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Fluoride toxicity: Physiological and biochemical consequences on seedling growth in *Vigna radiata*

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Abstract
Fluorine element occurs naturally. It is not considered as an essential element for growth of plants. Consumption of high amount of fluoride can cause toxic effects in plant and animals. Fluoride effect creates unbalancing in up-taking of other nutrients also. High concentration of fluoride shows phytotoxic effect on morphological and biochemical properties of plants. Fluoride acts as an inhibitor for the metabolic activities occurring during the seed germination process. Plant protein content also declines in the presence of high levels of fluoride. Current study shows fluoride toxicity effects on physiological and biochemical consequences during seedling growth in *Vigna radiata*

Keywords: fluoride effect, plant growth, total soluble sugar

Introduction

Fluoride has been proved to be beneficial in recommended doses and at the same time its toxicity at higher levels has also been well established (Dhar & Bhatnagar 2009) [15]. Fluoride is not an essential element and is therefore not required for plant growth (Sauerheber 2013) [25]. In human beings' consumption of high amount of fluoride creates dental and skeletal fluorosis (Srivastava, 2020: sharma *et al*, 2013). As per the WHO guidelines in drinking water fluoride level should be less than 1.5 mg/L (Brouwer *et al*, 1988: Gao *et al*, 2013) [7, 17]. High concentration of fluoride causes toxic effects on seedling growth (Hong *et al*, 2016: Choudhary *et al*, 2019: Yadu *et al*, 2016) [19, 11, 37]. Early seedling stages are important for a healthy plant growth (Derbali *et al*, 2020) [13]. During physiological growth, different concentrations of the fluoride also show toxic effect (Panda, 2015: Dey *et al*, 2012: Sharma & Kaur, 2018) [24, 14, 30].

High amounts of fluoride causes necrosis and chlorosis of leaf (Hill, 1969) [18]. Fluoride effect creates unbalancing in up-taking of other nutrients (Saini *et al*, 2013) [27]. It also causes negative effects on enzyme activities of the plants and results in growth inhibition (Camargo, 2003: Nagajyoti *et al*, 2010) [8, 23].

In India many states are affected by the fluoride toxicity including Rajasthan (Arif *et al*, 2014: Choubisa, 2001) [2, 9]. In Rajasthan main source of irrigation is bore well ground water. All 33 districts of Rajasthan are found to have high amounts of fluoride in ground water (Choubisa, 2018) [10]. Because of low rain fall, more ground water is required for the irrigation of crops therefore fluoride amount is continuously increasing in soil. High use of fertilizers has also elevated fluoride in the soil (Loganathan *et al*, 2003) [22].

Material and Methods

Seed germination studies

This study involves soaking of seeds in distilled water. After that, surface sterilization of seeds with 0.1% $HgCl_2$ solution for a minute was done then rinsed with distilled water 2-3 times and dried on blotting paper. The pre-sterilized Petri dishes were lined with filter paper over sterilized cotton pad. Ten healthy seeds of uniform size were kept at equal distance on the top of the filter paper in glass petri plates (100 mm diameter & 15 mm height). For each concentration of sodium fluoride three replicates were maintained. The solution of sodium fluoride of different concentration i.e., 10ppm, 40ppm, 70ppm, 100ppm were prepared and distilled water was used as control. As pre-treatment, 10.0ml of fluoridated water was added in each petri plate on the first day then, at regular intervals. During the entire experimental period, petri dishes were periodically moistened with the respective fluoride solution. Seeds were allowed to grow under laboratory conditions. Seedling emergence was studied after 48 hrs and measurement of different growth parameters was taken after 7 days.

Chlorophyll (Chl) estimation

1gm fresh leaves of seedling was weighed and finely cut. The pigment was extracted by grinding and homogenized in pestle mortar with 20- 40ml of 80% acetone, centrifuged at 10,000 rpm for 10 min. Supernatant was obtained after centrifugation and the volume was make up to 100 ml with 80% acetone.

This clear supernatant was then examined for total chlorophyll and carotenoid contents. The analysis of chlorophyll pigment was performed using spectrophotometer (Shimadzu) immediately after the solution was prepared. O.D. of the supernatant was recorded at 645 nm and 663 nm, while taking 80% acetone solvent as a reference (Arnon, 1949) [1]. The total chlorophyll was calculated by addition of the chlorophyll a and chlorophyll b values.

