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INTERACTIVE EFFECTS OF SALICYLIC ACID AND SODIUM FLUORIDE ON SOME BIOCHEMICAL ATTRIBUTES OF *PISUM SATIVUM* L.

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ABSTRACT: The aim of the present investigation was to ascertain the impact of salicylic acid on the productivity of the pea plant, *Pisum sativum* L., grown under sodium fluoride-induced stress. Several biochemical parameters (the photosynthetic pigments chlorophyll "a", chlorophyll "b", total chlorophyll, and carotenoids, total soluble proteins, total phenol content, proline content, and ascorbic acid content were studied in two varieties of *Pisum sativum* L. (RKS-510 and Classic) during the growth season 2015–2016, subjected to sodium fluoride (NaF) stress by the application of NaF, in concentrations of 50, 100, 150, and 200 ppm, as a soil drench on a biweekly basis and the biweekly exogenous application of salicylic acid as a foliar application, in concentrations of 100, 200 and 300 ppm, both alone and in combination with the NaF soil drench. Control plants were treated biweekly with tap water. The NaF caused toxic effects on the biochemical processes of the plants, with a dose-related gradual reduction in the biochemical attributes, which was alleviated by the salicylic acid. We concluded that the foliar application of salicylic acid increased the salt tolerance of the RKS-510 and Classic cultivars of *Pisum sativum* L.

Keywords: Biochemical attributes; Fluoride; *Pisum sativum* L.,Salicylic acid; Sodium fluoride, Stress.

INTRODUCTION

Salinity is one of the limiting environmental factors for soil fertility and plant production.[1] The basic cause of salinity-induced effects on the growth and development of plants is the accumulation of ions in the soil solution and ultimately in the plant cells.[2] Various fluoride sources and their impact on the plant biology have been well reported.[3] Fluoride is emitted into the environment through atmospheric emissions from volcanoes, seawater, weathering of rocks, and by the aluminum, glass, brick, pottery, and ceramic industries.[4] Fluoride in irrigation water can come through the high use of phosphate fertilizers and aerial deposition from ceramic industries and brick kilns.[5]

The fluoride ion negatively affects the process of photosynthesis and the metabolism of amino acids and proteins by disturbing membranes and the stromal enzymes related to carbon dioxide fixation and results in lowered chlorophyll concentrations[6] along with other physiological and biochemical disorders.[7]

Salicylic acid (SA) is a plant-originated, water-soluble, endogenous, antioxidant phenolic compound that enhances the growth of plants.[8] It is an important signaling molecule which modulates the responses of plants to environmental stresses.[8] The overall growth and photosynthetic capacity of plants under saline and stress conditions can be enhanced by the exogenous application of SA. Various physiological processes of plants are regulated by the activity of salicylic acid under saline conditions.[9] Salicylic acid promotes the salinity tolerance by accelerating the

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antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX).[10]

Pea (*Pisum sativum L.*) is an edible cool-season annual leguminous seed crop which is important for human nutrition. It is generally used as a fresh vegetable but is also preserved by canning and freezing.[11] Its seeds contain 18–20% dry matter, including 5–8% protein and 10–12% carbohydrate. Pea also contains various important antioxidants and phytochemicals which lower the level of free radicals and play an important role in the prevention of stomach cancer. Keeping the above production scenario and usefulness in mind it is urgent to strengthen production of pea.

The aim of the present investigation was to ascertain the impact of salicylic acid on the productivity of the pea plant, *Pisum sativum* L., grown under sodium fluoride-induced stress.

MATERIALS AND METHODS

Two varieties of *Pisum sativum* L., RKS-510 and Classic, were selected for the experiment which was conducted in the wire-house of the Botanical Garden, University of the Punjab, located at southern part of Lahore (74º21-00-E, 31º35-00-N), on a 21×15 m plot. Certified seeds of varieties RKS-510 and Classic were obtained from Punjab Seeds Corporation and Arain Seed Corporation, Lahore, respectively. Unhealthy, wrinkled, and infected seeds were carefully removed. Analytical grade chemicals were purchased from the market: sodium fluoride (NaF), salicylic acid (SA), acetone, phosphate buffer, Folin's mixture, Folin's Ciocalteu's phenol reagent, gallic acid, methanol, sodium carbonate, glacial acetic acid, sulphosalicylic acid, ninhydrin, Na-EDTA, oxalic acid, 2,6-dichlorophenol indophenol (DCPIP), potassium phosphate buffer, hydrogen peroxide (H_2O_2), guaiacol, and sulphuric acid (H_2SO_4).

Clay pots (25 cm diameter and 30 cm in length) were each filled with 5 kg of soil, constituted with sand and loam in a 1:3 ratio along with farmyard and leaf manure. The experiment was done in triplicate with a randomized complete block design (RCBD) to give similar environmental conditions. NaF, prepared in concentrations of 50, 100, 150, and 200 ppm, was used to create the stress condition. Salicylic acid, prepared in concentrations of 100, 200, and 300 ppm, was used to examine its ability to interact with and alleviate the NaF-induced stress. The seeds of both cultivars were soaked in clean tap water for 24 hours before sowing. Initially four seeds per pots were sown to raise the plants, but after germination only two healthy seedlings were selected for the further experimental procedures. The pots were examined on a regular basis to avoid attack by weeds and pathogens. The NaF and SA were applied biweekly (i.e., every two weeks) during the 2015–2016 growing season with the NaF being given as a soil drench (150 mL/pot) and the SA was applied exogenously (6 mL/pot) as a foliar spray. The control plants were given tap water biweekly.

