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# Some Responses of Inoculated Persian Clover with *Rhizobium* to $SO_2$ Pollution

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## ABSTRACT

**Sulfur dioxide ($SO_2$) is one of the most common and harmful air pollutants. High concentrations of $SO_2$ can cause stress and limit growth in plants. Some of the plants can resist stress by bacterial symbiosis such as *Rhizobium* symbiosis. *Rhizobium* is a beneficial bacterium that enhances plant growth and yield. To study the effects of $SO_2$ pollution on growth indexes, protein, proline and sulfur contents, 31 days old plants of *Trifolium resupinatum* (Persian clover), inoculated with native and standard *Rhizobium* were exposed to the different concentrations of $SO_2$ (0 as control, 0.5, 1, 1.5 and 2 ppm) for 5 consecutive days. Results showed that inoculation increased leaf area, leaf number, shoot height, root length, shoot fresh and dry weight and protein content of Persian clover but didn't show any significant effect on proline and sulfur contents. Different concentrations of $SO_2$ had a significant effect on leaf number, shoot height, root length, shoot fresh and dry weight, protein, proline and sulfur contents but didn't have effects on leaf area. 0.5 ppm concentration of $SO_2$ increased growth indexes and protein content. Proline and sulfur contents didn't change in 0.5 ppm. Increasing $SO_2$ decreased growth indexes and protein, and increased proline and sulfur contents. Interaction between *Rhizobium* inoculation and $SO_2$ treatment improved the stress effects of high concentrations of $SO_2$ on growth indexes, protein, proline and sulfur contents. It was therefore concluded that *Rhizobium* can increase tolerance and resistance of this plant to the abiotic stresses such as $SO_2$ pollution.**


**Keywords**: Air pollution, Persian clover, Protein, *Rhizobium*, Sulfur.

## INTRODUCTION

Sulfur appears in the life cycle of most plants. Most sulfur emitted to the atmosphere originates in the form of hydrogen sulfide from the decay of organic matter. These emissions slowly oxidize to Sulfur dioxide ($SO_2$) (Kitto and Stultz, 2005). Sulfur dioxide is one of the major air pollutants in industrialized areas (Anjali *et al*., 2012). Under atmospheric conditions, $SO_2$ is a reactive, acid gas that can be rapidly assimilated back to the environment. However, the combustion of fossil fuels, in which large quantities of $SO_2$ are emitted to relatively small portions of the atmosphere, can stress the ecosystem in the path of these emissions (Swain and Padhi, 2013). Sulfur dioxide annual worldwide emissions are approximately 160 million tons, nearly half of which are from industrial sources. The two principal industrial sources are fossil fuel combustion and metallurgical ore refining (Kitto and Stultz, 2005).

Tolerance of plants to $SO_2$ is related to its capacity to defend from the toxicity of active oxygen (Ceron-Breton *et al*., 2012). At low concentrations, $SO_2$ can be considered as a nutrient since sulfur is an essential macronutrient for plants. In general, plant exposure to $SO_2$ results in an increase in the sulfate content and a slight increase in the thiol

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content (mainly glutathione) of the shoot, since part of the $SO_2$ can be assimilated into organic sulfur compounds via sulfite. In most plants the predominant proportion of the organic sulfur is present in the protein fraction as cysteine and methionine residues (De Kok and Tausz, 2001). Sulfur is a structural component of amino acids such as methionine and cysteine. Methionine is the initiating amino acid in the synthesis of virtually all proteins. Cysteine, by virtue of its ability to form disulfide bonds, plays a crucial role in protein structure and in protein-folding pathways (Brosnan and Brosnan, 2006; Li and Yi, 2012). At high doses, $SO_2$ behaves as a toxic. This pollutant is deposited on the cuticle as dry deposition or to spread toward the inside of the leaves through the stomas. The rate of foliar uptake depends on the stomatal and the leaf interior (mesophyll) conductance toward $SO_2$ gas and its atmospheric concentration. There is a linear relationship between the uptake of $SO_2$ and the atmospheric concentration. Stomatal conductance is generally the limiting factor for uptake of $SO_2$ by the foliage, whereas the mesophyll conductance toward $SO_2$ is very high since the gas is highly soluble in the water of the mesophyll cells (Barker and Pilbeam, 2006). Inside the leaves, $SO_2$ entries in contact with water to be converted in bisulfite or/and sulfite. These radicals destroy chlorophyll, cause lipids peroxidation and damages in the chloroplast، causing affections in the photosynthetic activity (Ceron-Breton *et al*., 2012).

Plants, such as legumes can reduce this stress through symbiosis with Plant Growth Promoting Rhizobacteria (PGPR). Plant growth promoting rhizobacteria are a group of bacteria that enhance plant growth and yield via various plant growth promoting substances (Singh, 2013). Plant growth promoting rhizobacteria include bacteria residing in roots useful for plants in some conditions. *Rhizobium* is one of these bacteria. It is not obvious, which mechanism helps develop the growth of the plants by Rhizosphere bacteria in stress conditions, but some of the most important mechanisms have the ability to produce plant hormones, nitrogen fixing, confronting some plant pathogens by sidrophore production and antifungal compounds synthesis. Sidrophore production, phosphate solubilisation and other nutrients and ACC deaminase enzyme production are effective in reducing destructive effects of produced ethylene under stress (Baniaghil *et al.*, 2013). Legume plants were inoculated with rhizobial strains to enhance the nodulation and nitrogen fixation and finally to increase the growth, yield and resistance in stress conditions (such as $SO_2$ pollution) (Ahemad and khan, 2011).

