Comparison of fluoride effects on germination and growth of *Zea mays, Glycine max* and *Sorghum vulgare*.

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Running title: Effects of fluoride on crop plants.

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Abstract

BACKGROUND: Fluorosis is a disease caused by overexposure to fluoride (F). Argentina's rural lands have higher fluorine content than urban lands. Evidence confirms that plants grown in fluoridated areas could have higher F content. We compared F uptake and growth of crops grown in different F concentrations. The effect of 0-8 ppm F concentrations on maize, soybeans and sorghum germination (G) and growth was compared. After 6 days seeding, the G was determined, the roots and aerial parts lengths were measured, and vigor index (V) was calculated. F content was measured in each part of the plants. Controls with equal concentrations of NaCl were carried out.

RESULTS: Significant decrease in roots and aerial parts lengths, and in V of maize and soybeans plants was observed with F concentrations greater than 2 ppm. This was not observed in sorghum seedlings. Also, the amount of F in all crops augmented as F increases, being higher in roots and ungerminated seeds. Sorghum was the crop with the highest F content.

CONCLUSION: Fluoride decreased the G and growth of maize and soybeans and could influence on their production. Conversely, sorghum seems to be resistant to the action of F.

Key words: crop, fluoride, germination, *Zea mays*, *Glycine Max*, *Sorghum vulgare*.

Introduction

In the Argentina farming systems, maize (*Zea mays*), soybeans (*Glycine Max*) and sorghum (*Sorghum vulgare*) are the most important crops that sustain the economy of grain production. Argentina is one of the most important grain and oilseed producing countries in the world, playing a huge role in supplying the world market. The growth in planted and harvested areas, unit yields and grain production in Argentina has increased significantly in this decade.[1] With just over 30 million hectares occupied, the joint production of grains and oilseeds currently exceeds the threshold of 90 million tons. Argentina's total grains exports in 2015-16 are projected at 29.6 million tonnes, up from 26.3 million in 2014-15. Maize, sorghum and soybeans exports are forecast at 17.5, 1.8 and 27.6 million tonnes, respectively. Sorghum has also benefited from an Argentina-China agreement that has opened up Chinese markets for Argentina product.[2,3] At present, human consumption of grains of maize, soybeans and sorghum, and their products (oils and flour) is increasing, forcing the country to use rural lands without interruption. In addition, soybeans monoculture, which has been increased in recent years, contributes to poor soil nutrition. Therefore, adequate nutrition of these crops forms an essential aspect in the production yield and, therefore, one of the determinants of their profitability (Bolsa de cereales de Santa Fe (www.santafe.gov.ar)).

Fluoride in its elemental form (F_2) is a gaseous compound, however due to its high electronegativity, in nature is found exclusively in the form of fluorides. Among the elements of the earth's crust, fluoride is one of the most abundant and is found in minerals such as calcium fluoride (CaF_2), sodium fluoraluminate (Na_3AlF_6) and sodium fluoride (NaF).[4]

Previous studies done by our laboratory[5] showed that 28 % of the locations of the centre of Argentina exceeded the limit of fluoride (F) set by the World Health Organization (WHO, 1.5 ppm) in well water.[6] In turn, these values were consistent with a greater accumulation of F in rural lands in these same locations, which might be due to an enrichment as a result of agricultural activity. F can be added to land and hence waters as part of agrochemicals. Almost all of the phosphorus used in fertilizers comes from deposits of phosphorite ($Ca_{10}(PO_4)_6F_2$). Phosphoric fertilizers are produced by the action of sulfuric acid on phosphate minerals. These minerals contain a large amount of fluoride and, unable to be completely removed by the acid, fluoride remains in fertilizers.7 There is evidence that the use of phosphoric fertilizers in agriculture can increase the amount of F in soils.8 Fluorides, carbonates, phosphates and hydroxides may form complexes with neutral rare-earth elements with low solubility, resulting in low dissolved concentrations in the aqueous phase of ecosystems, acumulandose en los suelos.[9]

Comentario [Autor des1]: Esta en spañol!! jeje

There are studies which demonstrated a decrease in seed germination by adding F to water,[10] however, F concentrations used are 20 times higher than those measured in arable land in our country. There is also evidence that plants enrich its content in F depending on the content of this element in the soil in which they are grown,[11] but there is limited information on the effect of F on the growing of these crops that are marketed worldwide.

