Effect of Azolla extract on the salt tolerance of lentils during germination

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Abstract

Soil salinization is one of the threats that affect the germination, growth, and production of many crops. The present study has evaluated to what degree *Azolla piñnata* extracts can improve the salt tolerance of lentil (*Lens culinaris*, Giza 9) seeds. Germination percentage, salt tolerance, plumule, and radicle lengths and seedling vigor index were gradually reduced at 150 and 200 mM NaCl salt stress. While the catalase and ascorbic oxidase enzymes activities were gradually inhibited at the two levels of NaCl salt-stress, peroxidase and polyphenol oxidase activities were significantly enhanced. Total soluble sugar in salt-stressed lentils along with α-amylase activity were also lowered. The level of the phytohormones auxins, gibberellic acids, and zeatin highly decreased at 150 mM NaCl, while the abscisic acid level was not affected. On the other side, H_2O_2, lipid peroxidation, and proline levels drastically enhanced at 150 and 200 mM NaCl salt stress. Salt-stressed lentil extracts (2, 4, 6, and 8 mg ml^{-1}) had a high H_2O_2 scavenging activity percentage compared to unstressed ones. Fresh or boiled extract of *Azolla piñnata* alleviated all negative effects of 150 mM NaCl salt-stress on lentils by enhancing the activity of the antioxidant enzymes, H_2O_2 scavenging capacity, α-amylase activity, and elevating soluble sugars, proline, and phytohormones content (particularly gibberellic acid). The fresh extract was more effective than the boiled one to increase the NaCl stress tolerance of lentils. Germination was finally recovered by Azolla extracts which may be considered as a piece of evidence to support the role of the application of *Azolla piñnata* extracts as a biostimulant to improve germination and early growth of many species under salinity.

Keywords: antioxidant, *Azolla piñnata*, enzymes, lentil, oxygen, reactive, species

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Introduction

Ionic and osmotic components are the main effects of salt stress. Salt stress interrupts water content, nutrient balance, and the activity of various enzymes (Sharma et al., 2019). Moreover, under saline conditions, active oxygen forms (Reactive Oxygen Species) are produced and reacted with crucial macromolecules in the cell (oxidative stress) (Acosta-Motos et al., 2017). In such case, plant cell is protected by an integrated system which includes antioxidant compounds and enzymes such as catalase (CAT) and peroxidase (POX). These enzymes are the main

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active oxygen quenchers under salt stress (Seckin et al., 2017). Under such conditions, plants use unique strategies including physiological, biochemical, and molecular changes for adaptation (Acosta-Motos et al., 2017).

Germination represents a rich changeable stage that starts with water uptake during the plant life cycle. The main changes during this stage are hydrolysis of starch and protein, high-rate respiration, cell elongation, and synthesis of macromolecules (Bewley et al., 2013). This stage revealed the completion of crop establishment and yield in the field (Hasanuzzaman et al., 2013). Many authors including Aniat-ul-Haq and Agnihotri (2010); Sabaghnia and Janmohammadi (2015) studied the inhibitory effects of salinity on germination of Lentils.

The use of natural biostimulants of plants (PBs) is one of the approaches which are widely used to enhance the resistance of plants under abiotic stresses (Del Buono, 2021). Azolla (mosquito fern, duckweed fern, fairy moss, or water fern) is an aquatic fern (genus of seven species) and its family is Salviniaceae. Azolla is a weed in many parts of the world, entirely covering some bodies of water and the plant's common name is "mosquito fern". Azolla is a highly fertile plant. It doubles its biomass in 3-10 days, depending on conditions, and yield can reach 8-10 tons fresh matter/ha in Asian rice fields. Azolla is a potential fertilizer used as “green manure” in several countries in rice paddies to increase rice yields (Van Hove and Lejeune, 2002). Moreover, Azolla is a rich source of proteins, essential amino acids, vitamins (vitamin A, vitamin B12, Beta Carotene), growth promoter intermediaries, and minerals like calcium, phosphorous, potassium, ferrous, copper, magnesium, etc. It contains 25-30% protein, 10-15% mineral content and 7-10%, a combination of amino acids, bioactive substances, and biopolymers (Pillai et al., 2002). On the other hand, Azolla has very low carbohydrate and oil content (Bindhu, 2013; Mahanty et al., 2017). Of importance, Azolla acts as a source of some phytohormones such as auxins, gibberellins, and cytokinins that improve plant growth (Nevine and El-Shahat, 2017).

In the current study, the main purpose was the possibility of using Azolla piñnata extract to support the salt tolerance of lentils during germination and early seedling under NaCl salt-stress.

