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Metabolic responses of two *Helianthus annuus* cultivars to different fluoride concentrations during germination and seedling growth stages

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ABSTRACT

The present study was carried out to investigate the effect of sodium fluoride on two sunflower cultivars (*Helianthus annuus* (Fudek cv. and Earlyflower cv.) during germination and seedling growth stages. Increasing fluoride concentrations significantly decreased total protein and carbohydrate content, as well as amylase activity. However, proline content increased significantly with increasing fluoride concentration. Glutathione content and SOD activity significantly decreased while lipid peroxidation level significantly increased with increasing fluoride concentration. Increasing fluoride concentration in irrigated solution significantly decreased all pigments levels (chlorophyll a & b, total carotenoids and anthocyanins) in a seedling-stage experiment. Application of different concentrations of NaF during germination induced more severe effects than during the seedling stage. The earlyflower cv. was more sensitive to fluoride than Fudek cv.

KEYWORDS*:* Fluoride, Sunflower, SOD, GSH .lipid peroxidation, germination, seedlings.

INTRODUCTION

Fluorides are compounds containing the element fluorine (F) and is produced by the glass, aluminum, pottery, brick, and ceramic industries. Fumes from industrial areas around oil refineries, cement and phosphate fertilizer tend to increase the concentration of F in soils. High levels of F in soils may reduce crop yields (Moustafa *et al*. 1998). High exposures to fluorides from eating contaminated foods, breathing workplace air, or eating toothpaste can cause lung, skin and bone damage.

The observed symptoms in plants include depressed growth and development, chlorosis, decreased photosynthetic activity, abscission of leaves, flowers, or fruits, impaired cone and seed production, and necrosis (Posthumus 1983). Gaseous fluoride enters the leaf through the stomata and generally leaves are most sensitive when they are young and still expanding (Alan 2001). The rate of symptoms appears to depend on the weather and the time of exposure to the fluoride. Moustafa *et al*. (1998) reported that high levels of F in acid soils reduce crop yield due to increasing aluminium and decreasing phosphorus uptake.

Fluoride has long been known as a potent metabolic inhibitor, interfering with the metabolism of proteins, lipids, and carbohydrates (Yu *et al*. 1987; Reddy *et al*. 1989; Mathews & Hold 1990; Reddy & Venugopal 1990). Sodium fluoride (NaF) inhibits amylase (Yu *et al*. 1988) and invertase activity (Yu 1997) in germinating mung bean (*Vigna radiata*) seeds. Changes in enzyme activity and intermediary metabolism caused by chronic fluoride exposure may lead to altered growth, development, and reproduction of the organism.

Among the many biochemical effects of fluoride is the generation of superoxide free radicals (Curnutte *et al*. 1979). Superoxide free radical ($O_2^{-}\cdot$) is produced from O_2 by both natural and anthropogenic processes. It is produced naturally during mitochondrial respiration, upon exposure to UV-B radiation, and during an immune response by phagocytozing cells (Flohe *et al*. 1977; Cohen & Chovaniec 1978; Palenik *et al*. 1991).

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The anthropogenic processes of $O_2^{-}\cdot$ production are caused mainly by the action of various environmental pollutants such as NO_2, CN^-, and the herbicides paraquat and nitrogen (Cohen & Chovaniec 1978; Hassan & Fridovich 1978; Orr & Hogan 1983; Kohen *et al*. 1986).

Fluoride has been shown to inhibit the activity of SOD. Wild & Yu (1998) concluded that NaF at low concentration caused a small but significant increase in SOD activity, whereas high concentrations decreased the enzyme activity. On the other hand, oxidative enzymes such as catalase, peroxidase, ascorbic acid oxidase, polyphenol oxidase and cytochrome oxidase are stimulated with fluoride (Lee *et al*. 1965). Yu (1997) reported that exposure to 1.0 mM NaF inhibited mung bean germination, as manifested by decreased root elongation, altered tissue fatty acids and soluble sugar composition. He also reported an identification of SOD activity in mitochondrial preparations from mung bean seedlings and that NaF inhibited the enzyme *in vivo*.

The present work was carried out to investigate the effect of different fluoride concentrations on some metabolic products of two common cultivars of sunflowers grown in Egypt (*Helianthus annuus* cv. fudek and cv. earlyflower). The study was also conducted to evaluate the sensitivity of the germination and seedling-growth stages to fluoride treatments.

MATERIALS AND METHODS

Seeds of two sunflower (*Helianthus annuus*) cultivars Fudek and Earlyflower were obtained from the Agricultural Research Centre, Giza, Egypt, and were used in all experiments.

In the germination experiment, seeds of sunflower cultivars were surface-sterilized with 1% (v/v) sodium hypochlorite for 3 minutes, rinsed with distilled water and germinated in the dark in 12-cm diameter Petri-dishes on filter paper moistened with 0.0, 0.2, 0.5, 1.0 and 5.0 mM sodium fluoride. The germinating seeds were collected 24, 48 and 72 hours post fluoride treatment.

In the seedling-stage experiment, seeds were grown in plastic pots (15 cm height, 10 cm diameter) half-filled with pre-sieved sandy loam soil. All pots were watered up to saturation, kept in the open air and irrigated regularly every two days. Fourteen days after seed soaking, the pots were randomly divided to five groups regularly irrigated every two days with half-strength Hoagland solution containing 0.0, 0.2, 0.5, 1.0 and 5.0 mM NaF. Seedlings were harvested two-weeks after the beginning of NaF treatments.