Decreased pregnancy[12,13] and lactation[14,15] in cattle from areas with high fluoride content in water and land was described. It has also been detected fetal abnormalities and abortions in women living in areas of fluorosis.[16] In recent years, researchers found in southern Argentina sources of water and vegetables with high fluoride content, associated with dental fluorosis and decreased pregnancy in cattle and red deer.[17,18] There are no studies that evaluate the amount of fluoride ingested by grazing animals eating vegetables grown in areas with high F content in soil.

The F in larger amounts than recommended by WHO can lead to a clinical condition known as fluorosis. Dental fluorosis is a defect of the teeth marked by increased porosity of the enamel and can get to the point where the teeth begin to erode and crumble.[19] Skeletal fluorosis can cause calcifications in the ligaments, immobility, muscle atrophy and neurological problems related to spinal cord compression.[20] Furthermore, little is known about the amount of F that could be ingested through vegetables and its relationship to human health. The incorporation of F through plants could contribute to worsening fluorosis as it would constitute an extra source of intake of F. In addition, a negative impact on the production caused by soil with F could have a low profitability on crops grown in those areas.

For all of the above, the aim of this study was to evaluate and compare the effect of different concentrations of F in soil, similar to those found in the lands of central Argentina, on the germination, growth and F-enrichment of crops of *Zea mays*, *Glycine Max* and *Sorghum vulgare*.

Materials and Methods

Crops treatments

Commercial seeds of *Zea mays* (maize), *Glycine max* (soybeans) and *Sorghum vulgare* (sorghum) (Syngenta Agro, Argentina) were treated with different concentrations of fluoride (added as sodium fluoride (NaF)) in the substrate: 0.08, 0.4, 0.8, 2, 4 and 8 mg F Kg^{-1} of substrate (ppm). As controls the same concentrations of chloride (added as sodium chloride (NaCl)) and distilled water (0 ppm) were used. Fifty of each crop seeds were sown in plastic containers of 23 x 23 x 12 cm, sand was used as substrate and seeds were planted at 2 cm deep and 1 cm between seeds. A container for each concentration of F and a control container with the respective NaCl concentration was prepared. The containers were kept at 25 ± 2 °C with alternating 16 h light and 8 h dark. The detailed experiment was repeated 2 times for each crop. After 6 days of treatment, the emerged seedlings were counted for determination of germination (G). Then, all seedlings were carefully removed and washed thoroughly with distilled water, and length of aerial part and root, thickness of roots and F content were measured. With these data the vigor index (V) and F transfer rate (FT) were calculated.

Standard germination test (G)

This procedure is used to determine the percentage of a batch of seeds that are capable of producing a normal seedling germination under optimal conditions.[21] The seeds were sown as detailed above. After 6 days, the normal seedlings that emerged above the substrate were counted. Normal seedlings are those with the potential to develop successfully in plants when grown in good soil and under favourable conditions of moisture, temperature and light (Análisis de semillas. (www.analisisdesemillas.com.ar)).The G was defined as the number of seedlings expressed as a percentage of total sown seeds:

$$G=\frac{numberofemergedplants}{numberofseedssown}*100$$

Measurements of seedling growth

The growth of seedlings was analysed through the length of the aerial part and root. The length of roots and aerial parts was measured using the Image-J 1.46a software (National Institutes of Health, USA). Briefly, the seedlings were placed on a dark background and leaves and roots were deployed. A photograph was obtained along with a pattern of known length. This pattern was used for calibration of Image-J and then aerial part and the roots of each seedling were measured as detailed in Figure 1.

Roots thickness measurements

Image-J 1.46a software was used (National Institutes of Health, USA). Briefly, the seedlings were placed on a dark background and leaves and roots were deployed. A photograph was obtained along with a pattern of known length. This pattern was used for calibration of Image-J and then root thickness was measured at 1 cm from where the root begins (Figure 2).

Fluoride measurement

F was measured in roots, aerial part and ungerminated seeds. Six days after treatment, the seedlings were removed, washed thoroughly with distilled water, and then the samples were dried to constant weight at 37 °C. The dried sample was used for F measurement. Total fluorine was measured by direct potentiometry using an ion selective electrode Orion 94-09 and a reference electrode of Ag/AgCl. Prior to the measurement, acid labile fluorine was isolated from 100 mg of the sample by isothermal distillation by treatment of samples with phosphoric acid 98 w/w at 60 °C for 1 day. During this time, the hydrofluoric acid released from the sample is recovered by sodium hydroxide placed in the cup of the distillation chamber. Subsequently, the sodium hydroxide trap is adjusted to pH 5.5 with 17.5 mol/l acetic acid.[22] Standards in the range of 0.8 to 19 ppm were simultaneously processed. Results are expressed as parts per million of dry weight of the sample.

