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Fluoride stress and the antioxidative efficiency of soybean seedlings

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ABSTRACT

Soybean [*Glycine max* (L.) Merr.] seedlings were grown in presence of NaF (0-10.0 mM) solution for seven days in laboratory conditions. Seedling length, biomass and some physiological parameters were determined in order to determine the effect of fluoride on the antioxidative efficiency during the early period of seedling growth and to ascertain the mechanism of fluoride toxicity in plants. At lower concentration of fluoride the seedling length and seedling biomass were unaffected but towards higher concentrations, both these parameters decreased in comparison to the control sample. The soluble protein content of the cotyledonary leaf tissue increased but total chlorophyll content decreased with increase in the fluoride in the medium. Superoxide dismutase and catalase activities initially increased and then decreased, but peroxidase activity was almost unaffected. The lipid peroxidation level was found to decrease with increase in fluoride concentration which indicated that oxidative stress was not imposed due to fluoride toxicity and antioxidative efficiency of the tissue was probably maintained by some other mechanisms, not investigated in this study, in presence of fluoride. Thus, on the basis of the results it can be presumed that fluoride has toxic effect on plant during early seedling growth and the mechanism of toxicity might be due to alteration in physiological processes, other than the antioxidative system.

1. Introduction

Fluorine is a natural trace element and is widely distributed in nature. It belongs to halogen group and is known to be one of the most reactive of all chemical elements. It is rarely found in its elemental state in nature and occurs in the reduced (fluoride) form in combination with other minerals. It can combine directly at elevated temperatures with all elements other than oxygen and nitrogen (Banks and Goldwhite, 1966). Sodium fluoride is the most important of the alkali fluorides. Approximately 0.08% of the earth's crust is fluoride compounds. The minerals like fluorspar, rock phosphate, cryolite, mica, apatite, hornblende etc. contain significant amount of fluoride (Murray, 1986). The surface water concentration of fluoride generally ranges from 0.01 to 0.3 mg/L (ATSDR, 1993); ground water concentration ranges from 0.02 to 1.5 ppm (Fleischer, 1962) and the sea water contains more fluoride than the fresh water ranging approximately from 1.4 to 1.5 ppm (Bowen, 1966; Carpenter, 1969). The ambient air of the areas in the vicinity of emission sources have fluoride level of 2-3 $\mu g/m^2$ but the areas not in the direct vicinity of the sources contain less than 0.1 $\mu g/m^2$ (IPCS, 2002). In urban areas the ambient air level of fluoride is slightly higher than the rural areas. Fluoride gets deposited into the soil from several anthropogenic sources like phosphate fertilizers or atmospheric pollution due to industrial activities and burning of fossil fuels. From the soil it enters into the plants through the root system along with the mineral nutrient absorption and is transported to the transpiratory organs, mainly the leaves via xylematic flow where it is known to cause adverse effects (Elloumi *et al*., 2005). Fluorides have many adverse effects on human health including the skeletal fluorosis and dental fluorosis (Grynpas, 1990; IPCS, 2002).

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In plants it has also been reported to be toxic when taken up from the soil. Decrease in seed germination percentage, reduction in root and shoot length, decrease in vigour index, total chlorophyll content, reducing sugar and starch content etc. are some of the physiological anomalies that have been reported in plants due to fluoride toxicity (Elloumi *et al.*, 2005; Kumar *et al*., 2009; Bhargava *et al*., 2010). Imposition of oxidative stress in plants is a most common consequence in plants exposed to any type of stress situation. But reports on the state of the oxidative metabolism in plants under fluoride toxicity are rare. However few reports like those of Kumar *et al*. (2009) are available on the effect of fluoride on oxidative metabolism in plants. In this study, therefore, attempts have been made to investigate the effect of fluoride on the antioxidative efficiency of soybean seedlings.