Biochemical analysis was performed in triplicate on healthy leaves of the plants for the photosynthetic pigments chlorophyll "a", chlorophyll "b", total chlorophyll, and carotenoids, total soluble proteins, total phenol content, proline content, and ascorbic acid content, ascorbate peroxidase (APX) activity, and peroxidase (POX) activity).

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The plant pigments were measured according to the processes of Amon[12] and Taylor and Davies,[13] respectively. The plant leaves were cut into 0.5 cm segments and the extraction done with soaking in 80% acetone overnight at –10ºC. Then, for five min, the extract was centrifuged at 14000×g and by using a spectrophotometer (Hitachi-220, Tokyo, Japan) the absorbance of the supernatant was read at 480, 645, 663 nm, respectively.

The chlorophyll "a", chlorophyll "b", total chlorophyll, and carotenoids were measured by using following formula:

$$Chlorophyll\ a\ \left(\frac{mg}{g}FW\right) = (0.0127\ \times OD\ 663) + (0.00269\ \times OD\ 645)$$

$$Chlorophyll\ b\ \left(\frac{mg}{g}FW\right) = (0.0229\ \times OD\ 645) + (0.00468\ \times OD\ 663)$$

Where:

V = Volume of the extract (ml)

W = Weight of fresh leaf tissue (g)

Carotenoids: A^{car}/Em x 100 (g ml^{-1})

A^{car} = OD 480 + 0.114 (OD 663) – 0.638 (OD 645)

$E^{100\%}$ cm = 2500

$$Total\ Chlorophyll\ \left(\frac{mg}{g}FW\right) = (0.0202\ \times OD\ 645) + (0.0082\ \times OD\ 663)$$

For the estimation of the soluble protein, the method of Lowry et al.[14] was used. By mixing phosphate buffer (0.1 M, pH 7, 1:4 (w/v) in a pre-chilled pestle and mortar, frozen leaves were crushed. For 1 g of plant leaves, 4 mL of phosphate buffer was added. Then the sample was centrifuged at 10,000 rpm at 4ºC for 10 minutes. Into 0.4 mL of supernatant, 0.2 ml Folin's mixture was poured and it was left in the test tube at room temperature for 15 minutes. Then for the development of color, 0.2 mL of Folin's Ciocalteu's Phenol reagent was added. It was then mixed and placed at room temperature for 45 minutes. By using a spectrophotometer, the absorbance at 750 nm was measured. The amount of soluble protein was described by the standard curve.

Folin-Ciocalteu reagent was used to examine the total phenols in the extracts. The standards of gallic acid equivalents (GAE mg/g) were used as gallic acid to determine total phenols.[15] By using methanol, 0.01, 0.02, 0.03, 0.04, and 0.05 mg/g concentrations of gallic acid were prepared. In methanol, 0.1 mL of plant extract was also prepared. To all the test tubes of 0.5 mL samples, 2 mL of 7.5% sodium carbonate and 2.5 mL of Folin-Ciocalteu reagent was added. By using parafilm, the test tubes were sealed and placed at room temperature for 30 minutes. The

absorbance was measured at 760 nm by using a spectrophotometer. The Folin-Ciocalteu reagent gives a blue color after the reaction due to its sensitivity to the reducing compounds including polyphenols. Thus, the total phenol content was examined.[16]

By following the ninhydrin method of Bates et al.[17] the proline content was measured spectrophotometrically. In 3 mL of 3% sulphosalicylic acid, fresh leaf samples (300 mg) were homogenized. The filtrate was poured into test tubes and placed in a water bath at 100ºC and then reacted with 1 mL each of acid ninhydrin and glacial acetic acid for 1 hour. By using L-pro as a standard, the absorbance was measured spectrophotometrically at 520 nm after the extraction of the mixture with toluene.

Ascorbic acid: From leaf samples in Na-EDTA and oxalic acid, the content of ascorbic acid was assessed and with 2,6-dichlorophenol indophenol (DCPIP) dye their concentrations were measured. The absorbance was measured at 520 nm spectrophotometrically and ascorbic acid was measured by following the reduction method of Keller and Schwager.[18]

Data obtained from the pot experiment was then used to calculate the treatment mean, the standard error and Duncan's Multiple Range Test, as described by Steel and Torrie.[19] For this purpose, the software package Costat (version 3.03) was employed using the computer facility of the laboratory.

RESULTS

The data given in Tables 1–4 shows the biochemical attributes of the pea varieties RKS-510 and classic. The biochemical parameters studied for both varieties were chlorophyll "a", chlorophyll "b", total chlorophyll, and carotenoids, total soluble proteins, total phenol content, proline content, and ascorbic acid content.