Calculation of vigor index (V)

Vigor is a property that affects the behaviour of the seed, both germination and establishment as its ability to withstand stresses throughout its life (training, stay in the field, harvest, profit, storage, planting). The deterioration degree or severity of the genetic deficiency are inversely proportional to seed vigor. Vigor index (V) was calculated using the following equation:

$$V=(\text{rootlength}+\text{aerialpartlength}) * G$$

This index was calculated using the length of the root and aerial part of each seedling and the G of seeds with equal treatment.

Calculation of fluoride transfer rate (FT)

Transfer rate is a measure of the transfer of a compound from one part to another of the same plant. In this work the amount of F transferred from the roots to the aerial part of the seedlings was calculated. FT was calculated using the following equation:

$$FT=\frac{\text{aerialpartFconcentration}}{\text{rootFconcentration}}$$

FT greater than 1 indicated F transference from root to aerial part of the plant, otherwise, F was accumulated in the root.

Statistical Analysis

R 2.14.1 software was used in the analysis of results.[23] The results of germination were analysed using equal proportions test. Lengths, V, fluoride content and FT were compared using two-way ANOVA. Differences between groups were evaluated with post test LSD. In all tests a significance level of 5% was used.

Results

Treatments were performed with concentrations of 0, 0.08, 0.4, 0.8, 2, 4 and 8 ppm of F in the substrate. The statistical analysis revealed that no significant differences were observed for the concentrations 0.08, 0.4 and 0.8 ppm of F, for any parameter or for any crop studied, compared with F 0 ppm or with the same concentrations of the controls (0.08, 04 and 0.8 ppm). Therefore, the results for 2 ppm of F or higher were shown, to simplify the understanding of the results.

Comentario [Autor des2]: Yo agregaria de NaCl, aunque ya lo dijiste antes no viene mal repetirlo.

Standard germination test (G)

Table 1 shows a significant decrease in G of maize seedlings treated with 4 and 8 ppm of F respect to the control. On soybeans, a significant decrease of G at 8 ppm of F respect to its control was observed. However, sorghum G did not differ from its control for any of the F concentrations used. These results indicate that the G of maize and soybeans were the most affected by F in the substrate, which could affect their production and profitability. A lower germination suggests a lower grain production in these crops. However, as sorghum was not affected it could be planted on soils with high fluoride.

Table 1. Germination (%) of the three crops treated with 2, 4 and 8 ppm of NaF or NaCl (control). Data are shown as the means of seeds with equal treatment. At least one letter equally between two cells indicate no significant differences between group means. Grey cells show the most relevant changes. Equal proportions test, p<0.05.

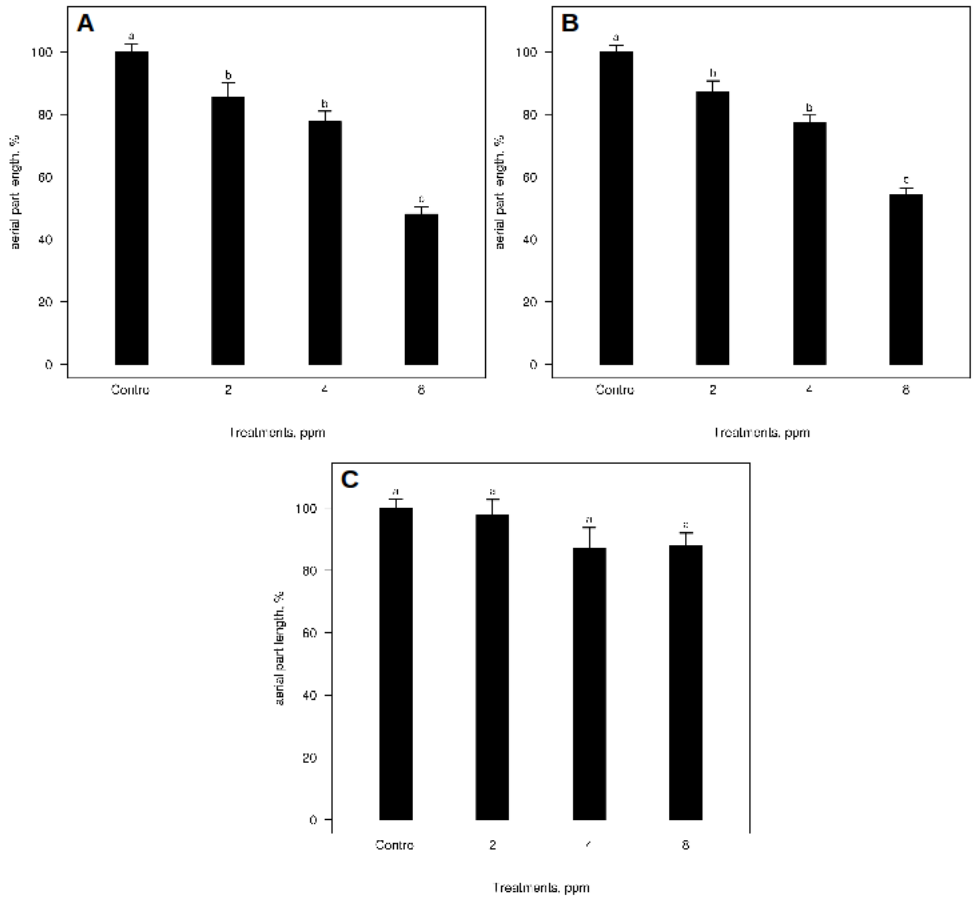
Crop/ ppm of substrate		**2**	**4**	**8**
Maize	Control	96^{a}	78^{a}	94^{a}
	NaF	88^{a}	50^{b}	42^{c}
Soybeans	Control	90^{a}	88^{a}	90^{a}
	NaF	86^{a}	84^{a}	48^{b}
Sorghum	Control	86^{a}	82^{a}	76^{a}
	NaF	86^{a}	66^{a}	80^{a}