Forage legumes have been important livestock feed for centuries as pasture, soilage and conserved forage. Because of the large number of species, their wide adaptation to soil and climatic conditions, and their general ability to reseed readily, *Trifolium* is one of the two most important legume genera in livestock agriculture. Persian clover (*Trifolium resupinatum* L.), one of the important species of genus *Trifolium* (Erdemli *et al*., 2007) has a maximum growth in subtropical climates (Ates, 2011).

In the present study, effects of $SO_2$ pollution on growth parameters, proline and sulfur contents and protein production of inoculated *Trifolium resupinatum* with *Rhizobium* were evaluated.

## MATERIALS AND METHODS

### Bacterial Culture and Inoculant Preparation

Sample Persian clover plants were collected form Arak farm lands and native strain of *Rhizobium* was extracted from roots of these plants (Swift and Bignell, 2001). Standard strain of *Rhizobium* (*Rhizobium meliloti* PTCC 1684) was prepared in lyophilized form (freeze dried bacteria). The YMA (Yeast extract Mannitol Agar) medium was prepared without agar (Liquid YMA: Mannitol, 10 g $l^{-1}$; $K_2HPO_4$, 0.5 g $l^{-1}$; yeast extract, 1 g $l^{-1}$; $MgSO_4$, 0.2 g $l^{-1}$; NaCl,





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0.1 g $l^{-1}$) and standard strain of *Rhizobium* was activated in this medium. Optimum concentration of *Rhizobium* to stimulate clover growth is about $10^5$ to $10^6$ cells $mL^{-1}$ (Caetano-Anolles *et al*., 1988), so *Rhizobium* was prepared in this concentration.

### Seed Preparation and Inoculation

The seeds of Persian clover (*Trifolium resupinatum* L. cv. Alashtar Lorestan) were prepared from Arak Agriculture Research Center. After sterilization of seeds (Wang and Oyaizu, 2009), they were inoculated with native and standard inoculums under vacuum and ambient temperature for 2 hours. After germination of clover seeds, they were transferred to plastic pots containing 2 L of Half-Hoagland solution (without nitrogen) (Millner and Kitt, 1992). N-free nutrient solution can encourage plants for the establishment symbiosis with *Rhizobium*. Pots were oxygenated by the air compressor. Each pot was considered as a treatment. Each treatment included three replicates. Clover plants were maintained under 12 hours photoperiod, at 25°C during day and 20°C during night provided with fluorescent lamps at Arak University greenhouse. The nutrient solution changed every five days (Bashan *et al*., 1989).

### $SO_2$ Treatment

$SO_2$ gas was prepared from Shazand Petrochemical Co. and was injected in different concentrations 0 (as control), 0.5, 1, 1.5 and 2 ppm into 31 days old plants. Gas injection was performed by syringe for 5 days and 2 hours daily to closed plastic containers (Agrawal *et al.*, 1985).

### Measurement of Growth Parameters

Every 9 days, growth parameters including shoot height, root length, leaf area and leaf number were measured. Leaf area was measured with a grid paper by counting grid. After the final harvest of 41 days old plants, shoot fresh weight (including stems and leaves) were measured. Dry weight of shoot was obtained by drying samples in an oven for 24 hours at 75°C until reaching a constant weight.

### Proline and Protein Determination

Proline colorimetric determination was performed according to Bates *et al*. (1973) based on proline's reaction with ninhydrin. Protein content was measured using the method of Bradford (1976). Bovine serum albumin was used as a standard.

### Sulfur Determination

Total sulfur content was analyzed by the gravimetric method (Chapman and Pratt, 1973): A known fresh weight (about 0.5 g) of leaves was pulverized and digested with 1:1 HCl. The mineralized and diluted solution was treated with $Mg(NO_3)_2$ to oxidize sulfur to sulfate quantitatively. By addition of $BaCl_2$ the corresponding barium sulfate was obtained, which was determined by gravimetric analysis and total sulfur was calculated from that.

### Statistical Analysis

All data were analyzed by variance analysis using SPSS 16. Experiments were tested using a completely randomized design in factorial form in three replicates. The data was represented as the means ± SE.

## RESULTS

### Effects of Bacterial Inoculation and $SO_2$ Pollution on Growth Parameters

The results of analysis of variance showed that the effect of *Rhizobium*





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inoculation on leaf area, leaf number, shoot height, root length (Table 1), and shoot fresh and dry weight (Table 2) of clover plants in developmental periods of 9, 18 and 27 days (before gas treatment), 36 and 41 days (after gas treatment) (Table 2) were statistically significant.