Materials and Methods

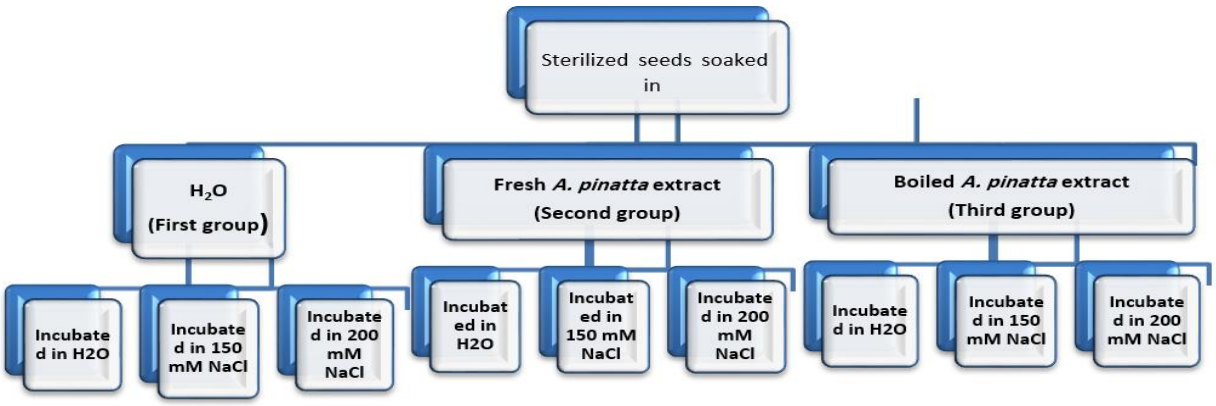
Seeds of Lens culinaris (Giza 9) and *Azolla piñnata* in its nutrient solution were obtained from Agricultural Research Center, Giza, Egypt.

Preparation of Azolla extract

Azolla piñnata (*A. piñnata*) mass was washed with distilled water to be free from any undesirable contaminants. Then, the mass was put on filter paper to remove the excess washing water. Ten percentage (10%) *A. piñnata* extract was prepared according to Bindhu (2013) (with some modifications). Ten grams (10 g) of *A. piñnata* mass was stirred in 100 ml distilled water for 30 min with or without boiling. Then, the extract was filtered (1 kg in 1 liter considered as 100% concentration of the *A. piñnata* extract). Then, it was preserved in a refrigerator at 4 ℃.

Germination experiment

Uniform seeds were surface sterilized with 0.2% (w/v) $HgCl_2$ solution for 5 min with frequent shaking and then thoroughly washed with deionized water. Fresh and boiled Azolla extracts were called F and B, respectively. Sterilized seeds were divided into three groups (Map 1). The first group of seeds was soaked for 12 h in distilled water, the second group was soaked in F extract, and the third group was soaked in B extract. After soaking, twenty-five seeds of each group were incubated at 25 ℃ in petri dishes on filter paper with 5 ml distilled water (as control) or 5 ml NaCl (150 mM or 200 mM), and each petri dish was repeated for three times. Percentage of germination, salt tolerance, plumule, and radicle lengths, seedling vigor Index, some antioxidant enzymes, and metabolic activities were measured after five days of germination. Vigor index of the seedlings was estimated according to the following formula (Abdul-Baki et al., 1973):



Map 1. Representation of the different treatments

Seedling Vigor Index = Total Seedling Length (cm) x Germination %

Salt tolerance was calculated using the following ratio (Kaymakanova, 2009):

Salt Tolerance = A/B x 100

Where:

A = germination in treated seedlings

B = germination in control plants

Biochemical Analysis

Enzyme activity

Extraction of antioxidant enzymes

Plant material was homogenized in a pre-chilled pestle and mortar with 10 ml of cold 0.2 M phosphate buffer (pH 6.2). The homogenate was filtered through cheese cloth and then centrifuged for 10 min at 6000 rpm at 4 ℃. The supernatant was used for enzymes assay (Castillo et al., 1984).

Antioxidant enzyme assay

Catalase (EC 1.11.1.6) activity was measured according to Góth (1991) method with some modifications. Crude extract with H_2O_2 (65 mM H_2O_2 in potassium phosphate (KP) buffer pH 7.4) were incubated for 4 min**.** Then, the reaction was stopped by adding ammonium molybdate (4 g L^{-1}). The residual of H_2O_2 was determined by the absorbance at 405 nm (6405 UV/Vis). Catalase activity was expressed as µg H_2O_2 destroyed g^{-1} fresh weight min^{-1}.

Peroxidase (EC 1.11.1.7) activity was assayed by Sreenivasulu et al. (1999) method with some modifications. Assaying mixture contained 0.1 M KP buffer (pH 6.0), 0.018 mM guaiacol, H_2O_2 (30%), and crude enzyme extract. The change in the absorbance was read at 436 nm for 30 s up to 3 min (6405 UV/Vis). The peroxidase activity was expressed as µg g^{-1} fresh weight min^{-1}.

Ascorbic oxidase (EC. 1.10.3.3) activity was estimated by Hammerschmidt et al. (1982). One milliliter of 0.2 M phosphate buffer (pH 6.2) and 0.2 ml of 1 mM ascorbic acid were added to 0.2 ml of enzyme extract in a clean quartz cuvette of a UV spectrophotometer (6405 UV/Vis). The volume in the cuvette was brought to 3.0 ml. After the initial absorbance (A_o) was read. The absorbance was recorded after 30 s intervals up to 3 min at 265 nm. The ascorbic oxidase activity was expressed as µg g^{-1} fresh weight min^{-1}.