Aerial part length

The length of the aerial part of the seedlings of maize and soybeans (Figure 3, A and B) treated with concentrations equal or greater than 2 ppm of F was significantly lower than their controls. With the concentration of 8 ppm, the aerial part was also significantly decreased in relation to 2 and 4 ppm for both crops. By contrast, the length

Comentario [Autor des3]: No se le
los ejes y las letras estan dificiles de
distinguir.

of the aerial part of the sorghum did not change compared to controls (Figure 3, C). Fluoride not only affects crop germination of maize and soybeans, but does not allow their normal growth. This indicates an increased susceptibility of these crops to fluoride, which is not observed for sorghum.

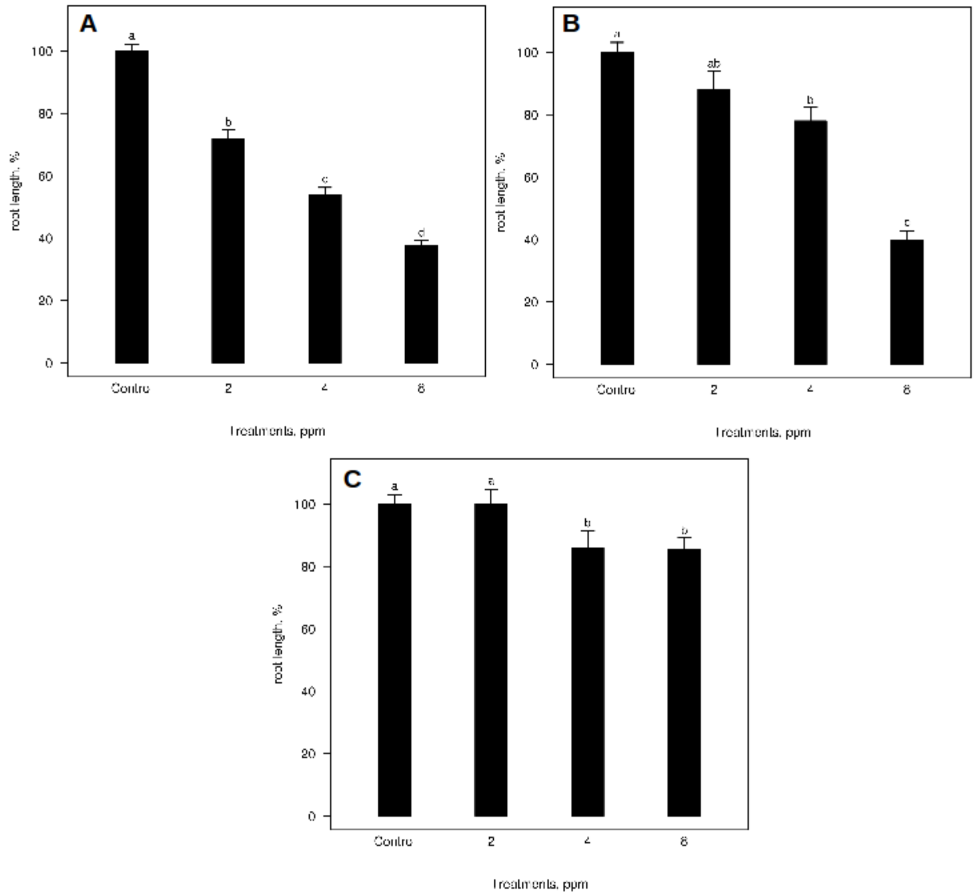


Root length

