

STUDIES ON THE AQUEOUS FLUORIDE INDUCED STRESSES IN GROWTH AND PROTEASE ENZYME ACTIVITY OF AMARANTHUS DUBIUS

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Abstract

When individuals eat cabbage, carrots, spinach, tea, wheat and other Indian foods, fluoride is increasingly entering the human food and drink chain. Therefore, food fluoride concentration should not be ignored when estimating total fluoride intake. The amount of fluoride in food is influenced by the concentration of fluoride in soil and irrigation water. Therefore, utilizing sodium fluoride with different concentrations in the water used to irrigate the plant *Amaranthus dubius*, the current study evaluated the induced effects of water-soluble fluoride on protease enzyme activities from leaves, stems, roots and seeds. The findings demonstrated that on the 55th day of growth, *Amaranthus dubius* irrigated with 50 ppm sodium fluoride had a high amount of protease (4.82 $\mu\text{g}/\text{mg}/5\text{min}$) in its leaves and a lesser amount (0.06 $\mu\text{g}/\text{mg}/5\text{min}$) in its roots of the control, which receives only water. The amount of protease increases when concentration of sodium fluoride increases. Protease levels ($\mu\text{g}/\text{mg}/5\text{min}$) in the leaves of all experimentally challenged *Amaranthus dubius* ranged from 0.06 $\mu\text{g}/\text{mg}/5\text{min}$ to 4.82 $\mu\text{g}/\text{mg}/5\text{min}$ (lowest in roots to maximum in leaves) on an expected time scale of 15 to 55 days.

Keywords: *Amaranthus dubius*, concentration, exposure, enzyme, fluoride, protease.

Introduction

Fluoride is a frequent, non-biodegradable and highly reactive environmental pollutant, as is well known. An ecosystem's fluoride (F^-) level has risen during the last 20 years as a result of numerous natural and man-made processes (Fuge 2019). Apatite, biotite, cryolite, epidote, fluorite, fluorapatite, fluormica, hornblende, scheelite, topaz and tremolite are some of the primary natural resources of F^- that can be found in soil and water. Examples of atmospheric emissions that naturally release F^- into the atmosphere include volcanic eruptions and rock weathering. Jha et al. (2011) and Gadi et al. (2016) claim that there is a current surge in anthropogenic activities associated with industry and agriculture. These activities either directly contribute to the addition of F^- through the use of phosphate fertilizers, pesticides and irrigation with fluorinated water or indirectly through air emissions from burning coal, refining oil, making bricks, making aluminum and other industries. Low levels of F^- encourage the calcification of hard tissues and help avoid dental cavities. However, excessive exposure levels, ingestion and accumulation of F^- in humans and animals cause skeletal, non-skeletal and dental fluorosis (which manifests as neurological and brain damage, infertility and gastrointestinal and urinary problems) (Shahab et al. 2017; Choubisa 2018). The Indian Standards (IS) and World Health Organization (WHO) have recommended levels of F^- in drinking water of 1.5 and 1.0 mg L^{-1} respectively.

In addition to humans (Choubisa 2001, Choubisa 2012), animals (Choubisa 1999, Choubisa 2013) and other species (Weinstein et al., 2004) prolonged exposure to fluoride (F^-) has numerous negative effects. Plant physiology is primarily impacted by F^- contaminated dust, water, soil and gases through altered leaf physiology. The concentration of F^- as well as the duration and frequency of F^- exposure determine the severity of the signs of F^- damage to plants, which can be either acute or chronic (Choubisa et al., 2013). When fluorine gradually permeates the subcellular components of plants, it changes the metabolic pathways that are sensitive to fluorine, making them toxic to the element. Extended exposure to F^- is linked to observable damage to the folia. Additionally, fluoride buildup hindered photosynthesis. The main ways that F^- affects photosynthesis are by preventing the Hills reaction, breaking down chloroplasts and reducing the synthesis of chlorophyll. The amount of chlorophyll is also decreased and the plant's photosynthetic system is weakened. Eventually, these resulted in less CO_2 being produced and absorbed (Yamauchi et al., 1983, Domingues et al., 2011). The enzyme protease activity in IR-64 was marginally elevated by increasing fluoride concentrations (Banerjee Aditya et al., 2019).