2. Materials and methods

Soybean (*Glycine max* (L.) Merr.) seeds were collected from the local market and were germinated on moist tissue paper in Petri dish after surface sterilization with 3% freshly prepared filtered solution of commercial bleaching powder. The germinated seeds were transferred on to nylon nets stretched over small plastic containers, containing 200 ml of different concentrations of sodium fluoride (0.5 mM, 1.0 mM, 5.0 mM and 10.0 mM) prepared in 1/10 strength Hoagland solution. 200 ml of 1/10 strength Hoagland solution, without NaF, was taken in another plastic container as control. 5 germinated seeds were kept on each container and the seedlings were allowed to grow in the laboratory condition at 28 ± 2^0C with 8 h light/16 h dark cycle. 7-day old seedlings were collected and root and shoot lengths were measured. Fresh weight of the seedlings recorded and dry weight was taken after keeping the seedlings in oven at 80^0C for 48 h.

In another set of experiment, the seedlings were collected for analysing different physiological parameters. Total chlorophyll was extracted with 80% acetone from cotyledonary leaf tissue and the content was determined following the method of Arnon (1949). Buffer soluble protein from the cotyledonary leaf tissue was extracted with sodium phosphate buffer, 50 mM, pH 7.5 and the supernatant was mixed with equal volume of 20% trichloroacetic acid (TCA) solution and kept for overnight in order to precipitate the protein. The pellets were washed successively with alcohol, alcohol: chloroform in a proportion of 3: 1, chloroform and finally with ether. Finally the pellets were resuspended with 0.3N NaOH for overnight. The samples were then centrifuged and supernatants collected as protein extract. Soluble protein content was measured following the method of Lowry *et al*. (1951).

The cotyledonary leaf tissue was homogenised under ice-cold condition with sodium phosphate buffer, 0.05 M, pH 7.4 for superoxide dismutase (SOD) and pH 7.5 for catalase and peroxidase. The homogenates were centrifuged at 17, 000 × *g* for 10 min at -4^0C and the supernatants were collected and used for enzyme assay after suitable dilution. The activity of SOD (EC 1.15.1.1) was assayed following the method of Das *et al*. (2000). The catalase activity was measured following the method of Aebi *et al*. (1983) and peroxidase activity was assayed as described by Kar and Feierabend (1984). The level of lipid peroxidation was determined by measuring the malondialdehyde following the method of Heath and Packer (1968).

All the experiments were done at least for three times with three replicates in each time. The mean values are presented and the standard deviations are indicated.

3. Results and discussion

It was observed that the lower concentrations of NaF did not have any adverse effect on the growth of the seedlings. There was no appreciable change in shoot length up to 1.0 mM and root length up to 5.0 mM of NaF in the medium. At 5.0 mM concentration, the shoot length was reduced by 63% which further increased to 71% at 10.0 mM in comparison to the same of the control seedlings (Fig. 1). But the root length was almost unaffected up to 5.0 mM of NaF and at 10.0 mM, the root length was decreased by 41% in comparison to the root of control seedlings. The fresh weight of the seedling was not significantly changed up to 1.0 mM NaF and at 5.0 mM, it was reduced by 28% which further increased to 58% at 10.0 mM of NaF in comparison to the fresh weight of the control seedlings (Fig. 2). The dry weight was not significantly altered up to 5.0 mM NaF and it was decreased by 22% at 10.0 mM NaF in comparison to the same of the control seedlings. Thus, at lower concentrations of fluoride the growth in terms of seedling length, fresh weight and dry weight of the seedling was unaffected and towards higher concentrations it was reduced. The soluble protein content was almost unaffected due to fluoride and rather it increased slowly with increase in the fluoride concentration in the medium and at 10 mM NaF it was increased by 58% in comparison to the control tissues (Table 1). But the total chlorophyll content of the cotyledonary leaf tissue was decreased by 35% in the sample grown in presence of 10 mM NaF in comparison to the control sample (Table 1). Elloumi *et al*. (2005) have reported that there was decrease in chlorophyll content along with other metabolite levels like starch and sugar in almond seedlings grown in presence of fluoride. They have also found the reduction in dry matter of root system which

