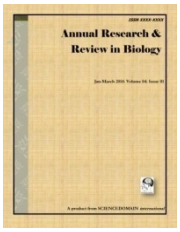

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Effect of Exogenous Application of Brassinolide on Growth and Metabolic Activity of Wheat Seedlings under Normal and Salt Stress Conditions

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Authors' contributions

This work was carried out in collaboration between all authors. Author SSE designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author SAAH managed the analyses of the study 'managed the literature searches. Both read and approved the final manuscript.

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ABSTRACT

The present study was carried out to show the effect of brassinolide on normal and salt stressed wheat plant. Grains of wheat (*Triticum aestivum* sakha 93) were divided into four groups: seeds supplemented with 20 ml distilled water (control); seeds treated with NaCl solutions (25, 50, 100, and 200 mM); seeds supplemented with 0.1, 0.5, 1 and 2 mgL^{-1}brassinolide and seeds treated with a combination of $1mg.L^{-1}$brassinolide and the mentioned NaCl concentrations. Root and shoot lengths, fresh and dry weights were measured as indicators for growth and biomass assessments while carbohydrates, proteins, amylase and protease were estimated as indicator for metabolic activity. Treatment of wheat seeds with different concentrations of brassinolide particularly $1mgL^{-1}$ causes a significant increase in growth parameters, carbohydrate fractions, total soluble proteins in root and shoot and the hydrolytic enzymes amylase and protease. Gradual increase in NaCl concentrations from 25mM to 200mM sharply decreased growth compared with the control. Results of this investigation showed that $1mgL^{-1}$brassinolide counteracted the inhibitory effects of salinity.

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1. INTRODUCTION

Brassinolide is one of the brassinosteroids which are steroidal plant hormones essential for plant growth and development [1]. They are a group of naturally occurring polyhydroxy steroids initially isolated from *Brassica napus* pollen in 1979. Research on brassinosteroids has revealed that they elicit a wide spectrum of morphological and physiological responses in plants which include: stem elongation, leaf bending and epinasty [2]. Besides their role in promoting plant growth activities, they also have physiological effect on the growth and development of plants [3,4]. In addition, brassinosteroids can regulate gene expression and stimulate a variety of physiological responses including changes in the enzymatic activities, membrane potential, photosynthetic activity,DNA, RNA and protein synthesis and changes in the balance of other endogenous phytohormones[4].Brassinosteroids are also effective in reducing abiotic stresses such as salinity, moisture, drought, low and high temperature [5,6,7].

High level of salts in the soil can often cause serious limitations to agricultural production and land development. Salinity is among the major environmental stresses that adversely affects plant growth and metabolism. Salts inhibit plant growth by osmotic stress, specific ion toxicity, ion imbalance and oxidative stress [5]. The main factors that contribute to this problem are the arid and semiarid climates and the salt load in the water used for irrigation. In Egypt, about one third of the cultivated land suffers from soil salinity due to low precipitation (<25mM annual rainfall), poor drainage systems and irrigation with saline water [8]. The soil salinity may cause several deleterious effects on growth and development of plants at physiological and biochemical level [9]. These effects can be due to low osmotic potential of soil solution, specific ion effects, and nutritional imbalances or combined effect of all these factors [10,11].

Wheat (*Triticum aestivum*) is an important winter cereal crop in Egypt. As reviewed by [8], Egypt supplies only 40% of its annual domestic demand for wheat. As many of the cereal crops, salinity tolerance of wheat varies not only among genotypes but also according to growth stage [8,12,13]. Wheat as many other crops respond to salinity by delayed and reduced germination percentage [14], reduced root length, callus size, coleoptile length and seedling growth [15,16,17,18].

Brassinolide was found to alleviate the harmful effect of salt stress by increasing levels of antioxidant enzymes, endogenous growth hormones and induced the appearance of salt defense proteins [17,19]. The present investigation was carried out to study the effect of brassinolide on growth and some metabolic activities of normal and salt-stressed wheat seedlings.

2. MATERIALS AND METHODS

Grains of wheat (*Triticum aestivum* sakha 93) were obtained from the Egyptian Ministry of Agriculture, Egypt. Seeds were soaked in sodium hypochlorite for 20 min, rinsed severally with distilled water. The seeds were germinated in petri dishes (15 seeds in each dish), and then divided into four groups: the seeds of the first group were supplemented with 20 ml distilled water. The second group was irrigated with NaCl solutions of the following concentrations (25, 50, 100, and 200 mM). The third group was supplemented with

brassinolide with the following concentrations (0.1, 0.5, 1 and 2 mg.L^{-1}). The fourth group was treated with a combination of 1mg.L^{-1} brassinolide and the mentioned NaCl concentrations. After 10 days, seedlings were collected, ten plants were taken from each treatment divided into roots and shoots, their lengths, fresh weight were recorded then dried in an oven at 60ºC to a constant weight, and the following evaluations were carried out.