Leaf area, leaf number, shoot height, root length and shoot fresh and dry weight of inoculated plants with *Rhizobium* increased significantly compared to non-inoculated plants. For example leaf area, leaf number, shoot height, root length and shoot fresh and dry weight in inoculated 41 days old plants with native *Rhizobium* increased by 68.6, 35.5, 25, 28, 75.32 and 79.10% respectively (Table 3). Effects of different concentrations of $SO_2$ on leaf number, shoot height, root length (Table 1) and shoot fresh and dry weight (Table 2) in 41 days old plants was statistically significant but did not show a significant effect on leaf area. Shoot height, root length and shoot fresh and dry weight of 41 days old plants showed a significant increase in 0.5 ppm concentration of $SO_2$ but decreased significantly in high concentrations compared to control plants. Leaf number of 41 days old plants increased significantly with 0.5 ppm of $SO_2$, but was not different with higher concentrations of $SO_2$ (Table 4).

Interaction between bacterial inoculation and $SO_2$ on shoot height, root length and shoot fresh and dry weight was statistically significant but didn't show a significant effect on leaf area and leaf number (Tables 1 and 2). 30.6% and 23.2% reduction in shoot height and root length of non-inoculated plants was changed to 1% increase in inoculation with native and

**Table 1.** Analysis of variance effects of bacterial inoculation and $SO_2$ gas on leaf area, leaf number, shoot height and root length of clover plants in developmental periods of 9, 18 and 27 days (before gas treatment), 36 and 41 days (after gas treatment).

| Treatment | Index | | | | |
|---|---|---|---|---|---|
| | Age (Day) | Leaf area | Leaf number | Shoot height | Root length |
| Bacterial Inoculation | 9 | 19.05* | 24* | 119** | 30.18** |
| | 18 | 37.84** | 30.33** | 82.41** | 51.7* |
| | 27 | 136.4** | 30.33** | 104.8** | 97.08** |
| | 36 | 163.36** | 232.75** | 398.1** | 222.6** |
| | 41 | 152.74** | 110.38** | 239.7** | 277.3** |
| $SO_2$ gas | 36 | $1.22^{ns}$ | 8** | 277.9** | 142.1** |
| | 41 | $1.1^{ns}$ | 5.24* | 266.7** | 193.4** |
| Interaction between inoculation and $SO_2$ | 36 | $2.04^{ns}$ | $1.18^{ns}$ | 10.6** | 8.09** |
| | 41 | $1.51^{ns}$ | $0.88^{ns}$ | 6.09** | 9.28** |

*= $P\leq 0.05$; **= $P\leq 0.01$, ns= Not significant.

**Table 2.** Analysis of variance effects of bacterial inoculation and $SO_2$ gas on fresh shoot weight, dry shoot weight, protein, proline and sulfur of 41 days old clover plants.

| Treatment | Index | | | | |
|---|---|---|---|---|---|
| | Fresh shoot weight | Dry shoot weight | Protein | Proline | Sulfur |
| Bacterial Inoculation | 131.67** | 140.9** | 24.18** | $37.42^{ns}$ | $0.979^{ns}$ |
| $SO_2$ gas | 304.19** | 277.6** | 55.86** | 636.42** | 311.74** |
| Interaction between inoculation and $SO_2$ | 15.47** | 17.22** | 2.02* | 7.62** | 8.55** |

*= $P\leq 0.05$; **= $P\leq 0.01$, ns= Not significant.





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**Table 3.** Effects of bacterial inoculation on growth parameters and protein content in developmental periods of 9, 18 and 27 days (before gas treatment) and 36 and 41 days (after gas treatment). Similar letters indicate not significantly different according to Duncan's test.

| Index | Inoculation | | | |
|---|---|---|---|---|
| | Age (Day) | No-inoculation | Inoculation with native *Rhizobium* | Inoculation with standard *Rhizobium* |
| Leaf area ($cm^2$) | 9 | $1.30^{c}$±0.6 | $2.50^{a}$±0.1 | $2.03^{b}$±0.1 |
| | 18 | $5.72^{c}$±0.2 | $9.80^{a}$±0.2 | $7.40^{b}$±0.5 |
| | 27 | $19.71^{c}$±0.2 | $29.35^{a}$±0.07 | $27.41^{b}$±0.7 |
| | 36 | $24.30^{c}$±0.7 | $34.20^{a}$±0.3 | $30.71^{b}$±0.2 |
| | 41 | $28.20^{c}$±0.8 | $38.22^{a}$±0.3 | $34.91^{b}$±0.2 |
| Leaf number | 9 | $0.67^{c}$±0.3 | $1.33^{a}$±0.3 | $0.99^{b}$±0.1 |
| | 18 | $1.67^{c}$±0.3 | $3.32^{a}$±0.3 | $2.32^{b}$±0.3 |
| | 27 | $3.71^{c}$±0.3 | $7.33^{a}$±0.3 | $5.30^{b}$±0.3 |
| | 36 | $5.71^{c}$±0.1 | $9.80^{a}$±0.2 | $7.90^{b}$±0.2 |
| | 41 | $7.00^{c}$±0.3 | $11.80^{a}$±0.2 | $10.33^{b}$±0.3 |
| Shoot height (cm) | 9 | $6.11^{c}$±0.2 | $8.91^{a}$±0.1 | $7.20^{b}$±0.1 |
| | 18 | $7.93^{c}$±0.2 | $11.40^{a}$±0.1 | $10.03^{b}$±0.2 |
| | 27 | $10.20^{c}$±0.1 | $13.31^{a}$±0.1 | $11.70^{b}$±0.2 |
| | 36 | $13.61^{c}$±0.4 | $11.04^{a}$±0.8 | $16.32^{b}$±0.6 |
| | 41 | $16.11^{c}$±0.6 | $20.10^{a}$±0.8 | $18.31^{b}$±0.7 |
| Root length (cm) | 9 | $7.90^{c}$±0.4 | $10.82^{a}$±0.1 | $9.33^{b}$±0.2 |
| | 18 | $10.22^{c}$±0.3 | $13.22^{a}$±0.2 | $11.71^{b}$±0.2 |
| | 27 | $12.60^{c}$±0.1 | $15.37^{a}$±0.1 | $13.72^{b}$±0.1 |
| | 36 | $15.50^{c}$±0.4 | $20.40^{a}$±0.8 | $18.20^{b}$±0.6 |
| | 41 | $17.40^{c}$±0.5 | $22.30^{a}$±0.8 | $20.40^{b}$±0.7 |
| Fresh shoot weight (g) | 41 | $0.77^{c}$±0.09 | $1.35^{a}$±0.2 | $1.11^{b}$±0.6 |
| Dry shoot weight (g) | 41 | $0.067^{c}$±0.01 | $0.12^{a}$±0.02 | $0.10^{b}$±0.02 |
| Protein (mg $g^{-1}$ FW) | 41 | $4.92^{c}$ ± 0.5 | $6.74^{a}$ ± 0.4 | $5.53^{b}$ ± 0.4 |

