2 treatments for plant

SO_2 gas prepared from Shazand Petrochemical Co. injected in different concentrations 0 (as control), 0.5, 1, 1.5 and 2 ppm into 35 days plants. Gas injection was performed by syringe for 5 days and 2 h daily to closed plastic containers (Agrawal *et al.*, 1985).

Enzyme assays

Extraction: Leaf fresh materials (0.1 g) was powdered by liquid nitrogen and homogenized in 1 ml of 50 mM phosphate buffer (pH 7) containing 1 mM ethylene diamine tetra acetic acid (EDTA) by a homogenizer into microtubes. Insoluble materials removed by Beckman refrigerated centrifuge at 13000 g for 20 min at 4°C, and the supernatant used as the source of enzyme extraction.

Assays: All enzyme assays were performed at 4°C and activities of the enzymes were determined with a spectrophotometer (PG T80 UV/VIS, Oasis Scientific Inc.).

Superoxide dismutase (SOD) assay Superoxide dismutase activity was measured by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium according to the method described by Giannopolitis and Ries (1977), using a reaction mixture (3 mL) containing 50 mM phosphate buffer (pH = 7.8), 13 mM methionine, 75 µM nitro blue tetrazolium, 20 µM riboflavin, 0.1 mM EDTA and 100 µl of the enzyme extract in absence of light. The reaction mixtures were illuminated for 15 min under fluorescent light. One unit of superoxide dismutase activity is defined as the amount of enzyme required to cause 50% inhibition of nitroblue tetrazolium reduction, which was monitored at 560 nm.

Catalase (CAT) assay

Catalase activity was assayed by measuring the initial amount of H_20_2 disappearance using the method of Cakmak and Marschner (1992) in a reaction mixture containing 2 ml of 25 mM phosphate buffer (pH 7.0, containing 10 mM H_20_2) and 100 µl of the enzyme extract. Decomposition of 1 µmol H_2O_2/min is equal to one unit of catalase activity.

Guaiacol peroxidase (GPOX) assay

Guaiacol peroxidase activity was measured by Polle *et al*. (1994). The reaction mixture contained 100 mM phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM H_20_2 and 50 µl of the enzyme extract. Activity was determined by increasing absorbance at 470 nm due to guaiacol oxidation.

Measurement of DPPH-radical scavenging activity

For determination of DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity used of Abe *et al*. (1998) method. Leaf fresh materials

(100 mg) was powdered by liquid nitrogen, homogenized in 1 ml of 90% ethanol and then maintained at 4°C for 24 h. Insoluble materials removed by centrifuge at 3500 g for 5 min. 20 µl of extracting solution was mixed with 800 µl of DPPH (0.5 mM in ethanol). The absorbance of the resulting solution was measured at 517 nm after 30 min in darkness. The antiradical capacity (three replicates per treatment) was expressed as IC_{50} (mg ml^{-1}), the antiradical dose required to cause a 50% inhibition. A lower IC50 value corresponds to a higher antioxidant capacity of plant extract (Patro *et al.*, 2005). The ability to scavenge the DPPH· radical was calculated by:

$$I\% = \frac{A0 - A1}{A0} \times 100$$

Where A0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min.

Statistical analysis

All data were analyzed by variance analysis using SPSS 16. Experiments were tested using completely randomized design in factorial form (in three replicates). Mean comparisons were conducted using one way and univariate tests.

Results

Study of root morphology of inoculated plants indicated pink nodules on the roots of these plants.

A) Results of bacterial inoculation and SO_2 pollution on total antioxidants

Effect of bacterial inoculation

In this study, the total antioxidant activities (I %) and capacities (IC_{50}) of the samples were determined by DPPH-radical scavenging activity test. The results showed that bacterial inoculation had no significant effect on total antioxidant activity and capacity. Rhizobium inoculation did not cause stress conditions for plant, so the plant is normally and changes in the antioxidant system is not created.

