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ORIGINAL ARTICLE

Growth Attributes and Pigmentation of Fenugreek Under Fluoride Toxicity

Jyotsana Bhati[1]*, Rekha Vijayvergia[1] and Anil Kumar[1]

[1] *Department of Botany, University of Rajasthan, Jaipur 302004, India*

*E-Mail: ***jyotsnasbhati@gmail.com***

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Indiscriminate and exorbitant use of chemicals and other xenobiotics like fluoride (F) is increasing in everyday life. The present investigation aimed to assess the phytotoxic effects of F on fenugreek (*Trigonella foenum-graecum*). Seeds of fenugreek were treated with 2.5 mM, 5 mM, 7.5 mM, and 10 mM concentrations of F. Toxic effect of F was observed as a decrease with an overall poor health of the seedlings. The highest applied concentration of F (10 mM) was toxic to the extent that seeds were not germinated. After a slight increase, a reduction in the root-shoot length, root-shoot biomass, number of nodules, and fresh and dry weight of nodules was observed with increasing concentrations of F. Furthermore, leaf area and relative water content in the leaf showed a progressive reduction with an increment in F concentration. Plants treated with F showed that pigments like chlorophyll a, chlorophyll b, and total chlorophyll were decreased under F toxicity. Detrimental impacts of F toxicity were found to be highly significant *(p=0.001)* for nodule dry weight, leaf area, chlorophyll a, and, total chlorophyll content.

Key words: Anthocyanin, chlorophyll contents, fluoride toxicity, growth, Trigonella foenum-graecum

Fluorine is the 13th most abundant element in the crust of our planet. It is a useful non-metal in pharmaceuticals, agrochemicals, and other industries as well as for organisms, especially humans. It is crucial for the mineralization of bones and the formation of dental enamel, therefore, added to toothpaste, mouthwashes, or other dental products and in water supplies. However, F shows adverse impacts on organisms above the threshold. The permissible concentration of F in the water varies according to different agencies i.e. 1.5 ppm according to the World Health Organization (WHO) and 1 ppm according to the Indian Council of Medical Research (ICMR). Fluorosis caused by the excess of F is endemic to at least 25 nations, including India (UNICEF). Fluoride can be enriched in soils through natural or anthropogenic sources such as rock weathering, phosphate fertilizers, coal combustion, etc. (Kumar, 2020). Increased concentration of F disrupts soil structure and brings changes in physico-chemical parameters such as soil pH which may not be suitable for particular plants or soil enzymes. Natural fluoride in most soils ranges from about 10–1000 ppm but only soluble and exchangeable fluoride fractions in soil are biologically important for plants (Kamaluddin and Zwiazek, 2003). Although fluorine is not an essential element for plant growth (Mackowiak *et al.,* 2003), physiological anomalies and crop yield reduction are consequences of F toxicity which creates oxidative stress and influences cellular metabolism and homeostasis (Banerjee and Roychoudhury, 2020). F decreases agricultural harvests by half as most crop plants are highly sensitive to it (Pelc *et al.,* 2020).

Fenugreek (*Trigonella foenum-graecum* L.) is a herbaceous, annual plant belonging to the family Fabaceae. It is a highly sensitive plant to F (Ambika and Sumalatha, 2005). India is the native and largest producer of the plant and the state of Rajasthan accounts for 80% of fenugreek production in India. This plant is used as a vegetable, condiment, and supplement in cereals. It is also an ingredient in traditional medicine. It is used in ailments related to the digestive, respiratory, and reproductive systems. Remedy against high cholesterol levels, inflammation, gastrointestinal ailments, hypertension, sexual problems and rheumatism, stomach disorders, fever, anemia, and constipation, and for stimulation of appetite, and as a hypocholesterolemic (Abdel-Barry *et al.,* 1997).

Keeping the above facts of crop production, profitability, food security, and human health of useful plants in view, the effects of different concentrations of NaF on the growth performance, biomass yield, and pigment synthesis in fenugreek grown in F-contaminated soil were studied in the present investigation.

