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Manganese induced changes in growth, chlorophyll content and antioxidants activity in seedlings of broad bean (*Vicia faba* L.)

Author Details

Shashi K. Arya (*Corresponding author*)
Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi - 221 005, India
e-mail: shashiarya03@gmail.com

B.K. Roy
Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi - 221 005, India

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Abstract

The effect of manganese(Mn) on broad bean (*Vicia faba* L.) was studied with regard to growth, Mn accumulation in root and shoot, chlorophyll, proline content and peroxidase activity. Seeds were treated with Mn (10, 20, 40, 80,120,160 μM) and grown hydroponically up to 15 days. Manganese level in both root and shoot increased progressively in response to increasing concentration and it was high in roots (13 fold) over the shoots (8 fold). The reductions in root (52%) and shoot (62.92%) development were evident for the maximum Mn concentration (160 μM). The chlorophyll amount gradually declined with increasing Mn concentrations and attained its maximum (42%) at 160 μM. By contrast, the guaiacol peroxidase activity was high (71%) along with the accompanying rise in proline content (75%) in shoots of the highest Mn concentration (160 μM). However, there was about 2 fold increase in total glutathione content at 40 μM than the basal level and further declined to 21.65 μg g^{-1} fresh wt. at 160 μM Mn. The alterations in overall reflected Mn concentration- dependent changes in the parameters studied. The results suggest that the plant *Vicia faba* L. copes with Mn exposure through enhanced production of antioxidants.

Key words

Vicia faba L., Manganese (Mn), Chlorophyll, Peroxidase, Proline, Oxidative stress

Introduction

Manganese (Mn) is essential micronutrients for the normal growth and development of plant and also serves as activator for enzymes involved in tricarboxylic acid cycle. However, Mn is toxic when in excess and consequently it represents an important factor in environment contamination and causes various phytotoxic effects (Pitman, 2005). Excess concentration of some heavy metals in soil are increasing due to the application of fertilizers, mining practices and sewage sludge that exert toxic effects on plants (Marschner, 1995). It is also involved in both oxygen radical productions as component of water splitting complex in PSII and oxygen radical detoxification via its role in SOD activity (Pitman, 2005). Higher accumulation of metal ions in plant interferes with activities of several enzymes essential for metabolic processes (Lindon and Teixeira, 2000). Manganese with particular to the higher concentrations showed the symptoms of toxicity such as appearance of brown spots, chlorosis and necrosis of leaves (McCain and Markley, 1989). It has been indicated that the degree of cell damage under heavy metal stress depends on the rate of ROS formation and on the efficiency and capacity of detoxification and repair mechanisms. It has been indicated that the degree of cell damage under heavy metal stress depends on the rate of ROS formation and on the efficiency and capacity of detoxification and repair mechanisms. Various reports documents that excess manganese (Mn) is responsible for oxidative burst with production of reactive oxygen species (ROS) like superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$) (Demirevska-Kepova *et al*., 2004; Boojar and Goodarzi, 2008). In these circumstances, cells are protected from reactive oxygen species by the combined action of enzymatic antioxidant systems like production of superoxide dismutases, catalases, peroxidases and synthesis of nonenzymatic antioxidants like ascorbate, glutathione, phenolic compounds and phytochelatins (Arora *et al*., 2002). Accumulation of proline is well documented in plant cells against various stress (Mishra and Agrawal, 2006). Proline is polyfunctional antioxidant that acts as osmotic adjustment, cryoprotectant, metal chelation and scavenging of $OH^\bullet$ (Wang and Bolen, 2006). Regardless of this, there is no conclusive evidence that proline accumulation really contributes to metal tolerance.

Similarly, the enhanced synthesis of peroxidase (POD) has been observed in plant as a general response to excess heavy metals and various stresses (Breda *et al*., 1993). The peroxidase enzyme is reported in wide range of biological functions such as indole acetic acid (IAA) catabolism, pathogen defense and scavenging of toxic peroxides by oxidation (Demirevska-Kepova *et al*., 2004). Glutathione (γ-Glu–Cys–Gly) function as a cellular protectant and signalling molecule for stress and also the major reservoir of non-protein reduced sulfur in plants (Foyer and Noctor, 2001). Both glutathione and phytochelatins (polymers of γ-Glu–Cys) chelate heavy metals such as cadmium, copper, manganese, arsenic facilitating their sequestration in the vacuole (Cobbett, 2000) and play defensive role in stresses. It has been illustrated that reduced glutathione (GSH) directly quenches ROS species and considered as an important non-enzymatic antioxidant (Alscher, 1989). It has been evidenced that GSH serve as a precursor for phytochelatins (PCs) synthesis under heavy metal stress (Nagalakshmi and Prasad, 2001; Pawlik- Skowronska *et al*., 2007).