Effect of SO_2 pollution

The DPPH radical scavenging activity and capacity indicated no significant change in 0 and 0.5 ppm concentrations of SO_2. Increasing SO_2 stress changed I% and IC_{50} significantly. I% increased and IC_{50} decreased in high concentration of SO_2 (1, 1.5 and 2 ppm) as compared with control plant. I% was at its highest value in 2 ppm of SO_2 with 80.58% increase in comparison with the controls (Fig. 1a). IC_{50} was at its lowest value in 2 ppm with 44.13% decrease in comparison with the controls (Fig. 1b).

Interaction effect of bacterial inoculation and SO_2 pollution

To understand the protective function of antioxidants against SO_2 stress, alfalfa plants were treated with native and standard *Rhizobium meliloti* followed by measurement of the level of I% and IC_{50} (Table 1). Interaction effect of bacterial inoculation and SO_2 pollution on I% and IC_{50} was significant as compared with control plants (no bacteri, no SO_2). Increasing SO_2 stress significantly increased I% and decreased IC_{50} of alfalfa leaves compared to the control in the experiment. Inoculation of alfalfa plant with native and standard *Rhizobium meliloti* significantly reduced the stress effects of high concentrations of SO_2 on I% and IC_{50}. Among bacterial inoculation and SO_2 pollution treatments, noninoculated plants under 2 ppm SO_2 (-R 2) and inoculated plants with native *Rhizobium meliloti* under 0 ppm SO_2 (R_n 0) showed higher and lower level of I% respectively. I% was lower in inoculated treatments compared with control treatments. Interaction between bacterial inoculation and SO_2 pollution showed that they had significant effect ($p \leq 0.01$) on IC50. Increasing SO_2 doses significantly decreased IC_{50}.

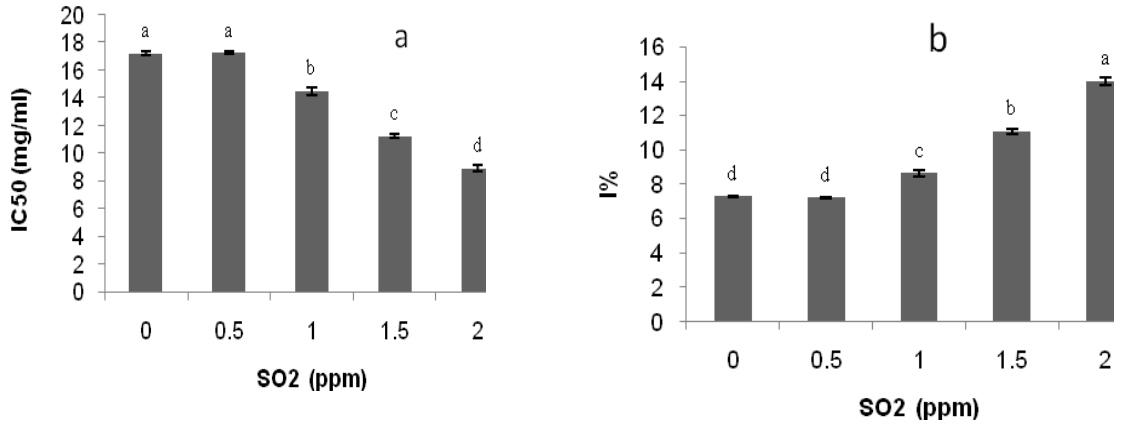

Fig. 1. (a) Antioxidant capacities (IC_{50}) and (b) antioxidant activities (I %) in the leaves of alfalfa under SO_2 pollution in 45 days plants. Data are mean ± SE. Different letters indicate that the mean value is significantly different ($p< 0.01$).

The IC_{50} in the leaves of noninoculated alfalfa plant under 2 ppm SO_2 (-R 2) was 54.24% lower than control treatments (-R0). The level of decreasing in IC_{50} was slightly higher in inoculated plants. Interaction effect of bacterial inoculation and SO_2 pollution had shown that decline in IC_{50} on noninoculated plants under 2 ppm SO_2 (-R2) was 54.24% but on inoculated plants with native and standard *Rhizobium meliloti* under 2 ppm SO_2 (R_n2 and R_s 2) was 45.93 and 46.16% respectively. IC_{50} levels were lower in inoculated plants than noninoculated plants (Table 1).