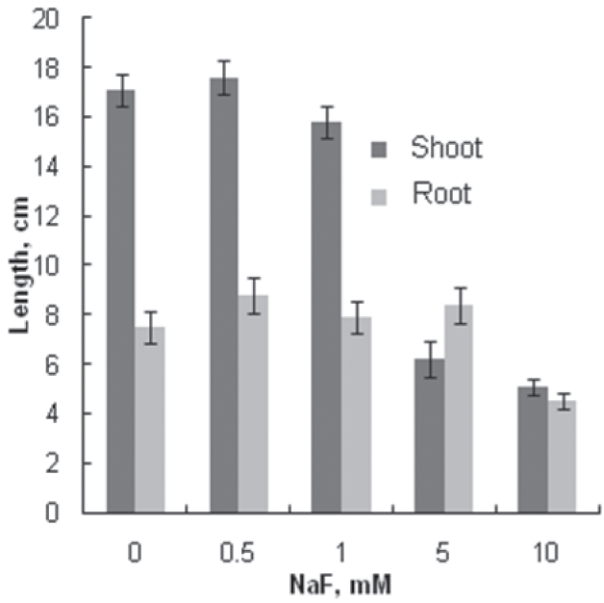

Fig. 1 Changes in the shoot length and root length of 7 days old soybean seedlings grown in presence of NaF in the laboratory condition.

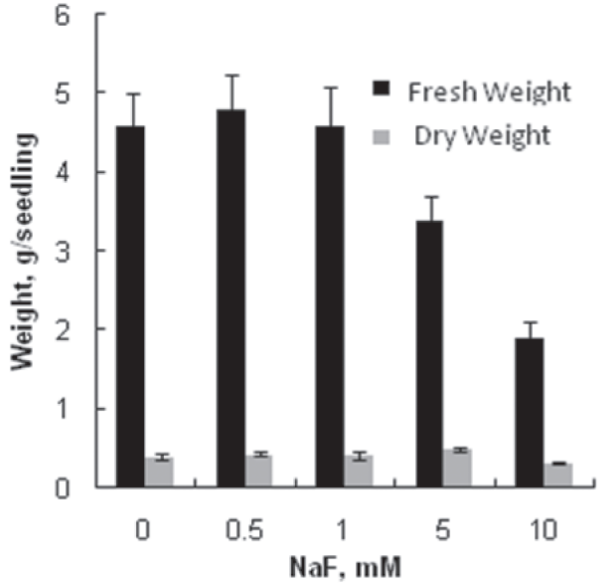

Fig. 2 Changes in the fresh weight and dry weight of 7 days old soybean seedlings grown in presence of NaF in the laboratory condition.

Table 1

Changes in the total chlorophyll content, soluble protein content and lipid peroxidation level in cotyledonary leaf tissue of 7-days old *Glycine max* seedlings grown in presence of NaF

NaF(mM)	Total chlorophyll(mg/g FW)	Soluble protein(mg/g FW)	MDA(nmol/g FW)
0	0.33 ± 0.021	25.1 ± 1.9	14.4 ± 1.5
0.5	0.34 ± 0.029	26.2 ± 2.4	8.8 ± 0.9
1.0	0.275 ± 0.019	28.4 ± 3.0	9.6 ± 1.1
5.0	0.29 ± 0.03	30.6 ± 2.6	6.7 ± 0.5
10.0	0.215 ± 0.02	38.3 ± 3.2	5.5 ± 0.43

accumulated large amount of F. Here in this study also we have found decrease in chlorophyll content of the cotyledonary leaf tissue and decrease in seedling length as well as seedling mass towards higher concentration of the NaF in the medium. Therefore, it is evident from this study that fluoride has toxic effect in the soybean seedling. On account of increase in the protein content of the seedling with increase in the fluoride concentration it may be presumed that perhaps within this short period of assessment, i.e., 7 days, the measured protein was nothing but the hydrolysed product of the reserved protein of the cotyledonary tissue and not the protein that the plant synthesized. Effect of F on protein content can only be assessed only when the seedlings would be allowed to grow for a longer period.

Oxidative stress is a situation during which the rate of generation of reactive oxygen species exceeds the capacity of endogenous antioxidative protective system to scavenge them off. Prolonged exposure of the organism to oxidative stress situation is often been reported to be lethal. The antioxidative system plays a very vital role in reducing the deleterious effect of reactive oxygen species. This system includes antioxidative enzymes like superoxide dismutase (SOD), catalase and peroxidases and low molecular weight antioxidants like ascorbate, carotenoids, reduced glutathione, flavonoids, α-tocopherol etc. (Halliwell and Gutteridge, 2007). In this work even though the levels of low molecular weight antioxidant was not measured, but the activities of antioxidative enzymes like SOD, catalase and peroxidase were measured and the results are presented in Fig. 3, 4 and 5. It was found that the SOD activity was almost unaffected at 0.5 mM NaF, rather it was increased slightly in comparison to the control sample (Fig. 3). But at 1.0 mM concentration, the activity was decreased significantly and there was almost