**Table 4.** Effects of $SO_2$ pollution on leaf number, shoot height, root length, shoot fresh and dry weights of 41 days old plants. Similar letters indicate not significantly different according to Duncan's test.

| | Different concentrations of $SO_2$ (ppm) | | | | |
|---|---|---|---|---|---|
| Index | 0 | 0.5 | 1 | 1.5 | 2 |
| Leaf number | $9.42^{b}$±0.6 | $10.54^{a}$±0.8 | $9.45^{b}$±0.3 | $9.00^{b}$±0.8 | $9.21^{b}$±0.7 |
| Shoot height (cm) | $19.77^{b}$±0.5 | $21.82^{a}$±0.9 | $18.25^{c}$±0.4 | $16.33^{d}$±0.5 | $14.81^{e}$±0.6 |
| Root length (cm) | $21.62^{b}$±0.7 | $23.70^{a}$±1.1 | $19.61^{c}$±0.6 | $18.31^{d}$±0.5 | $16.90^{e}$±0.6 |
| Fresh shoot weight (g) | $1.60^{b}$±0.2 | $1.81^{a}$±0.1 | $0.85^{c}$±0.07 | $0.65^{d}$±0.04 | $0.51^{e}$±0.03 |
| Dry shoot weight (g) | $0.14^{b}$±0.02 | $0.15^{a}$±0.01 | $0.08^{c}$±0.01 | $0.06^{d}$±0.03 | $0.05^{e}$±0.002 |

standard *Rhizobium* (Table 5). Similar results were observed in 36 days old plants

## Effects of Bacterial Inoculation and $SO_2$ Pollution on Protein Content

In this study, inoculation had a significant effect on protein content (Table 2) and caused increase in the total protein content. The highest levels of protein content were observed in inoculation plants with native *Rhizobium* (36.99% increase) compared to non-inoculated plants. In inoculation with standard *Rhizobium,* protein content increased by 12.93% compared to non-inoculated plants (Table 3). Protein content changed significantly with different concentrations of $SO_2$ (Table





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**Table 5.** Effects of interaction between *Rhizobium* inoculation and $SO_2$ on shoot height, root length, shoot fresh and dry weight of in 41 days old plants. Similar words indicate not significantly different according to Duncan's test.