B) Bacterial inoculation and SO_2 pollution on antioxidant activity (SOD, CAT, GPOX).

Effect of bacterial inoculation

The results showed that inoculation did not impose any significant effect on antioxidant activity (SOD, CAT, GPOX) and there was no significant difference among inoculated and noninoculated plants.

Effect of SO_2 pollution

There was significant difference among treatments under different concentrations SO_2 pollution in SOD, CAT and GPOX activity. The change in SO_2 concentration caused increased in antioxidant activity (Fig. 2).

Superoxide dismutase activity

SOD activity was not different under the influence of 0 and 0.5 ppm of SO_2, but increased significantly with higher concentrations of SO_2.). The SOD activity in the leaves of alfalfa at 1, 1.5 and 2 ppm of SO_2 pollution was 48.96%, 86.15% and 135.12% higher than 0 ppm of SO_2 pollution treatments, respectively (Fig. 2a).

Catalase activity

SO_2 pollution effect on CAT activity was significant. Compared with the control, CAT activity was affected by SO_2 treatments, was highest in the 2 ppm dose whereas being no change in other doses. The CAT activity in the leaves of alfalfa plant under 2 ppm 59.37% higher than control treatments (0 ppm) (Fig. 2b).

Guaiacol peroxidase activity

The antioxidant activity of GPOX was not different under the influence of 0 and 0.5 ppm of SO_2 pollution but increased in higher doses of SO_2 pollution. Among SO_2 pollution treatments, 2 ppm showed higher level of GPOX activity. The GPOX activity in the leaves of alfalfa at 1, 1.5 and 2 ppm of SO_2 pollution was 23.24, 57.73 and 77.91% higher than 0 and 0.5ppm of SO_2 pollution treatments, respectively (Fig. 2c).