MATERIALS AND METHODS

The experimental plant *Trigonella foenum-graecum* L. is one of the important legumes of this region. Seeds of certified variety RMT-305 were obtained from the Rajasthan Agricultural Research Institute, Durgapura, Jaipur. The experiment was set up during November at the Department of Botany, University of Rajasthan, Jaipur where the photoperiod was 10 h and the average temperature was 29.1°C. One and a half kg of soil was filled in pots. Homogenized soil was treated with sodium fluoride (NaF) with concentrations of 2.5, 5, 7.5, and 10 mM. The different concentrations were prepared separately by taking the corresponding amount (calculated based on molecular weight) of chemicals per kilogram of air-dried soil. No other supplement nutrients were applied to the soil. The experimental soil was sandy loam type with a slightly alkaline pH (7.12). The physicochemical characteristics of the experimental soil are presented in Table 1. Pots without added F constituted the control. The experiment was replicated three times. Seeds of uniform size and weight of fenugreek were surface sterilized, and soaked in water. 10 seeds were sown equidistantly at 1.5 cm deep in each pot. Watering (50 mL) was done on alternate days. Pots were maintained in uniform conditions of humidity and temperature. After establishment, seedlings were thinned to 6 in each pot. After 30 days, trifoliate leaf tissue was analyzed for pigment contents. Plants were harvested after 40 days for biomass and nodules were counted. Nodules were dried at 50°C in an oven for 48 h for the measurements of dry weight of nodules.

Estimation of pigment contents

Chlorophyll a, b, total chlorophyll, and carotenoids

Chlorophyll a, b, total chlorophyll, and carotenoids were determined after the method of Arnon (1949) using acetone as an extracting agent.

Anthocyanin

Anthocyanin estimation was carried out by the method of Mehrtens *et al.* (2005).

Determination of leaf area

The leaf area was determined by the weight method as described by Kumar and Aery (2023).

Determination of relative water content (RWC)

RWC in the leaf was determined by the method described by Mali and Aery (2008).

Statistical analysis

The data were analyzed for determining the one-way analysis of variance (ANOVA) using OriginPro software and graphs were prepared using MS Excel. The standard deviation, coefficient of determination (R^2), and regression equation (Y) were also computed. Significant differences in means were established by Tukey's post hoc test *($p<0.05$).* All data are expressed as means of three biological replicates.

RESULTS

The application of F to the soil resulted in a downturn in the growth aspects of the fenugreek plant (Fig. 1). The Highest applied concentration of F (10 mM) was toxic to the level, rendering the soil inhospitable for seed germination. After a slight increase at 2.5 mM of F concentration, maximum decrement in root and shoot length was recorded at 7.5 mM of F concentration which was 19.59% and 40.17% lower, respectively, than the control (Fig. 2). Similarly, at first, plant biomass exhibited an uptick then it witnessed a downturn. Minimal levels of root and shoot biomass were noted at 7.5 mM of F and were 64.43% and 31.52% lower respectively, when compared to the control (Fig. 2).

Root nodules were also observed in the present investigation as fenugreek is a leguminous plant. The maximum number of nodules was found to be highest for the concentration of F at 2.5 mM, which was 53.59% higher, in comparison to the control (Fig. 2). After reaching a peak at 2.5 mM, the number of nodules again decreased and only slightly varies from 5 mM to 7.5 mM. A similar trend was recorded in the case of fresh and dry weight of nodules. After an increase of 27.92% at 2.5 mM of F concentration, the reduction was recorded. Minimal decrement in fresh and dry weight of nodules at 7.5 mM of F concentration was 41.52% and 59.64% lower, respectively, as compared to the control (Fig. 2).

Additionally, RWC in the leaf progressively declined with increasing concentrations of NaF (Fig. 2). RWC was decreased by 2.82% for 2.5 mM of soil F while higher concentrations of soil F (7.5 mM) resulted in a 14.82% decrease for RWC in the leaves, as compared to the control.

When compared with the control plants, leaf area (LA) was also steadily lessened (Fig. 2). For LA, the most significant drop was found at the 7.5 mM F concentration, which was 54.70% lower, as opposed to the control.