Mature seeds of faba bean are a good sources of protein (about 25% in dried seeds) and also having starch, cellulose, vitamin C and minerals . Therefore, it has an increasing importance for human and animal diet. Due to high yield, smaller seeds, less anti-nutritional factors and high adaptation ability to modem agriculture has made this plant more attractive for farmers, feed and food manufactures industries (Duc, 1997).

In the present communication, we have planned to investigate the accumulation of Mn in root and shoot and to know the effect of different Mn concentrations on growth rate, chlorophyll content and enzymatic (guaiacol peroxidase) and non- enzymatic (proline and glutathione) antioxidants in *V. faba* seedlings.

Materials and Methods

Plant material and treatment: Seeds of *V. faba* L. (Fabaceae) obtained from Institute of Agricultural Sciences, BHU, Varanasi, India were pre- soaked overnight and germinated on moist filter paper. Seedlings were transplanted into plastic pots containing 3 kg acid washed sand saturated with 250 ml of nutrient solution. The composition of nutrient medium used as described by Peters and Mayne (1974). Manganese was supplied as manganese sulphate ($MnSO_4$) with a common range (10, 20, 40, 80, 120, 160 mM). Seedlings (six treatments in three replicates and control) were placed in growth chamber at 25°C temperature, 75±5 relative humidity, a photon flux density of 270 m mol $m^{-1}s^{-1}$ and a 16 hr photoperiod. The pH of the nutrient solution was adjusted to 5.5± 0.2. Fifteen days old Mn treated and control plants were used for the study of growth and biochemical analysis because upto this period seedlings attained full growth (Keltjensa and van Beusichema, 1998).

Determination of Mn and relative growth: Plants were harvested and separated into shoots and roots and soaked in 0.2% ethylenediaminetetra acetic acid (EDTA) for 2 hr and dried in oven at 80°C for 24 hr. One gram dried samples were wet digested in HNO_3: $HClO_4$ (5:2 v/v) and Mn content determined by atomic absorption spectrophotometer (PE-100 Perkin Elmer Boston USA). Relative root and shoot growth were determined according to the method of Ouzounidou *et al*. (1992).

Chlorophyll estimation: Extraction and estimation of chlorophyll was done in leaf according to the method of Arnon (1949). Fresh leaf samples (250 mg) of treated and control plants were immersed in 20 ml of 80% acetone and stored overnight. Next day, samples were crushed by mortar and pestle and centrifuged at 5000 rpm for 10 min. The volume of the supernatant was maintained up to 25 ml with 80% acetone. Absorbance was taken at 663 and 645 nm using UV- VIS 117 Systronics spectrophotometer and total chlorophyll amount was calculated according to Witham *et al*. (1971).

Guaiacol peroxidase assay: Guaiacol peroxidase (GPOD) activity was assayed in shoot according to the method of Zhang and Kirkham (1994) with some modification. One gram shoot of treated and control plants were homogenized in 8 ml of 0.05 M phosphate buffer (pH 7.0). The homogenates were centrifuged at 15'000X g for 20 min at 4°C and supernatant was used for the assay of GPOD activity. The enzyme activity was assayed in 60 mM l^{-1} posphate buffer (pH 6.1) mixed with 28 μM l^{-1} guaiacol and 5 μM l^{-1} H_2O_2. Oxidation of guaiacol measured by increase in absorbance at 470 nm. Protein content was determined according to the method of Lowry *et al*. (1951).

Determination of proline content: Proline content in shoot was determined by the method of Bates *et al*. (1973) with certain modifications. Shoot samples (0.5 g) were homogenized in 10 ml 30% sulphosalicylic acid. The homogenate was centrifuged at 10'000X g for 15 min. Supernatant was boiled for 1 hr after adding acetic acid and acid ninhydrin and absorbance was taken at 520 nm (UV- VIS 117 Systronics spectrophotometer).

Estimation of total glutathione content: Root and shoot samples were homogenized in mixture of 6% metaphosphoric acid pH 2.8, 1mM EDTA, and 10% PVPP. After centrifugation, total GSH content was determined in acid soluble extracts according to the method of Griffith (1980) and Anderson *et al*. (1992) with slight modification. After neutralizing with 500 mM potassium phosphate buffer pH 8.0 and incubating with 10 mM 5-5' dithio-bis (2- nitrobenzoic acid), GSH and GSSG were assayed where GSSG was used as standard.

Statistical analysis: All experiments were carried out with three independent repetitions in triplicate. Values were expressed as mean± standard deviation (SD). ANOVA was done by using software SPSS ver. 10 and graphs were prepared by Sigma Plot ver. 8.