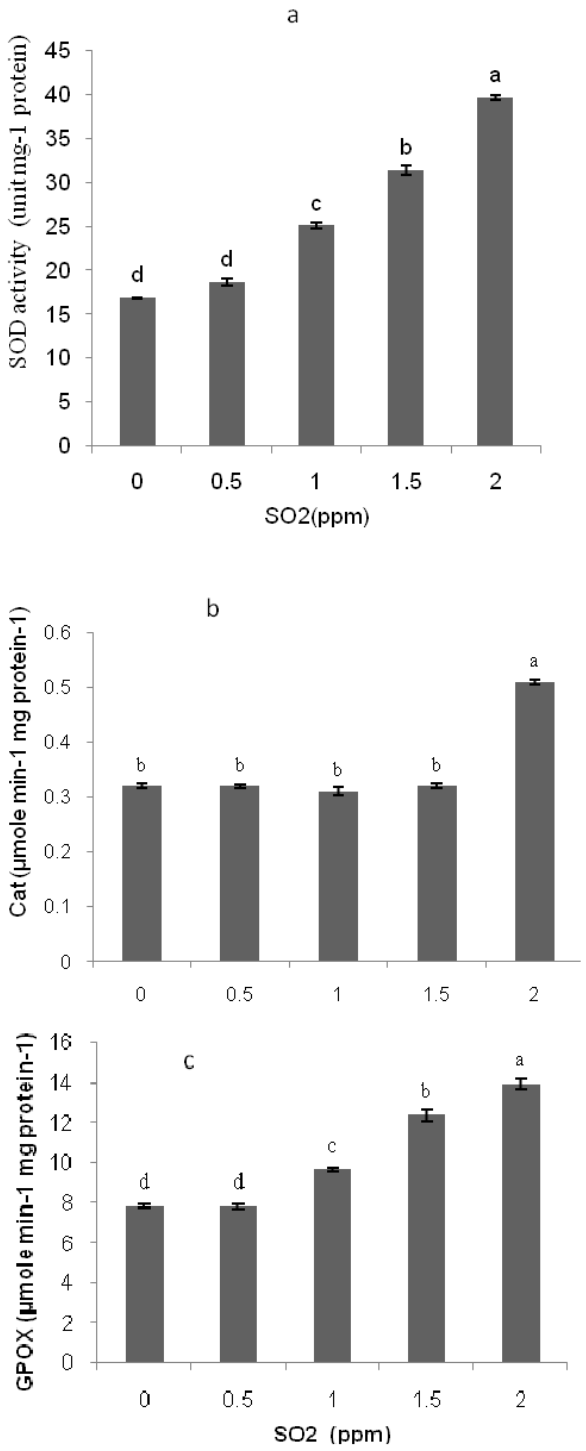

Fig. 2. SOD (a), CAT (b) and GPOX(c) activity in the leaves of alfalfa under SO_2 pollution in 45 days plants. Data are mean ± SE. Different letters indicate that the mean value is significantly different ($p< 0.01$).

Table 1. Means comparison of interaction effect of bacterial inoculation (no-inoculation (-R), inoculation with native (R_n) and standard (R_s) *Rhizobium meliloti*) and SO_2 pollution (0, 0.5, 1, 1.5 and 2 ppm) on values of antioxidant capacities (IC_{50}) and antioxidant activities (I %) in 45 days plants. Similar words indicate not significantly different according to Duncan's test. The data are the means of three replicates ±SE and comparisons were performed separately for each index.

Bacterial inoculation	SO_2 treatments (ppm)	IC_{50} (mg/ml)	I%
Noninoculation (-R)	0	$17.44^{a} \pm 0.27$	$7.21^{g} \pm 0.11$
	0.5	$17.32^{a} \pm 0.22$	$7.18^{g} \pm 0.09$
	1	$13.33^{c} \pm 0.20$	$9.38^{e} \pm 0.14$
	1.5	$10.64^{e} \pm 0.03$	$11.74^{c} \pm 0.03$
	2	$7.98^{g} \pm 0.04$	$15.66^{a} \pm 0.13$
Inoculation with native *Rhizibium* (R_n)	0	$17.11^{a} \pm 0.27$	$7.31^{g} \pm 0.11$
	0.5	$16.99^{a} \pm 0.12$	$7.35^{g} \pm 0.05$
	1	$15.03^{b} \pm 0.10$	$8.32^{f} \pm 0.05$
	1.5	$11.63^{d} \pm 0.04$	$10.74^{d} \pm 0.04$
	2	$9.43^{f} \pm 0.06$	$13.25^{b} \pm 0.08$
Inoculation with standard *Rhizibium* (R_s)	0	$17.24^{a} \pm 0.18$	$7.25^{g} \pm 0.07$
	0.5	$17.30^{a} \pm 0.16$	$7.23^{g} \pm 0.06$
	1	$15.16^{b} \pm 0.11$	$8.24^{f} \pm 0.05$
	1.5	$11.64^{d} \pm 0.10$	$10.74^{d} \pm 0.09$
	2	$9.39^{f} \pm 0.06$	$13.32^{b} \pm 0.09$

Interaction effect of bacterial inoculation and SO_2 pollution on antioxidant activity

Interaction of bacterial inoculation and SO_2 gas on antioxidant activity of SOD, CAT and GPOX was statistically significant. Combination of SO_2 pollution and bacterial inoculation had shown that the highest levels of SOD, CAT and GPOX activity were obtained at noninoculated plants under 2 ppm SO_2 (-R 2). Inoculated plants under high concentrations of SO_2 (1, 1.5 and 2 ppm) showed antioxidant activity lower than noinoculated plant. For instance, noninoculated plant under 2 ppm SO_2 gas treatment showed 84.37% increase in the CAT activity but inoculated plant under 2 ppm SO_2 gas treatment showed 43.75% increase in the CAT activity. The rate of increase in antioxidant activity under SO_2 pollution stress in inoculated plant was lower than in the noninoculated plant (Fig 3).