| Inoculation | $SO_2$ (ppm) | Shoot height (cm) | Root length (cm) | Shoot fresh weight (g) | Shoot dry weight (g) |
|---|---|---|---|---|---|
| No-inoculation | 0 | $18.3^{d}\pm0.1$ | $19.4^{de}\pm0.3$ | $0.9^{e}\pm0.05$ | $0.08^{d}\pm0.004$ |
| | 0.5 | $18.4^{d}\pm0.3$ | $19.1^{de}\pm0.4$ | $1.36^{c}\pm0.2$ | $0.1^{c}\pm0.02$ |
| | 1 | $16.7^{e}\pm0.4$ | $17.4^{f}\pm0.3$ | $0.65^{fg}\pm0.07$ | $0.06^{ef}\pm0.007$ |
| | 1.5 | $14.5^{f}\pm0.1$ | $16.3^{g}\pm0.1$ | $0.5^{gh}\pm0.01$ | $0.05^{fg}\pm0.002$ |
| | 2 | $12.7^{g}\pm0.40$ | $14.9^{h}\pm0.2$ | $0.4^{h}\pm0.08$ | $0.04^{g}\pm0.001$ |
| Inoculation with native *Rhizobium* | 0 | $21.6^{b}\pm0.3$ | $24^{b}\pm0.3$ | $1.5^{b}\pm0.2$ | $0.12^{b}\pm0.02$ |
| | 0.5 | $24.7^{a}\pm0.6$ | $27.3^{a}\pm0.7$ | $1.71^{a}\pm0.1$ | $0.14^{a}\pm0.01$ |
| | 1 | $19.8^{c}\pm0.2$ | $21.7^{c}\pm0.3$ | $1.1^{d}\pm0.2$ | $0.1^{c}\pm0.01$ |
| | 1.5 | $19.1^{d}\pm0.3$ | $20.1^{d}\pm0.3$ | $0.8^{ef}\pm0.05$ | $0.07^{de}\pm0.005$ |
| | 2 | $18.5^{e}\pm0.2$ | $19.7^{e}\pm0.3$ | $0.6^{g}\pm0.02$ | $0.05^{fg}\pm0.008$ |
| Inoculation with standard *Rhizobium* | 0 | $19.3^{c}\pm0.2$ | $21.4^{c}\pm0.2$ | $1.4^{b}\pm0.09$ | $0.12^{b}\pm0.009$ |
| | 0.5 | $22.4^{b}\pm0.4$ | $24.5^{b}\pm0.6$ | $1.4^{b}\pm0.06$ | $0.12^{b}\pm0.02$ |
| | 1 | $19.2^{d}\pm0.2$ | $19.9^{c}\pm0.6$ | $0.8^{f}\pm0.03$ | $0.07^{de}\pm0.002$ |
| | 1.5 | $18.8^{e}\pm0.1$ | $19.7^{d}\pm0.2$ | $0.6^{g}\pm0.02$ | $0.05^{f}\pm0.002$ |
| | 2 | $18.4^{f}\pm0.1$ | $19.6^{f}\pm0.2$ | $0.5^{gh}\pm0.01$ | $0.04^{fg}\pm0.001$ |

2). It showed a significant increase in 0.5 ppm concentration of $SO_2$ but decreased significantly in high concentrations of $SO_2$ compared to control plants (Figure 1-a). Interaction between bacterial inoculation and $SO_2$ on total protein content was statistically significant (Table 2). The highest level of protein content was obtained from inoculated plants with native *Rhizobium* under 0.5 ppm of $SO_2$. The lowest level of protein content was obtained from non-inoculated plants exposed to 2 ppm of $SO_2$ (Figure 1-b).

## Effects of Bacterial Inoculation and $SO_2$ Pollution on Proline Content

The results showed that *Rhizobium* inoculation had no significant effect on free proline content but proline content changed with $SO_2$ stress (Table 2). Free

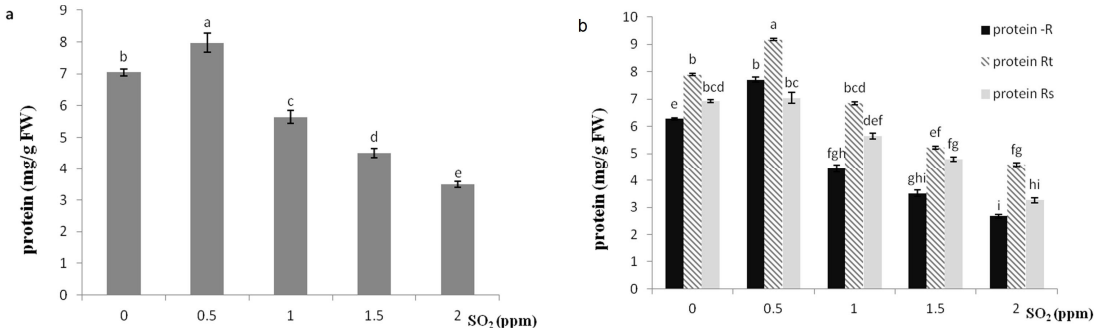


**Figure 1.** Effects of different concentrations of $SO_2$ (a) and interaction between different $SO_2$ and bacterial inoculation [without inoculation (–R); inoculation with native (Rt), and standard *Rhizobium* (Rs)] (b) on total protein content of 41 days old Persian clover. Means followed by different letters are significantly different as determined by Duncan's test.





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proline content indicated no significant change in 0 and 0.5 ppm of $SO_2$. Increasing $SO_2$ stress caused an increase in proline content. The largest increase in proline content compared to control plants was observed in plants exposed to 2 ppm. The increase in proline content in 1, 1.5 and 2 ppm was 1.42, 2.14 and 2.93-fold compared to controls respectively (Figure 2-a). Interaction between bacterial inoculation and $SO_2$ on free proline content was statistically significant (Table 2). The highest level of proline content was obtained from non-inoculated plants exposed to 2 ppm of $SO_2$ (Figure 2-b).

## Effects of Bacterial Inoculation and $SO_2$ Pollution on Sulfur Content

The results showed that *Rhizobium* inoculation had no significant effect on total sulfur content (Table 2). Effects of $SO_2$ on total sulfur content of Persian clover were statistically significant (Table 2). Sulfur content indicated no significant change in 0 and 0.5 ppm of $SO_2$. Sulfur content increased with increasing $SO_2$ concentrations (Figure 3-a). Interaction between bacterial inoculation and $SO_2$ on total sulfur content was statistically significant (Table 2). The highest level of sulfur content was obtained from non-inoculated plants exposed to 2 ppm of $SO_2$ (Figure 3-b).