Numerous studies have been published on F^- uptake and how it affects different plant species. Conversely, little is known regarding the effects of F^- poisoning on the protease activity in the leaves, stems, roots and seeds of plants that are commonly cultivated by Indian farmers in this region. Therefore, the purpose of this study was to understand and assess how fluoride poisoning in water affects the enzyme protease activity in the important crop *Amaranthus dubius*. The study's findings are important and useful for academics, farmers and agricultural experts.

Materials and Methods

Red lettuce cultivars from *Amaranthus dubius* seeds were used in the experiment under natural soil bed and weather circumstances; certified seeds were obtained from the agriculture department of the Tamilnadu government in Tirunelveli. NaF crystals were dissolved in distilled water to create a stock solution of 1000 mg/L. Seven plastic pots measuring eight inches in diameter were chosen for this study. 500 grams of combined cow dung and 4,000 grams of rich soil were added to each pot, which was then left for three days. After being moist for eight hours, twenty verified *Amaranthus dubius* seeds were planted in each pot with adequate space between them. 50 milliliters of purified water and 50 milliliters of sodium fluoride solutions at varying concentrations 1, 2, 5, 10, 25, and 50 ppm were used to irrigate the plants in the morning for the control and treatment samples. Each therapy continued for fifty-five days. The germination process was completed in fifteen days. Measurements of the fluoride intake, height, fresh weight and number of leaves on the control and treated samples were made every five days after two saplings were safely removed from these pots. After 55 days, the experiment was terminated and the quantity of seeds extracted from the plants that were harvested was recorded.

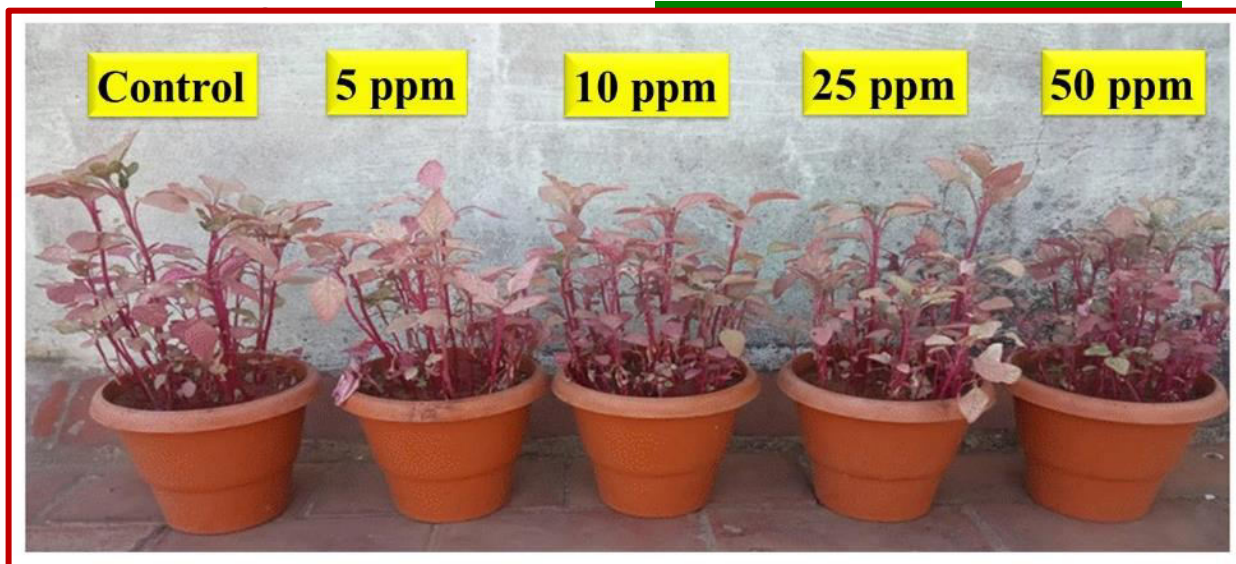


Fig.1 In pot studies, the enzyme protease activity was investigated using different sodium fluoride concentrations, including control, 5 ppm, 10 ppm, 25 ppm and 50 ppm on *Amaranthus Dubius*.