Under the influence of F exposure, a decline in pigments was also documented. In the present study, the chlorophyll contents were observed to be the lowest at 7.5 mM of the applied dose of F and showed 84.33%, 56.16%, and 77.71% reduction over the control for chlorophyll a, chlorophyll b, and total chlorophyll respectively (Fig. 3).

Other accessory pigments like anthocyanin and carotenoids initially exhibited a surge and reduced subsequently. In this experiment, reduction at 7.5 mM of F concentration was 86.68% for carotenoids against the control (Fig. 3). Furthermore, anthocyanin was recorded minimum at 7.5 mM of F concentration which was 20.65% lower than the control (Fig. 3). Adverse impacts of excessive fluoride were found to be significant *($p=0.05$)* for shoot length, root biomass, nodule fresh weight, and carotenoids content, while it was highly significant *($p=0.001$)* for nodule dry weight, leaf area, chlorophyll a, and, total chlorophyll amount.

Table 1: Characteristics of the experimental soil.

Parameter	Value
pH	7.2
Organic carbon (%)	0.3
Conductivity (µS/cm)	65.7
Total dissolved solids (ppm)	31.16
Salinity (psu)	0.03
Resistivity (Ω)	15.63
Total K (kg h^{-1})	178
Total P (kg h^{-1})	86
Total S (mg kg^{-1})	15.7
Total Zn (mg kg^{-1})	0.64
Total Fe (mg kg^{-1})	4.60
Total Cu (mg kg^{-1})	0.32
Total Mn (mg kg^{-1})	2.20

Figure 1. Effect of different F concentrations on growth performance of *T. foenum-graecum*.