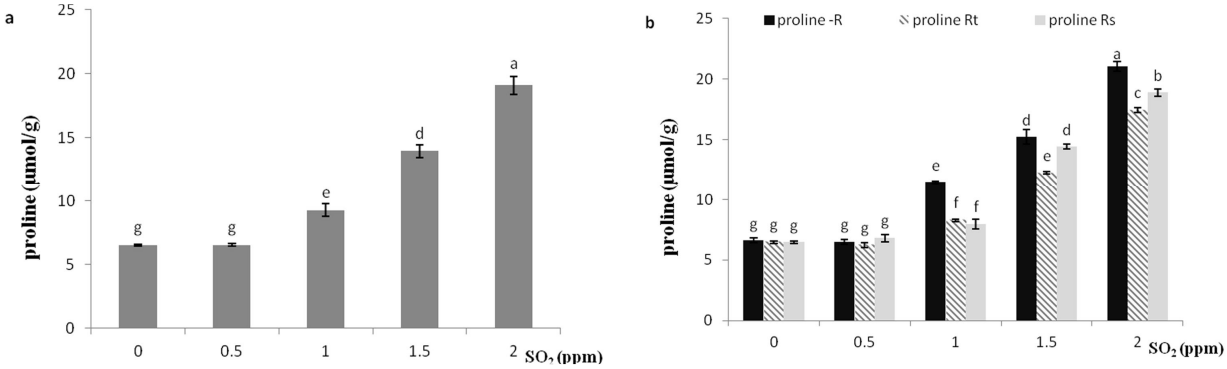


**Figure 2.** Effects of different concentrations of $SO_2$ (a) and interaction between $SO_2$ and bacterial inoculation [without inoculation –R; inoculation with native (Rt), and standard *Rhizobium* (Rs)] (b) on free proline content of 41 days old Persian clover. Means followed by different letters are significantly different as determined by Duncan's test.

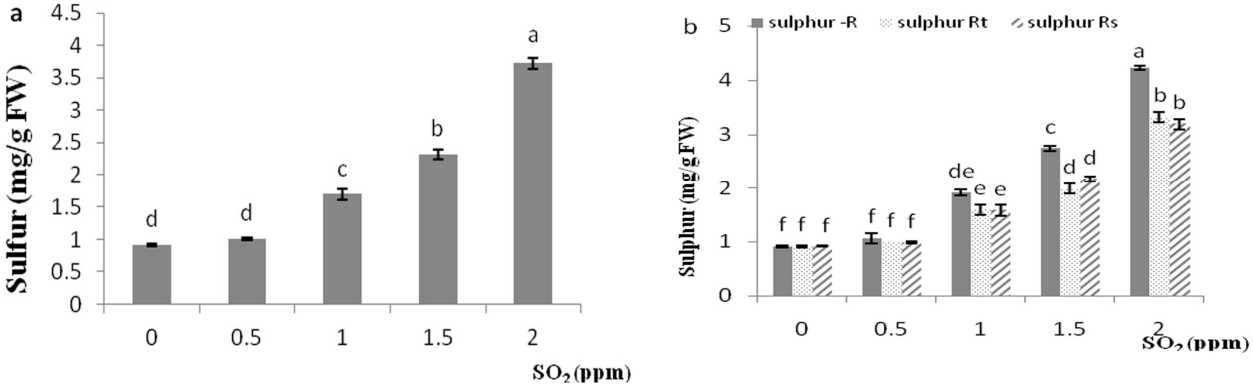


**Figure 3**. Effects of different concentrations of $SO_2$ (a) and interaction between $SO_2$ and bacterial inoculation [without inoculation –R; inoculation with native (Rt), and standard *Rhizobium* (Rs)] (b) on total sulfur content of 41 days old Persian clover. Means followed by different letters are significantly different as determined by Duncan's test.





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## DISCUSSION

Plant growth promotion by rhizobacteria can occur directly and indirectly. There are several ways by which plant growth promoting bacteria 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 that enhance plant growth at various stages of development. Indirect growth promotion occurs when PGPR promotes plant growth by improving growth restricting conditions. This can happen directly by producing antagonistic substances, or indirectly by inducing resistance to pathogens (Timmusk, 2003). Nitrogen is a major component of the chlorophyll, amino acids, energy-transfer compounds, and it is a significant component of nucleic acids such as DNA. In symbiosis between plant and bacteria, rhizobial bacteria are able to reduce atmospheric $N_2$ into ammonia by the nitrogenase enzyme. The ammonia as the NH4+ ion is assimilated into glutamate and transported predominantly as the amino acids asparagine and glutamine by amide-exporters, or as the ureidesallantoin and allantoic acid via the ureide-expor, and exchanged for photosynthates from the host plants (Saliou-Sarr *et al*., 2015). If the absorbable form of nitrogen (nitrate) for plants is available for roots (for example by Hoagland nutrient solution), the plant has no need to establish a symbiosis with rhizobia. Therefore symbiosis should be encouraged with *Rhizobium* and the effect of *Rhizobium* on plants should be observed, also N2 should be removed of nutrient solution.