Data Analysis

Fluoride intake and translocation factor:

The roots and stems of plants were separated and dissolved in varying amounts of 0.1 M perchloric acid. An ion-selective electrode was used to test for water extractable fluoride in plant roots and stems. The translocation factor (TF) of F^- in the *Amaranthus Dubius* was calculated using the formula below (Iram et al. 2016).

$$TF = (C_{Stem}/C_{Root})$$

Where C_{Stem} = fluoride concentration in the stem of the plant (mg/kg) and

C_{Root} = fluoride concentration in the roots of the plant (mg/kg)

Table 1 Impact of varying sodium fluoride concentrations (mg/Kg) on *Amaranthus dubius* plant roots and stems.

Analysing data	Control*	1 ppm	2 ppm	5 ppm	10 ppm	25 ppm	50 ppm
Root (F mg/Kg)	NA	4.141	4.918	5.516	6.476	8.826	11.206
Stem (F mg/Kg)	NA	2.031	2.482	3.344	4.139	6.124	8.568
Translocation factor	-	0.4904	0.5046	0.6062	0.6391	0.6915	0.7645
Number of seeds	231	211	202	183	159	137	116

*-NA – Not applicable

Analysis of protease enzyme activity

Preparation of crude enzyme extract

The root, leaf, stem and seed of *Amaranthus dubius* collected were crushed separately using 20mM phosphate buffer (pH 7.5), 0.1% tween 20 detergent and protease cocktail inhibitor. The obtained crude pulp was on centrifugation in a table top centrifuge machine under cooled condition at 10,000 rpm for 10 minutes at 4°C. 40% ammonium sulphate was

added to the crude soup obtained; protein precipitated. The precipitate was dissolved by adding 20 mM tris buffer and used it for further analysis.

Estimation of protease activity assay (Folin and Ciocalteu, 1927)

The substrate in this test was β -casein. Tyrosine and other peptide fragments are released when protease breaks down casein. The amount of tyrosine released following the proteolytic activity is calculated using a tyrosine standard calibration curve. The 0.18 mg/mL tyrosine stock solution was mixed with deionized water to create a range of tyrosine standard solutions at various concentrations (5–50 μ g/mL). The protease activity was also measured by mixing 1 milliliter of the extract with 1 milliliter of pure casein hydrolysate (20 mg mL⁻¹). The mixture was incubated for 30 minutes at 30 degrees before the assay was prepared in compliance with Banerjee et al. (2019a).

Estimation of protease activity (μ g/mg/5min) from leaf of *Amaranthus dubius*

The findings demonstrated that on the fifteenth day of growth, *Amaranthus dubius* watered with 1 ppm of sodium fluoride (1.04 μ g/mg/5min) and *Amaranthus dubius* watered with 2 ppm of sodium fluoride (1.25 μ g/mg/5min) had the highest levels of protease activity in their leaves, followed by *Amaranthus dubius* (0.92 μ g/mg/5min) that received only water (control). The *Amaranthus dubius* watered with 50 ppm sodium fluoride showed a significant difference from the control on the 55th day of growth (3.74 μ g/mg/5min - 4.82 μ g/mg/5min of protease activity).