These were calculated by observing the change in the observations at different wavelengths as per the protocol and then applying the formula. The total chlorophyll content of the plant was expressed in mg/g fresh weight.

$$\text{Total chlorophyll (a+b)} = 20.2\, A_{645} + 8.02\, A_{663}\ \text{mg/l}$$

To calculate chlorophyll content on fresh weight basis, the following formula was used:

$$\text{Total chlorophyll (mg/g)} = \frac{20.2\ A_{645} + 8.02\ A_{663} \times V}{a \times 1000 \times w}$$

$$\text{Chlorophyll a (mg/l)} = 12.70\ A_{663} - 2.69\ A_{645}$$

$$\text{Chlorophyll b (mg/l)} = 22.90\ A_{645} - 4.68\ A_{663}$$

Where, A_{645} = Absorbance at 645 nm
A_{663} = Absorbance at 663 nm
A = Length of path of light in the cell (1.0 cm)
V = Volume of extract (ml)
W = Fresh weight of the sample (g)
The amount of chlorophyll was expressed in terms of mg/g fresh weight.

Determination of proline content
Excised seedling tissue (0.5g) was crushed with mortar and pestle in 5 ml of 3% sulfosalicylic acid and homogenate was centrifuged at 13,000g for 10 min at 4oC. To 2.0 ml of supernatant 2 ml freshly prepared ninhydrin reagent and 2 ml of glacial acetic acid was added. This reaction mixture was boiled at 100oC for 1h. after completion of reaction, the tubes were placed in ice and 4 ml toluene was added and this was vigorously shaken to extract the chromophore. Absorbance was taken at 520 nm in UV-Visible Spectrophotometer (UV-1800, Shimadzu) using toluene as a blank. Proline concentration was determined using standard curve prepared with known concentration of proline and their respective absorbance.

Peroxidase enzyme (POD) assay
For this enzyme assay 1 gm of each plant material (chilled in deep freeze at -200C) was homogenized with a mortor & pestle in 10 ml chilled sodium phosphate buffer (0.2 M, pH 7.0) and further centrifuged at 10,000 rpm for 15 min. at 40C. The supernatant was used for POD assay. The Reaction Mixture was prepared immediately in a total volume of 4 ml having 2ml of 0.2M sodium phosphate buffer (pH 7.0), 1 ml of enzyme extract and 0.5 ml of 1% guaiacol. Then 0.5 ml of 0.5 % H2O2 was added to 3.5 ml of reaction mixture. The reaction time was recorded immediately after adding the enzyme extract. The increase in absorbance was recorded at 470 nm at 1 min intervals up to 3 min with a UV-Visible spectrophotometer. The activity of POD enzyme was expressed as ΔA470 nm per minute per gram fresh weight.

Catalase enzyme (CAT) assay
Plant extract was prepared by homogenizing 1 gm of each sample (chilled in deep freeze at-200C) in 50mM chilled sodium phosphate buffer (7.0) and further centrifuged at 10,000 rpm for 15 min. at 40C. The supernatant was used for CAT assay. The Reaction Mixture was prepared immediately in a total volume of 3 ml having 2.7 ml of 50mM sodium phosphate buffer (pH 7.0), 0.1 ml of enzyme extract. Then 0.2 ml of 200mM H2O2 was added in reaction mixture. The decrease in absorbance was recorded at 410 nm up to 3 min intervals with a UV-Visible spectrophotometer. One unit was expressed as ΔA470 nm per minute per gram fresh weight.

Protein estimation
The test sample (50 mg each) were separately homogenized in 10 ml of cold 10% trichloroacetic acid (TCA) for 30 min and kept at 4^0C for 24 hours. These mixtures were centrifuged separately and supernatants were discarded. Each of the residues was re-suspended in 10 ml of 5% TCA and heated at 80^0 C on a water bath for 30 minutes. The samples were cooled, centrifuged and supernatants were again discarded. The residue was then washed with distilled water, dissolved in 10 ml of 1N NaOH, and left overnight at room temperature. Each of the above (1 ml) was taken and the total protein content was estimated using the spectrophotometer through method of Lowry *et al.,* (1951). A regression curve was prepared. A stock solution of BSA (Sigma Chem. Co., St. Louis, USA) was prepared in 1N NaOH ($1 mgl^{-1}$). Eight concentrations (ranging from 0.1 to 0.8 mgl^{-1}) were separately measured in test tube and volume of each sample was made to 1 ml by adding distilled water. To each, 5 ml of freshly prepared alkaline solution (Prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of 0.5 % $CuSO_4.5H_2O$ in 1% Sodium potassium tartarate) was added and kept at room temperature (35°C) for 10 minutes. In each sample 0.5 ml of Folin-Ciocalteau reagent (commercially available reagent was diluted with equal volume of distilled water just before use) was added rapidly with immediate mixing and optical density of each sample was measured after 30 minutes at 750 nm using spectrophotometer against blank. Three replicates of each concentration were taken and average value was plotted against their respective concentrations to compute regression curve.
All samples were processed in the same manner and the concentration of the total protein content in each sample was calculated by referring the optical density of each sample with standard curve.