The length of the roots of maize seedlings treated with 2, 4 and 8 ppm of F significantly decreased compared to controls (Figure 4 A). The length of the F-treated roots also decreased significantly between the different concentrations of F.

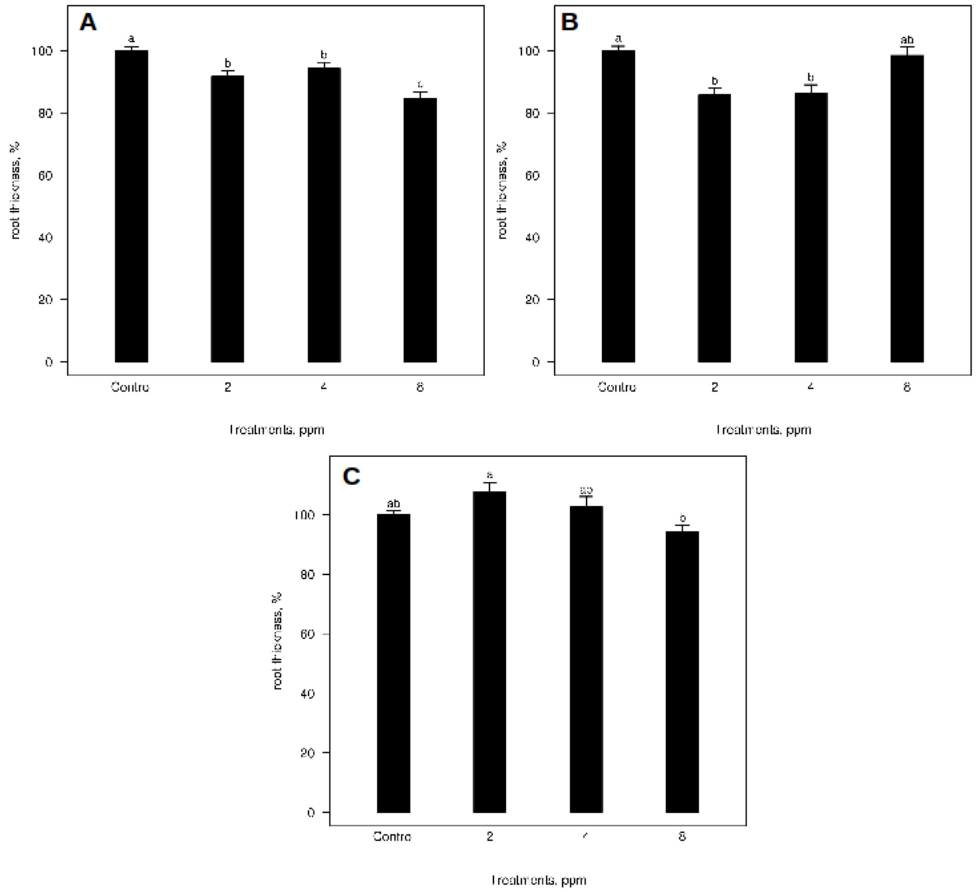
The roots of soybeans and sorghum seedlings (Figure 4, B and C) treated with 4 and 8 ppm of F decreased significantly compared to the control. The soybeans seedlings treated with 8 ppm of F also significantly reduced their roots over 2 and 4 ppm, however, this effect was not observed in sorghum.

Lower total growth (aerial part and root) suggests that it will be difficult the normal development and grain production of plants. Although root length in sorghum decreased compared to controls, it was not affected as the roots of maize and soybeans. While at 8 ppm F sorghum root decreased by 15 %, soybeans and maize decreased it by 63 %.