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Table 1. Plant pigment of *Pisum sativum* L., variety RKS-510, measured at 44 days after sowing (DAS) using different sodium fluoride and salicylic acid concentrations. Values are mean±SE. (NaF=sodium fluoride; SA=salicylic acid; NaF-SA=sodium fluoride+salicylic acid)

Treatment	Plant pigment			
	Chlorophyll "a" (mg/g fresh wt)	Chlorophyll "b" (mg/g fresh wt)	Carotenoid (mg/g fresh wt)	Total chlorophyll (mg/g fresh wt)
Control	1.69ab ± 0.06	0.42a ± 0.015	0.58a ± 0.012	1.66a ± 0.017
NaF-50	1.56a-c ± 0.02	0.35a ± 0.012	0.52a ± 0.006	1.59ab ± 0.009
NaF-100	1.48a-d ± 0.01	0.31a ± 0.009	0.45a ± 0.012	1.50ab ± 0.009
NaF-150	1.41a-d ± 0.01	0.28a ± 0.015	0.40a ± 0.012	1.44ab ± 0.012
NaF-200	1.29a-d ± 0.006	0.22a ± 0.006	0.37a ± 0.009	1.31ab ± 0.006
SA-100	1.73a ± 0.01	0.48a ± 0.006	0.64a ± 0.009	1.70a ± 0.012
SA-200	1.68ab ± 0.006	0.43a ± 0.012	0.59a ± 0.006	1.64a ± 0.006
SA-300	1.39a-d ± 0.009	0.38a ± 0.09	0.53a ± 0.009	1.52ab ± 0.012
NaF-50+ SA-100	1.61a-c ± 0.015	0.40a ± 0.006	0.57a ± 0.012	1.64a ± 0.006
NaF-50+ SA-200	1.55a-c ± 0.009	0.34a ± 0.009	0.53a ± 0.009	1.60a ± 0.02
NaF-50+ SA-300	1.48a-d ± 0.006	0.29a ± 0.015	0.46a ± 0.006	1.54ab ± 0.006
NaF-100+ SA100	1.57a-c ± 0.009	0.35a ± 0.009	0.49a ± 0.012	1.55ab ± 0.009
NaF-100+ SA-200	1.49a-c ± 0.009	0.30a ± 0.006	0.46a ± 0.012	1.52ab ± 0.009
NaF-100+ SA-300	1.23b-d ± 0.006	0.25a ± 0.015	0.40a ± 0.009	1.43ab ± 0.003
NaF-150+ SA-100	1.49a-c ± 0.006	0.33a ± 0.006	0.47a ± 0.009	1.49ab ± 0.009
NaF-150+ SA-200	1.39a-d ± 0.006	0.29a ± 0.009	0.44a ± 0.09	1.43ab ± 0.009
NaF-150+ SA-300	1.18cd ± 0.006	0.22a ± 0.015	0.38a ± 0.012	1.37ab ± 0.012
NaF-200+ SA-100	1.35a-d ± 0.012	0.25a ± 0.006	0.40a ± 0.009	1.39ab ± 0.009
NaF-200+ SA-200	1.28a-d ± 0.009	0.20a ± 0.012	0.38a ± 0.009	1.32ab ± 0.012
NaF-200+ SA-300	1.01d ± 0.05	0.16a ± 0.019	0.21a ± 0.019	1.12b ± 0.10

Within each parameter, values not followed by the same lower case letter are significantly different with Duncan's multiple range test and p=0.05. a-c=abc; a-d=abcd

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Table 2. Plant pigment of *Pisum sativum* L., variety Classic, measured at 44 days after sowing (DAS) using different sodium fluoride and salicylic acid concentrations. Values are mean±SE. (NaF=sodium fluoride; SA=salicylic acid; NaF-SA=sodium fluoride+salicylic acid)

Treatment	Plant pigment			
	Chlorophyll "a" (mg/g fresh wt)	Chlorophyll "b" (mg/g fresh wt)	Carotenoid (mg/g fresh wt)	Total chlorophyll (mg/g fresh wt)
Control	1.62ab ± 0.015	0.39a ± 0.015	0.55a ± 0.012	1.64a ± 0.007
NaF-50	1.53a-c ± 0.015	0.32a ± 0.012	0.48a ± 0.015	1.56a ± 0.015
NaF-100	1.42a-d ± 0.006	0.27a ± 0.017	0.42a ± 0.012	1.48ab ± 0.00
NaF-150	1.38a-d ± 0.009	0.24a ± 0.020	0.38a ± 0.012	1.42ab ± 0.006
NaF-200	1.22a-d ± 0.018	0.18a ± 0.018	0.35a ± 0.003	1.29ab ± 0.006
SA-100	1.69a ± 0.015	0.43a ± 0.009	0.60a ± 0.012	1.59a ± 0.052
SA-200	1.61ab ± 0.020	0.40a ± 0.015	0.56a ± 0.003	1.59a ± 0.006
SA-300	1.35a-d ± 0.015	0.35a ± 0.015	0.51a ± 0.003	1.49ab ± 0.009
NaF-50+ SA-100	1.57a-c ± 0.019	0.36a ± 0.006	0.53a ± 0.009	1.61a ± 0.009
NaF-50+ SA-200	1.50a-c ± 0.009	0.31a ± 0.003	0.50a ± 0.012	1.57a ± 0.019
NaF-50+ SA-300	1.45a-d ± 0.006	0.26a ± 0.006	0.44a ± 0.013	1.47ab ± 0.027
NaF-100+ SA100	1.54a-c ± 0.015	0.32a ± 0.006	0.47a ± 0.003	1.51ab ± 0.003
NaF-100+ SA-200	1.44a-d ± 0.015	0.26a ± 0.009	0.43a ± 0.015	1.48ab ± 0.012
NaF-100+ SA-300	1.19b-d ± 0.012	0.22a ± 0.006	0.37a ± 0.021	1.40ab ± 0.006
NaF-150+ SA-100	1.44a-d ± 0.024	0.29a ± 0.009	0.42a ± 0.006	1.45ab ± 0.015
NaF-150+ SA-200	1.34a-d ± 0.007	0.25a ± 0.017	0.41a ± 0.09	1.39ab ± 0.012
NaF-150+ SA-300	1.11cd ± 0.015	0.20a ± 0.009	0.36a ± 0.020	1.34ab ± 0.023
NaF-200+ SA-100	1.30a-d ± 0.009	0.22a ± 0.006	0.37a ± 0.021	1.37ab ± 0.024
NaF-200+ SA-200	1.27a-d ± 0.009	0.19a ± 0.006	0.35a ± 0.012	1.29ab ± 0.015
NaF-200+ SA-300	1.00d ± 0.058	0.13a ± 0.012	0.19a ± 0.009	1.05b ± 0.076

