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Induction of arsenic stress tolerance in *Capsicum annum* through *Trichoderma harzianum* sand mix method

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Abstract

The research was performed at Nusrat Jahan College Rabwah Pakistan to overcome arsenic stress in two verities "Sanam and Ghotki" of *Capsicum annum* through *Trichoderma harzianum* sand mix method. Seeds of mentioned chili varities and *Trichoderma* fungus were taken from National Agricultural Research Centre (NARC), Pakistan. Cups of sand were mixed with *Trichoderma harzianum* at the rate of 2×10^7 CFU. Chili seeds after surface sterilization through mercuric chloride were sown in cups of fungal treated sand. Arsenic oxide (AsO 1: 1mg/L and AsO 2: 2mg/L) stress was applied after one week of sowing. Seedlings were harvested after 30 days of sowing and were preserved in 50mM potassium phosphate buffer. Roots, shoots and leaves were separately preserved. The preserved samples were subjected to different biochemical tests. This study has revealed *Trichoderma* sand mix method very effective method to eliminate Arsenic oxide stress by generating ROS damaging proteins.

Key words: Green chilies, arsenic, *Trichoderma harzianum*, seedlings, sand mix.

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1. Introduction

Arsenic is one of the most toxic heavy metal. It mostly exists in conjugation with various minerals. It is also present in pure crystalline forms. Arsenic pollution is result of many different types of anthropogenic activities as well as natural phenomena. Increasing arsenic pollution is constantly contaminating water supplies and soils biochemistry. As a result, crops like rice, wheat, beans, turmeric, chillies, onions, carrots, reddish and many more have been reported to reserve arsenic contents in their edible regions [1]. Large concentrations of arsenic in any plant can hinder normal growth mechanism, ceases seed germination, and retard increase in the plant heights. It also reduces the rate of flowering and fruiting as well as number of grains [2]. Arsenic present in plants disturbs various metabolic activities in the cells such as associations with sulphohydryl groups and additions, deletions of phosphate groups from Adenosine triphosphate (ATP). Arsenic develops free radicals and Reactive oxygen species (ROS) that cause. oxidative damages [3]. In order to overcome such adverse effects of arsenic stress, healthy preventive or treating measures are being worked out on many scientific platforms but among all measures, biological means of controlling arsenic pollution are important as there are no threats of chemical contaminations or side effects. *Trichoderma harzianum* is soil inhabitant ascomycete fungus, that forms dirty green colored colonies. Many researches have shown that this fungus act as growth promoting agent in different plants [4]. Many species of these fungi are known for eliminating different biotic as well as abiotic diseases of plants. Capsicums are one of the major components of humans' routine diet. These provide flavor to food also many vitamins and phenolic compounds that work as antioxidants. Concentrations of antioxidants are dependent on multiple factors such as age, growing scenarios and variations in genotypes and varieties [5].

The present research work has been planned considering edible importance of chilies which are being contaminated with alarming levels of arsenic. The reduction of arsenic contamination to chilies was studied using *Trichoderma harzianum* sand mix method.

2. Materials and methods

The present study was conducted at Department of Botany, Nusrat Jahan College Rabwah, Pakistan. The experimental design was to induce stress tolerance in chillies of two varities "SANAM" and "GHOTKI" taken from NARC PAKISTAN against arsenic oxide (1 mg/l, 2 mg/l) through *Trichoderma harzianum* sand mix method. This fungus was also taken from NARC. Cups of sand were mixed with *Trichoderma harzianum* at the rate of 2×10^7 CFU. Chili seeds of both varieties after surface sterilization through mercuric chloride were sown in cups of fungal treated sand. Arsenic oxide (AsO 1: 1 mg/l and AsO 2: 2 mg/l) stress was applied after one week of sowing. Seedlings were harvested after 30 days of sowing and were preserved in 50 mM potassium phosphate buffer. Roots, shoots, and leaves were separately grinded and then centrifuged at 14000 rpm for 15 minutes. After centrifugation supernatants were subjected to different biochemical tests.

2.1. Total soluble proteins

Concentration of total soluble proteins was estimated using the Bradford [6] with few amendments. The 1 ml supernatant was reacted with 2 ml Bradford Reagent and incubated for 15-20 min. The reading was measured at 595 nm.

2.2. Ascorbate peroxidase activity (APX)

The APX working was measured using the method of Asada and Takahashi [7]. The reaction solution (1600µl) was comprised of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 400 µl of enzyme extract. The absorbance was taken at 290 nm against the blank and the enzyme activity was represented in Umg^{-1} protein (U=change in 0.1 absorbance min-1 mg^{-1} protein).