Results and Discussion

Manganese accumulation in shoot and root increased corresponding to the increasing Mn concentration in the nutrient solution. Shoot and root of *V. faba* seedlings showed significant differences ($p<0.01$ and $p<0.05$) in the rate of Mn accumulation to the different concentrations (Table 1). The total amount of Mn

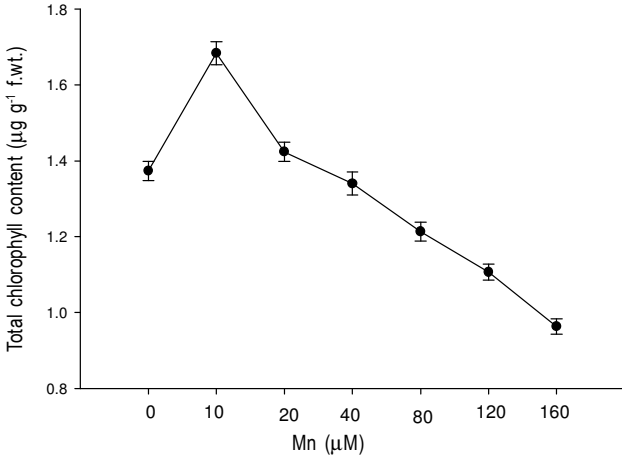

Fig. 1: Effect of Mn treatments on total chlorophyll concentration in leaves of *V. faba*. Values are mean of three replicates ± SD

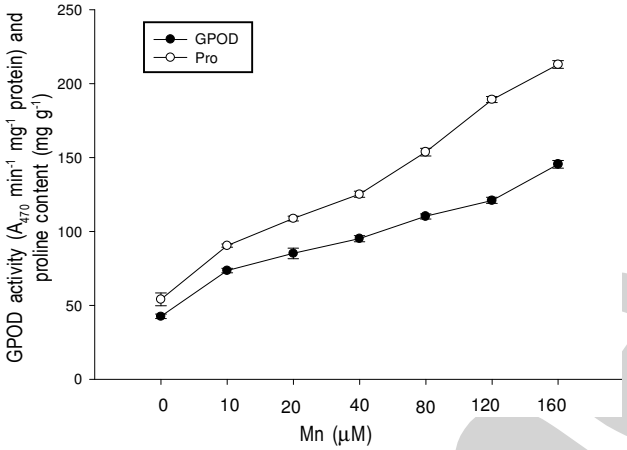

Fig. 2: Effect of different Mn concentrations on guaiacol peroxidase (GPOD) activity and proline content in shoots of *V. faba.* Values are mean of three replicates ± SD

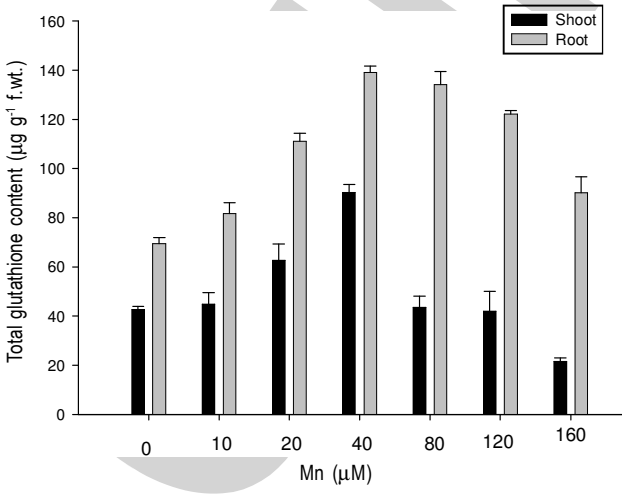

Fig. 3: Mn induced changes in total glutathione content in shoots and roots of *V. faba*. Values are mean of three replicates ± SD

accumulation was comparatively lower in shoot than root. At lowest concentration of Mn (10 mM) shoot shared only 1 fold and further increased by 4-8 fold at higher Mn concentrations (40-160 µM) compared to control. Root in immediate contact with Mn accumulated 3-13 folds higher to the increasing concentrations of Mn (10-160 µM). The effect of Mn on the growth was concentration dependent. A reduction (20 and 21.35%) in shoot and root length initiated at the lowest (10 µM) concentration and it was apparently higher in shoot (89.89%) and root (97.3%) at highest concentration of Mn (160 µM) (Table 1).

During the present investigation, reduction in the growth of *V. faba* plant in terms of root and shoot elongation at excess of Mn has been considered as one of the immediate toxic effect like other heavy metals in plants (Fecht- Christoffers *et al*., 2003). The deleterious effect of Mn was more pronounced on the root growth of *V. faba* than on the shoots (Doncheva *et al*., 2005). In comparison, less reduction of shoot than root may account for indirect effect in response to inadequate uptake or less translocation of Mn. Such reduction in growth have been attributed to interference of Mn with photosynthesis (Henriques, 2003). Reduction in root and shoot growth is completely consistent with results of previous worker (Shenker *et al*., 2004).