Within each parameter, values not followed by the same lower case letter are significantly different with Duncan's multiple range test and p=0.05. a-c=abc; a-d=abcd; b-d=bcd

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Table 3. Biochemical parameters of *Pisum sativum* L., variety RKS-510, measured at 44 days after sowing (DAS) using different sodium fluoride and salicylic acid concentrations. Values are mean±SE. (NaF=sodium fluoride; SA=salicylic acid; NaF-SA=sodium fluoride+salicylic acid; fr. wt=fresh weight)

Treatment	Biochemical Parameter			
	Total soluble proteins content (mg/g fr. wt)	Total phenol content (mg/g fr. wt)	Proline content (mg/g fr. wt)	Ascorbic acid content (mg/g fr. wt)
Control	1.59ab ± 0.012	1.33b-e ± 0.015	0.73a ± 0.012	0.53a ± 0.006
NaF-50	1.25b-e ± 0.006	1.46a-d ± 0.018	0.82a ± 0.015	0.60a ± 0.006
NaF-100	0.98d-h ± 0.012	1.56a-c ± 0.015	0.88a ± 0.006	0.66a ± 0.006
NaF-150	0.82e-h ± 0.015	1.65ab ± 0.017	0.92a ± 0.009	0.74a ± 0.009
NaF-200	0.65gh ± 0.012	1.86a ± 0.009	1.03a ± 0.012	0.81a ± 0.012
SA-100	1.89a ± 0.017	1.13c-g ± 0.012	0.71a ± 0.009	0.50a ± 0.009
SA-200	1.54a-c ± 0.006	1.06d-g ± 0.017	0.68a ± 0.006	0.46a ± 0.018
SA-300	1.05d-g ± 0.015	1.00d-h ± 0.006	0.62a ± 0.018	0.42a ± 0.006
NaF-50+ SA-100	1.34b-d ± 0.006	1.21c-f ± 0.015	0.75a ± 0.015	0.55a ± 0.015
NaF-50+ SA-200	1.27b-e ± 0.009	1.14c-g ± 0.015	0.70a ± 0.009	0.46a ± 0.018
NaF-50+ SA-300	1.13c-f ± 0.009	1.06d-g ± 0.019	0.64a ± 0.015	0.42a ± 0.006
NaF-100+ SA100	1.03c-f ± 0.009	1.00d-h ± 0.015	0.80a ± 0.013	0.58a ± 0.012
NaF-100+ SA-200	1.02d-f ± 0.009	0.94e-h ± 0.018	0.76a ± 0.015	0.56a ± 0.006
NaF-100+ SA-300	0.86e-h ± 0.012	0.91e-h ± 0.012	0.68a ± 0.006	0.53a ± 0.009
NaF-150+ SA-100	0.95d-h ± 0.006	0.85f-h ± 0.015	0.79a ± 0.006	0.68a ± 0.009
NaF-150+ SA-200	0.87e-h ± 0.01	0.80f-h ± 0.012	0.71a ± 0.012	0.61a ± 0.015
NaF-150+ SA-300	0.77f-h ± 0.009	0.74f-h ± 0.012	0.95a ± 0.023	0.56a ± 0.015
NaF-200+ SA-100	0.72f-h ± 0.10	0.79f-h ± 0.009	0.88a ± 0.025	0.77a ± 0.012
NaF-200+ SA-200	0.65gh ± 0.015	0.67gh ± 0.012	0.80a ± 0.019	0.71a ± 0.009
NaF-200+ SA-300	0.56h ± 0.009	0.53h ± 0.009	0.13a ± 0.012	0.65a ± 0.012

Within each parameter, values not followed by the same lower case letter are significantly different with Duncan's multiple range test and p=0.05. a-c=abc; b-d=bcd; b-e=bcde; c-f=cdef; d-f=def; d-h=defgh; e-h=efgh; f-h=fgh.