The following parameters were measured in both germination and seedling-stage experiments except photosynthetic pigments, which were measured in the seedling-stage experiment only.

The total carbohydrate contents were extracted in test tubes containing dry matter with 5 ml of 2.5 N HCl. The tubes were placed in boiling water bath for 3 hours. Glucose was estimated colorimetrically at 630 nm by the anthrone method, as described by Hedge & Hofreiter (1962).

Invertase activity was assayed in a total volume of 500 µL of reaction medium containing 10 mM sodium acetate buffer (pH 4.5), 20 mM sucrose, and enzyme extract. The reaction was conducted at 30 °C for 30 min, and were terminated by adding 500 µL of potassium phosphate buffer (pH 7.5) and boiling for 1 min (Xu *et al*. 1996). Glucose was quantified by anthrone method (Hedge & Hofreiter 1962).

Activities of amylase(s) were determined colorimetrically as described by Afifi *et al.* (1986). The amylase activity was estimated by measuring the decreasing of starch-iodine complex at 660 nm/15 min.

Antioxidant-enzyme extracts were prepared by homogenizing the sunflower plant in a prechilled mortar in 20 ml chilled extraction buffer (pH 7.5). Extracts were then centrifuged at 6000 rpm for 20 min at 5 °C. Enzyme assays were conducted immediately following extraction. Superoxide Dismutase Activity (SOD) was measured by the photochemical method as described by Winter *et al.* (1975). Assays were carried out under illumination. One unit SOD activity is defined as the amount of enzyme required to cause 50% inhibition of the rate of p-nitro blue tetrazolium chloride reaction at 560 nm.

Glutathione content was determined spectrophotometrically following the method described by Griffith (1980).

Estimation of lipid peroxidation was assayed spectrophotometrically using TBA-MDA assay. Lipid peroxides were extracted with 5 ml of 5% (w/v) metaphosphoric acid and 100 µL of 2% (w/v in ethanol) butyle hydroxytoluene. An aliquot of the supernatant was reacted with thiobarbituric acid 95°C and cooled to room temperature. The resulting thiobarbituric acid malondialdehyde adduct was extracted with 1-butanol (Minotti & Aust 1987).

Total protein content was extracted with trichloroacetic acid and NaOH and estimated spectrophotometrically according to Lowery *et al.* (1951). Proline content was extracted with sulphosalycilic acid and estimated spectrophotometrically in the fresh shoot system according to Sadasivam & Manickam (1991). Chlorophyll a, b and carotenoids were estimated in the fresh plant leaves according to the procedure of Metznert *et al.* (1965). Anthocyanins were extracted and quantified from leaf tissue by extracting with 350 µL of 18% 1-propanol, 1% HCl, and 81% water. The amount of anthocyanins in the resulting extract was quantified spectrophotometrically. The values were reported as $A_{535} - 0.25\ (A_{650})\ g^{-1}$ fresh weight (Lange *et al.* 1970).

Analysis of variance (ANOVA) and correlation tests were performed on all data using SPSS (version 8). Significance was determined at a level of $p<0.05$. Means are plotted ± SE. There were 3 replicates of each treatment.

RESULTS

Fluoride treatment induced sharp significant decreases in total carbohydrate content at both germinating and seedling stages (Figs 1a & b, 2). The decrease in carbohydrate content was found to be concentration dependent.

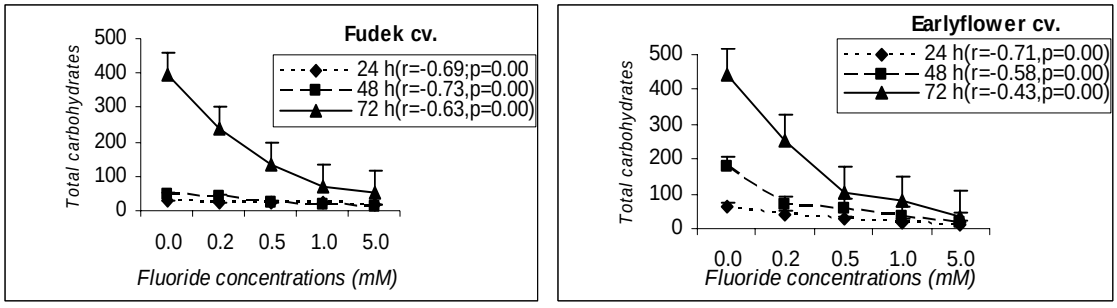


Figure 1 (a & b): Effect of different fluoride concentrations (mM) on total carbohydrate content (mg glucose/100g fresh wt) of both sunflower cultivars at 24, 48 and 72 hours post germination.

There were significant decreases in invertase and amylase activities at the germination (Figs 3 & 4) and seedling stages (Fig 5) of the two sunflower cultivars. It was also noticed that the inhibition of both enzymes increased with increasing fluoride concentration.