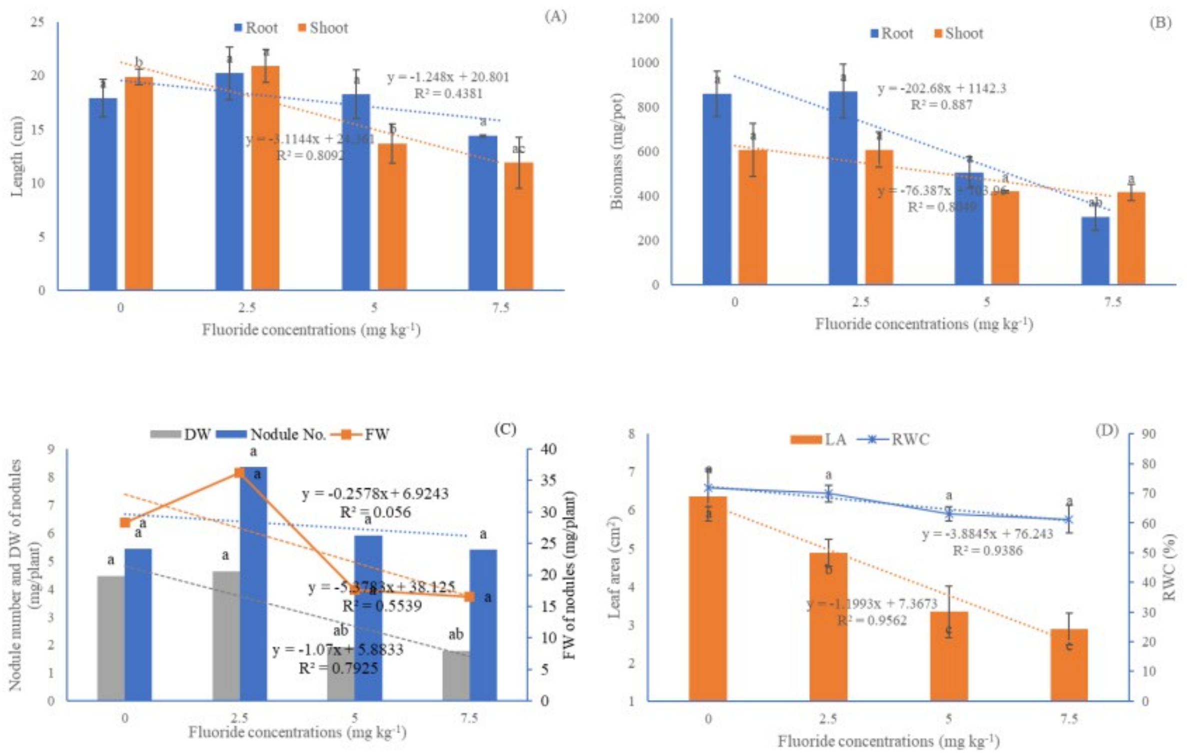

Figure 2. Effect of different F concentrations on growth and biomass production of *T. foenum-graecum* (A) Root-shoot length (B) Root-shoot biomass (C) Number, fresh weight, and dry weight of nodules (D) Leaf area and relative water content. Different letters over bars and lines indicate significant difference between treatments according to Tukey's test ($p<0.05$). The dotted lines show linear regression between applied F doses and the parameter studied. (Error bars showing the standard deviation)

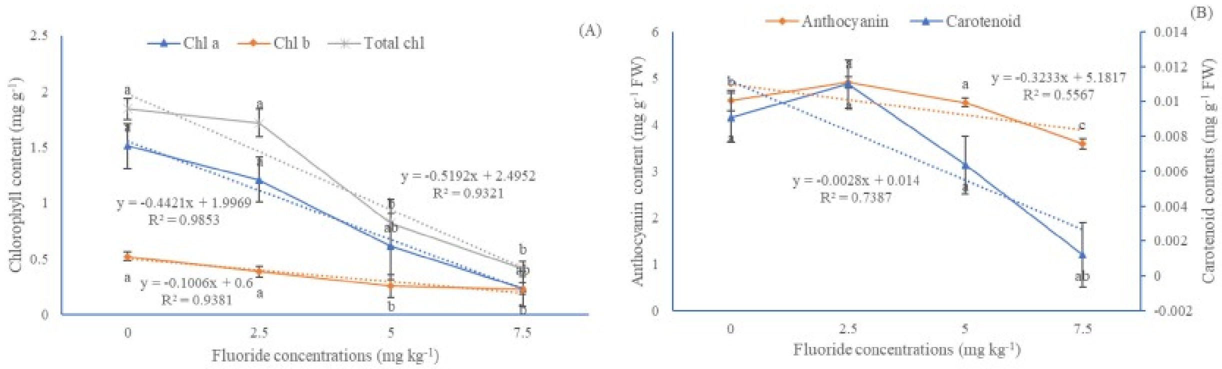

Figure 3. Effect of different F concentrations on photosynthetic and accessory pigments of *T. foenum-graecum* (A) Chlorophyll a, chlorophyll b, and total chlorophyll contents (B) Anthocyanin and carotenoid contents. Different letters over lines indicate significant difference between treatments according to Tukey's test ($p<0.05$). The dotted lines show linear regression between applied F doses and the parameter studied. (Error bars showing the standard deviation)