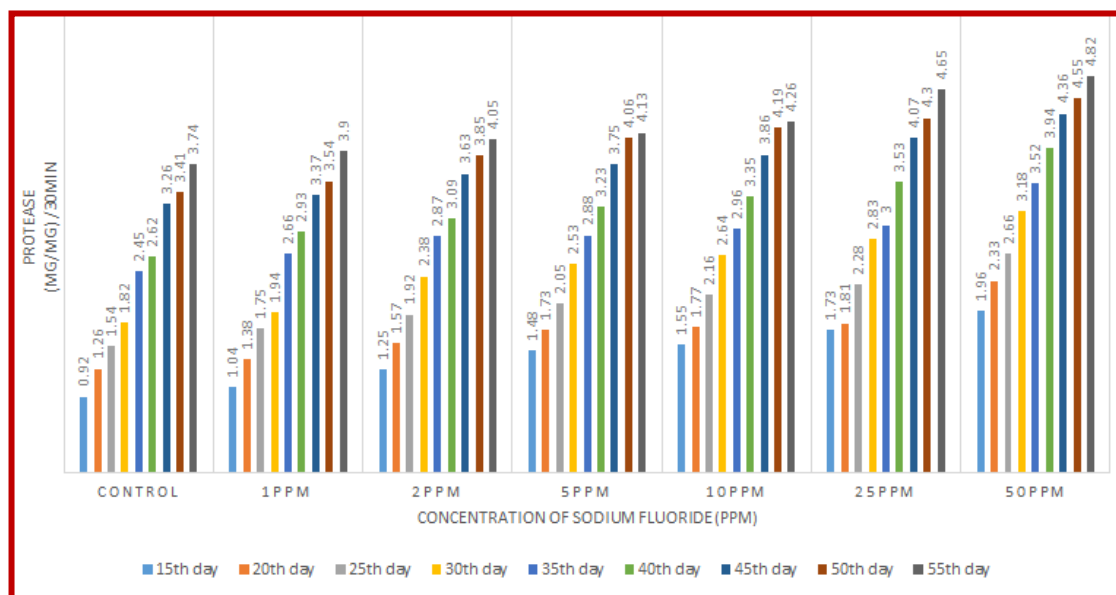


Fig.2 Protease activity (μ g/mg/5min) from the leaves of *Amaranthus dubius* watered with various concentrations of sodium fluoride and harvested on various days

Protease activity rises with increasing sodium fluoride concentration in every experimental setup. With each day of plant growth, the rate of protease activity likewise rises. Table 2 and figure 2 present the findings.

Table 2 Protease activity (μ g/mg/5min) from the leaves of *Amaranthus dubius* watered with various concentrations of sodium fluoride and harvested on various days

Days	Concentrations of sodium fluoride (ppm)						
	Control	1ppm	2ppm	5ppm	10ppm	25ppm	50ppm
15	0.92	1.04	1.25	1.48	1.55	1.73	1.96
20	1.26	1.38	1.57	1.73	1.77	1.81	2.33

25	1.54	1.75	1.92	2.05	2.16	2.28	2.66
30	1.82	1.94	2.38	2.53	2.64	2.83	3.18
35	2.45	2.66	2.87	2.88	2.96	3.00	3.52
40	2.62	2.93	3.09	3.23	3.35	3.53	3.94
45	3.26	3.37	3.63	3.75	3.86	4.07	4.36
50	3.41	3.54	3.85	4.06	4.19	4.30	4.55
55	3.74	3.90	4.05	4.13	4.26	4.65	4.82

Estimation of protease activity ($\mu\text{g}/\text{mg}/5\text{min}$) from stem of *Amaranthus dubius*

According to the study's findings, *Amaranthus dubius* showed low levels of protease activity ($0.08\mu\text{g}/\text{mg}/5\text{min}$) in its stems on the fifteenth day when it was administered only water (control). Next came *Amaranthus dubius*, which on the fifteenth day of growth was watered with 1 ppm of sodium fluoride ($0.12\mu\text{g}/\text{mg}/5\text{min}$) and *Amaranthus dubius*, which was watered with 2 ppm of sodium fluoride ($0.18\mu\text{g}/\text{mg}/5\text{min}$). Remarkably, on the 55th day of plant growth, there was a notable difference between the control and the *Amaranthus dubius* that was irrigated with 50 ppm sodium fluoride ($0.98\mu\text{g}/\text{mg}/5\text{min}$ - $2.27\mu\text{g}/\text{mg}/5\text{min}$ of protease activity). In every experimental configuration, the rate of protease activity rises with each day of plant growth and the amount of protease activity increases as sodium fluoride concentration rises. Table 3 and figure 3 present the findings.

Table 3 Protease activity ($\mu\text{g}/\text{mg}/5\text{min}$) from *Amaranthus dubius* stems that were watered with different sodium fluoride concentrations and harvested on different days

Days	Concentrations of sodium fluoride (ppm)						
	Control	1ppm	2ppm	5ppm	10ppm	25ppm	50ppm
15	0.08	0.12	0.18	0.27	0.36	0.44	0.55
20	0.25	0.26	0.35	0.46	0.62	0.95	1.05
25	0.32	0.35	0.47	0.52	0.84	1.02	1.17
30	0.46	0.48	0.55	0.65	0.96	1.13	1.33
35	0.53	0.55	0.62	0.74	1.12	1.28	1.57
40	0.68	0.77	0.84	0.93	1.25	1.35	1.83
45	0.75	0.82	0.95	1.16	1.37	1.58	2.06
50	0.84	1.05	1.15	1.35	1.51	1.83	2.14
55	0.98	1.12	1.24	1.45	1.68	2.02	2.27