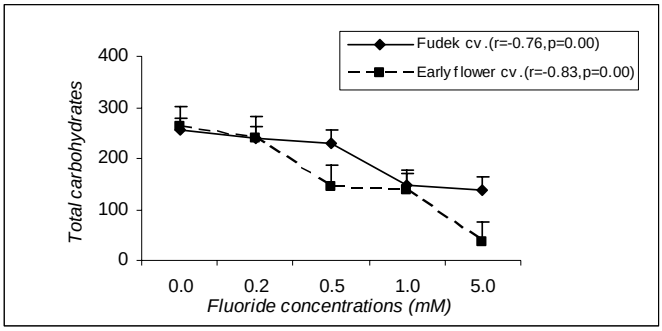


Figure 2: Effect of different fluoride concentrations (mM) on total carbohydrates content (mg glucose /100g fresh wt.) of both sunflower cultivars at seedling stage.

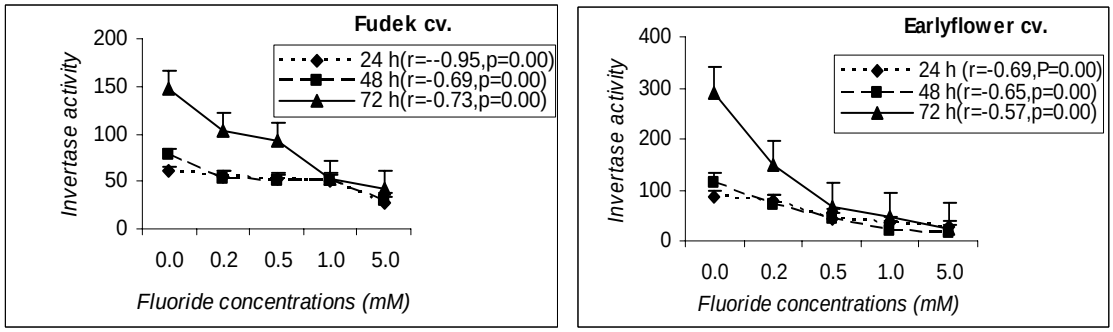


Figure 3 (a & b): Effect of different fluoride concentrations (mM) on Invertase activity (unit/g tissue) of both sunflower cultivars at 24, 48 and 72 hours post germination.

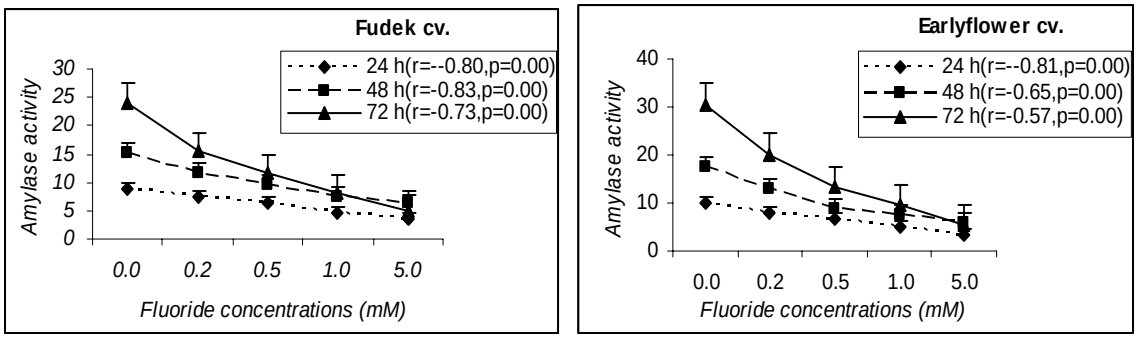


Figure 4 (a & b): Effect of different fluoride concentrations (mM) on amylase activity (unit/g tissue) of sunflower plants at 24, 48 and 72 hours post germination.

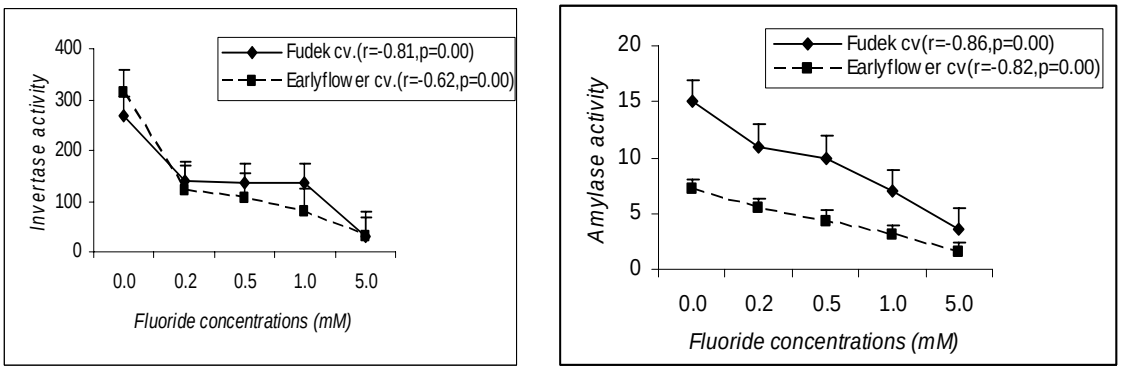


Figure 5 (a & b): Effect of different fluoride concentrations (mM) on Invertase and amylase activities (unit/g tissue) of both sunflower cultivars at the seedling stage.