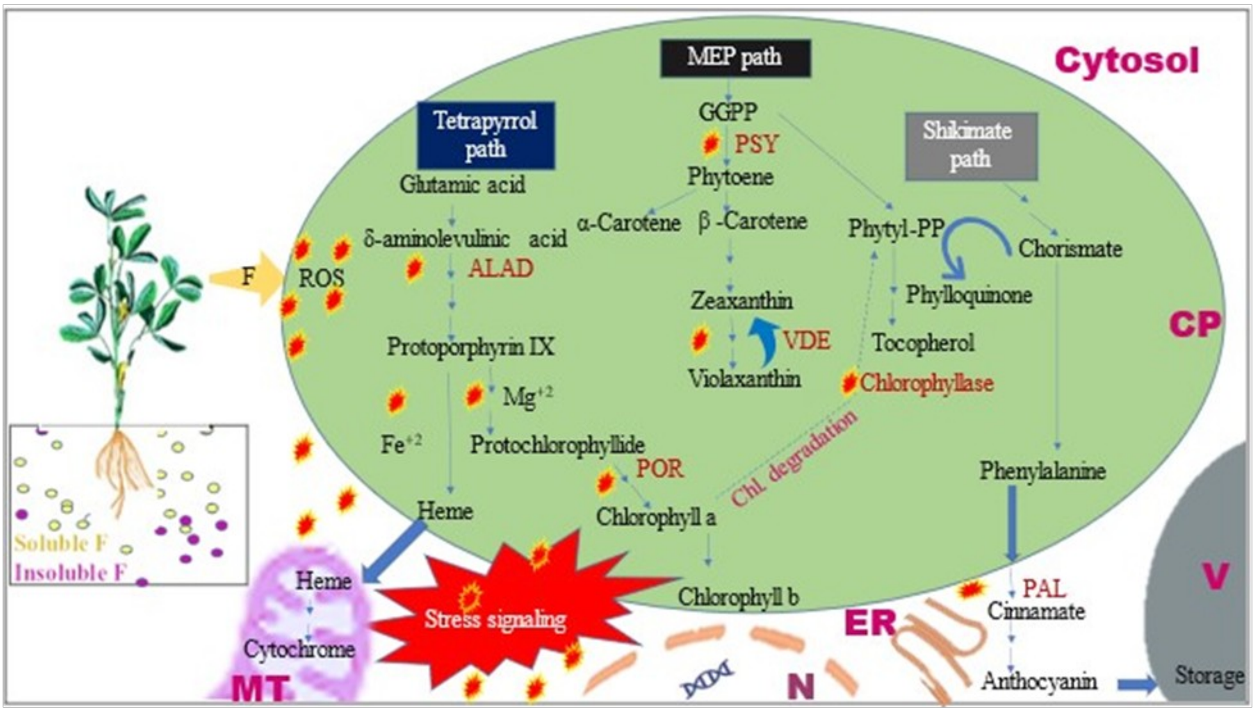

Figure 4. Mechanism of F toxicity and ROS on photosynthetic and accessory pigments. ALAD: δ-aminolevulinic acid dehydratase; POR: Protochlorophyllide oxidoreductase; MEP: Methylerythritol 4-phosphate; GGPP: Geranylgeranyl diphosphate; PSY: Phytoene synthase; VDE: Violaxanthin de-epoxidase; Phytyl pp: Phytyl diphosphate; PAL: Phenylalanine ammonia-lyase; CP: Chloroplast; MT: Mitochondria; N: Nucleus; ER: Endoplasmic Reticulum; V: Vacuole

DISCUSSION

Water-soluble forms of fluorine can combine with sodium in clay and soil and the resultant sodium fluoride (NaF) can influence physiological, biochemical, and structural activities in plants (Bhat *et al.,* 2023). The tolerance of plant species to different concentrations of fluorine compounds varies with soil F, duration of the exposure, soil pH, the dominant wind direction, vegetation structure, plant species, variety, growth conditions, age, or plant parts of the same species.

Fluoride accumulation in soil eventually diminishes agricultural production due to the lowering of growth or biomass production. F also adversely affects plant growth and yield and root and shoot lengths, number and size of leaves, and seed set as reported in radish (Singh *et al.,* 2013). The yield loss of soybean reached 30% at 375 mg kg^{-1} or more F levels (Bustingorri *et al.,* 2015). In the present experiment, a 10 mM concentration of F was found to be toxic to the extent that seeds were not germinated. Burgohain and Chowardhara (2022) also reported that F treatment caused 13% to 43.10% suppression of germination in fenugreek seeds from 2.0 mM to 6.0 mM. F also had a significant detrimental impact on other parameters such as seedling length, germination index, relative germination energy, and seedling vigor index, etc. of the plant. In a like manner, F also adversely affected the germination of other plants such as wheat (Pelc *et al.,* 2020), maize, and soybeans (Fina *et al.,* 2016). The reason behind this might be the accumulation of soluble salts after the evaporation of water which subsequently, hardens the soil surface and interrupt the germination process (Kumar and Chopra, 2012). F also prevents the dephosphorylation of phytin by inhibiting the enzyme phytase and retards the supply of inorganic phosphate during germination (Hong *et al.,* 2016). F acts as a metabolic inhibitor and it also impairs germination while reducing the activity of amylase and lipase (Yu, 1996).