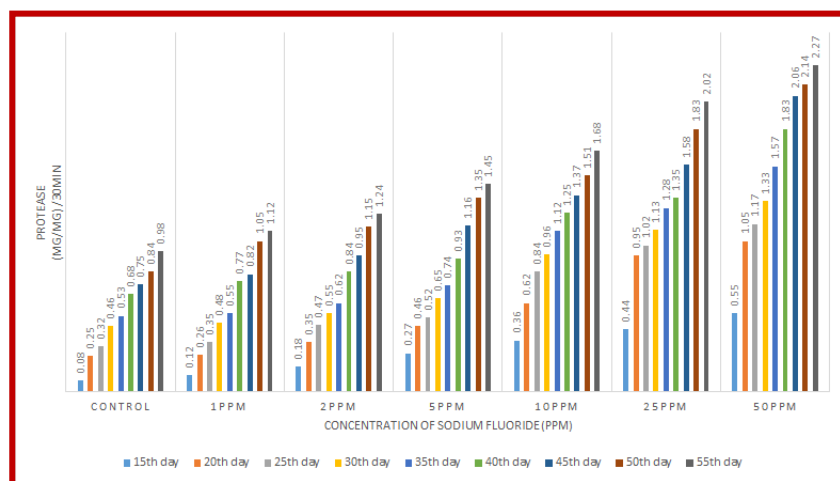


Fig.3 Protease activities ($\mu\text{g}/\text{mg}/5\text{min}$) from *Amaranthus dubius* stems that were watered with different sodium fluoride concentrations and harvested on different days

Estimation of protease activity ($\mu\text{g}/\text{mg}/5\text{min}$) from root of *Amaranthus dubius*

Amaranthus dubius exhibited low levels of protease activity ($0.06\mu\text{g}/\text{mg}/5\text{min}$ – $0.63\mu\text{g}/\text{mg}/5\text{min}$) in all experimental roots on the fifteenth day of the study and the amount rose as the number of days increased. Significantly different from the control, *Amaranthus dubius* watered with 50 ppm sodium fluoride on the 55th day of growth exhibited the highest protease activity ($2.06\mu\text{g}/\text{mg}/5\text{min}$) followed by 25 ppm sodium fluoride ($1.85\mu\text{g}/\text{mg}/5\text{min}$). The amount of protease activity rises with increasing sodium fluoride concentration in all experimental setups and the rate of protease activity likewise rises with each day of plant growth. Table 4 and figure 4. document the results.

Table 4 Protease activity ($\mu\text{g}/\text{mg}/5\text{min}$) from the roots of *Amaranthus dubius* watered with various concentrations of sodium fluoride and harvested on various days

Days	Concentrations of sodium fluoride (ppm)						
	Control	1ppm	2ppm	5ppm	10ppm	25ppm	50ppm
15	0.06	0.08	0.13	0.17	0.36	0.57	0.63
20	0.09	0.12	0.25	0.33	0.45	0.76	0.96
25	0.13	0.20	0.36	0.48	0.58	0.83	1.03
30	0.20	0.26	0.47	0.55	0.63	1.07	1.15
35	0.29	0.32	0.56	0.69	0.81	1.16	1.33
40	0.35	0.46	0.64	0.73	0.95	1.38	1.49
45	0.42	0.53	0.72	0.86	1.07	1.52	1.62
50	0.56	0.69	0.96	1.05	1.18	1.70	1.89
55	0.78	0.93	1.03	1.16	1.27	1.85	2.06