Polyphenol oxidase (EC 1.10.3.1) activity was determined by the method described by Mayer and Harel (1979) with slight modifications. The assaying mixture contained buffered catechol solution and crude enzyme extract. The change in absorbance at 495 nm was followed for 30 s up to 5 min (6405 UV/Vis). The enzyme activity was expressed as µg g^{-1} fresh weight min^{-1}.

Extraction and assay of α- amylase enzyme

About 4 g of plant material was homogenized with 20 ml of sodium phosphate buffer, pH 7.5. The homogenate was vortexed and centrifuged for 10 min at 20,000 g and the supernatant was used for α- amylase assay (Sangeetha, 2013). α- amylase (E.C. 3.2.1.1) activity was determined by Rick and

Stegbauer (1974) method. One milliliter of 1.0% soluble starch was incubated at 37 ℃ in phosphate buffer (pH 7.0) as a substrate with enzyme extract (1 ml) for 30 min. After incubating mixtures, 1 ml of 3,5-dinitrosalicylic acid as a color reagent was added to the previous mixture and boiled in a water bath for 10 min. Then, the mixtures were cooled in an ice bath and the volume was increased to 10 ml with distilled water. The color intensity of the reaction mixture was recorded at 546 nm by a spectrophotometer (CE1010). The activity of α- amylase enzyme was expressed as μg g^{-1} protein min^{-1}.

Determination of metabolites

Total soluble sugar content was measured by Umbreit et al. (1959) method, using anthrone reagent (2 g L^{-1} H_2SO_4). Total soluble protein content was assessed according to Lowry et al. (1951) by using Folin-phenol (copper sulfate reagent in an alkaline medium). Proline content was measured by Bates et al. (1973) method. Sulfosalicylic acid (3%) was used for proline extraction, and the color reagent acid-ninhydrin was used for proline determination.

Estimation of H_2O_2 content and lipid peroxidation

Hydrogen peroxide (H_2O_2) content was estimated by Gong et al. (2008) method. Plant material (0.3 g) was ground with 4 ml of 0.1% (w/v) TCA in an ice bath. This mixture was centrifuged at 12,000 g at 4 °C for 15 min. Then, 0.5 ml of the supernatant was mixed with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml KI (1 M). Absorbance of the mixture was recorded at 390 nm and the H_2O_2 content was calculated by using a standard curve as mM g^{-1} fresh weight (CE 1010).

Monodehydroascorbate (MDA) amount was measured by the thiobarbituric acid (TBA) reaction to determine the level of lipid peroxidation as described by Heath and Packer (1968). Fresh plant material (0.5 g) was homogenized in 10 ml of 5% trichloroacetic acid (TCA). Then, the mixture was centrifuged at 15000 g for 10 min. Four milliliters of thiobarbituric acid (0.5%) were added to 2 ml of the supernatant. At 95 ℃, the previous mixture was heated for 30 min. Then, the mixture was cooled in an ice bath and centrifuged at 10,000 g for 10 min. The absorbance of the supernatant was recorded at 532 and 600 nm. The non-specific absorbance was subtracted at 600 nm, and the MDA content was calculated as μM of MDA g^{-1} fresh weight (CE 1010).

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of the plant extract was determined by a modified method of Oyedemi et al. (2010). Various concentrations of plant material extract were prepared in distilled water (2, 4, and 6 mg L^{-1}) from stock solution 8 mg L^{-1}. Four milliliters of each concentration were added to 0.6 ml of 4 mM H_2O_2 in 0.1 M phosphate buffer (pH 7.4). The mixture was incubated at room temperature for 10 min and the absorbance was recorded at 230 nm against a blank solution (plant material extract only) (6405 UV/Vis). The percentage of hydrogen peroxide scavenging activity was calculated from the following relation:

H_2O_2 scavenging activity % = [(Abs control - Abs sample)] / (Abs control) x 100

Determination of phytohormones content

Phytohormone analyses were performed at Arid Land Agricultural Research (ALAR), Faculty of Agriculture, Ain Shams University.

Extraction

The extraction of phytohormones was performed according to Shindy and Smith (1975) method. Plant material (5 g) was homogenized with cold 80% methanol. The homogenate was then placed in a flask with 20 ml methanol for 24 hours at 0 ℃. Then, the homogenate was filtered through Whatman filter paper no. 42 under vacuum. The residue was transferred to a flask with methanol and stirred for 30 min and filtered again. The method was repeated, and the combined extracts were evaporated to the aqueous phase by the rotatory evaporator.

Estimation of phytohormones

To estimate the amounts of alkaline hormones, the aqueous phase pH was adjusted to 8.6 and partitioned 3 times with equal volumes of ethyl

Table 1
Effect of fresh (F) and boiled (B) extracts of *Azolla piñnata* on germination percentage (%), salt tolerance, plumule and radicle length (cm), plumule/radicle length ratio, and seedling vigor index (SVI) of lentil (Lens culinaris) seeds (5-d-olds) under control and NaCl salt-stress conditions (0, 150 mM and 200 mM).