Root thickness

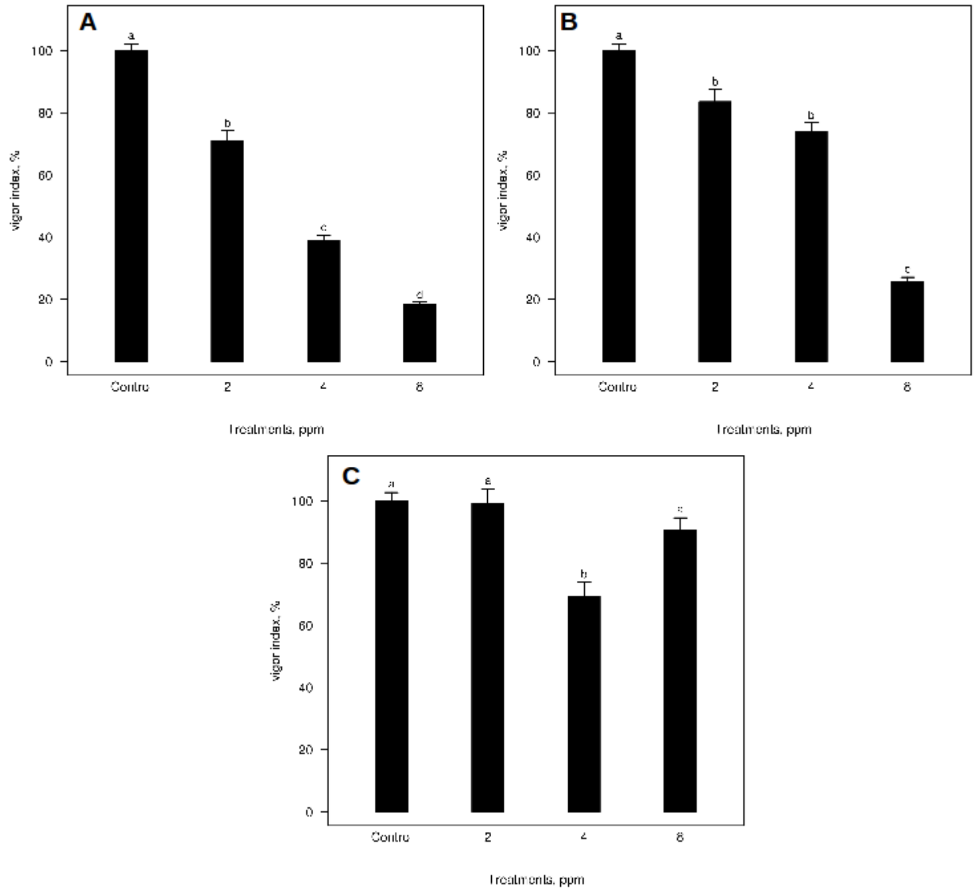
The thickness of the roots of maize seedlings treated with 2, 4 and 8 ppm of F was significantly lower than controls, being, additionally, significantly lower in plants treated with 8 ppm than 2 or 4 ppm (Figure 5, A). In the case of soybeans, root thickness decreased significantly in concentrations of 2 and 4 ppm of F respect to the control, without changes at 8 ppm (Figure 5, B). Conversely, it can be seen that the thickness of the roots of sorghum seedlings treated with F did not differ from their controls (Figure 5, C).



Vigor index (V)

The V decreased significantly at concentrations of 2, 4 and 8 ppm of F compared to controls for maize and soybeans crops (Figure 6, A and B). In turn, a significant decrease of V at 8 ppm with respect to the other F concentrations in the substrate was observed. However, a significant decrease only at 4 ppm of F with respect to control was observed in sorghum seedlings. This is probably due to the significant decrease in root length with a lower G of sorghum observed at this concentration of F in the substrate. Additionally, it can be seen that at 8 ppm of F in the substrate, while sorghum had a V of 91%, soybeans of 26% and maize only of 18%.

Measures of germination, plant length, root thickness and vigor index show that maize and soybeans are susceptible to F on the substrate, while sorghum seems to be resistant to this ion.



Fluoride content

F content was measured only in those seedlings treated with F in the substrate and compared with respect to 0 ppm of F. In Table 2, a significant accumulation of F in those seeds that did not germinate (NS) is observed for all crops investigated. In turn, maize seedlings treated with 8 ppm had a significant increase in the concentration of F in roots, which could not be observed in soybeans and sorghum.

Finally, it can be seen that the NS of sorghum incorporated significantly greater amount of F with respect to the other parts of the plant and other crops. More interestingly is that the amount of fluoride in the plant concentrates almost 8 times compared to the amount of F in the substrate.