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Table 4. Biochemical parameters of *Pisum sativum* L., variety Classic, measured at 44 days after sowing (DAS) using different sodium fluoride and salicylic acid concentrations. Values are mean±SE. (NaF=sodium fluoride; SA=salicylic acid; NaF-SA=sodium fluoride+salicylic acid; fr. wt=fresh weight)

Treatment	Biochemical Parameter			
	Total soluble proteins content (mg/g fr. wt)	Total phenol content (mg/g fr. wt)	Proline content (mg/g fr. wt)	Ascorbic acid content (mg/g fr. wt)
Control	1.53ab ± 0.009	1.28bcd ± 0.015	0.69a ± 0.009	0.49a ± 0.009
NaF-50	1.20b-f ± 0.006	1.40bc ± 0.012	0.79a ± 0.015	0.56a ± 0.012
NaF-100	0.94d-h ± 0.009	1.52ab ± 0.006	0.81a ± 0.009	0.62a ± 0.006
NaF-150	0.79e-h ± 0.019	1.59ab ± 0.012	0.88a ± 0.012	0.71a ± 0.012
NaF-200	0.62gh ± 0.012	1.81a ± 0.009	0.99a ± 0.033	0.78a ± 0.012
SA-100	1.83a ± 0.020	1.01c-e ± 0.015	0.68a ± 0.018	0.46a ± 0.015
SA-200	1.46a-c ± 0.021	0.91d-f ± 0.009	0.63a ± 0.006	0.42a ± 0.006
SA-300	0.98d-g ± 0.017	0.98c-e ± 0.013	0.58a ± 0.012	0.39a ± 0.009
NaF-50+ SA-100	1.28b-d ± 0.019	1.08c-e ± 0.012	0.71a ± 0.017	0.50a ± 0.009
NaF-50+ SA-200	1.21b-f ± 0.009	1.07c-e ± 0.026	0.66a ± 0.027	0.41a ± 0.012
NaF-50+ SA-300	1.08c-g ± 0.012	0.96c-e ± 0.029	0.60a ± 0.012	0.38a ± 0.009
NaF-100+ SA100	0.98d-g ± 0.006	0.97c-e ± 0.032	0.76a ± 0.026	0.54a ± 0.009
NaF-100+ SA-200	0.96d-h ± 0.021	0.91d-f ± 0.020	0.71a ± 0.018	0.51a ± 0.009
NaF-100+ SA-300	0.78e-h ± 0.012	0.87d-f ± 0.017	0.64a ± 0.009	0.50a ± 0.009
NaF-150+ SA-100	0.91d-h ± 0.009	0.81ef ± 0.009	0.83a ± 0.012	0.61a ± 0.009
NaF-150+ SA-200	0.82d-h ± 0.015	0.76ef ± 0.022	0.73a ± 0.012	0.57a ± 0.015
NaF-150+ SA-300	0.74f-h ± 0.024	0.71ef ± 0.015	0.69a ± 0.015	0.51a ± 0.006
NaF-200+ SA-100	0.68gh ± 0.009	0.75ef ± 0.023	0.90a ± 0.018	0.72a ± 0.009
NaF-200+ SA-200	0.61gh ± 0.009	0.63ef ± 0.012	0.85a ± 0.027	0.69a ± 0.009
NaF-200+ SA-300	0.49h ± 0.015	0.48f ± 0.015	0.76a ± 0.020	0.62a ± 0.006

Within each parameter, values not followed by the same lower case letter are significantly different with Duncan's multiple range test and p=0.05. a-c=abc; b-d=bcd; b-f=bcdef, c-e=cde; c-g=cdefg; d-f=def; d-g=defg; d-h=defgh; e-h=efgh; f-h=fgh.

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Tables 1 and 2 show the amount of photosynthetic pigments, i.e., chlorophyll "a", chlorophyll "b", total chlorophyll, and carotenoids, in both varieties of pea plant. They decreased with increases in the concentrations of sodium fluoride. The plants treated with a high fluoride concentration lacked a dark green color due to a deficiency of plant pigments.

For variety RKS-510, at 50 ppm sodium fluoride, chlorophyll "a" was decreased at 1.56 mg/g fr. wt, compared to the control value of 1.69 mg/g fr. wt. The highest reduction was observed in the 200 ppm NaF group with 1.29 mg/g fr. wt. The chlorophyll "a" content was observed at a maximum in the SA-100 group with 1.73 mg/g fr. wt. With the increasing NaF concentrations of 50, 100, 150, and 200 ppm, the chlorophyll "a" levels decreased with values of 1.56, 1.48, 1.41, and 1.29 mg/g fr. wt. The decreases were not statistically significant compared to the control group (Table 1).

For variety Control, at 50 ppm sodium fluoride, chlorophyll "a" was decreased at 1.53 mg/g fr. wt, compared to the control value of 1.62 mg/g fr. wt. The highest reduction was observed in the 200 ppm NaF group with 1.29 mg/g fr. wt. The chlorophyll "a" content was observed at a maximum in the SA-100 group with 1.22 mg/g fr. wt. With the increasing NaF concentrations of 50, 100, 150, and 200 ppm, the chlorophyll "a" levels decreased with values of 1.53, 1.42, 1.38, and 1.22 mg/g fr. wt. The decreases were not statistically significant compared to the control group (Table 2).

For variety RKS-510, at 50 ppm sodium fluoride, chlorophyll "b" was decreased at 0.35 mg/g fr. wt, compared to the control value of 0.42 mg/g fr. wt. The highest reduction was observed in the 200 ppm NaF group with 0.22 mg/g fr. wt. The chlorophyll "b" content was observed at a maximum in the SA-100 group with 0.48 mg/g fr. wt. With the increasing NaF concentrations of 50, 100, 150, and 200 ppm, the chlorophyll "b" levels decreased with values of 0.35, 0.31, 0.28, and 0.22 mg/g fr. wt. The decreases were not statistically significant compared to the control group (Table 1).