Total soluble sugars
The dried experimental plant material (50 mg each) was homogenized in pestle and mortar with 20 ml of 80% ethanol separately and left overnight. Each sample was centrifuged at 1200 rpm for 15 minutes; the supernatants were collected separately and concentrated on a water bath using the method of Loomis and Shull (1973). Distilled water was added to make up the volume up to 50 ml and processed further for quantitative analysis. One ml of aliquot of each sample was used for the estimation of carbohydrates using the phenol sulphuric acid method of Dubois *et al.,* (1951). A standard regression curve of standard sugar (glucose) was prepared. A stock solution of glucose ($100 \mu g\ ml^{-1}$) was prepared in distilled water. From this solution, 0.1 to 0.8 ml was pipette out into eight separate test tubes and volume was made up to 1 ml with distilled water.
These tubes were kept on ice; 1 ml of 5% phenol was added in each tube and shaken gently. 5 ml of concentrated sulphuric acid added was rapidly poured so that the steam hits the liquid and tubes were gently shaken during the addition of the acid. Finally the mixture was allowed to stand on water bath at 26-30^0C for 20 minutes. The characteristic yellow orange colour was developed. The optical density was measured at 490 nm using spectrophotometer, after setting for 100% transmission against a blank (distilled water). Standard regression curve was computed between the known concentration of glucose and their respective optical density, which followed Lambert Beer‘s Law. All samples were analyzed in the same way as described above and contents of total soluble

sugars were calculated by computing optical density of each of the samples with standard curve.

For the statistical analysis, three replicates were maintained for each treatment. All data were subjected to general linear model multi factorial analysis has been applied for looking the fluoride effect on different parameters like concentration, germination, shoot length, root length, fresh weight, dry weight, chlorophyll content, MDA content, catalase, peroxidase, proline, SOD activity, Total soluble sugar and protein. We select fluoride concentration as independent variable and germination, shoot length, root length, fresh weight, dry weight, chlorophyll content, MDA content, catalase, peroxidase, proline, SOD activity, Total soluble sugar and protein used as dependent variable.

Results

For *Vigna radiata* all variables like germination, shoot length, root length, fresh weight, dry weight, chlorophyll content, MDA content, catalase, peroxidase, proline, SOD activity, total soluble sugar and protein significantly influenced by the concentration of fluoride. That is show in Table1 and 2.

Table 1: Multiple factorial analysis for fluoride as independent variable with all others dependent variables

Tests of between-subjects effects						
Source	**Dependent variable**	**Type III sum of squares**	**df**	**Mean square**	**F**	**Sig.**
Concentration	Germination	2978.216	4	744.554	757.174	.000
	Shoot length	62.203	4	15.551	183.669	.000
	Root length	13.256	4	3.314	169.314	.000
	Fresh weight	26575.067	4	6643.767	1.780E3	.000
	Dry weight	546.267	4	136.567	157.577	.000
	Chlorophyll	5.371	4	1.343	23.694	.000
	MDA	1597.383	4	399.346	210.256	.000
	Catalase	51388.147	4	12847.037	1.926E3	.000
	Peroxidase	3413.871	4	853.468	236.987	.000
	Proline	6487.004	4	1621.751	455.377	.000
	SOD	8246.743	4	2061.686	714.540	.000
	Total Soluble Sugar	5176.423	4	1294.106	507.758	.000
	Protein	1183.764	4	295.941	301.981	.000

At mean difference is significant at the 0.05 level.