Table 2. F content (ppm) of aerial part (A), root (R) and non-germinated seed (NS) of seedlings treated with 0, 2, 4 and 8 ppm of NaF on the substrate. Data are shown as the means of seedlings with equal treatment. At least one letter equally between two cells indicate no significant differences between group means. * Indicates significant differences compared with the other crops of the same ppm of NaF. Grey cells show the most relevant changes. Three-way ANOVA, LSD post test, $p<0.05$.

Crop	**part/ppm**	**0**	**2**	**4**	**8**
Maize	**A**	0.03^{c}	0.05^{c}	0.22^{c}	0.33^{c}
	R	0.30^{c}	0.30^{c}	1.01^{c}	4.39^{b}
	NS	0.79^{c}	0.95^{c}	3.02^{bc}	8.38^{a}
Soybeans	**A**	0.02^{b}	0.02^{b}	0.06^{b}	0.70^{b}
	R	0.58^{b}	0.11^{b}	0.02^{b}	0.26^{b}
	NS	0.02^{b}	0.38^{b}	0.17^{b}	27.07^{a}
Sorghum	**A**	0.07^{b}	0.05^{b}	0.02^{b}	0.15^{b}
	R	0.05^{b}	0.57^{b}	0.37^{b}	1.82^{b}
	NS	1.12^{b}	$1.14^{b,*}$	$7.90^{b,*}$	$62.55^{a,*}$

Fluoride transfer rate (FT)

The FT did not differ among treatments of any crop investigated nor among crops (Table 3). One can see that this index was always less than 1 for maize and sorghum, indicating that F was not transferred to the aerial part but accumulated in roots and seeds of F-treated seedlings. However, for the soybeans treated with 4 and 8 ppm of F in the substrate the FT was higher than 1, indicating that part of the F was transferred to the aerial part of these seedlings. This is important because if F is transferred to the aerial part could be incorporated into the edible part of the plant.

Table 3. F transfer rate of maize, soybeans and sorghum treated with 2, 4 and 8 ppm of NaF in the substrate. Data are shown as the means of seedlings with equal treatment. Grey cells show the most relevant changes. Two-way ANOVA, $p>0.05$.

Crop/ppm	**2**	**4**	**8**
Maize	0.17	0.31	0.23
Soybeans	0.20	2.83	2.72
Sorghum	0.08	0.07	0.08

Discussion and conclusions

The study of the concentrations of F in productive lands of Argentina and its effects on the growth of own crops in the region, is an important fact to keep in mind when grains are produced. Previous work showed that the total fluorine content of rural land (7.67 ppm) was significantly greater than the fluorine content in urban land in these same locations (5.9 ppm),[5] confirming that this increase may be due to an intensive agriculture activity in that area.

The results observed in our work shows that not all crops are affected in the same way with the concentration of F in the substrate. In Table 4 we summarise the results found in priority order for crop production and profitability.

Table 4. Summary of variables of germination and growth of soybeans, maize and sorghum treated with fluoride in the substrate. "0" no effect, "+" positive effect, "-" negative effect, "--" very negative effect.

Crop	G	V	Aerial part length	Root length	Root thickness
Soybeans	-	--	-	--	-
Maize	--	--	-	--	-

Sorghum	0	-	0	-	0

Maize and soybeans crops appear to be more sensitive to the action of F in the ground. However, sorghum appears to be more resistant to the action of this ion. The maize and soybeans growth decreased significantly from 2 ppm of F in the substrate, compared to their controls. This was evidenced by a significant decrease in germination, in the length of aerial parts and roots, in root thickness and vigor index. The V is an index that takes into account both plant growth and germination, so that a decrease in each of these parameters reveals a V much lower in F-treated maize and soybeans than controls, indicating a significant loss of vigor of the seedlings that may impede their development as adult plants. Some studies also show phytotoxicity in plants because of F.24 In watermelon plants, F 20-200 ppm decreased their growth, vigor index and the content of carotenoids and chlorophylls.25

By contrast, the sorghum crop was unchanged in the germination, in the length of the aerial part or in the roots thickness. Only a decrease was observed in root length and V at 4 ppm of F, changes that were not as important as in maize and soybeans. At 8 ppm of F in the substrate sorghum had a V of 91 %, soybeans of 26 % and maize of 18 %, indicating that the last two species probably are mostly affected by F and would not be capable of generating adult normal plants. These differences between crops make one suspect that the growth of sorghum on soils with high amount of F would be feasible without causing production losses.