In this study, leaf area, leaf number, shoot height, root length, shoot fresh and dry weight and total protein content of Persian clover increased significantly in inoculation with *Rhizobium*. All these indexes showed a greater increase in inoculation with native *Rhizobium* compared with standard *Rhizobium*. Inoculation alfalfa with *Rhizobium* resulted in significant increase in shoot dry weight, total dry weight and leaf area of plants compared to control plants (Askari and Hosseinkhani, 2012). Keneni *et al*. (2010) studied the characterization of acid and salt tolerant rhizobial strains isolated from faba bean. In this study, the native *rhizobial* strains isolated from the plants of Northern Ethiopia tolerated a higher salt concentration (5% NaCl) than the exotic *rhizobial* strains. Both native and exotic strains failed to grow at pH 4 and 4.5 levels in the laboratory conditions. In the soil adjusted to pH 4-7, all the native *rhizobial* strains persisted while those of the exotic strains failed to survive at pHs below 5.5. They reported that native rhizobial strains have been found to be superior to the exotic strains in stimulating growth, dry matter yield, nodulation and nodule wet weight of faba bean.

Increase of protein content following *Rhizobium* inoculation was observed in *Cicer arietinum* (Aslam *et al.*, 2010). In this study, proline and sulfur contents didn't change with *Rhizobium* inoculation. Proline accumulates in many plant species in response to environmental stress (Szabados and Savoure´, 2009). So *Rhizobium* inoculation did not cause stress conditions for the plant, so the plant is normal and changes do not occur in the proline content.

Damage to plants is an important consequence of atmospheric $SO_2$. The phytotoxicity of $SO_2$ depends on its concentration and on the duration of exposure and is influenced by sulfur status in plants. A low dose of sulfur can even be useful to plants since sulfur is important for plants and can help to alleviate other nutrient deficiencies in plants, such as that of Fe. Sulfur is also a structural component of amino acids, proteins, vitamins and chlorophyll. Sulfur enhances the development of nodules and nitrogen fixation, and also affects carbohydrate metabolism. However, high doses can be harmful (Muneer *et al*., 2014). Gaseous pollutants, particularly $SO_2$, enter plants





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through the stomata by the process of photosynthesis and respiration. $SO_2$ reacts with water on the cell walls inside leaves; by transfer and assimilation, the resulting sulfate, react with other compounds and are transported to various parts of plants. If plants are exposed to air pollutants for a long time or the pollutant concentrations exceed a critical threshold, plants may be injured. Plant injury is usually cumulative in nature, reducing growth and yield and accelerating senescence (Zhang *et al.*, 2013). In this study, shoot fresh and dry weight, shoot height and root length of Persian clover increased in 0.5 ppm of $SO_2$ but decreased in 1, 1.5 and 2 ppm. $SO_2$ can cause positive effects on physiological and growth characteristics of plants at very low concentrations, especially in sulfur-deficient soils. Mostly, however, increased uptake of $SO_2$ causes toxicity and reduced growth and productivity (Swanepoel *et al.*, 2007). Shoot fresh and dry weight, shoot height and root length of *Calendula officinalis* decreased under 1, 1.5 and 2 ppm concentrations of $SO_2$ (Wali *et al.*, 2007). In this study, leaf number increased in 0.5 ppm of $SO_2$ but indicated no significant change in higher concentrations of $SO_2$. The number of leaves on a plant is relatively responsive to changes in the external environment. It may increase in response to $SO_2$ to counteract a reduced photosynthetic efficiency (Murray, 1985). Leaf number of *Calendula officinalis* increased in 0.5 ppm of $SO_2$ but decreased in 1 and 2 ppm of $SO_2$ (Wali *et al.*, 2007). Nayak *et al.* (2015) studied the effect of air pollution (including $SO_2$) on five different plant species i.e., *Tectona grandis*, *Saroca asoca*, *Terminalia catapa*, *Sizygium cumini* and *Cassia fistula*. They reported that the growth indexes such as plant height, diameter, number of leaves per plants and leaf area of all studied species decreased significantly under air pollution.

In this study, free proline content indicated no significant change in 0 and 0.5 ppm but increased in 1, 1.5 and 2 ppm concentrations of $SO_2$. In many plants, free proline accumulates in response to the imposition of a wide range of biotic and abiotic stresses. As a compatible molecule in cell, proline possesses the ability to mediate osmotic adjustment, stabilize subcellular structures and scavenge free radicals. Besides, proline accumulation may reduce stress-induced cellular acidification or prime oxidative respiration to provide energy for recovery (Tan *et al.*, 2008). Proline content increased in *Populus Robusta* exposed to $SO_2$ pollution (Karolewski, 1989).