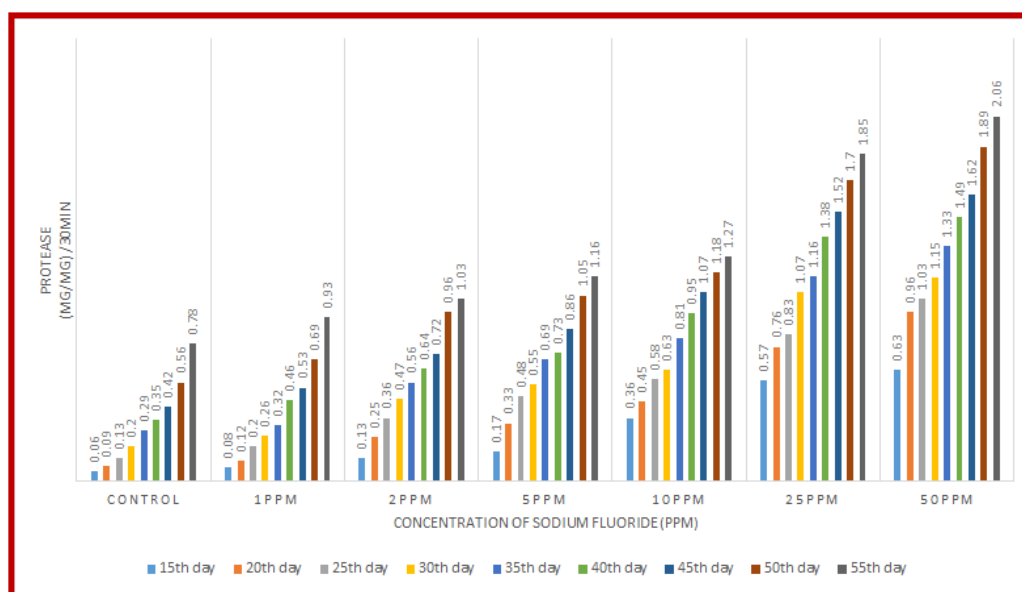


Fig.4 Protease activity ($\mu\text{g}/\text{mg}/5\text{min}$) from the roots of *Amaranthus dubius* watered with various concentrations of sodium fluoride and harvested on various days

Estimation of protease activity ($\mu\text{g}/\text{mg}/5\text{min}$) from seed of *Amaranthus dubius*

Protease activity ($\mu\text{g}/\text{mg}/5\text{min}$) was estimated from the seeds gathered from every experimental setup and the findings are shown in table 5 and figure 5. Protease activity was strong ($3.12 \mu\text{g}/\text{mg}/5\text{min}$) in *Amaranthus dubius* seeds that were irrigated with 50 ppm sodium fluoride. While the plant did not get sodium fluoride throughout the entire research, the control group's protease activity was modest ($2.0 \mu\text{g}/\text{mg}/5\text{min}$). Protease activity rises in proportion to sodium fluoride concentration.

Table 5 Protease activity ($\mu\text{g}/\text{mg}/5\text{min}$) from the seeds of *Amaranthus dubius* watered with various concentrations of sodium fluoride

S.no	Concentration of sodium fluoride (ppm)	protease activity ($\mu\text{g}/\text{mg}/5\text{min}$)
1	Control	2.00
2	1ppm	2.23
3	2ppm	2.46
4	5ppm	2.53
5	10ppm	2.65
6	25ppm	2.86
7	50ppm	3.12