2.3. Total phenolics contents

Total phenolics were evaluated with the help of Folin-Ciocalteu protocol (Wolfe *et al*. 2003) [8] with few amendments. The samples were mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were shaken for fifteen seconds and were permitted to stand for 30 min at 40 ˚C to develop color. Then absorbance was taken at 765 nm using a spectrophotometer.

2.4. MDA contents

Malondialdehyde (MDA) was determined in accordance to method proposed by Dhindsa *et al.* [9]. In the 2 ml TCA, added 2 ml of 0.6 % thiobarbituric acid. It was heated at 100 degree centigrade for 20 minutes in water bath. After heating immediately cooled for 20 minutes and then centrifuged at 10000 rpm for 10 minutes. The resulting color was measured at 532 nm using a spectrophotometer.

2.5. Hydrogen peroxide concentration

H_2O_2 concentration was determined according to the protocol of Velikova *et al*. [10]. The 0.1 ml of supernatant was added to 0.1 ml of 10 Mm potassium phosphate buffer (pH 7.0) and 1M IKI. The absorbance was measured at 390 nm.

3. Results and discussions

3.1. Total soluble proteins

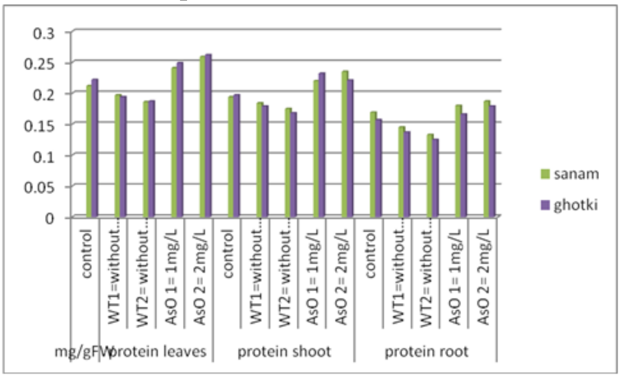

Figure 1: Mean values of protein concentrations of chillies at different treatments

According to Figure 1, protein concentration enhanced many folds in both varieties of chillies, in leaves, shoots and roots at 1 mg/L and 2 mg/L of arsenic oxide stress. This increase in protein amount was because of the fact that *Trichoderma* application has helped chilies in overcoming both stress levels of arsenic oxide. WT (WT = without *Trichoderma*) group was found unable to resisit toarsenic stress and as a result protein contents decreased as compared to control.

3.2. Ascorbate Peroxidase Activity (APX)

The results of Figure 2 show that in all parts of sanam and ghotki chillies of *Trichoderma*, APX activity was increased at both levels of arsenic stress as compared to control group. On the other hand WT set showed decline in APX activity because in this group *Trichodrma* was not mixed in sand thus this group was unable to raise APX activity to reduce stress levels.

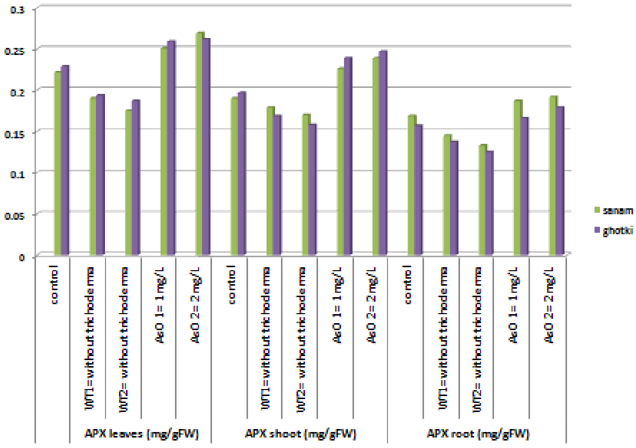

Figure 2: Mean values of APX activity of chillies after different treatments.