In this study, protein content showed a significant increase in 0.5 ppm concentration of $SO_2$ but decreased significantly in high concentrations of $SO_2$ (1, 1.5 and 2 ppm). Stimulation of sulfur-containing amino acids synthesis by $SO_2$ causes increase in protein content in low concentrations of $SO_2$ (Sardi, 1981). Such a reduction in the protein content of $SO_2$-treated leaves might have resulted from decreased photosynthesis or inhibition of protein synthesis, or enhanced protein degradation (Singh *et al.*, 1985). Sardi (1981) reported that exposure to 150 µg m-3 of $SO_2$ increased the protein content of both soybean and pea by stimulation of the synthesis of amino acids containing sulfur, but exposure to 500 or 1,000 µg m-3 of $SO_2$ had inhibitory effects on protein synthesis. Ibrahim and Almohisen (2014) investigated the effect of air pollution ($SO_2$, $NO_2$ and $O_3$) on four legume species (*Pisum sativum*, *Vicia faba*, *Glycine max* and *Vigna sinensis*). The results demonstrated that free amino acids and proline contents gradually increased and total protein content





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declined in the plant's leaves as pollutants increased.

In this study, total sulfur content indicated no significant change in 0 and 0.5 ppm concentrations of $SO_2$ but increased in higher concentrations. Sulfur is one of the essential elements plants require for growth and reproduction. Sulfur is essential for organic molecules synthesis, including amino acids such as cysteine and methionine, which are then incorporated into proteins (Rennenberg and Herschbach, 1996). Total sulfur content increased in *Trifolium repens* (Murray, 1985) and *Vicia faba* (Agrawal *et al.*, 1985) under $SO_2$ treatment.

In this study, interaction between inoculation and $SO_2$ treatment showed significant effect on shoot height, root length, shoot fresh and dry weight, total protein, free proline and total sulfur content. *Rhizobium* can increase plant resistance to biotic and abiotic stress factors by production of the phytohormones, enzymes, nitric oxide, osmolytes, organic acids and antibiotics (Dimkpa *et al.*, 2009). Inoculation *Phaseolus vulgaris* under osmotic stress with *Rhizobium tropici* and *Rhizobium gallicum* increased plant tolerance to the stress. The results showed better tolerance of *Phaseolus vulgaris* to osmotic stress when inoculated with the native *R. gallicum* (Sassi-aydi *et al.*, 2012).

## CONCLUSIONS

Stress resistance in plants has been related to better growth of plants. Low concentration of $SO_2$ (0.5 ppm) doesn't create stress conditions in Persian clover therefore proline content doesn't alter in this concentration. This concentration has a positive effect on growth indexes, protein and sulfur contents. In higher concentrations of $SO_2$ (1, 1.5 and 2 ppm), proline content increases but growth indexes, protein and sulfur contents decrease with increasing stress intensity. *Rhizobium* inoculation of Persian clover significantly reduced the negative effects of high concentrations of $SO_2$ on growth indexes, protein, sulfur and proline content.

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## برخی از پاسخ‌های شبدر ایرانی تلقیح‌یافته با ریزوبیوم به آلودگی $SO_2$

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## چکیده

دی‌اکسیدگوگرد یکی از آلاینده‌های مضر و معمول هوا است. غلظت‌های بالای $SO_2$ می‌تواند سبب ایجاد تنش و محدودیت رشد در گیاهان شود. بعضی از گیاهان با برقراری همزیستی با باکتری‌هایی مثل ریزوبیوم در برابر تنش مقاومت می‌کنند. ریزوبیوم یک باکتری مفید است که رشد و محصول گیاه را افزایش می‌دهد. برای مطالعه اثرات آلودگی $SO_2$ روی شاخص‌های رشدی و محتوای پروتئین، پرولین و گوگرد گیاه *Trifolium resupinatum* (شبدر ایرانی) تلقیح‌یافته با ریزوبیوم بومی و استاندارد، گیاهان ۳۱ روزه در معرض غلظت‌های مختلف $SO_2$ (۰ به عنوان شاهد، ۰/۵، ۱، ۱/۵ و ۲ ppm) برای ۵





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روز متوالی قرار گفتند. نتایج نشان داد که تلقیح باکتریایی، سطح و تعداد برگ، طول بخش هوایی، عمق ریشه، وزن تر و خشک بخش هوایی و محتوای پروتئین شبدر ایرانی را افزایش داد اما هیچ تاثیر معنی‌داری بر محتوای پرولین و گوگرد نشان نداد. غلظت‌های مختلف $SO_2$ اثر معنی‌داری را بر تعداد برگ، طول بخش هوایی، عمق ریشه، وزن تر و خشک بخش هوایی، محتوای پروتئین، پرولین و گوگرد داشت ولی بر سطح برگ اثری نداشت. غلظت ppm ۰/۵ $SO_2$ شاخص‌های رشد و محتوای پروتئین را افزایش داد. محتوای پرولین و گوگرد در غلظت ppm ۵/ تغییری نکرد. افزایش غلظت $SO_2$ شاخص‌های رشد و محتوای پروتئین را کاهش و پرولین و گوگرد را افزایش داد. برهمکنش بین تلقیح باکتریایی و تیمار $SO_2$ اثرات تنش غلظت‌های بالای $SO_2$ را بر شاخص‌های رشد، پروتئین، پرولین و گوگرد بهبود بخشید. بنابراین ریزوبیوم می‌تواند تحمل و مقاومت این گیاه به تنش‌های غیرزیستی مثل آلودگی $SO_2$ را افزایش دهد.





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