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Arsenic induced eco-physiological changes in Chickpea (*Cicer arietinum)* and protection by gypsum, a source of sulphur and calcium

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ABSTRACT

World food security is threatened by low production of food grains due to adverse impacts of abiotic stressors on plant health. Metalloids, including arsenic (As), interfere with plant growth and metabolism. In recent past, As has emerged as a severe threat to animal and plant health due to its rapidly increasing concentration in the environment. The influence of gypsum (calcium sulphate, $CaSO_4 \cdot 2H_2O$), a sulphur (23.3%) and calcium (18.6%) source, on As-induced stress was studied in chickpea gram (*Cicer arietinum*). Plants were raised in pots containing 150 g Soilrite™ with following combinations of 1% (w/w) gypsum and As (50 ppm): Control, Arsenic (As), 1% gypsum and 1% gypsum + 50 ppm As. Fifteen-days-old plants were subjected to treatments and several physiochemical parameters were studied at two different time intervals (10 and 20 days after treatment, DAT). Arsenic was found to increase oxidative stress, lesser in presence of gypsum. Arsenic treatment decreased activities of SOD, GR, APX and CAT but an increase was noted in the presence of gypsum, except CAT. Gypsum greatly helped plants in acquiring higher levels of proline, ascorbate, glutathione, non-protein thiols and phytochelatins. More importantly, gypsum helped in maintenance of cellular redox during As stress. Biomass accumulation was also improved in presence of gypsum. Hence, it is clear that sulphur and calcium source (gypsum) might play either an indirect role (reducing plant As uptake) or a direct role (by strengthening the cellular defence) that might be through upregulation of S-defence pathway (glutathione, phytochelatins and non-protein thiols) for protection against As.

1. Introduction

Sulphur (S) is an essential macro-nutrient (Lee and Korban, 2002) required for proper plant growth and good yield (Randazzo, 2009). Sulphur is necessary for important cellular functions including disulphide bond formation, heavy metal and arsenic detoxification (Mallick et al., 2013), iron-sulphur (Fe-S) cluster-mediated electron transport, metabolism of secondary products (Saito, 2000), vitamin co-factors (Hell and Hillebrand, 2001) and redox cycle. Sulphur increases availability of other essential plant nutrients including nitrogen and phosphorus and help in better yields (Salvagiotti et al., 2009).

The deficiency of sulphur in agricultural soil is observed all over the world (Haneklaus et al., 2007). This deficiency of sulphur in soil is accounted for causing stress in plants and decreasing the quality and quantity of plant product. Therefore, sulphur deficient soils require sulphur fertilizers to overcome different kinds of stresses including heavy metals. The efficiency of sulphur transporter might be other factor that decides the S-use efficiency of the plants (Takahashi et al., 1997). Gypsum [$Ca(SO_4) \cdot 2H_2O$] is an important source of sulphur (23.28%) and calcium (18.62%), widely used in agricultural lands. Toxic heavy metals affects metabolism of plants; and a simultaneous S deficiency might result in poor yield and low resistance against abiotic stresses. However, optimal sulphur level helps plant in modulation of proteome and synthesis of S-rich compounds helping in detoxification (Dixit et al., 2015). On the other hand, calcium (Ca) is an essential macronutrient which plays multiple roles in plant growth, development and signalling

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besides mitigating arsenic toxicity in rice seedlings (Rahman et al., 2016).

Arsenic (As), a non-essential heavy metal, is toxic even at relatively low concentrations because it is readily absorbed and translocated rapidly in plants. Plant growth and physiology is disturbed due to arsenic toxicity (Gusman et al., 2013). This toxicity leads to the over-production of reactive oxygen species (ROS) (Gupta et al., 2013). So produced ROS are responsible for damage to cell membrane, proteins, DNA replication and repair. The major ill-effect of ROS toxicity is the alteration of chlorophyll synthesis (Bashir et al., 2015) which results in slow rate of photosynthesis and harvesting chl a/chl b protein complex II (Qureshi et al., 2010), and interference with rubisco activation. Decreased rate of photosynthesis leads to poor crop yields and it has been observed that As inhibits photosynthesis (Song et al., 2013). Arsenic has also shown effects on enzyme catalysis, water balance and carbohydrate and nitrogen metabolism (Finnegan and Chen, 2012).

Arsenic activates the sulphur assimilation pathway responsible for the synthesis of cysteine, a precursor of glutathione (GSH) biosynthesis. GSH (a non-protein thiol) acts as an important antioxidant in reducing As-induced stress (Noctor et al., 2011; Rai et al., 2011). GSH also helps in phytochelatin (PC) synthesis, which has a major role in As detoxification (Kumar et al., 2015). Overexpression of PC synthase in *Arabidopsis thaliana* has also shown enhanced As tolerance (Li et al., 2004).

Plants are provided with an efficient mechanism for protection against ROS and peroxidation reactions. The majority of ROS-scavenging mechanisms in plants involve superoxide dismutase (SOD), which is found in almost all cellular compartments, the water–water cycle in chloroplasts, and the ascorbic acid (AsA)-glutathione (GSH) cycle in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes, in which APX and GR play crucial roles. Catalase (CAT) removes H_2O_2 in peroxisomes. Since S is required for amino acid and protein synthesis, S-treated plants have better anti-oxidative response and tolerance (Bashir et al., 2013). As alters sulphate assimilation pathways and antioxidant system in plants (Rai et al., 2011) which suggests the existence of a general adaptive response to an increase in cellular demand for reduced S operating through PCs, non-protein thiols and antioxidant enzyme activity (Dixit et al., 2015). Therefore, availability of S in plants plays a crucial role for protection against As. Specifically in case of gypsum, it has also been shown that it incorporate arsenic (Zhang et al., 2015) which in turn may reduce the availability of arsenic to the plants.