Figure 7 shows that increasing sodium fluoride concentration induced a significant decrease in SOD activity at germination stage in both cultivars when compared with their control values. Increasing the time of exposure decreased this effect. However, after 72

hours, the two cultivars had nearly restored their SOD activity in the germination-stage experiment. Sodium fluoride exhibited the same effect on SOD activity during the seedling-stage experiment, but the two cultivars did not restore SOD activity (Fig 9a)

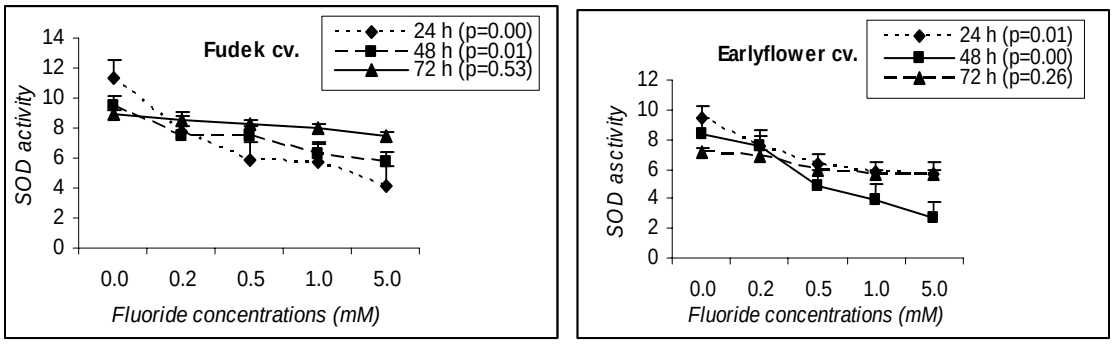


Figure 7 (a & b): Effect of different fluoride concentrations (mM) on SOD activity (unit/g tissue) of both sunflower cultivars at 24, 48 and 72 hours post germination.

Moreover, increasing fluoride concentration induced a significant decrease in glutathione content throughout the experimental period as well as during the two experimental stages (Figs 8, 9b)

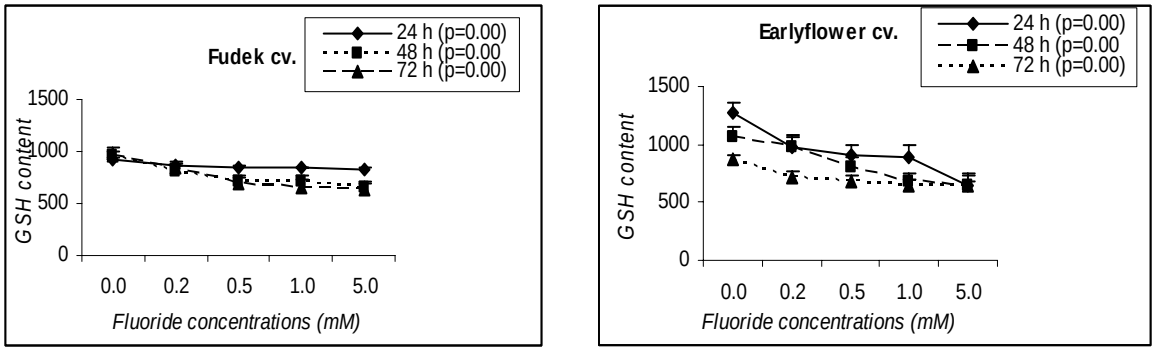


Figure 8 (a&b): Effect of different fluoride concentrations (mM) on GSH content (µg/g tissue) of both sunflower cultivars at 24, 48 and 72 hours post germination.

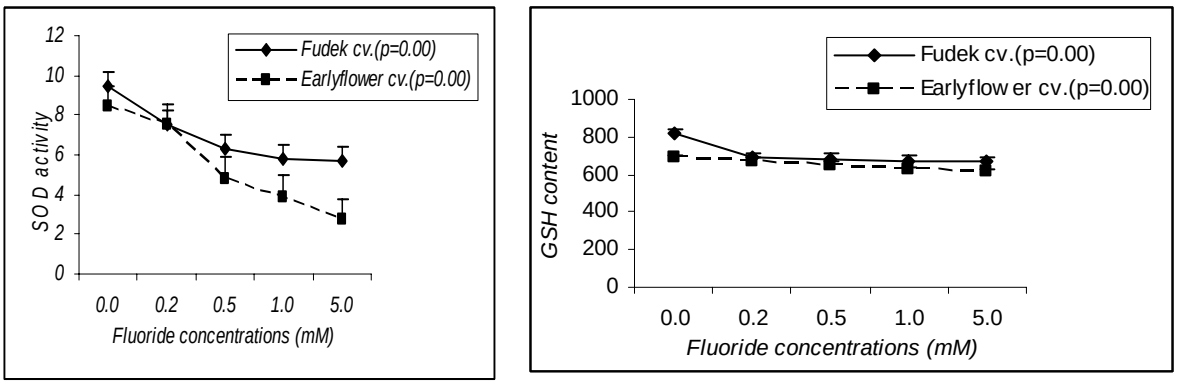
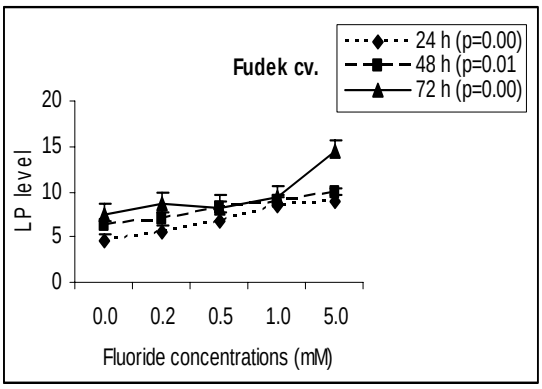


Figure 9 (a&b): Effect of different fluoride concentrations (mM) on SOD activitiy (unit/g tissue) and Glutathione content (µg/g tissue) of both sunflower cultivars at the seedling stage.