The analysis of the concentration of F in seedlings shows that all crops analysed are capable of incorporating the F from the substrate, accumulating mainly in roots and ungerminated seeds. In turn, not only they incorporated the F from the substrate, but this ion concentrates on the seedlings. Seedlings treated with 8 ppm of F in the substrate, had concentrations of F of 8 (maize), 27 (soybeans) and 62.5 mg kg^{-1} of dry weight (sorghum) in ungerminated seeds, concentrating F almost 8 times. There are other papers where a similar accumulation of F in vegetables of fluorosis areas26,27 or in plants irrigated with F is shown.[24] Wheat seeds irrigated with fluoridated water retained the highest amount of F, coincident to our results.28 Sorghum was able to take more of F from the substrate, so that not only could be planted on soils with high F content but would have potential properties to act as a bioremediator of these soils, which could be rotated between crops most affected with F as maize and soybeans. Several screenings of areas rich in F are conducted to find vegetables resistant to F so as to be used as phytoremediators.29 These results could be due to the different susceptibility of the crops to F, so that some plants would be resistant or sensitive to F action. Some works demonstrated that strains of mice that accumulate more F in bone and have higher circulating F levels are also resistant to the effects of F.30

The knowledge that fluoride affects the growth of maize and soybeans may be an important factor to take into account to identify the better areas to plant these crops. Although the results indicate a decrease in growth and length of seedlings, it is necessary to evaluate the effect of fluoride concentrations on the field production of these crops. A smaller vigor index and a shorter length allows to speculate that production would be lower.

These results are also important for food production. The edible portion of maize and soybeans is the grain, which is used for both human consumption and breeding of certain animals. A study in Brazil showed that cereals and commercial biscuits that are mostly consumed by children, contain large amounts of F. When consumed just once per day, cereals and beverages might supply up to 25 % of the maximum recommended daily fluoride intake (0.07 mg F kg^{-1} body weight) for a 2-year-old child (12 kg).31 Studies are at the moment carried out to determine the fluoride content both in the plant and in the grain, in experiments conducted in the field. This knowledge is important because if maize and soybeans in soils with high content of F may produce high-F grains, could be an extra source of F for human beings or animals. F intake above certain limits leads to fluorosis with negative effects on the musculoskeletal and endocrine systems. In China there are many cases of fluorosis due to the intake of crops irrigated with highly fluoridated water.32

This work demonstrated that there are certain crops that are more susceptible to the amount of fluoride in soil such as maize and soybeans, which could directly affect on the nutrition of the population. However, there are other crops more resistant to these conditions (such as sorghum) and could be used as phytoremediators of soils high in F. Not all crops are affected the same way by F, or incorporated the same amount of F from the substrate on which they are grown.

Acknowledgements

This work was founded by Secretaría de Estado, Ciencia, Tecnología e Innovación of Santa Fe Province, Argentina without commercial interests. We thank María Florencia Paleari for technical assistance.

Conflict of interest

The author declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Figure captions

Figure 1. Photograph of a sorghum seedling after 6 days. Measurements of length of aerial part and root are shown.

Figure 2. Photograph of seedling root of sorghum after 6 days of treatment. Measurement of root thickness is shown.

Figure 3. Length of the aerial part (expressed as % of control group taken as 100 %) of maize (A), soybeans (B) and sorghum (C) seedlings treated with 2, 4 and 8 ppm of

NaF and controls. Data are shown as percentages relative to control group. At least one letter equal between two bars indicates no significant differences between group means. Two-way ANOVA, LSD post test, $p<0.05$.

Figure 4. Root length (expressed as % of control group taken as 100 %) of maize (A), soybeans (B) and sorghum (C) seedlings treated with 2, 4 and 8 ppm of NaF and controls. Data are shown as percentages relative to control group. At least one letter equal between two bars indicates no significant differences between group means. Two-way ANOVA, LSD post test, $p<0.05$.

Figure 5. Root thickness (expressed as % of control group taken as 100 %) of maize (A), soybeans (B) and sorghum (C) seedlings treated with 2, 4 and 8 ppm of NaF and controls. Data are shown as percentages relative to control group. At least one letter equal between two bars indicates no significant differences between group means. Two-way ANOVA, LSD post test, $p<0.05$.

Figure 6. Vigor index (expressed as % of control group taken as 100 %) of maize (A), soybeans (B) and sorghum (C) seedlings treated with 2, 4 and 8 ppm of NaF and controls. Data are shown as percentages relative to control group. At least one letter equal between two bars indicates no significant differences between group means. Two-way ANOVA, LSD post test, $p<0.05$.

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