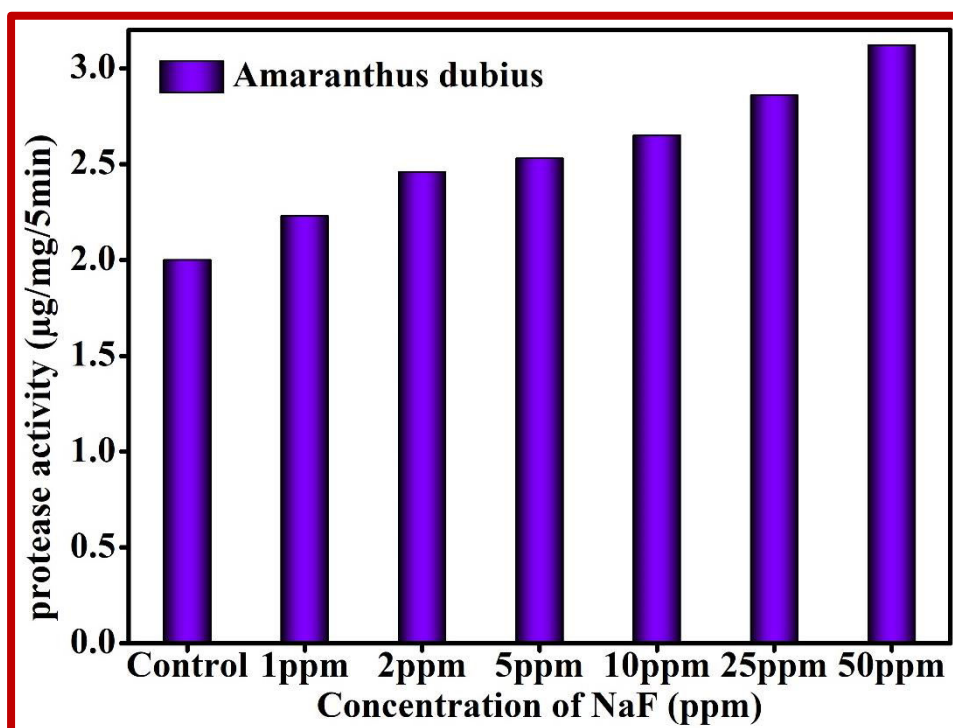


Fig.5 Protease activity ($\mu\text{g}/\text{mg}/5\text{min}$) from the seeds of *Amaranthus dubius* watered with various concentrations of sodium fluoride

Table 6 Other parameters of *Amaranthus dubius* watered with various concentrations of sodium fluoride

NaF (ppm)	Root length (cm)	Number of leaves	Height (cm)	Biomass (g)
Control	20	20	30.2	7.32
50 ppm	10.8	10	20.2	6.19

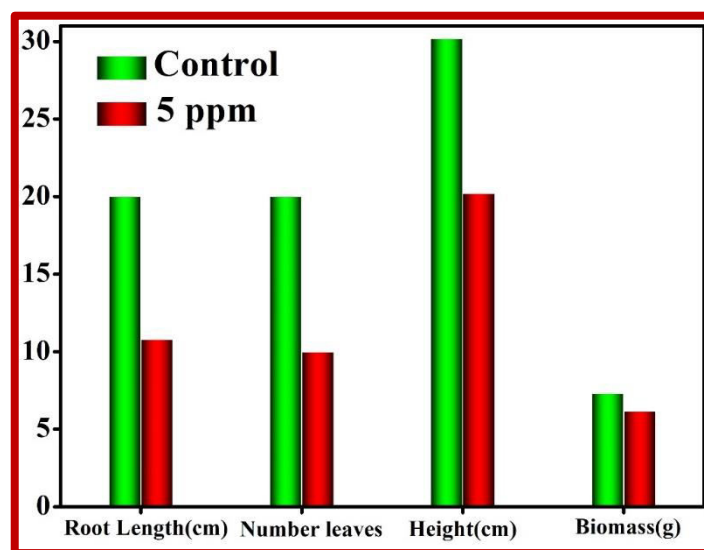


Fig.6 Other parameters of *Amaranthus dubius* watered with various concentrations of sodium fluoride

Results and Discussions

The effects of fluoride toxicity on height, fresh weight, number of leaves, seed yield and fluoride uptake in *Amaranthus dubius* were investigated for control and various concentrations of sodium fluoride, ranging from 1, 2, 5, 10, 25, and 50 ppm. Fig. 1 shows the results of the fluoride toxicity pot experiments conducted on *Amaranthus dubius*. The control group had the highest results, while the greater concentration of NaF (50 ppm) produced the lowest outcomes in all aspects except fluoride uptake.

The additional parameters of *Amaranthus dubius* irrigated with different sodium fluoride concentrations are displayed in table 6 and figure 6. The control plants exhibited the maximum height (cm), maximum number of leaves per plant and higher fresh weight (g)/plant, whereas the plants treated with 50 ppm concentrated NaF showed the lowest results. The control group exhibited the highest seed yield while the group treated with 50 ppm NaF showed the lowest seed yield.

Fluoride uptake and translocation factor

Plants do not really need the fluoride chemicals. The findings of earlier research on fluoride intoxication in plants showed that eating of high fluoride compound concentrations was associated with this condition. As shown in Fig. 7, the fluoride uptake in *Amaranthus dubius* has risen with increasing NaF concentrations of 1 to 50 ppm.