Lipid peroxidation level was significantly increased with increasing fluoride concentration and time of exposure in both the germinating and seedling stages in both cultivars (Figs 10a&b, 11).



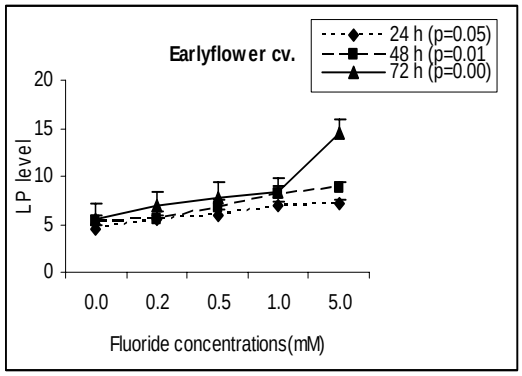
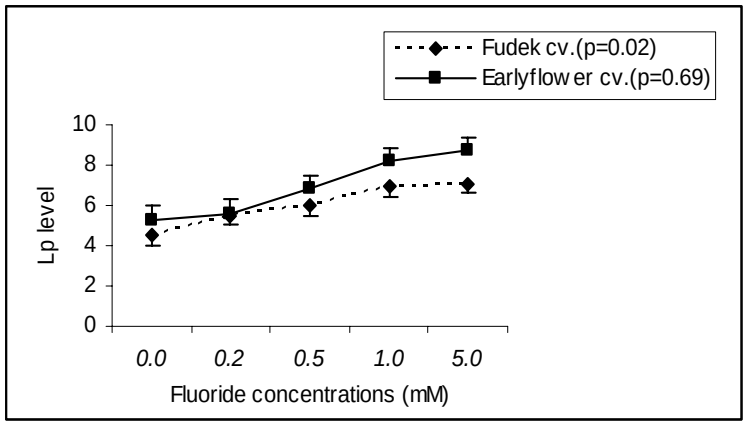
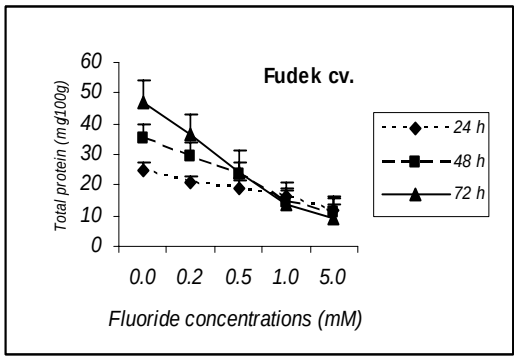


Figure 10 (a&b): Effect of different fluoride concentrations (mM) on lipid peroxidation levels (µg/g tissue) of both sunflower cultivars at 24, 48 and 72 hours post germination.

Figure 11: Effect of different fluoride concentrations (mM) on Lipid peroxidation levels (µg/g tissue) of both sunflower cultivars at the seedling stage.



The total protein content at both germination and seedling stages for both cultivars was significantly decreased at all fluoride concentrations (Figs 12a&b). The decrease in protein content was time dependent, and negatively correlated with fluoride concentrations.



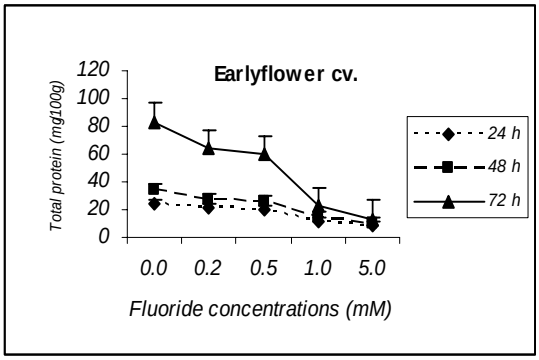
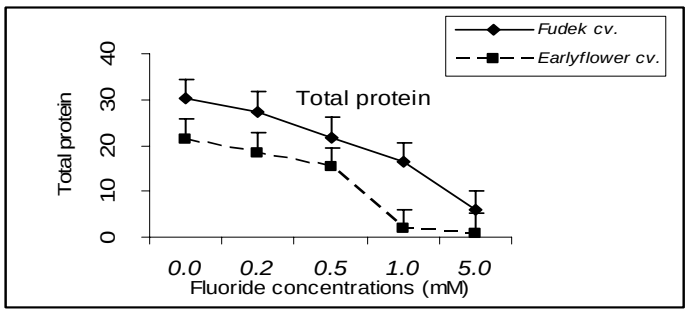
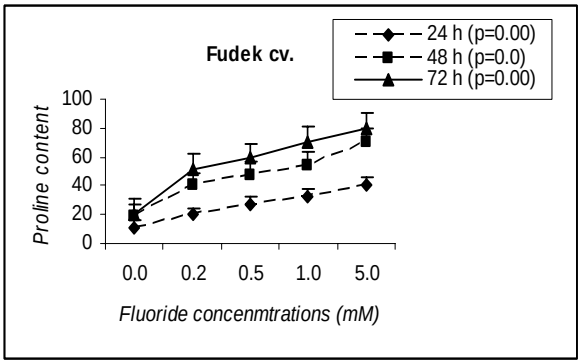


Figure 12 (a&b): Effect of different fluoride concentrations (mM) on total protein content (mg /100g fresh wt.) of both sunflower cultivars at 24, 48 and 72 hours post germination.