Discussion

Various abiotic stresses lead to the overproduction of reactive oxygen species (ROS) in plants which are highly reactive and toxic and ultimately results in oxidative stress. However, the cells are equipped with excellent antioxidant defense mechanisms to detoxify the harmful effects of ROS. ROS are now also considered as key regulatory molecules vital for cells, but they cause cellular damage when produced in excess or when the antioxidant defense system is not properly functioning. Therefore, the concentration of ROS in cell must be controlled (Gill and Tuteja, 2010). After entering SO_2 to the leaf, oxidation of sulphite to sulphate occurs in the chloroplast. This oxidation gives rise to formation ROS such as O_2^- (Arora *et al*, 2002; Li and Yi, 2012). When SO_2 is converted to sulphite in aqueous solution, it is a nucleophilic agent that is able to attack in numerous substrates by opening S—S bridges. This reaction, so called sulphitolysis, causes the inactivation of proteins like thioredoxins and, as a consequence, causes a severe reduction in plant growth or even cell death. Plants manage the internal sulphite concentration, i.e. they control the: (1) uptake of the gas by the laminar boundary layer, the cuticle or the guard cells and (2) rate of its metabolic conversion by feeding into sulphur assimilation stream for production of cysteine, or reoxidation into sulphate. Apoplastic peroxidases were described to detoxify sulphite.