Table 2: Showing fluoride toxic effect on seedling growth parameter [Moong]

Conce.	Germination %	Shoot length	Root length	Total seedling length	Fresh wt.	Dry wt.	Seed vigour index
Control	100	9.11±0.02	4.2±0.1	13.3±0.12	182.33±0.58	27±1.00	1332.00±0.57
10 ppm	100	8.2±0.20	4.08±0.20	12.28±0.36	170.66±0.58	25.33±0.5	1229.00±0.50
40 ppm	84.033±1.25	5.77±0.40	3.03±0.05	8.80±0.44	117±4.00	17.00±1.00	726.70±0.33
70 ppm	71.00±1.00	4.5±0.45	2.30±0.20	6.80±0.66	94.00±1.00	14±1.00	475.50±0.30
100 ppm	66.33±1.52	3.9±0.10	1.83±0.05	5.73±0.12	75.00±1.00	12±1.00	387.30±0.60

Table 3: Showing fluoride toxic effect on biochemical parameters [Moong]

	Chlorophyll	MDA content	Catalase	Peroxidase	Proline	SOD	Total soluble sugar	Protein
Control	5.43±0.30	5.4±0.30	202±3.4	30±1.2	25.5±1.08	21.3±1.39	36±0.59	54.0±1.3
10 ppm	4.57±0.25	11.5±1.22	172±2.1	41±1.3	43.0±2.62	32.1±0.36	41.60±1.06	49.0±0.70
40 ppm	4.030.20±	22±1.66	113±2.7	55±3.0	53.6±1.96	45.8±3.12	54.1±1.1	39.0±0.80
70 ppm	3.84±0.20	26.8±1.33	72±2.1	74±2.2	74.3±2.06	63.0±1.44	75.8±3.03	36.0±0.91
100 ppm	4.0±0.20	34.07±1.86	46±2.4	40±1.0	82.9±1.31	87.6±0.75	83.40.95±	30.0±1.2

Findings of seed germination and seedlings growth experiment show a decreasing trend in root length and shoot length with increasing sodium fluoride concentration. At 100ppm fluoride concentration 66% germination was found and root length and shoot length were reduced to 3.9cm and 1.8cm than that of control Figure 1, Figure 2, Figure 3 respectively. In case of fresh weight and dry weight similar trend was observed. Fresh weight and dry weight were found 75mg, 12mg at 100ppm concentration of fluoride respectively as shown in Figure 4 and Figure 5. Chlorophyll content and catalase activity was also found to decrease with the concentration of fluoride shows in Figure 6 & Figure 8. Other than morphological parameters biochemical parameters like MDA, proline, peroxidase activities, SOD and total soluble sugar contents were also found to increase with increasing fluoride concentration (Figure 7, 9, 10, 11, 12). But protein content in leaves of seedlings showed a gradual decrease with increasing fluoride concentration. At 100 ppm fluoride concentration protein content was nearly 29 mg g^{-1} in fresh weight, less than that of control (Figure *13).* Micrograph study showed the adverse effect of fluoride on anatomical structure of root, shoot, chlorophyll and leaf. Increased level of MDA, proline, peroxidase activities, SOD and total soluble sugar contents showed toxic effect on seedling stage of *Vigna radiata.*