Seeds pre-soaked in	NaCl (mM)	Germination %	Salt tolerance	Plumule length (cm)	Radicle length (cm)	Plumule /Radicle length ratio	Seedling Vigor Index (SVI)
H_2O	0	99.30 a	100.0 a	5.00 a	5.66 b	0.886 bc	1059 a
	150	80.00 c	80.50 c	0.93 f	1.66 d	0.586 d	202.6 d
	200	44.00 e	44.20 f	0.83 f	0.83 e	0.993 bc	73.30 e
Extract (F)	0	100.0 a	100.0 a	4.6 b	6.00 a	0.771 cd	1063 a
	150	100.0 a	97.90 b	2.93 c	2.13 c	1.360 a	506.6 b
	200	48.00 d	50.30 e	1.20 e	0.96 e	0.933 bc	92.80 e
Extract (B)	0	99.30 ab	100.6 a	4.7 b	5.66 ab	0.842 bc	1033 a
	15	97.30 b	100.6 a	2.00 d	1.93 cd	1.030 b	383.0 c
	200	44.00 e	59.00 d	0.900 f	0.63 e	1.410 a	76.6 e
LSD at 0.05		2.66	2.06	0.26	0.53	0.255	109.8

Different letters represent significant difference ($p<0.05$).

acetate. The ethyl acetate fractions were combined and evaporated until dryness. To estimate the concentrations of acidic hormones, the aqueous phase pH was adjusted to 2.8 and again partitioned 3 times with equal volumes of ethyl acetate. The combined acidic and alkaline fractions were injected into HPLC for acidic (auxins, gibberellins, and abscisic acid) and alkaline hormones (zeatin).

Statistical Analysis

Variations in the germination, growth parameters, enzymes activity and metabolite concentrations under different salt stress levels (0, 150, and 200 mM NaCl) and the treatment including soaking in distilled H_2O and fresh or boiled extract of *A. piñnata*) were carried out by one-way analysis of variance (ANOVA I), and mean values of 3 replicates were compared by LSD and Duncan's multiple comparison test at 5% probability level. When the differences were significant, a post-hoc test (Duncan test at $P<0.05$) was applied (SPSS, 2006).

Results

Germination and growth parameters

The impact of salt stress on germination, salt tolerance, and growth parameters of Lentil (Giza 9) seeds are depicted in Table 1. With increasing NaCl salt concentration, the germination percentage of seeds reduced by 19.3 and 55.3% at 150 and 200 mM of NaCl, respectively. The salt tolerance showed the same trend of seed germination percentage with the elevation of NaCl concentration. Some parameters as plumule and radicle lengths and seedling vigor index (SVI) significantly reduced under different levels of NaCl salt-stress. The plumule/radicle ratio was negatively affected at 150 mM NaCl salt-stress but was not affected at 200 mM NaCl. Soaking of seeds in *Azolla piñnata* (*A. piñnata*) extract F or B enhanced the germination percentage at 150 mM NaCl to be near the control (unstressed seeds). On the other hand, the *A. piñnata* extract F stimulated the germination percentage of seeds by 4% at 200 mM NaCl compared to control while the extract B did not affect the germination percentage at 200 mM NaCl. Salt tolerance showed a significant enhancement at 150 and 200 mM NaCl along with both extracts' treatment. Also, the plumule and radicle length was enhanced by treating seeds with F or B extracts at 150 mM NaCl. In addition, SVI was positively affected by *A. piñnata* extracts (F or B) at 150 mM NaCl salt-stress. The fresh extract of *A. piñnata* was more effective in enhancing germination percentage, plumule and radicle lengths, and SVI of lentils under NaCl salt-stress.

Some biochemical activities

Catalase (CAT) and ascorbic oxidase (AOX) activities reduced in lentils under the two levels of

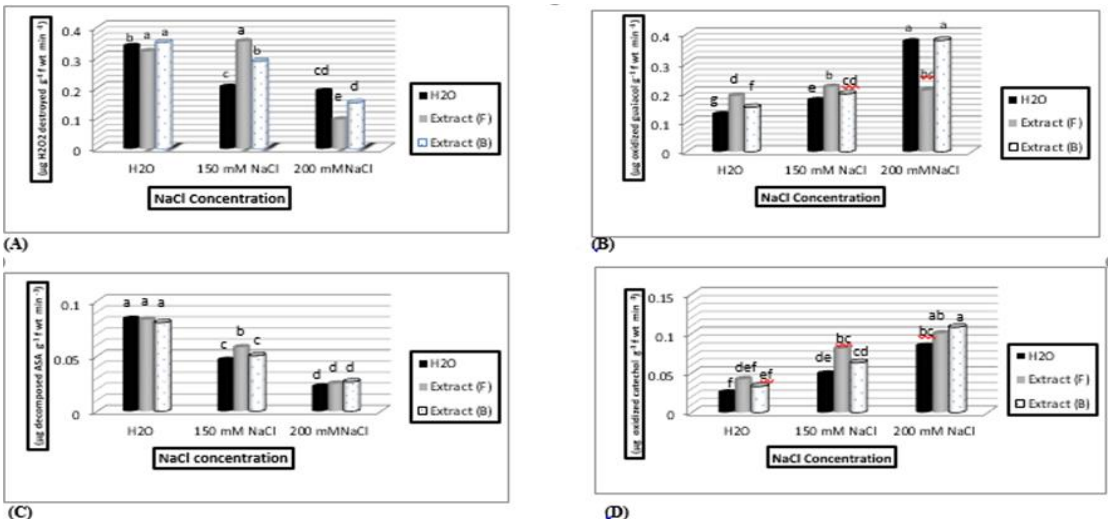


Fig I. Effect of fresh (F) and boiled (B) extract of *Azolla piñnata* on the activity of (A) Catalase (CAT), (B) Peroxidase (POX), (C) Ascorbic oxidase (AOX), and (D) Polyphenol oxidase (PPO) enzymes (µg g^{-1} f wt min^{-1}) of Lentil (*Lens culinaris*) seeds (5-d-old) under normal and NaCl salt stress (0, 150 and 200 mM); Columns with the same letters are insignificant.