For variety Control, at 50 ppm sodium fluoride, chlorophyll "b" was decreased at 0.32 mg/g fr. wt, compared to the control value of 0.39 mg/g fr. wt. The highest reduction was observed in the 200 ppm NaF group with 0.18 mg/g fr. wt. The chlorophyll "b" content was observed at a maximum in the SA-100 group with 0.43 mg/g fr. wt. With the increasing NaF concentrations of 50, 100, 150, and 200 ppm, the chlorophyll "b" levels decreased with values of 0.32, 0.27, 0.24, and 0.18 mg/g fr. wt. The decreases were not statistically significant compared to the control group (Table 2).

For variety RKS-510, the maximum value for the carotenoids was in the SA-100 group (0.64 mg/g fr. wt) compared to the control value of 0.58 mg/g fr. wt. With the increasing NaF concentrations of 50, 100, 150, and 200 ppm, the carotenoids levels decreased with values of 0.52, 0.45, 0.40, and 0.37 mg/g fr. wt. The decreases were not statistically significant compared to the control group (Table 1).

For variety Control, the maximum value for the carotenoids was in the SA-100 group (0.60 mg/g fr. wt) compared to the control value of 0.55 mg/g fr. wt. With the increasing NaF concentrations of 50, 100, 150, and 200 ppm, the carotenoids levels

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decreased with values of 0.48, 0.42, 0.38, and 0.35 mg/g fr. wt. The decreases were not statistically significant compared to the control group (Table 2).

For both varieties, the total chlorophyll content was also showed the same pattern of enhancement with SA and reduction with NaF. When NaF, in concentrations of 50, 100, 150, and 200 ppm) and SA, in concentrations of 100 and 200 ppm (but not 300 ppm), were applied in combination, the SA generally ameliorated the stress of the NaF as reflected in the total chlorophyll value. The overall amount of plant pigments was higher in the variety RKS-510 as compared to the variety Classic (Tables 1 and 2).

Table 3 (variety RKS-510) shows that total soluble proteins decreased as the concentration of NaF increased and two lowest protein content levels were observed with NaF-200 ppm and NaF-200+SA-300 at 0.65, and 0.56 mg/g fr. wt, respectively, due to the stress of both NaF and SA. Salicylic acid enhanced the amount of soluble proteins when applied exogenously. The control group and SA-100 showed the two highest soluble protein content levels (1.59 and 1.89 mg/g fr. wt), respectively, while the groups with 50, 100, and 150 ppm NaF showed gradually reducing levels (1.25, 0.98, and 0.82 mg/g fr. wt, respectively). All the treatments with NaF in combination with SA-100 ppm showed a low soluble protein content. SA-300 ppm also caused stress in the plants and lowered the soluble protein levels.

Table 4 (variety Classic) shows that NaF, at concentrations of 50, 100, 150, and 200 ppm, lowered the protein content, compared to the control value of 1.53 mg/g fr. wt, with values of 1.20, 0.94, 0.79, and 0.62 mg/g fr. wt, respectively. The lowering was significant ($p=0.05$) for the NaF concentrations of 100, 150, and 200 ppm. The control and SA-100 groups showed the two highest soluble protein content levels (1.53 and 1.83 mg/g fr. wt, respectively). The combinations of both NaF and SA showed a variable result. SA reduced the stress of NaF but an excess of SA also caused stress. The plants of the treatment of NaF-200+SA-300 showed the minimum protein content of 0.49 mg/g fr. wt due to the stress of both NaF and SA.

Phenols are compounds produced by plants in response to stress and they accumulate in plants. With elevated levels of salinity, phenols are produced in excess. The exogenous application of SA reduced the stress of NaF and also affected the production of phenols. For variety RKS-510, the maximum level of phenols was observed with the NaF-200 ppm group (1.86 mg/g fr. wt), a significant increase ($p=0.05$) compared to the control group which had a phenol content level of 1.33 mg/g fr. wt. For variety RKS-510 with treatment with 50, 100, and 150 ppm NaF, the corresponding total phenol content levels were 1.46, 1.56, and 1.65 mg/g fr. wt. Compared to the values with treatment with NaF, treatment with a combination of NaF and SA resulted in lower total phenol values (Table 3).

The total phenol content was less overall with variety Classic variety compared to variety RKS-510 (Table 4). For variety Classic, the maximum level of phenols was observed with the NaF-200 ppm group (1.81 mg/g fr. wt), a significant increase ($p=0.05$) compared to the control group which had a level of 1.28 mg/g fr. wt phenol content. For variety Classic with treatment with 50, 100, and 150 ppm NaF, the corresponding total phenol content levels were 1.40, 1.52, and 1.59 mg/g fr. wt.

The proline content was increased as the result of NaF stress. SA lowers the proline content of the pea plants in both the RKS-510 and Classic varieties but the decrease was more pronounced in the Classic variety (Tables 3 and 4). The highest proline content was measured in the NaF-200 group (1.03 and 0.99 mg/g fr. wt, for the RKS-570 and Classic varieties, respectively). These values were increased compared to the control values (0.73 and 0.69 mg/g fr. wt, for the RKS-570 and Classic varieties, respectively) but the increase was not statistically significant. With treatment with 50, 100, and 150 ppm of NaF, there was a gradual increase in the proline content (0.82, 0.88, and 0.92 mg/g fr. wt for the RKS-510 variety and 0.79, 0.81, and 0.88 mg/g fr. wt for the Classic variety, respectively).