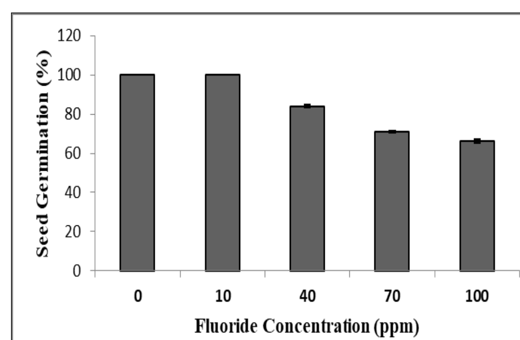

Fig 1: Fluoride effect on germination of *Vigna radiata*

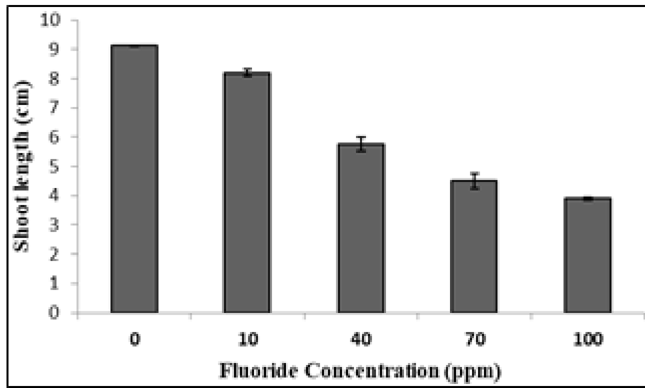

Fig 2: Fluoride effect on shoot length of *Vigna radiata*

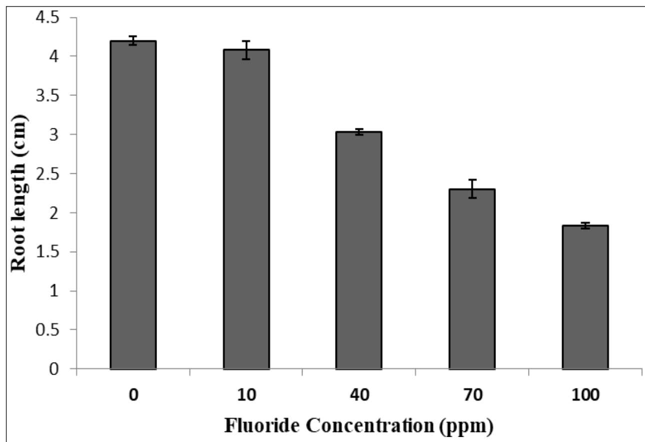

Fig 3: Fluoride effect on Root length of *Vigna radiata*

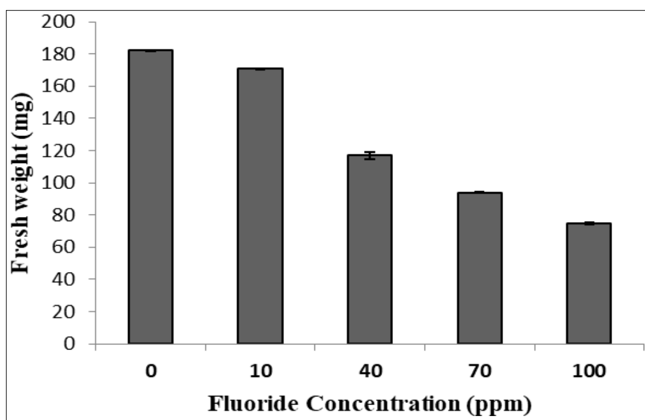

Fig 4: Fluoride effect on Fresh weight of *Vigna radiata*

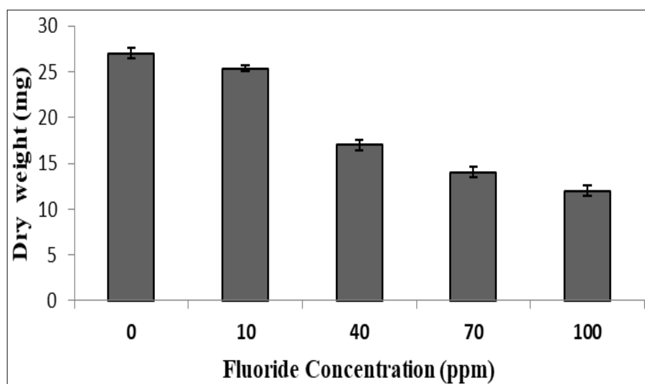

Fig 5: Fluoride effect on dry weight of *Vigna radiata*

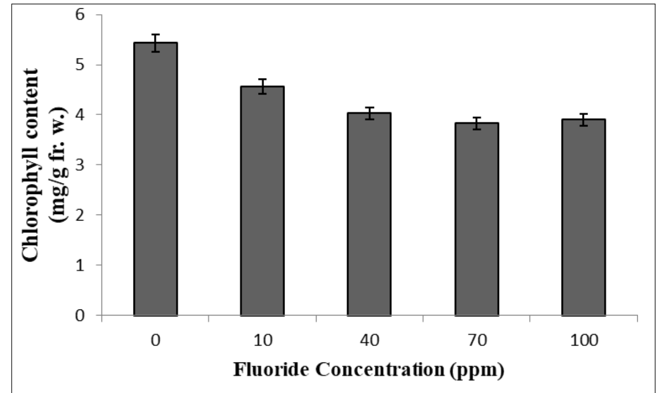

Fig 6: Fluoride effect on Chlorophyll content of *Vigna radiata*

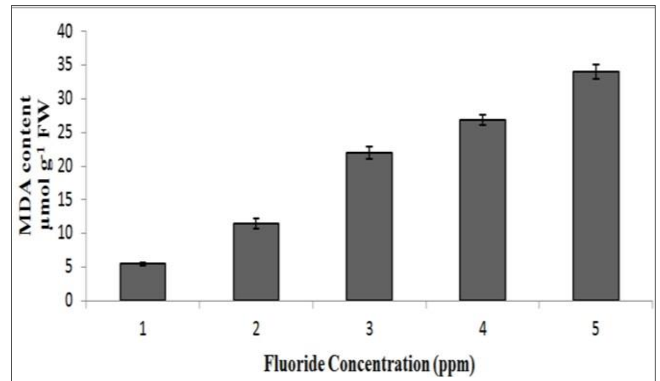

Fig 7: Fluoride effect on MDA content of *Vigna radiata*

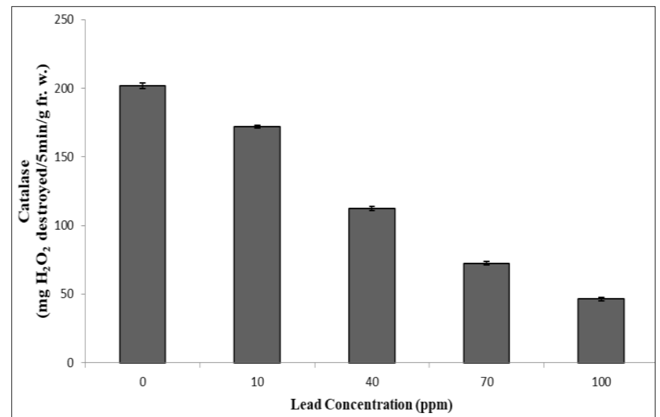

Fig 8: Fluoride effect on Catalase activity of *Vigna radiata*

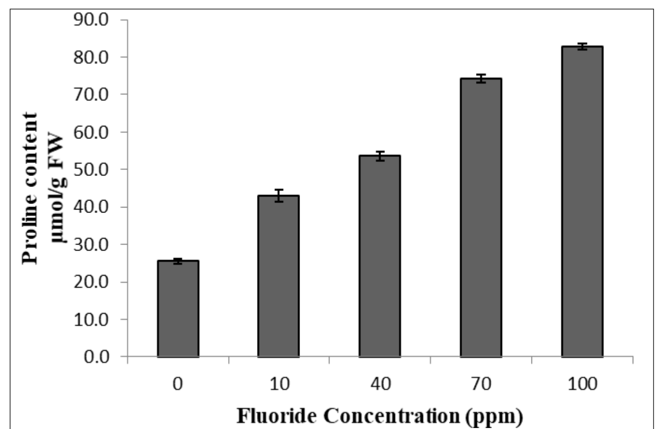

Fig 9: Fluoride effect on proline content of *Vigna radiata*

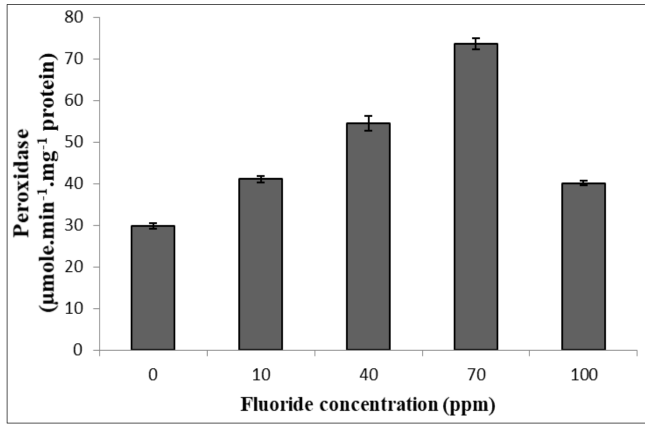

Fig 10: Fluoride effect on peroxidase activity of *Vigna radiata*

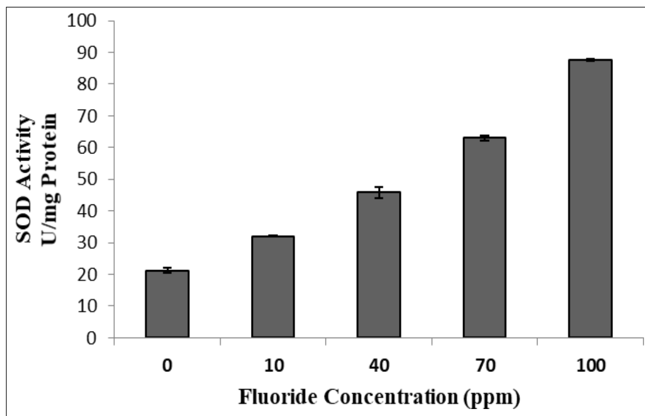

Fig 11: Fluoride effect on SOD activity of *Vigna radiata*

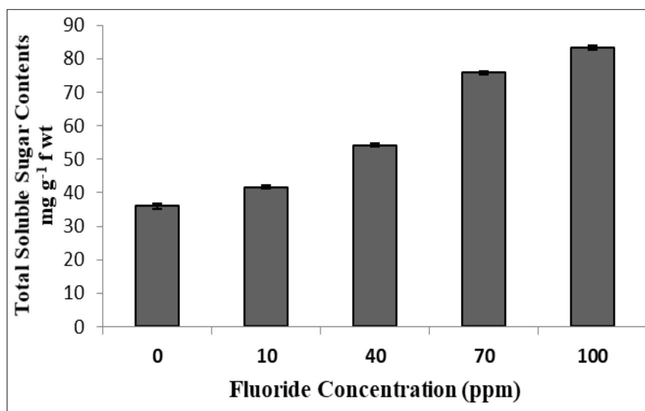