salt stress (150 and 200 mM NaCl) compared to control (Fig. I). On the other hand, the activities of peroxidase (POX) and polyphenol oxidase (PPO) of salt-stressed lentils enhanced compared to unstressed plants. The activities of CAT and POX enzymes were significantly enhanced by F and B extracts at 150 mM NaCl salt-stress, although extract F, only significantly increased the activities of AOX and PPO at 150 mM NaCl compared to untreated stressed lentils. At 200 mM NaCl, CAT and POX showed a negative response under the extract F treatment relative to the untreated group.

It was observed in the current results that soluble sugar content and α- amylase activity were dramatically decreased by increasing the salt stress level, which was more obvious at 200 mM NaCl (Fig. II). The α- amylase activity was recovered by treating the seeds with *A. piñnata* extract F and B at 150 mM NaCl. Consequently, the soluble sugar content increased to 2-folds at 150 mM NaCl salt-stress. The same treatments did not have any significant effect on α- amylase activity and sugar content at 200 mM NaCl salt-stress.

By increasing NaCl concentration, the H_2O_2 content and lipid peroxidation were elevated to their maximum levels at 200 mM NaCl (Table 2). At 150 mM NaCl, the treatment of Lentils by the *A. piñnata* extracts F or B decreased the content of H_2O_2 to restore its content in unstressed Lentils

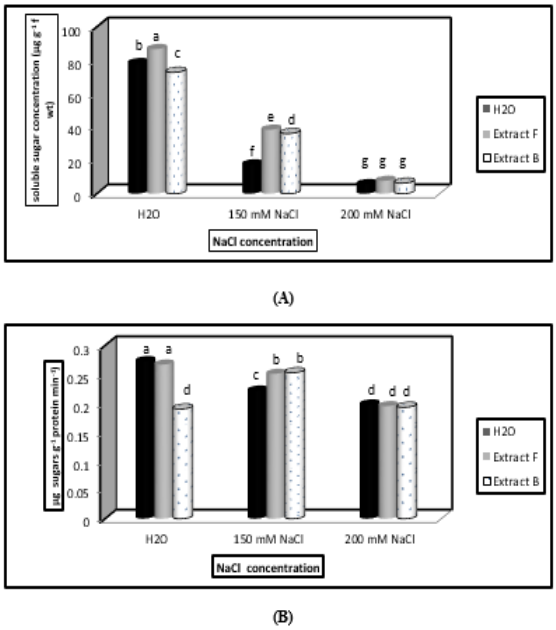


Fig II. Effect of *A. piñatta* extract F or B on (A) soluble sugar concentration (µg g^{-1} f wt) and (B) α-amylase activity (µg g^{-1} protein min^{-1}) of lentils; Columns with the same letters are insignificant.

(control). Extracts F and B showed the same effect in decreasing the lipid peroxidation with its product MDA at 150 mM NaCl salt-stress. Moreover, the accumulation of proline was also observed in salt-stressed lentils at 150 and 200 mM NaCl (Table 2). This accumulation of proline was enhanced by the treatment of seeds with both extracts of *A. piñnata* to reach its maximum level 114.49 µM g^{-1} f wt in lentils treated with extract F at 150 mM NaCl salt-stress.

Table 2
Effect of fresh (F) and boiled (B) extract of *Azolla piñnata* on H_2O_2 content (mM g^{-1} f wt), lipid peroxidation (µM of MDA g^{-1} f wt), and proline content (µM g^{-1} f wt)

Seeds pre-soaked in	NaCl (mM)	H_2O_2	Lipid peroxidation	Proline
H_2O	0	2.5 e	0.58 d	38.85 g
	150	2.9 bc	0.71 c	58.27 e
	200	3.2 a	1.11 a	62.54 e
Extract (F)	0	2.8 d	0.71 c	46.26 f
	150	2.4 e	0.64 cd	114.49 a
	200	2.5 e	0.99 b	94.62 c
Extract (B)	0	2.6 de	0.73 c	48.91 f
	150	2.5 e	0.64 cd	105.5 b
	200	3.0 b	1.02 ab	85.20 d
LSD at 0.05		0.2	0.12	7.41

Different letters represent significant difference (P<0.05).