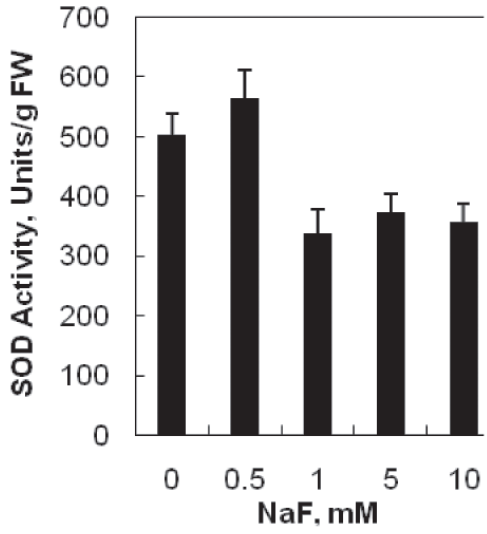

Fig. 3 Changes in the superoxide dismutase (SOD) activity in the cotyledonary leaf tissue of 7 days old soybean seedlings grown in presence of NaF in the laboratory condition.

no further change towards higher concentrations of NaF. This indicated that protection against superoxide radicals was maintained at 0.5 mM NaF and from 1.0 mM onwards there was probability of accumulation of higher amount of superoxide radical, since the SOD activity was decreased. In SOD catalysed reaction, H_2O_2 is always a byproduct which is a highly oxidising agent and there are also other metabolic sites which generate H_2O_2 (Elstner, 1982; Halliwell and Gutteridge, 2007). Therefore, protection against H_2O_2 is very vital and in aerobic cell catalase is known to be the principal enzyme responsible for H_2O_2 decomposition; of course peroxidases reduce the H_2O_2 level to some extent along with the co-oxidation of reduced co-substrates (Halliwell and Gutteridge, 2007).

Catalase activity was almost doubled at 0.5 mM NaF than the control tissue and then significantly reduced towards higher concentrations of F (Fig. 4). At 10 mM concentration, catalase activity was less than half of the control value. The increase in catalase activity at lower concentration might be in response to excessive generation of H_2O_2 as a result of SOD driven dismutation and/or other metabolic processes generating H_2O_2 in presence of fluoride. Thus, at this point the protection against H_2O_2 was enough as a result there was no inhibition in the growth of the seedlings, both in the forms of seedling length (Fig. 1) and seedling mass (Fig. 2). But towards higher concentration the activity declined which might be due to inhibition of catalase synthesis or degradation of catalase protein. McCune *et al*. (1964) have reported that the level of catalase was significantly higher in plants fumigated with fluoride after six days but not significantly different in the subsequent periods. This indicates that at initial period of stress due to fluoride exposure, the protective mechanism in the plant was enough in terms of elevated SOD and catalase activities as a result there was no effect on the growth of the seedlings. But towards higher concentrations, the activities of these two enzymes decreased and coincidentally the growth of the seedlings was also found to reduced. Peroxidase is another antioxidative enzyme which is responsible for decomposition of H_2O_2 along with co-oxidation of reduced co-substrate (Halliwell and Gutteridge, 2007). There are also cell wall bound peroxidases which are released under stress situation increasing the enzyme activity in assay condition and hence, increase in peroxidase activity is usually considered as an indicator of stress situation. In this study, guaiacol was taken as reduced co-substrate for determination of peroxidase activity and the results are presented in Fig. 5. It was found that excepting at 5.0 mM concentration of NaF, there was almost no change in peroxidase activity due to increase in F concentration in the medium. Even though at 5.0 mM NaF there was slight increase in peroxidase activity in comparison to the control sample, the trend of the activity of peroxidase in this study can not be attributed to its efficacy for H_2O_2 decomposition.