Cicer arietinum L. (Chickpea) is a legume of Fabaceae family and its seeds are highly rich in protein. In present study, we examine the effects of arsenic, and protection by gypsum (a sulphur and calcium source) against As-induced oxidative stress, changes in cellular antioxidant activity, contents of non-protein thiols, phytochelatins and photosynthetic pigments, and growth parameters in *Cicer arietinum*. This might be first report on such aspects of cellular and growth parameters showing protective role of gypsum against arsenic.

2. Materials and methods

2.1. Experimental design

Seeds of Chickpea (*Cicer arietinum)* were treated with 1% (v/v) sodium hypochlorite solution for 10 min followed by thorough washing in de-ionized water. The sandy soil containing organic manure was divided into four sets with final treatment as follows: i. Control, ii. Arsenic (50 ppm $NaAsO_2$, sodium arsenite), iii. Gypsum (1% w/w of soil) and iv. Gypsum + Arsenic. Chickpea seeds were allowed to germinate in moist soil for 5 days and seedlings were further grown in a growth chamber with following conditions: 16/8 h light/dark period under photon flux density of 150 ± 10 μmol photons m^{-2} s^{-1}, 25/20 °C day/night temperatures and 80% relative humidity. After a period of 10 and 20 days of plant growth, leaves were harvested and used immediately for biochemical parameter, leaf area, and fresh and dry weight analysis. Values for each biochemical and antioxidant parameter were calculated on dry weight (g^{-1} DW) basis.

2.2. Thiobarbituric acid reactive substances (TBARS)

The content of thiobarbituric acid reactive substances (TBARS) was estimated to measure the magnitude of oxidative stress (Heath and Packer, 1968). 1 g fresh leaves were ground in liquid nitrogen and mixed with 10 ml of 1% (w/v) TCA (tri-chloroacetic acid). Leaf paste was centrifuged at 9660*g* for 15 min. Supernatant (0.2 ml) was added with 0.8 ml 0.5% (w/v) thio-barbituric acid (TBA), vortex-mixed and heated at 99 °C for 30 min. The mixture was quickly cooled in an ice bath. Final volume was adjusted to final volume of 1 ml followed by a spin at 2817*g* for 10 min and absorbance of the supernatant was recorded at 532 nm and 600 nm. TBARS content was expressed in nmol g^{-1} dry weight (DW).

2.3. Superoxide dismutase (SOD) assay

Method of Dhindsa et al. (1981) was used for assaying SOD activity which depends on inhibition of photochemical reduction of NBT (nitroblue tetrazolium) by SOD. Assay mixture contained 1.5 ml of 0.1 M sodium phosphate buffer, pH 7.5, 13 mM of l-Methionine, 1% (w/v) PVP (polyvenypyrrolidone), 100 μl of 1 M Na_2CO_3 with equal amount of 2.25 mM NBT solution, 3 mM EDTA, 60 μM riboflavin and enzyme extract. Resultant mixture was incubated at 28 °C under two fluorescent lamps on 15 W. At completion of reaction, reaction tubes were covered with black clothes and absorbance was recorded at 560 nm. Percent inhibition of colour by SOD was plotted as a function of the volume of enzyme. A 50% decline in photochemical reduction of NTB was considered as one unit of enzyme activity and expressed as EU mg^{-1} protein min^{-1}.

2.4. Ascorbate peroxidase (APX) assay

The ascorbate peroxidase (APX) extraction and assay were performed as mentioned in Qureshi et al. (2007). Leaf material (0.2 g) was homogenised in 2 ml of ice-cold 0.5 M phosphate buffer (pH 7.8) containing 1% (v/v) Triton-X 100, 100 mM EDTA, pH 7.8 and 1% (w/v) PVP. Homogenate was spun at 6708*g* at 4 °C for 15 min. APX activity was measured in the supernatant. The enzyme assay mixture containing potassium phosphate buffer (0.1 M, pH 7.4), 0.3% (v/v) H_2O_2, 0.5 mM ascorbate, and 100 μl enzyme was used to initiate the APX reaction at 25 °C for 5 min. Enzyme kinetics was performed and fall in ascorbate concentration was monitored at 290 nm. A unit of ascorbate peroxidase (EU) is defined as the amount of APX required to oxidize 1 μmol of ascorbate min^{-1} at 25 °C.

2.5. Glutathione reductase (GR) assay

Enzyme extract was prepared as mentioned for APX in previous section. Method of Anderson (1985) was used to perform glutathione reductase (GR) assay. Reaction mixture contained 0.1 M potassium phosphate buffer (ph 7.2), 20 μM oxidized glutathione (GSSG) and 0.2 mM NADPH. The reaction was started by adding 0.1 ml enzyme extract and absorbance was recorded at 340 nm for 5 min/25 °C using enzyme kinetics. A unit of GR activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of GSSG min^{-1} at 25 °C.