Table 3.
Effect of fresh (F) and boiled (B) extracts of *Azolla piñnata* on the level of some phytohormones (auxin, gibberellins, cytokinin, and ABA) expressed as mg/100 g fw

Seeds pre-soaked in	NaCl (mM)	IAA	GA3	Zeatin	ABA	GA3/ABA
H_2O	0	1.45 b	28.21 a	0.153 b	0.033 a	854.8
	150	0.58 d	8.95 d	0.096 c	0.031a	288.7
Extract (F)	150	1.540 a	16.57 b	0.161 a	0.013 c	828.5
Extract (B)	150	0.83 c	9.86 c	0.163 a	0.020 b	758.4
LSD at 5%		0.103	0.926	0.008	0.007	

Different letters represent significant difference (p<0.05).

The H_2O_2 scavenging activity of lentils' extracts showed obvious increase at 150 and 200 mM NaCl salt-stress compared to control group (Fig. III). On the other hand, treating the lentils with extracts F or B enhanced the H_2O_2 scavenging activity mainly at 150 mM NaCl. The extract of 8 mg ml^{-1} concentration had the highest scavenging activity at 150 mM NaCl of seeds treated with F extract. However, these treatments decreased the H_2O_2 scavenging activity at 200 mM NaCl.

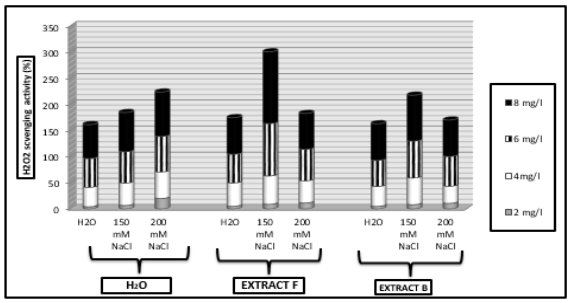


Fig III. Effect of *A. piñatta* extract F or B on H_2O_2 scavenging activity (expressed as %) in lentils

The levels of different phytohormones in lentils at 150 mM NaCl with or without the treatment with *A. piñnata* extract F or B are illustrated in Table 3. Estimation of the phytohormones was carried out at 150 mM only because the *A. piñnata* extract positively affected the germination and growth at this level of NaCl stress. All studied phytohormones of lentils were negatively affected at 150 mM NaCl salt-stress, except abscisic acid (ABA). The reduction percentages of hormonal content were 60.0, 68.2, and 37.2% for IAA, GA3, and zeatin, respectively. The treatment with extracts F and B enhanced hormonal levels in stressed lentils by 2-folds or more. Extract F was more effective than extract B in the increment of phytohormone levels. By the same way, GA/ABA ratio negatively and positively responded to salt stress and treatments, respectively. On the other side, by treating seeds with extract F and B, ABA content decreased compared with control.

Discussion

Germination and growth parameters

Salinity stress restricts the optimal growth and crop production especially in arid and semi-arid regions (Naveed et al., 2020). The negative impact of salt stress on the germination percentage and growth parameters detected in the current work has been recently established by many authors, e.g. Kamran et al. (2020) and Patel et al. (2020). During germination, the water uptake by seeds from highly saline soil solution declines. Moreover, ions accumulate in seeds under salinity conditions (Murillo-Amador et al., 2002). The inhibitory impact of high salt concentrations on plumule and radicle lengths under high salt concentrations may be due to the high solute potential in the medium leading to firstly, low water absorbance and secondly, reduction of cell division and differentiation, and lastly, reduction in the plumule and radicle lengths (Keshavarzi, 2011). One of the obvious effects of salinity stress on germination is that it disturbs water uptake by seeds leading to the change in plant hormones and enzymes levels so that seedling progress are repressed (Bor et al., 2003; Younis et al., 2008). The highest reduction of lentils SVI at 200 mM NaCl stress was also recorded by Djanaguiraman et al. (2006) in rice genotypes and Keshavarzi (2011) in savory (*Satureja hortensis*). This reduction in seedling vigor may be attributed to the high solute potential in the soil which is correlated with high ion concentration (Kader and Jutzi, 2004).

Azolla extracts improved the germination percentage and growth of lentils under salt stress conditions, mainly at 150 mM NaCl. The same trend was observed by many authors as Bhindu (2013) and Tejaswini et al. (2015). They found that the Azolla extracts applied on *Pisum sativum* (with 5, 10 and 20%) and on *Phaseolus vulgaris* (as of 25 g and 10 g) enhanced the germination percentage and plumule and radicle lengths. Finally, the salt tolerance of lentils was stimulated along with Azolla treatments due to the elevation of tissue tolerance and osmoregulation which were observed also by Chakraborty et al. (2016) in salt-stressed *Brassica napus*.