In the present study, F application at a relatively low concentration (2.5 mM) slightly increased the biomass and growth of fenugreek plants. Higher F concentrations were found to have derailed growth prospects for fenugreek and it reduced root-shoot length (Fig. 2A), and their biomass (Fig. 2B). The results were similar in F-treated chickpeas (Yadu *et al.,* 2017). Similarly, Bhat *et al.* (2023) reported that fenugreek seeds treated with NaF (50 & 100 ppm) had the lowest shoot and root length among different sodium halides in the following order; NaF > NaI > NaCl. A significant decline in fresh and dry weight of duckweed was also noted in a concentration-duration dependent manner which might be due to disruption in metabolic pathways and adverse effects on the transport of nutrients, cell integrity, or enhanced accumulation of F (Sharma *et al.,* 2019). F-induced decrement in dry weights of tomato shoots and roots was also reported, however, it was not found in the case of oat which suggests that oat is tolerant of high concentrations of F either by excluding F at the root or by detoxifying F at the cellular level (Stevens *et al.,* 1998). Moreover, Treshow and Harner (1968) reported that when pinto bean and alfalfa plants were fumigated with F, growth was increased and internode development was higher as compared to the control plants. In the case of beans, both fresh and dry weights increased with F content up to about 200 ppm, and chlorosis or necrosis in foliar tissue was also not reported. Beyond 200 ppm, the weight of the plant diminished and leaves gradually became chlorotic at about 500 ppm of F. In the case of alfalfa, F initially increased the weight of alfalfa plants and then decreased it but the results are not statistically significant. However, the dry weight of plants beyond 500 ppm of F was only 63% of control plants in spite of no chlorosis or necrosis. According to Miller and Miller (1974), fumigation with HF resulted in disrupted activity of adenosine triphosphatase, which breaks down ATP and increases the respiration rate, while, injury of tissues might be correlated with reduced respiration.

The present investigation also suggested the detrimental impacts of F toxicity on nodules. Up to 2.5 mM of F concentration, the number, fresh weight, and dry weight of nodules increased but it decreased afterward (Fig. 2C). Likewise, Soam and Agarval (1990) reported the adverse impact of increasing NaF concentration on nodulation in faba bean. The ATPase of the peribacteroid membrane which encapsulates nitrogen-fixing bacteria is very sensitive to NaF (Domigan *et al.,* 1988). Additionally, a study by Trikha and Chundawat (2015) on rhizobacterial strains isolated from the root nodules and rhizosphere of chickpeas indicated movement of F from media to the bacterial cells and consequent interference with their growth. Symptoms of F toxicity on nitrogen fixation are not always visible and consequential as reported in alfalfa (Porter and Sheridan, 1981).

Fluoride interferes with metabolic processes in the cell and suppresses growth at higher concentrations by causing alteration in water balance (Zwiazek and Shay 1988). In the present study, RWC in the leaves of the fenugreek was reduced consecutively with all given concentrations of F (Fig. 2D). A decrement in RWC in the present experiment is concordant with reports on mung beans (Maitra *et al.,* 2013), olive (Zouari *et al.,* 2016), rice (Banerjee and Roychoudhury, 2020), and jack pine seedlings (Zwiazek and Shay, 1988). The decrement in RWC could be elucidated by osmotic stress, partial dehydration in cells, and deformation of vascular tissues due to F toxicity (Zouari *et al.,* 2016; Maitra *et al.,* 2013).