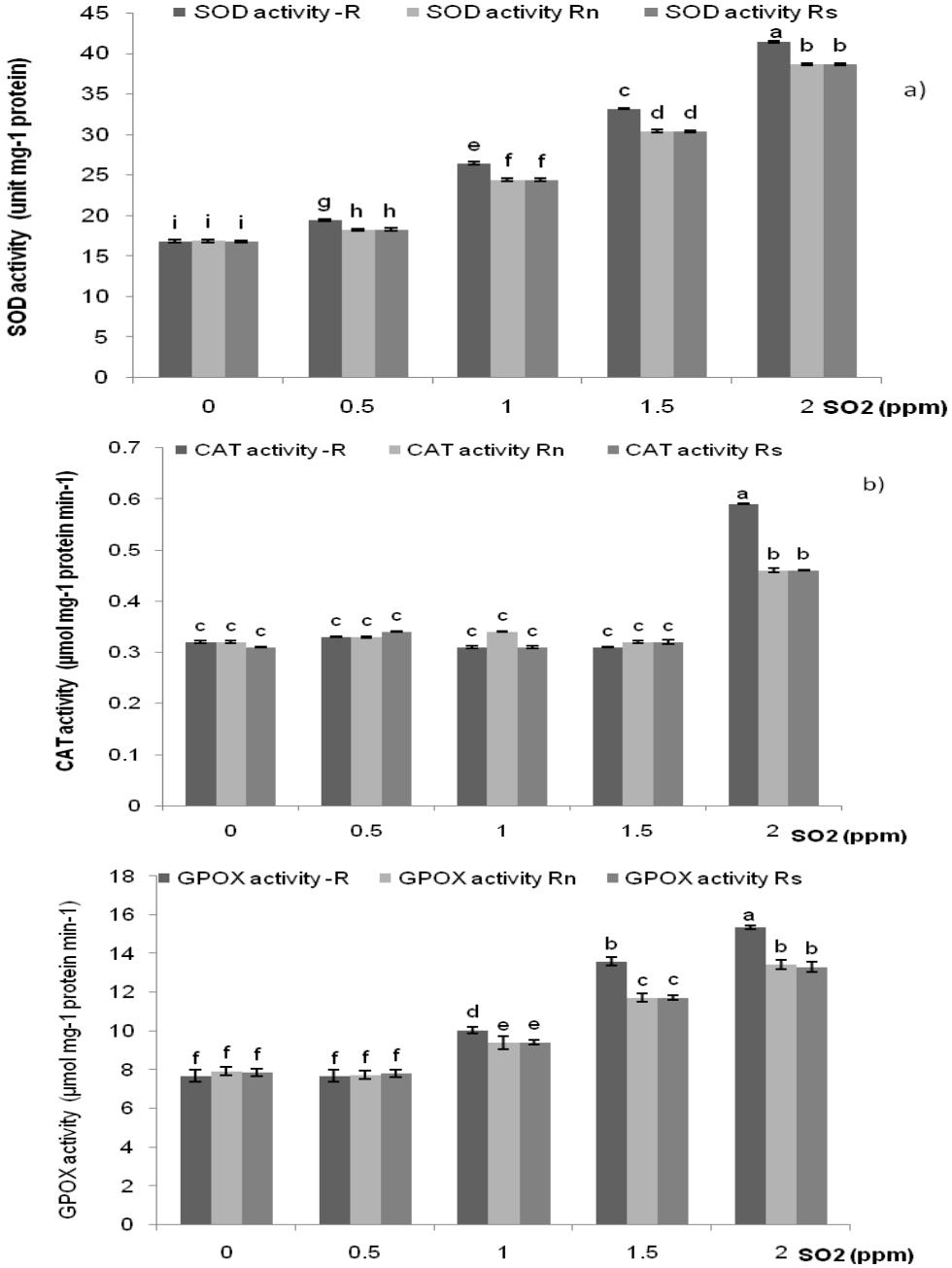

Fig 3. Interaction effect of bacterial inoculation and SO_2 pollutionon antioxidant activity of SOD (a), CAT (b) and GPOX (c) the leaves of alfalfa under SO_2 pollution in 45 days plants. Data are mean ± SE. Different letters indicate that the mean value is significantly different ($p < 0.01$).

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Non-enzymatic oxidation of sulphite, initiated by superoxide anions formed on the reduction site of the electron transport system in chloroplasts (Lang *et al*., 2007). sulphite oxidation proceeds via a radical chain reaction involving light-dependent photosynthetic electron transport, sulphite oxidizing activity associated with isolated thylakoid membranes by the enzyme sulphite oxidase (SO) that the plant enzyme is localized in peroxisomes. Molecular oxygen as the terminal electron acceptor for plant SO and showed that it converts molecular oxygen into hydrogen peroxide (Hänsch *et al*., 2006). SO plays a key role in protection of plants from the damaging effects of SO_2. SO is a housekeeping enzyme that oxidaze sulphite using H_2O_2 and different phenolic compounds. It is involved in the recycling of sulphur. It may also protect the thioredoxin system from damage (Lang *et al*., 2007). In such conditions, plants develop a high efficient antioxidant enzymatic defense system to increase tolerance to different stress factors (Gill and Tuteja, 2010).

In this study , values of IC_{50}, I% and antioxidant activity indicated no significant difference at 0.5 ppm of SO_2 as compared with control plants (exposed to 0 ppm) because may be stress conditions are not created in 0.5 ppm of SO_2, but indicated significant differences in higher concentration. In higher concentrations of SO_2 (1, 1.5 and 2 ppm), IC_{50} value decreased with increasing stress intensity but I% increased. Increasing of I% means more antioxidants has been produced with increasing stress intensity. Increase of DPPH-radical scavenging activity has been reported in many studies. DPPH-radical scavenging activity significantly increased as compared with control plants in *Oryza sativa* seedlings under heat shock treatment (Kang and Saltveit, 2002) and in *Cakile maritima* exposed to salinity stress (Ksouri *et al.*, 2007). Use of silicon and salinity on *Sorghum bicolor* increased DPPH-radical scavenging activity as compared with control plants (Kafi *et al.*, 2011).

Guaiacol peroxidase (GPOX) decomposes indole-3-acetic acid (IAA) and has a role in the biosynthesis of lignin and defence against biotic stresses by consuming H_2O_2 (Gill and Tuteja, 2010). The activity of GPOX varies considerably depending upon plant species and stresses condition. In this study, GPOX activity increased in 1, 1.5 and 2 ppm of SO_2 as observed in *Zizyphus mauritiana* and *Mangifera indica* (Rao and Dubey, 1990) exposed to SO_2 pollution.

Catalases (CAT) are tetrameric heme containing enzymes with the potential to directly dismutate H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stressed conditions. CAT has one of the highest turnover rates for all enzymes: one molecule of CAT can convert 6 million molecules of H_2O_2 to H_2O and O_2 per minute. CAT is important in the removal of H_2O_2 generated in peroxisomes by oxidases involved in

b-oxidation of fatty acids, photorespiration and purine catabolism (Gill and Tuteja, 2010). In this study, CAT activity indicated no significant difference at 0.5, 1 and 1.5 ppm concentration of SO_2 as compared with control plants but in 2 ppm in wheat plant under drought stress (Simova-Stoilova *et al.*, 2010). CAT activity decreased in *Zea mays* exposed to SO_2 pollution as compared with control plants. CAT activity of *Calandula officinalis* under high concentration of salinity (100 mM) increased in leaves but decreased in roots (Chaparzadeh *et al*., 2004).