Ascorbic acid is an antioxidant which plays an important role in protection against physiological stress. The ascorbic acid content was higher with increasing NaF concentrations as shown in Tables 3 and 4 for both the RKS-510 and Classic pea varieties. The highest ascorbic acid content was measured in the NaF-200 group (0.81 and 0.78 mg/g fr. wt for the RKS-510 and Classic varieties respectively. These values were increased compared to the control values (0.53 and 0.49 mg/g fr. wt, for the RKS-570 and Classic varieties, respectively) but the increase was not statistically significant. With treatment with 50, 100, and 150 ppm of NaF there was a gradual increase in the ascorbic acid content (0.60, 0.66, and 0.74 mg/g fr. wt for the RKS-510 variety and 0.56, 0.62, and 0.71 mg/g fr. wt for the Classic variety, respectively).

DISCUSSION

Fluorine is a widespread element in nature as the 13th most abundant element in the earth crust. The contamination of the natural environment with fluorine compounds has become a global problem.[20] Numerous investigations indicate that a high fluorine content adversely affects the growth and development of plants[21] causing leaf damage,[22] or decreasing the content of chlorophyll in the leaves.[23] Many kinds of fluorides are poisonous and present in acidic soils. These soils frequently have moderate to high levels of fluoride thus causing a stressed condition for plants. *Pisum sativum* L. is commonly used as fresh peas and dry pulses. Economically, it has been grown for the canning industry. The impact of fluoride stress on the productivity of pea plant has been determined by applying various concentrations of NaF.

A reduction in leaf area resulted in a decreased net photosynthetic rate and biomass accumulation.[24] The results of the present experiment show that the plant pigments tend to decrease gradually with increased NaF concentrations while the exogenous application of SA reduced the stress of NaF. The reduced plant pigments in the plants grown under NaF stress resulted in less productivity. The decrease in the photosynthetic pigments reduces the photosynthetic efficiency of *Pisum sativum*.

The present study demonstrated that the total soluble proteins were decreased by the gradual increase in the NaF concentration. This is consistent with the findings of Malik and Arya who found that the proteins were affected due to the fluoride-induced toxicity which was greater at the higher concentrations of 100 and 200 ppm.[25] The phenol content is important for inducing tolerance to biotic and abiotic stresses.[26] In the present study, changes in phenolic compounds was observed response to salinity stress. We found that the total phenol content increased as the concentration of sodium fluoride used in the treatment increased. The exogenous application of

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salicylic acid resulted in a marked effect and reduced the total phenol content. The results of present study are consistent with the studies by Amarowicz et al. [27] and Giannakoula et al.[28] who found that in lentil crops the level of phenolic compounds was increased under salinity stress. This increased phenolic content results in a stimulation of secondary metabolism under fluoride stress. Furthermore, phenolic compounds are involved in the defense against oxidative stress developing due to fluoride stress. It has been reported that the total phenols in leaves of *Simarouba glauca* increased significantly with increasing NaF treatments.[29]

Proline is a stress related dominant signaling molecule which is organic in nature and acts as a sink for energy. Under stress conditions, proline is mainly involved in stabilization of sub-cellular structures and facilitates osmotic adjustment. Furthermore, by acting as an enzymatic regulator, proline also controls the cell osmoregulation and protects the proteins during dehydration.[30] In the present study, the proline content was enhanced with increased NaF concentrations while the exogenous application of SA reduced the proline content. The main cause of higher the proline content under stress is the accelerated production of proline rich protein which enhances the tolerance capacity of the crop plant.[31]

CONCLUSIONS

In the present experimentation, sodium fluoride was continuously given to the RKS-510 and Classic cultivars of pea plant (*Pisum sativum* L.) on a biweekly basis and found to produce detrimental effects on some biochemical attributes. This is a matter of serious concern, from an agricultural viewpoint, as Pakistan is an agrarian country and its soils are being contaminated with a toxic load of NaF from various sources. Alhough plants are silent sufferers, they reveal their response in the form of a deprived growth pattern and a reduced yield as observed in present investigation. However. the foliar application of salicylic acid proved to be effective against the sodium fluoride stress. The exogenous application of salicylic acid successively reduced the effects of the sodium fluoride stress and enhanced the morphological and physiological parameters of *Pisum sativum*. However, at higher concentrations, the salicylic acid itself may cause a high level of stress in plants and was then less effective.