Fig 12: Fluoride effect on total soluble sugar of *Vigna radiata*

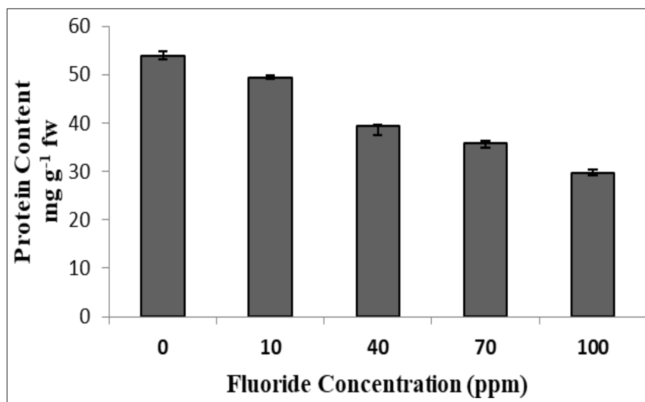

Fig 13: Fluoride effect on protein content of *Vigna radiata*

Discussion

High concentration of fluoride shows phytotoxic effect on morphological and biochemical properties of plants (Fornasiero, 2001: Sahariya *et al*, 2021) [16, 26]. Phytotoxic determining parameters also change due to effect of high fluoride concentration in soil or water (Stevens, 2000: Clausen, 2015) [34, 12]. Reduction in germination, root length, shoot length, and chlorophyll were observed due to unbalanced nutrient uptake at the seedling stage of *Vigna radiata* (Sharma *et al*, 2020) [29]. Decrease in fresh weight, dry weight and chlorophyll content because of declining metabolic activity in the presence of high fluoride concentration were reported by Kumar & Singh, 2015 [21] and Pawłowska, 2019 [25]. Fluoride acts as an inhibitor for the metabolic activities reported by during the germination process as a result protein content also declines. In plants during stress condition proline level increases for stability of the plant (Biczak *et al*, 2017) [6]. As well as proline, total soluble sugar, increase continuously with increasing concentration of fluoride content (Kant *et al*, 2006: Baunthiyal *et al*, 2014) [20, 5]. Total soluble sugar and proline increased level promote high tolerance ability in the plant during stress condition (Vardharajula *et el*, 2011: Asghari *et al*, 2020) [36, 3]. Under stress condition catalase activity decrease with increased fluoride concentration. SOD and peroxidase activity also increase gradually with increasing fluoride concentration (Banerjee & Roychoudhury, 2019: Singh & Roychoudhury, 2020) [4, 32].

Peroxidase slightly increase 0ppm to 70ppm fluoride consecetration but at 100ppm of fluoride concentration sharply decline. It reveals that plant under stress condition show peroxidase activity but at the high level plant reach at mortile phase.

Our results are in accordance with the findings of above mentioned authors. Therefore, on the basis of our study we can say that high concentration of fluoride shows negative effects on morphological and biochemical parameters of seedling growth. Statistically, also it was proved that all dependent variables were significantly affected with the increasing concentration of fluoride.

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