Biochemical activities

Under salinity stress, reactive oxygen species (ROS) are accumulated in higher levels than that occurred under normal condition; therefore, antioxidant system including antioxidant enzymes such as catalase (CAT) and peroxidase (POX) have a considerable role to exclude ROS by neutralization (Seckin et al., 2010). The increase in POX activity under NaCl salt-stress in lentils resembles that previously reported by Yaghubi et al. (2014). It was stated that salinity stress increases peroxidase activity by either promoting their gene expression (Mittal and Dubey, 1991), or by activating already formed peroxidase isoforms. Besides H_2O_2 scavenging, POX has other roles in plant cell; it takes part in cell wall biosynthesis and lignification. So, its high activity under salinity is correlated with plant growth (Lin and Kao, 2002). However, the current reduction of the CAT activity with increasing salt stress was also observed by Dash and Panda (2001) in their study on *Phaseolus mungo* seeds. This reduction in catalase activity under salinity stress may be due to the accumulation of salicylic acid, and this occurs in some species of plants under salt stress (Shim et al., 2003). Noctor and Foyer (1998) suggested that salt stress may enhance proteases to destroy catalase enzymes. So, catalase protein destruction exceeds its biosynthesis under salinity stress and finally, its activity would be reduced. Oxidase activity is also required to dissipate the reducing powers which are produced from electron transport chains in mitochondria during respiration (Hossain et al., 2017). The reduction of ascorbic oxidase (AOX) activity in lentils under salt-stressed may explain the role of AOX to enhance the ascorbic acid pool, which is considered an antioxidant defense system under salinity stress. Ascorbic is also required for dioxygenase activity which correlates to hormones biosynthesis such as gibberellins and ethylene (De Tullio and Arrigoni, 2007). So, the enhanced AOX activity has been linked with the reduction of stress tolerance (Garchery et al., 2013). Nevertheless, the induction of PPO by the salt stress was reported by Weisany et al. (2012) in soybean and Elhamid et al. (2014) in wheat. PPO is considered as an antioxidant enzyme (Pehlivan, 2017) owing to its role in antioxidant coordination by scavenging the

harmful free radicals formed during oxidative stress. Thus, PPO helps plants to resist such stress conditions. Numerous products of PPO activity, such as polyphenols and flavonoids, have an antioxidant role (Pehlivan, 2017).

The enhancement of the studied antioxidant enzymes POX, CAT, PPO, and AOX by Azolla application under 150 mM NaCl salt-stress indicated their vital role to quench ROS and protect macromolecules of the cell. Peroxidase along with CAT acts as a key detoxifier of excess H_2O_2 in the cell, and these enzymes' activities are influenced by the stress force (Sharma et al., 2012); the high activity of antioxidant enzymes acts as a barrier against the induction of oxidative stress under salinity (Harter et al., 2014). Some enzymes' expression may be regulated by NaCl salt stress as PPO in *Fragaria ananassa* (Jia et al., 2015). The authors suggested unique responses of PPOs to various stresses through different mechanisms. First, PPO oxidizes phenolics in the presence of O2, forming quinones, so it has a role to reduce various noxious metabolites that increase under stress (Mickky and Aldesuquy, 2017). Second, PPOs may link proteins and quinones or phenolics by ROS; developing a barrier against quinone toxicity. Third, it was known to act in defense mechanisms through signaling pathways. Also, previous results indicated that the level of ascorbic acid in germinated seeds is highly controlled and kept sufficient in normal conditions. The low concentration of ascorbic acid (1 and 2 mM) in seeds enhances their germination while a higher concentration (4 mM) inhibits seed germination. So, the reduction of AOX enzyme activity under salinity stress is in accordance with the production of ROS and their scavenging by ascorbate but its activity enhancement may be associated with the low concentration of ascorbic acid, and ROS are involved in the inhibition of seed germination (Ye et al., 2012).

Mobilization of soluble sugars by the hydrolysis of starch which is stored in cotyledons is one of the germination regulators of seeds and early growth (Gorham et al., 1985). The reduction of α-amylase of seeds germinated in salty medium was also observed by Adda et al. (2014). They showed that the reduction of α-amylase activity was more evident at 200 mM and after three days of germination in *Phaseolus vulgaris* L. cultivars (Cocorose and Djadida). The hydrolytic activity of α-amylase and availability of soluble sugar from complex starch are strongly affected by salinity especially at the early germination stage (Ashraf and Foolad, 2007; Dkhil and Denden, 2010). A fabulous relationship has been found between the reduction in α-amylase activity and the inhibition of GA biosynthetic pathway and its responsive genes expression during the early germination stage (Liu et al., 2018). This relation was clear in the current results. Equally, Appleford et al. (2006) reported in their study a correlation between the GA content reduction and the decline of transcription of α-Amy1 and α-Amy2 genes in wheat grain.

Azolla extracts increased α-amylase activity and the soluble sugar content of lentils under NaCl salt-stress compared to unstressed ones. Therefore, germination speed was highly related to starch hydrolysis (Hussain et al., 2015), supporting the improvement of the germination percentage and plumule and radicle lengths by Azolla extract under NaCl stress. This is because the stored carbohydrates in seeds are the main source of seed vigor, and carbohydrate is an appropriate substrate for other pathways which are critical to achieve germination (Adda et al., 2014).

In saline environments, the overproduction of ROS may disturb the plant lipid metabolism and cause damage to lipids in membranes by lipid peroxidation (LPO) (Sharma et al., 2012). The enhancement of lipid peroxidation was in accordance with the extensive H_2O_2 levels under NaCl salt concentrations. Similarly, Patil and Bhamburdekar (2015) found that different concentrations of NaCl (100, 150, and 200 mM) enhanced lipid peroxidase activity, increasing the lipid peroxidation during germination of soybean seeds up to 72 hrs.