REFERENCES

1 Khan MH, Panda SK. Alterations in root lipid peroxidation and antioxidative responses in two rice cultivars under NaCl-salinity stress. Acta Physiologiae Plantarum 2008;30(1):81.
2 Hasegawa PM, Bressan RA, Zhu J-K, Bohnert HJ. Plant cellular and molecular responses to high salinity. Annual Review of Plant Biology 2000;51(1):463-99.
3 Mishra PC, Sahu SK, Bhoi AK, Mohapatra SC. Germination performance and growth potential of selected crops grown in fluoride contaminated soil. Open Journal of Soil Science 2014;4:388-98.
4 Ahmad MN, Ahmad SS, Zia A, Iqbal MS, Shah H, Mian AA, Shah RU. Hydrogen fluoride effects on local mung bean and maize cereal crops from peri-urban brick kilns in South Asia. Fluoride 2014;47(4):315-9.
5 Gupta S, Banerjee S, Mondal SM. Phytotoxicity of fluoride in the germination of paddy (*Oryza sativa*) and its effect on the physiology and biochemistry of germinated seedlings. Fluoride 2009;42(2):142-6.
6 Istas JR, Alaerts G. The effect of fluorine and its compounds on plants. Revue de l'Agriculture 1974;27:487-96.
7 Gautam R, Bhardwaj N. Bioaccumulation of fluoride in different plant parts of *Hordeum vulgare* (barley) var. rd-2683 from irrigation water. Fluoride 2010;43(1):57-60.

8 Breusegem FV, Vranová E, Dat JF, Inzé D. The role of active oxygen species in plant signal transduction. Plant Science 2001;161(3):405-14.
9 Khan SU, Bano A, Gurmani AR. Abscisic acid and salicylic acid seed treatment as potent inducer of drought tolerance in wheat (*Triticum aestivum* L.). Pakistan Journal of Botany (Pakistan) 2012;44(1):43-9.
10 Noreen S, Ashraf M, Hussain M, Jamil A. Exogenous application of salicylic acid enhances antioxidative capacity in salt stressed sunflower (*Helianthus annuus* L.) plants. Pak J Bot 2009;41(1):473-9.
11 Ismail A, Tiong NW, Tan ST, Azlan A. Antioxidant properties of selected non-leafy vegetables. Nutrition & Food Science 2009;39(2):176-80.
12 Arnon DI. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiology 1949;24(1):1-15.
13 Taylor RF, Davies BH. Triterpenoid carotenoids and related lipids. Triterpenoid carotenoid aldehydes from *Streptococcus faecium* UNH 564P. Biochemical Journal 1976;153(2):233-9.
14 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 1951;193(1):265-75.
15 Lim YY, Lim TT, Tee JJ. Antioxidant properties of guava fruit: comparison with some local fruits. Sunway Academic Journal 2006;3:9-20.
16 Pourmorad F, Hosseinimehr S, Shahabimajd N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. African Journal of Biotechnology 2006;5(11):1142-5
17 Bates L, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Plant and Soil 1973;39(1):205-7.
18 Keller T, Schwager H. Air pollution and ascorbic acid. Forest Pathology 1977;7(6):338-50.
19 Steel RG, Torrie J. Analysis of variance III: factorial experiments. Principles and procedures of statistics: A biometrical approach. 2nd ed. New York: McGraw-Hill; 1980. pp. 336-76.
20 Czerny B, Put A, Mysliwiec Z, Juzyszyn Z. The influence of quercetin on some biochemical parameters in rats exposed to environmental contamination with fluorine compounds. Polish Journal of Environmental Studies 2000;9(3):157-62.
21 Joshi M, Bhardwaj N. Effect of fluoride on growth parameters and its accumulation in *Triticum aestivum* var. Raj. 3675. Fluoride 2012;45(3/2):297-301.
22 Doley D. Rapid quantitative assessment of visible injury to vegetation and visual amenity effects of fluoride air pollution. Environmental Monitoring and Assessment 2010;160(1-4):181-98.
23 Chakrabarti S, Patra PK, Mandal B, Mahato D. Effect of sodium fluoride on germination, seedling growth and biochemistry of bengal gram (*Cicer arietinum*). Fluoride 2012;45(3 Pt2):257-62.
24 Sakr M, El-Emery M, Fouda R, Mowafy M. Role of some antioxidants in alleviating soil salinity stress. J Agric Sci Mansoura Univ 2007;32:9751-63.
25 Malik RPS, Arya KPS. Effect of fluoride toxicity on the growth and yield of mungbean (*Vigna radiata* L.). Internat. J. Plant Sci 2008;3(2):419-20.
26 Wahid A. Physiological implications of metabolite biosynthesis for net assimilation and heat-stress tolerance of sugarcane (*Saccharum officinarum*) sprouts. Journal of Plant Research 2007;120(2):219-28.
27 Amarowicz R, Estrella I, Hernández T, Dueñas M, Troszyńska A, Kosińska A, Pegg RB. Antioxidant activity of a red lentil extract and its fractions. International Journal of Molecular Sciences 2009;10(12):5513-27.
28 Giannakoula A, Ilias I, Dragišić-Maksimović JJ, Maksimović V, ivanović BD. Does overhead irrigation with salt affect growth, yield, and phenolic content of lentil plants? Archives of Biological Sciences 2012;64(2):539-47.
29 Mali VV, Bhoite AS, Gaikwad DK. Effect of fluoride stress on phenolics of medicinally important plant *Simarouba glauca.* DC Indian J Adv Plant Res 2014;1:1-4.
30 Rontein D, Basset G, Hanson AD. Metabolic engineering of osmoprotectant accumulation in plants. Metabolic Engineering 2002;4(1):49-56.
31 Datta J, Maitra A, Mondal NK, Banerjee A. Studies on the impact of fluoride toxicity on germination and seedling growth of gram seed (*Cicer arietinum* L. cv. Anuradha). Journal of Stress Physiology & Biochemistry 2012;8(1):194-202.