Increasing fluoride concentration led to a significant increase in proline content during the two experimental stages in both sunflower cultivars (Fig 14a&b). The results also showed that proline content was higher in Earlyflower cv. than that in Fudek cv. The increase in proline content was time dependent, and positively correlated with fluoride concentrations.

Figure 13: Effect of different fluoride concentrations (mM) on total protein content (mg/100g fresh wt.) of both sunflower cultivars at the seedling stage.





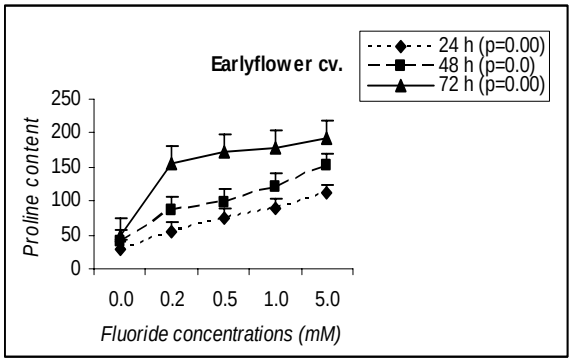
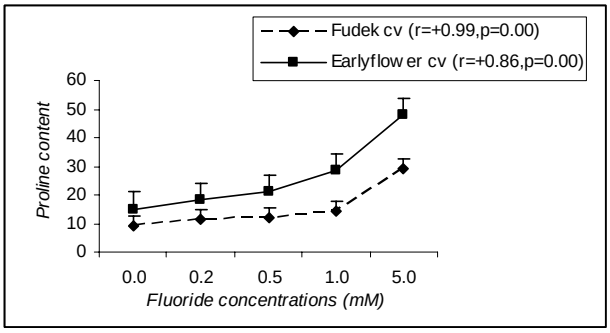
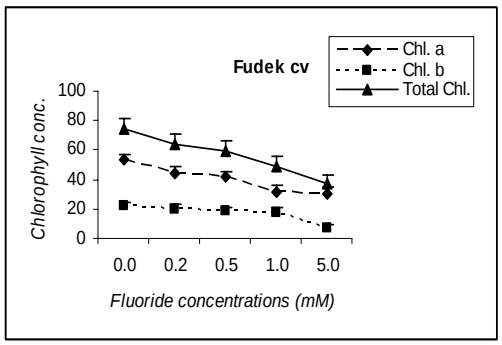


Figure 14 (a&b): Effect of different fluoride concentrations (mM) on proline content (µg /100g fresh wt.) of both sunflower cultivars at 24, 48 and 72 hours post germination.

Figure 15: Effect of different fluoride concentrations (mM) on proline content (µg /100g fresh wt.) of both sunflower cultivars at seedling stage.



In the seedling-stage experiment, application of the different fluoride concentrations induced significant decreases in chlorophyll a&b content. The decrease was highly significant at 1.0 and 5.0 mM sodium fluoride for Fudek cv. and at 0.5, 1.0 and 5.0 mM sodium fluoride for Earlyflower cv (Figure 16 a,b). Moreover, increasing fluoride concentration significantly decreased carotenoid and anthocyanin pigments in both cultivars (Figure 17 a,b).



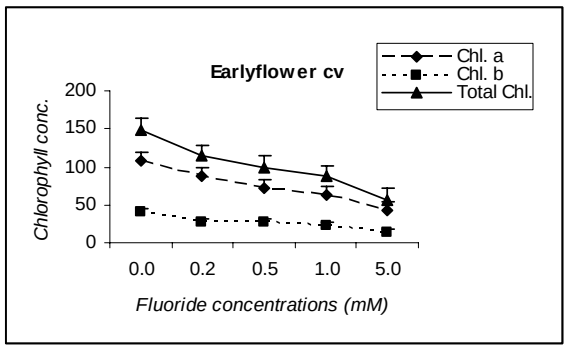


Figure 16 (a&b): Effect of different fluoride concentrations (mM) on chlorophyll a&b and total chlorophyll content (µg /100g fresh wt.) of both sunflower cultivars at the seedling stage.