In oxidative stress situation when higher amount of H_2O_2 and superoxide radicals are generated, they interact with each other in presence of transition metal ions forming hydroxyl radical, via Haber-Weiss reaction (Elstner, 1982). Hydroxyl radicals are known to be the potentially very toxic and unsaturated fatty acid components of membrane lipids are susceptible to its attack and are peroxidised by it. The result is the loss of membrane integrity and cellular architecture (Halliwell and Gutteridge, 2007). Therefore, increased lipid peroxidation is considered as an indicator of

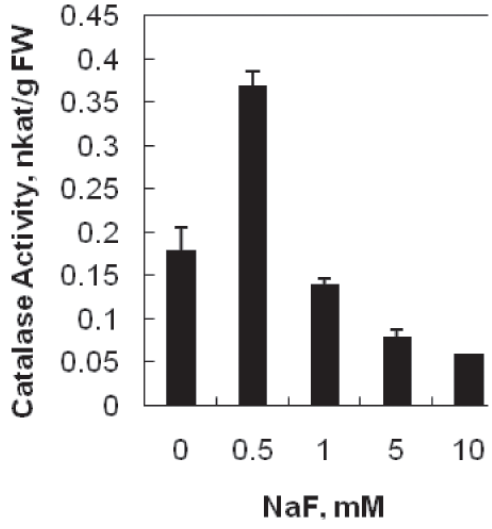

Fig. 4 Changes in the catalase activity in the cotyledonary leaf tissue of 7 days old soybean seedlings grown in presence of NaF in the laboratory condition.

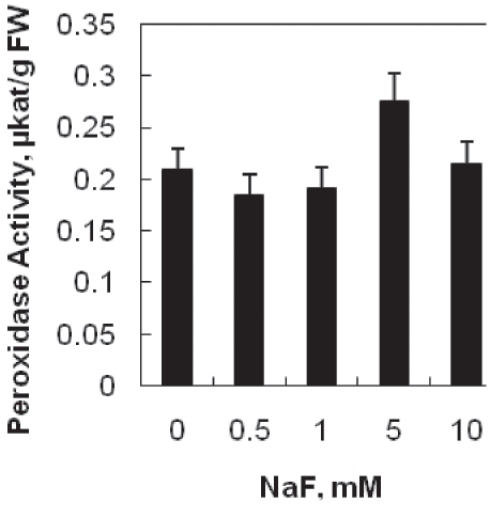

Fig. 5 Changes in the peroxidase activity in the cotyledonary leaf tissue of 7 days old soybean seedlings grown in presence of NaF in the laboratory condition.

prevalence of oxidative stress situation in aerobic cells (Kappus, 1985). Malondialdehyde (MDA) was measured in this study to determine the lipid peroxidation level in the tissues taking thiobarbituric acid as the reactive material and the results are presented in Table 1. It was found that the lipid peroxidation level was maximum in control sample and with increase in fluoride concentration in the medium the lipid peroxidation level decreased significantly and it reached almost at one third of the control value at 10.0 mM NaF. On the basis of decrease in lipid peroxidation level it can be presumed here that fluoride does not impose oxidative stress in developing soybean seedlings. In an earlier report on higher plants Yang and Miller (1963) have found decrease in the contents of sucrose, non-reducing sugar, total sugar and amino acid along with inhibition in the activities of some enzymes of carbohydrate metabolism in fluoride fumigated samples. Thus, alterations in these physiological parameters might be one of the mechanisms behind fluoride toxicity in plants.

On the basis of the results obtained in this study it can be presumed that due to exposure to fluoride the antioxidative system was not significantly affected. Even though towards higher concentrations of NaF the activities of SOD and catalase were decreased the oxidative stress was not imposed since there was no increase in lipid peroxidation level, rather it decreased. Probably towards higher concentrations of NaF the antioxidative efficiency of the tissue was protected by low molecular weight antioxidants or the enzymes involved in the ascorbate-glutathione pathway. Further studies in this aspect are essential for a meaningful conclusion. But decrease in the seedling length, biomass of the seedlings and total chlorophyll level of the cotyledonary leaf tissues towards higher concentrations of the fluoride indicated that fluoride had toxic effect on plant during early seedling growth and the mechanism of toxicity might be due to alteration in physiological processes other than antioxidative system. Therefore, in highly fluoride polluted areas it is not advisable to irrigate the crop plants with contaminated water.

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