Total chlorophyll content also varied with Mn levels. An increase of 22% in total chlorophyll was observed in the range of 10-20 mM treatments and at the further amendments (40 mM) induced a further slight decline of 2% and accompanied by a significant decrease (30-43%) ($p<0.005$) at the increasing concentrations (80-160 µM) of Mn (Fig. 1).

Furthermore, decrease in total chlorophyll content under Mn exposure suggested the possibility of adverse effect on photosynthesis and plant metabolism as evidenced with findings of chlorosis, browning and necrosis of young leaves (Heniriques, 2003). The decreasing trend of chlorophyll content corresponding to increasing concentrations of Mn reported here in are similar to earlier observations with respect to some important crops (Asrar *et al*., 2005). It implies the possibility of Mn induced iron (Fe) deficiency and damage of chlorophyll by displacing Mg required for chlorophyll biosynthesis at higher concentrations (Hauck and Spribille *et al*., 2002).

The activity of GPOD in treated plants progressively increased with increasing concentrations as compared to control (Fig. 2). At 10 mM concentration a sudden increase of 42% in GPOD activity was observed and increasing trend (55-71%) was maintained in response to the higher treatments (40-160 µM). All such treatments induced GPOD activity and amount varied among the treatments significantly ($p<0.001$).

All the concentrations of Mn used here stimulated and enhanced the GPOD activity. Such activity of GPOD may eventually reduce the cell metabolic damage by simultaneous induction during plant growth (Fecht- Christoffers *et al*., 2003). In the present result, pattern of increase of GPOD during period

Table - 1: Effects of different manganese (Mn) concentrations on accumulation and root and shoot length in *V. faba* seedlings

Concentration (µM)	Mn accumulation (µg g^{-1}dry weight)		Length (%) of control	
	Root	Shoot	Root	Shoot
0	151.92±3.22	102.94±3.51	100.0000	100.0000
10	405.75±3.49	125.51±2.37	84.2700	92.0000
20	648.82±3.70	253.10±2.96	72.3400	83.6700
40	938.03±3.52	425.51±3.65	52.8100	75.4500
80	1228.31±3.18	602.44±5.90	37.2000	61.9800
120	1589.89±2.86	692.26±3.37	21.3500	50.5400
160	1983.67±4.11	801.82±2.23	13.8700	39.7800

Values are mean of three replicates ± SD

of Mn stress suggests that possibly GPOD is playing a role as an important scavenger of H_2O_2 and confirm the expression of Mn toxicity or Mn induced oxidative stress similar to the other heavy metals (Demirevaska-Kepova *et al*., 2004; Singh *et al*., 2009). It is clear that excess Mn doses used are affecting growth to an extent that display a degree of resistance due to suppression of Mn induced oxidative stress in presence of antioxidative enzymes.

Fig. 2 shows Mn induced proline accumulation in shoots. It has been revealed that the proline content substantially increased with increasing Mn concentrations. Induction of proline initiated at lower Mn level (10 mM) by 40% increase than control and this trend continued and went up to 57-75% increase at the higher concentrations (40-60 mM).In this investigation, level of proline depends on stress intensity, growth and plant-age (Piquery *et al*., 2000). The maximum extent of growth pattern and proline accumulation as recorded suggested that proline scavenges free radical and chelates Mn displays tolerance in *V. faba* (Alia *et al.,* 2001).

Fig. 3 shows the total glutathione content in roots and shoots of Mn treated and untreated plants of *V. faba*. Total GSH content was higher in roots than shoots. The content was increased from 10-40 µM and then declined with increasing concentration of Mn (80-160 µM) in both roots and shoots. Highest content was recorded in shoot (90.27 µg g^{-1}fresh weight) and in root (139.16 µg g^{-1}fresh weight) at 40 µM as compared to control. Minimum content in root (90.13 µg g^{-1} fresh weight) and in shoot (21.65 µg g^{-1} fresh weight) was observed at highest Mn concentration (160 µM). Increase in total glutathione content was significant with increase in concentration and duration. In our findings the total glutathione content increased initially upto 40 µM Mn and then declined with increase in concentrations of Mn (Fig. 3). The reduction in total glutathione content might contribute to synthesis of phytochelatins (Nagalakshmi and Prasad, 2001). Initiation of metal induced GSH synthesis as antioxidant supports the results of previous worker (Arya *et al*., 2008).

On the basis of results obtained, it can be concluded that *V. faba* exhibited physiological and visual symptoms of Mn toxicity. It is clear that *V. faba* is capable of tolerating excess Mn by enhancing antioxidative systems.

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