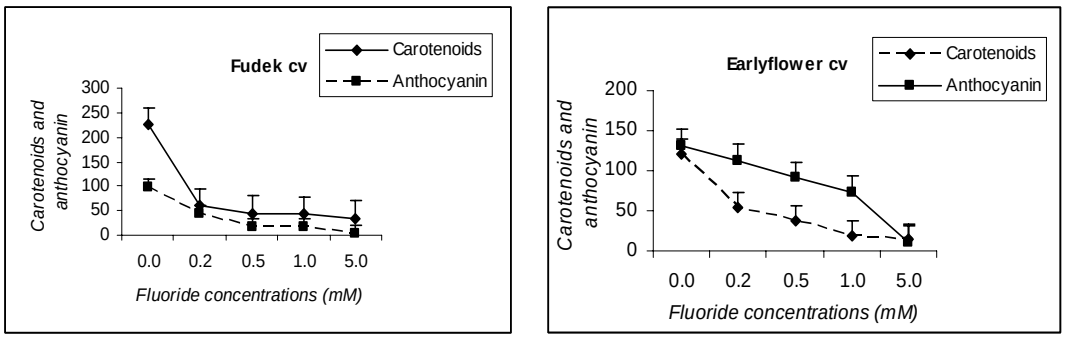


Figure 17 (a&b): Effect of different fluoride concentrations (mM) on carotenoid and anthocyanin content (µg /100g fresh wt.) of both sunflower cultivars at the seedling stage.

DISCUSSION

In the present study, the total carbohydrate content in the two sunflower cultivars was significantly decreased at all fluoride concentrations. The decrease in total carbohydrate contents might be attributed to the effect of F on inhibiting photosynthesis during seedling growth or conversion of fats into carbohydrate during seed germination. Fluoride inhibits photosynthesis by limiting CO_2 utilization. The present findings are in agreement with Warburg (1962) and Bhatnager & Bhatnager (2000). Vennesland (1963) reported an immediate burst of CO_2 on addition of sodium fluoride to acid-grown *Chlorella*. Asthir *et al*. (1998) found that culturing for five days in the presence of fluoride reduced the amount of wheat grain starch, whereas contents of total free sugars, particularly sucrose, and soluble protein increased.

It was found that fluoride inhibited the activities of invertase and amylase (Figs. 3-6), as found by other researchers (Asthir *et al.* 1998; Yu *et al.* 1988; Yu 1997). Fluoride often inhibits enzymes that require such cofactors as Ca^{2+}, Mg^{2+}, and Mn^{2+} ions. Thus the inhibition of amylase and invertase activities can be attributed, in part, to removal of the cofactor Ca^{2+}, Mg^{2+} and Mn^{2+} ions (Wilde & Yu 1998). Magnesium is the activator of more than 300 enzymes, while fluorine is known as their inhibitor. Changes in enzyme activity and intermediary metabolism caused by fluoride exposure leads to altered growth, development and reproduction (Machoy-Mokrzynnska 1995).

The activity of SOD is negatively correlated with increasing fluoride concentration, and thus high fluoride concentrations are likely to inhibit SOD (Figs. 7, 9a). This could be attributed to the fact that production of $OH^{\cdot}$ and $^{\cdot}O_2^{-}$ radicals depends on fluoride concentration. Superoxide radicals prevail at high fluoride concentrations, whereas at low concentrations there is dominance of hydroxyl radicals generated in the Haber-Weiss reaction with the participation of Fe^{2+} as well as H_2O_2 and $^{\cdot}O^{2-}$ (Zhao *et al*. 1989; Wang *et al.* 1997).

In the present study, there was a decrease in SOD activity at the seedling-growth stage (Fig. 9a). These findings disagree with Wilde & Yu (1998), who stated that the mitochondrial SOD activity increased with age of seedling: they claim that as growth continues during germination, the demand for respiration increases, and so the number of mitochondria increases with age.

It was found that increasing F concentrations decreased GSH content at both growth stages (Figs. 8 & 9b). The essential changes in the activities of SOD and glutathione enzymatic systems may take place as an adaptive response of the plant cell to the investigated stress-factor impact. The toxicity of free radicals and H_2O_2 is greater if fluoride can impair the production of free radical scavengers such as ascorbic acid and

glutathione, or the functioning of protective enzymes such as ascorbate peroxidase and SOD (Wilde & Yu 1998; Grishko & Syshcheikov 1999).

Lipid peroxidation levels were increased with increasing fluoride concentration and time of exposure (Figs. 10 & 11). Superoxide free radicals have the potential to cause adverse effects on biomolecules. They can damage membrane lipids through lipid peroxidation, and cause enzyme inactivation and DNA-strand breakage (VanHemmen & Meuling 1975; Lorentzen & Tso 1977; Brawn & Fridovich 1981). The simultaneous production of oxygen occurs by photolysis of water. Substitution of chloride ions, appearing in the photosystem II, by fluoride ions inhibits the photo-oxidation of water and increases production of new radical forms in protein chains of this system, which is unable to commence the process of photolysis (Baumgarten *et al.* 1990).

Fluoride treatments decreased the protein content in both germinating and seedlings of sunflower cultivars at all time of exposures (Figs. 12 & 13). This could be attributed to the ability of fluoride to modify the ratio of free nucleotides and that of RNA, to decrease the rate of RNA synthesis, and/or to enhance ribonuclease activity. As a result, it affects protein synthesis negatively (Bhatnager & Bhatnager 2000). Asthir *et al.* (1998) concluded that wheat grains respond to fluoride-mediated disruption of carbon metabolism by a compensatory effect on nitrogen metabolism.