Metalloenzyme Superoxide dismutase (SOD) is the most effective intracellular enzymatic antioxidant which is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress. Various environmental stresses often lead to the increased generation of ROS, where, SOD has been proposed to be important in plant stress tolerance and provide the first line of defense against the toxic effects of elevated levels of ROS. The up-regulation of anti-oxidative enzymes, for example, superoxide dismutases (SODs), is a general response to different abiotic stress conditions. The SOD_s remove O_2^{*-} by catalyzing its dismutation, one O_2^{*-} being reduced to H_2O_2 and another oxidized to O_2. It removes O_2^{*-} and hence decreases the risk of OH^* formation via the metal catalyzed Habere Weiss-type reaction (Gill and Tuteja, 2010). In this study, SOD activity increased significantly in 1, 1.5 and 2 ppm of SO_2 as compared with control plants. Similar results have been reported in other studies. SOD activity indicated a significant increase in *Phaseolus vulgaris* (Bernardi *et al.*, 2001) exposed SO_2 pollution.

In this study, inoculation with native and standard *R. meliloti* had no significant effects on values of IC_{50}, I%, GPOX, SOD and CAT activity, as reported by Gabballah and Gomaa (2005). Their study showed that inoculation of two cultivars of *Vicia faba* with *Rhizobium* had no significant effects on SOD activity.

Interaction of inoculation and SO_2 treatment in this study was significant. Indeed *Rhizobium* inoculation under SO_2 condition showed significant effect on the values of IC_{50}, I%, GPOX, SOD and CAT activity. Rhizobacteria such as *Rhizobium* can promote plant growth and by mechanisms that used for this growth promotion can reduce stress conditions for plants. Plant growth promotion by rhizobacteria can of SO_2 indicated a significant increase. CAT is an antioxidant that is activated in severe stress conditions (Gill and Tuteja, 2010). Various studies have reported different results from the CAT activity. Increase in CAT activity indicated occur directly and indirectly. There are several ways by which PGPR can affect plant growth directly, e.g. by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, production of siderophores that solubilize and sequester iron, or production of plant growth regulators (hormones) that enhance plant growth at various stages of development. Indirect growth promotion occurs when PGPR promote plant growth by improving growth restricting conditions. This can happen directly by producing antagonistic substances, or indirectly by inducing resistance to biotic and abiotic stresses (Timmusk, 2003). Indeed can be concluded that bacterium has been reduced antioxidant activity and capacity by reducing the effects of high concentrations of SO_2 and stress conditions. Different studies have expressed different conclusions about the interaction between bacterial inoculation and stress. Inoculation of Lettuce under salinity stress with *Rhizobium* sp. and *Serratia* sp.decreased enzyme activity, including glutathione reductase and ascobate peroxidase, with increasing salinity stress (Han and Lee, 2005). A clear decline in SOD activity in two cultivars of faba bean was observed with increasing salinity stress. Use of *Rhizobium* inoculation and sodium benzoate increased SOD activity in faba bean plants under salinity (Gabballah and Gomaa, 2005).

Conclusion

The induction of the cellular antioxidant machinery is important for protection against ROS. Overexpression of ROS scavenging enzymes like SOD, CAT, and GPOX resulted in abiotic stress tolerance in various crop plants due to efficient ROS scavenging capacity. Low concentration of SO_2 (0.5 ppm) doesn't create stress conditions in alfalfa therefore activity and capacity of most of antioxidants don't alter in this concentration. In higher concentrations of SO_2 (1, 1.5 and 2 ppm), activity and capacity of alfalfa antioxidants increase with increasing stress intensity. Inoculation of alfalfa plant with native and standard *Rhizobium meliloti* significantly reduced the negative effects of high concentration of SO_2 on antioxidants activity and capacity.

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