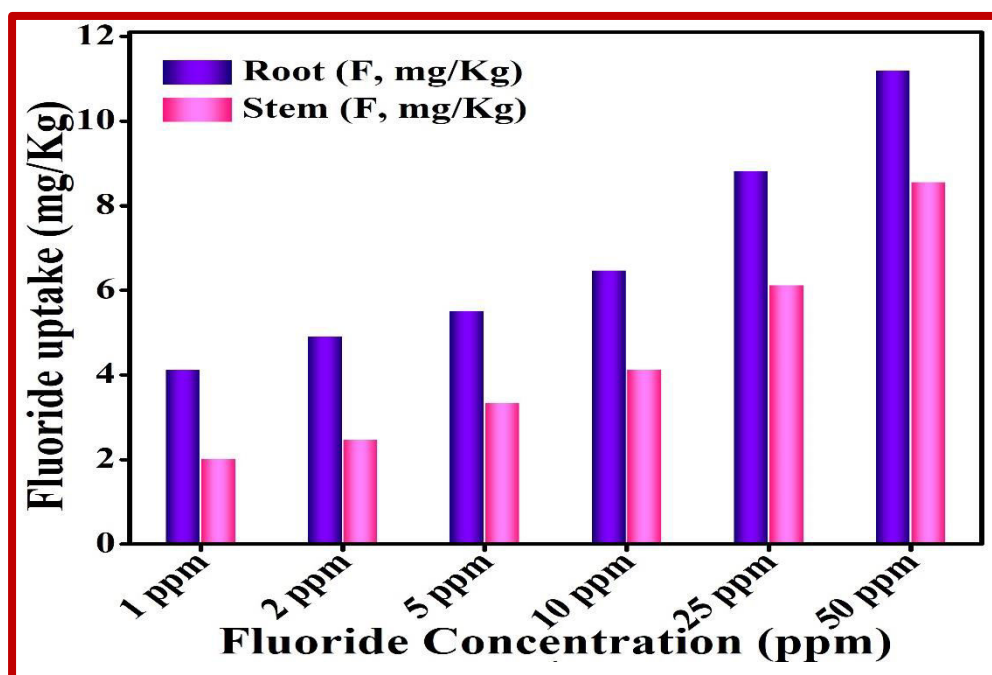


Fig.7 Fluoride uptake analysis of *Amaranthus dubius* roots and stem by using various concentration of sodium fluoride such as 1ppm, 2ppm, 5ppm, 10ppm, 25ppm and 50 ppm. At varying concentrations of 1, 2, 5, 10, 25, and 50 ppm sodium fluoride solutions, the root part's fluoride uptake ranged from 4.141, 4.918, 5.516, 6.476, 8.825, and 11.206 mg/kg, while the stem part's results ranged from 2.031, 2.482, 3.344, 4.139, 6.124, and 8.568 mg/kg. The translocation factor for the *Amaranthus dubius* plant ranges from 0.4904 to 0.7645, as shown in Table 1. The findings showed that the root portion's uptake of fluoride

Analysis of protease enzyme activity ($\mu\text{g}/\text{mg}/30\text{min}$) in leaf, stem, root and seed of *Amaranthus dubius* watered with various concentrations of sodium fluoride

The results of the study show that *Amaranthus dubius* showed minimal levels of protease activity ($\mu\text{g}/\text{mg}/5\text{min}$) in its leaves, stems, roots and seeds when it was administered only water (control). Comparatively, on the 55th day of growth, there was a significant difference between the control and the *Amaranthus dubius* that was watered with 50 ppm of sodium fluoride. In all experimental setups, protease activity increases as sodium fluoride concentration rises. The protease activity is high in the leaves. The study also suggests that when the amount of sodium fluoride in the water used to irrigate the plant rises, the protein content would decrease. These findings were supported by the findings of Debska et al. (2012) who stated that the over abundance of fluoride in plant tissues has been demonstrated to protease activity increases during abiotic stress, resulting in proteolytic degradation and protein inactivity under stress circumstances. Reactive oxygen species (ROS) production typically declines under stressful environments, including pathogen attacks, illnesses, toxicity and food shortages. This results in oxidative alterations of macromolecules such proteins, carbohydrates, unsaturated fatty acids and DNA (Tola et al., 2021).

Conclusions

Fluoride can affect plant protease activity by boosting lipoxxygenase and protease activity, which speeds up protein breakdown. This is due to the production of reactive oxygen

species like hydrogen peroxide and superoxide radical that are triggered by fluoride toxicity. The significance of the results of this study for safe agriculture is mainly to avoid the use of groundwater containing high amounts of F^- for spinach cultivation. To ensure the safety and quality of the entire food chain, *Amaranthus dubius* seedlings should not be grown in regions with high soil F^- content or in groundwater infested with F^- .

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