The present data also showed that increasing fluoride concentration increased proline accumulation in sunflower tissues (Figs. 14 & 15). Proline accumulation in response to stress is widely reported, and may play a role in stress adaptation within the cell, which is of great interest to those studying stresses in plants (Gibon *et al.* 2000). The rapid accumulation of amino acids during salinity stress suggests that these compounds may be acting as sinks for excess N in relation to the decreased growth occurring during the imposed stress (Dubay & Pessarakli 1995). Amino acids also play a role in osmotic adjustment, and serving as available sources of carbon and nitrogen. Several explanations for the accumulation of free amino acids and amides under stress have been suggested. These include stimulation *de novo* synthesis, inhibition degradation of amino acids, impairing protein synthesis, and/or enhanced protein degradation (Ranieri *et al*. 1989; Gibon *et al.* 2000).

The results of the present experiment showed an inhibition in photosynthetic pigments of sunflower seedlings treated with different fluoride concentrations (Figs. 16 & 17), as in Bhatnager & Bhatnager (2000) who noticed that fluoride inhibited the chlorophyll pigments of marine algae. On the other hand, Kamaluddin & Zwiazek (2003) found that long-term exposure of *Populus tremuloides* Michx roots to NaF did not significantly affect leaf chlorophyll content but decreased net photosynthesis. The inhibition of photosynthetic pigments in the present study could be an indirect result of the toxic effect of fluoride ion in Mg deficiency, where it reduces Mg absorption by plants (Marier 1984). The fluoride ion also interferes with the biological activity of Mg^{+2} (Guminska 1985).

We conclude that the application of NaF to the two *Helianthus* cultivars during the germination stage induced more severe effects than application during the seedling stage. The Early flower cultivar was more sensitive to fluoride than the Fudek cultivar.

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الملخص العربى

الإستجابات الأيضية لصنفين من عباد الشمس لتركيزات مختلفة من الفلورايد أثناء مرحلتى الإنبات و نمو البادرة.

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أجري هذا البحث على صنفين مختلفين من نبات عباد الشمس (هيليانسس أنس ايرفلور) و (هيليانسس أنس فيودك) حيث أجريت هذه التجربة على مرحلتين عمريتين مختلفتين لنبات عباد الشمس و هما مرحلة الإنبات و مرحلة نمو البادرة. ففي مرحلة الإنبات تم انبات هذه البذور على ورق ترشيح مبتل بالماء لمدة يوم واحد ثم تم اضافة المعاملات المختلفة من الفلوريد(0.2و 0.5و 1و 5ملى مول) لمدة 24و48 و72 ساعة. أما بالنسبة لمرحلة نمو البادرة تم وضع هذه البذور في أصص بلاستيكية يصل طولها إلى 15 سم و عرضها إلى 10 سم, وتم ريهم لمدة أسبوعين بطريقة منتظمة , ثم بعد ذلك بدأت معاملة النباتات بالفلوريد بنفس التركيزات السابقة لمدة أسبوعين آخرين. وقد أظهرت النتائج أن اضافة الفلوريد إلى ماء الري تسبب في تقليل محتوى الكربوهيدرات الكلية وكذلك النشاط الأنزيمي لكل من الأميليز و الأنفرتيز في كل من الصنفين المذكورين سواء في مرحلة نمو البادرة أو في مرحلة الإنبات. وقد اوضحت النتائج ايضا أنه باضافة الفلوريد بتركيزات مختلفة في خلال الفترات 24 و 48 و 72 ساعة ادى إلى انخفاض المحتوى البروتيني للبذور المستنبتة . كما بينت النتائج العملية أن الصنف (ايرفلور) كانت نسبة الأنخفاض فيه أكبر من الصنف (فيودك) ونفس النتائج تم الحصول عليها فى مرحلة نمو البادرة. كما أشارت النتائج إلى أن زيادة تركيز الفلوريد المضاف يؤدي إلى زيادة تراكم البرولين في البذور المستنبتة في كلا الصنفين محل الدراسة لنبات عباد الشمس وقد كان محتوى صنف الايرفلور من البرولين اكبر من صنف فيودك. كما أشارت النتائج إلى انخفاض محتوى الكلوروفيل في أوراق الصنفين مع ملاحظة أن نسبة الانخفاض كانت أعلى في الصنف (ايرفلور) عن الصنف (فيودك). أما بالنسبة لمستوى فوق اكسدة الدهون فقد زادت نسبته في النبات في كلا المرحلتين العمريتين المذكورين مع زيادة تركيز الفلوريد المضاف للصنفين . لوحظ ايضا أن SOD له اسلوب مغاير حيث انه يزداد في حالة التركيزات القليلة كما في التركيز 0.2 و 0.5 ميللي مولر من الفلوريد , ويقل كلما زاد تركيز الفلورايد المضاف كما في حالة التركيز 5.0 و1.0 ميللى مولر من الفلوريد اما بالنسبة الى الجلوتاثيون يقل كلما زاد تركيز الفلوريد المضاف لكلا الصنفين فى كل من المرحلتين العمريتين للنبات . من ذلك يتضح أن الصنف (ايرفلور) اكثر حساسية للفلورايد مقارنة بالصنف (فيودك) كما وجد أيضا أنه كلما كان النبات أكبر عمرا زادت قدرته على مقاومة التأثير المدمر للفلورايد. و يمكن التحذير من أن مركبات الفلورايد الناتجة من المخلفات الصناعية وعادم السيارات تتراكم بصورة كبيرة داخل انسجة النبات وهذا بدوره يؤدي إلى تسمم المستهلك بصورة غير مباشرة, كما أن لتراكم هذا العنصر تأثير سيئ على نمو وأيض النباتات.