Fluoride accumulation in leaves, which can be either gaseous or transported from roots, causes a decrease in leaf area which was corroborated by electrolytic leakage and lower water content as reported in the leaf of aspen seedlings (Kamaluddin and Zwiazek, 2003). In the present investigation, a significant and progressive reduction in leaf area was observed (Fig. 2D). F visibly affects plant growth by causing chlorosis, burning of leaves, leaf necrosis, and pigmentation levels as reported in maize (Ghaffar *et al.,* 2020). In the present investigation, different pigments were drastically influenced by high F concentrations in the soil. The content of chlorophyll a, chlorophyll b, and total chlorophyll was progressively reduced with incremental concentrations of F (Fig. 3A). The pigmentation level reported in the present study is in congruence with the observations reported in barley (Sachan and Lal, 2018) and watermelon (Ram *et al.,* 2014), where the content of all i.e. total chlorophyll, chlorophyll a, and chlorophyll b showed a significant reduction. Similar observations were recorded in F-stressed plants viz; sunflower (Saleh and Abdel-Kader, 2003), and paddy (Sakuntala and Patra, 2013). On the contrary, treatment with lower concentrations of NaF showed stimulation in total pigment content in chickpeas (Sachan and Lal, 2018) and wheat (Tomar and Aery, 2000) whereas it decreased at subsequent higher levels of NaF.

F higher than the threshold of plants adversely affects the uptake or translocation of several important cofactors required for the effective functioning of different enzymes. Reduced leaf Mg^{+2} and Fe^{2+} content in F-treated almond seedlings also led to decreased photosynthesis as affirmed by the comparatively low levels of chlorophyll, reducing sugars and starch in the leaves (Elloumi *et al.,* 2005). Other possible causes behind the reduction of chlorophyll pigments may be the altered activity of delta-aminolevulinic acid dehydratase (ALAD; required in chlorophyll synthesis), an increase in the activity of the chlorophyll degrading enzyme chlorophyllase, and lipid peroxidation in the chloroplast membrane (Ghaffar *et al.,* 2020; Sharma *et al.,* 2019) (Fig. 4).

F toxicity results in excessive levels of superoxide radical or other reactive oxygen species (ROSs) which subsequently damage primary metabolites, membrane lipids, enzyme activities, integrity of DNA strands, and plant nutritive processes (Chakrabarti and Patra, 2013; Kumar and Aery, 2016). In response to oxidative stress exerted by F, plants increase the synthesis of protective osmolytes and antioxidants which efficiently scavenge ROSs by inducing the expression of genes like phenylalanine ammonia-lyase (PAL), anthocyanin synthase (ANS), and polyphenol oxidase (PPO) encoding enzymes required for flavonoids, anthocyanins, and phenolics respectively (Banerjee *et al.,* 2021). The present investigation also showed an initial increase (up to 2.5 mM of F concentration) in anthocyanin and carotenoids (Fig. 3B). Stress in plants positively influenced the concentration of these accessory pigments, implying their role in tolerance mechanisms in plants against stress (Kholiya and Kumar, 2023). It might be mediated through enzymes such as phytoene synthetase (PSY) and violaxanthin de-epoxidase (VDE) which helps dissipate excess energy through the xanthophyll cycle as reported with stress caused by heavy metals (Zhang *et al.,* 2020). Several prenylquinones such as phylloquinone K1 and α-tocopherol etc. are found in thylakoids and chloroplast membranes and perform safeguarding functions similar to carotenoids, especially against oxidative stress (Joyard *et al.,* 2009) (Fig. 4).

Results indicate that higher concentrations of F lead to malfunctioning of germination, biochemical, and growth peculiarities in fenugreek. Higher than 2.5 mM of F concentrations in soil could be threatening to the plants. Furthermore, biochemical investigation acknowledges impairment of photosynthetic machinery through unfavorable impact on pigments like chlorophyll a, b, total chlorophyll, carotenoids, and anthocyanins. F toxicity in fenugreek was also corroborated by a diminution in leaf area and relative water content in the leaf and other growth parameters of the plant. This study will be further utilized for assessment of dose-dependent response of fenugreek to F application. As fenugreek is used as forage for animals and as condiments in Indian cuisines, it is significant to evaluate the toxic impacts of F especially in areas where F is beyond safe or permissible limits.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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