Azolla extracts application reduced H_2O_2 content and lipid peroxidation by inhibiting peroxidation of unsaturated fatty acids of phospholipid (in the plasma membrane) and causing a drop in malondialdehyde production (MDA), and the free

radical damage to cell membranes under stress conditions.

H_2O_2 is directly decomposed by CAT activity or linked with glutathione reductase, and it might be removed by glutathione (GSH) oxidation-reduction reactions. All these mechanisms to remove H_2O_2 from a cell protect it from damage (Hung et al., 2005). The elevation of the scavenging capacity of lentils at 150 and 200 mM NaCl salt-stress as compared with the control is related to the high production of ROS.

Applying Azolla extracts exhibited a powerful antioxidant activity as evidenced by the increment of scavenging potential of the lentil extract. So, Azolla may provide protection for lentils against induced oxidative stress under salinity, owing to the antioxidant compounds and enzymes of the extract. The antioxidants of lentil extract acts as donors of electrons (Ebrahimzadeh and Nabavi, 2009). However, different studies suggested that H_2O_2 is a key signal mediating many sequences of reactions. Desikan et al. (2001) proved by the evidence that H_2O_2 regulates the expression of some genes as a central signaling mediator. So, it is known for its potential biological functions.

Proline acts as a ROS scavenger under drought stress besides its significance in protein and membrane structure determination (Ashraf and Foolad, 2007). Proline content was accumulated in lentils under salt stress (150 and 200 mM NaCl) and its accumulation reached its maximum level by Azolla treatments. In many plant species, the stress tolerance mechanism was associated with the accumulation of proline. Proline acts as an osmoprotectant along with sucrose, polyols, trehalose, GB, and alanine betaine. Proline can defend functional cells under stress. Many reports have pinpointed the role of osmoprotectants in different stresses such as drought (Karimi et al., 2018) and salinity (Wang et al., 2004). Thus, the germination rate, growth, and development may be stimulated by those osmoprotectants under salinity stress (Sharma et al., 2019). Again, it was suggested that the expression of salt responsive genes may be regulated by proline, improving plant tolerance under stress conditions (Khedr et al., 2003). Khedr et al. added that there is a relation between the enhancement of antioxidant enzymes such as CAT and POX and proline under salt stress in some species e.g., *Pancratium maritimum*.

Phytohormones stimulate the biosynthesis of osmolytes in plants growing under environmental stress conditions such as cytokinin (Sharma et al., 2019). Levels of different phytohormones in plants/seeds are reduced by exposure to stress conditions. The reduction of phytohormones (auxin, gibberellin, and cytokinin) under 150 mM NaCl salt-stress was observed. Similarly, Egamberdieva et al. (2009) confirmed the reduction of auxins under salinity stress. This observation encourages the researchers to apply different hormones such as auxins, gibberellins, and cytokinins to alleviate the harmful effects of salt stress on plants or seeds during growth and germination. Other authors, e.g. Horváth et al. (2015) and Zhang et al. (2016) observed the negative effect of salt stress on the biosynthesis pathways of GA during seed germination and seedling stages. In the present study, the reduction in GA content was very noticeable under salinity stress. Many authors including Yuan et al. (2011) and Colebrook et al. (2014) stated that GA inactivation under salinity stress is regulated by some genes, e.g. the genes that encode DELLA proteins. Also, the GAs/ABA ratio was negatively affected by 150 mM NaCl stress. This result was in harmony with Shu et al. (2017). They found that NaCl treatment inhibited soybean seed germination by decreasing the ratio of GA/ABA, as GA content decreased while ABA level increased. The enhancement of plant phytohormones by Azolla extract may be the key in the impact of the extract on the germination process of lentils. Azolla as a biostimulant may work via elevation of hormone and nutrient concentrations under normal and stressful conditions. This way, Azolla increases seed germination and improves the early growth of the plant under water stress (Lana, 2009). Biostimulants as algae, arbuscular mycorrhizal fungi (AMF), fungi, and bacteria improve salt stress tolerance acting as bioactive compounds by enhancing germination and growth parameters (shoot and root length and fresh and dry weight) and plant efficiency and production (Yakhin et al., 2017). Of importance, salt stress

studies, have shown that application of the biostimulant (Stimulate®) which contains a mixture of various hormones such as 0.009% cytokinin, 0.005% gibberellin, and 0.005% auxin had a positive effect on plants under salinity stress, so this role was attributed to the hormonal effect (Zhang, 1997).

Conclusion

NaCl stress had a negative effect on germination percentage, salt tolerance, plumule and radicle length, SVI, α-amylase, soluble sugar content, and hormonal levels in lentils. On the other hand, salt stress induced proline and H_2O_2, contents and lipid peroxidation and H_2O_2 scavenging activity. Antioxidant enzymes system differentially responded to salt stress. Azolla extract as a biostimulant enhanced seed germination and plumule length via improving ROS detoxification, α-amylase enzyme, and hydrogen peroxide scavenging capacity. Azolla also induced the contents of proline phytohormone and soluble sugars. Finally, Azolla extracts positively induced the germination of lentils under salinity stress, especially the fresh extract at 150 mM NaCl salinity.

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