3.3. Total phenolic contents

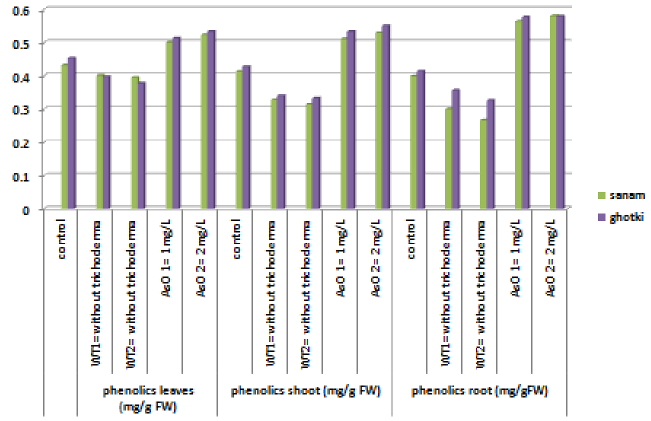

Figure 3: Mean values of total phenolics content of chillies at different treatments

At both stress levels of arsenic, *Trichoderma* sand mixed group showed increase in phenolics contents as compared to WT group and control group. All parts, roots, leaves and shoots of sand mixed group showed enhancement in phenolics contents.

3.4 MDA contents

Table 1: Mean values of MDA content in roots, shoots and leaves of green chillies

Unit: umol/gFW	SANAM	GHOTKI
Control Leaf	0.139	0.143
WT1 = without trichoderma	0.145	0.156
WT2 = without Trichoderma	0.152	0.162
AsO 1 = 1mg/L	0.126	0.132
AsO 2 = 2mg/L	0.118	0.126
Control Shoot	0.132	0.127
WT1= without trichoderma	0.142	0.167
WT2 = without trichoderma	0.165	0.172
AsO 1 = 1mg/L	0.125	0.124
AsO 2 = 2mg/L	0.119	0.106
Control Root	0.104	0.121
WT1= without trichoderma	0.139	0.147
WT2 = without trichoderma	0.147	0.159
AsO 1 = 1mg/L	0.109	0.112
AsO 2 = 2mg/L	0.096	0.103

According to Table 1 *Trichoderma* sand mixed group of chillies showed decline in MDA contents as compared to non-treated group in all parts of seedlings of both verities, proving that this fungus application prevents chillies from oxidative damage.

3.5 Hydrogen peroxide concentration

Table 2: Mean values of H_2O_2 concentration in roots, shoots and leaves of green chillies

Unit: umol/gFW	SANAM	GHOTKI
Control Leaf	0.121	0.104
WT1= without Trichoderma	0.147	0.139
WT2 = without Trichoderma	0.159	0.147
AsO 1 = 1mg/L	0.112	0.109
AsO 2 = 2mg/L	0.103	0.096
Control Shoot	0.127	0.132
WT1= without Trichoderma	0.167	0.142
WT2 = without Trichoderma	0.172	0.165
AsO 1 = 1mg/L	0.124	0.125
AsO 2 = 2mg/L	0.106	0.119
Control Root	0.143	0.139
WT1= without trichoderma	0.156	0.145
WT2 = without trichoderma	0.162	0.152
AsO 1 = 1mg/L	0.132	0.126
AsO 2 = 2mg/L	0.126	0.118

According to Table 2, hydrogen peroxide concentration was reduced at both levels of stress in *Trichoderma* sand mix group as compared to WT group in all parts of seedlings of both varieties of chillies.

Output of this study reveals that *Trichoderma harzianum* fungus is resistant to arsenic oxide contamination at levels of 1 mg/L and 2 mg/L. Being itself resistant to this heavy metal contamination, this fungus has effectively combated arsenic oxide stress in both Ghotki and Sanam varieties of green chilies. *Trichoderma harzianum* sand mix method has proven to be very positive and effective biological treatment of preventing green chilies and other plants from harms of arsenic oxide. Increase in protein content of *Trichoderma* sand mix chilies even under stress of arsenic and reduction in protein concentrations of chilies without *Trichoderma* clearly indicated that this fungus has triggered auxins and other hormones which promote healthy growth and biomass of chilies along with developing tolerance against arsenic stress. *Trichoderma harzianum* has activated defense mechanism against arsenic stress by raising levels of phenolic compounds and peroxidases as is evident by results of total phenolics contents and ascorbate peroxidase activity. These phenolics compounds and peroxidases act as ROS scavenging substances because they trigger different ROS deteriorating proteins such as glutathione – S-transferase, etc. Thus, due to activation of ROS damaging species MDA content and hydrogen peroxide concentration is reduced as a result all *Trichoderma* treated chilies remained unaffected from oxidative damages.

4. Conclusions

Following important conclusions can be withdrawn from the present study:

- *Trichoderma harzianum* fungus is resistant to arsenic oxide contamination.
- Being itself resistant to this heavy metal contamination, this fungus has effectively combated arsenic oxide stress in both Ghotki and Sanam varieties of green chilies.

- *Trichoderma harzianum* sand mix method has proven to be very positive and effective biological treatment of preventing green chilies

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