2.1 Estimation of Carbohydrates and Proteins

100 mg of dried fine powder material was obtained from either root or shoot, were added to 5 ml borate buffer (28.63g boric acid + 29.8g potassium chloride + 3.5g sodium hydroxide in one liter solution, pH 8.0) and kept overnight at room temperature before centrifugation. The residue was washed several times with distilled water and oven dried at 60ºC. Borate buffer extract was used for estimation of direct reducing value (DRV), total reducing value (TRV) and soluble protein. The method of Naguib [20] was adopted for the estimation of polysaccharides in the residue.

Total soluble protein content was determined as described by Bradford [21]. 0.1ml of borate buffer extract was mixed with 5 ml of Coomasie blue reagent, shacked well, and incubated at room temperature for 3-5 minutes. A 3ml cuvette was used to measure the absorbance at 595 nm against a blank (0.1ml borate buffer pH 8.0 and 3 ml of the reagent).
The concentration of a protein was calculated as mg g-1 dry weight.

2.2 Sample Extraction and Protease Enzyme Activity

A sample of 0.5 g from seedlings was homogenized in 50 mM Na-acetate buffer (pH 4.7) containing 2 mM cystein and 1 mM EDTA, then stirred for 1 hour and centrifugated at 10,000 rpm for 30 min. The supernatant was used as a raw extract for protease assay. Protease activity was estimated colorimetrically using the method described by Ong and Gauchery [22]. Enzyme extract (0.025 ml) was added to 1.75 ml of 0.5% casein in an appropriate buffer and incubated at 37ºC for 60 min. Thereafter, 3 ml of 5% trichloroacetic acid was added and left to stand at room temperature for 30 min, then filtered, 5 ml of 0.4 N NaOH and 1 ml of diluted phenol reagent (1:2 v/v) were added to the filtrate (1 ml) alternatively. After color development (about 30 min), the optical density (O.D) was determined at 660 nm every 60 minutes interval. One unit of protease was chosen to equal 1.0 Δ O.D 660/60 min at 37ºC.

2.3 Amylase Enzyme Activity

The assay of α-amylase activity was carried out according to a modification of the method of Das and Sen-Mandi [23]. 0.25 ml of enzyme extract in (50 mM Na-acetate buffer containing 10 mM CaCl2 and 1 M NaCl at pH 5.5) was added to 0.5 ml of 1% soluble starch in Na-acetate buffer (50 mM Na-acetate; 1 M NaCl at pH 5)serving as the substrate. 1 ml of alkaline 3, 5 dinitro-salicylic acid was added and the reaction was terminated by heating at 100ºC for 15min. The mixture was allowed to cool, diluted with 10 ml distilled water and the absorbance was measured at 546 nm.

2.4 Statistical Analysis

Each of the previous experiments was conducted using complete randomized blocks design. All the obtained data was statistically analyzed (three replicates each) by the methods described by Cochran and Cox [24].

3. RESULTS

Data in Table (1) showed that wheat growth was affected by the increase in NaCl concentration which caused a gradual decrease in all the measured growth parameters (root and shoot length, fresh and dry weights) compared to control samples reaching its maximum at 200mM. Treatment of wheat plant with different concentrations of brassinolide (0.1, 0.5,1 and 2mg.L^{-1}) caused an increase in root parameters (length, fresh and dry weights). Shoot length and fresh weight were improved with increasing brassinolide concentration reaching its maximum at 1 mg.L^{-1}then decreased with 2 mg.L^{-1} compared to control samples. Based on the above mentioned results, 1mg.L^{-1}brassinolide concentration was chosen to be mixed with the tested NaCl concentration. Addition of 1mg.L^{-1} brassinolide to the 50 and 100 mM NaCl caused an increase in root length. It has no positive effect on samples treated with 200mM.Addition of 1mg.L^{-1} BR caused an increase in both roots and shoots fresh and dry weight even with samples treated with 200 mM NaCl.

Brassinolide alone is not effective on root monosaccharides and disaccharides at 0.1 and 0.5 (lower content compared to control) but, 1mg.L^{-1}stimulate an increase in the content compared to control samples (Table 2) while 2mg.L^{-1} has an inhibitory effect on the content of monosaccharides. On the other hand, increase in brassinolide concentration leading to a gradual increase in the content of polysaccharides reaching its maximum at 1mg.L^{-1}but BR at 2mg.L^{-1} caused a reduction in the amount of polysaccharides. Addition of 1mg.L^{-1}brassinolide in the presence of salt significantly increased the content of the three measured fractions (mono, di and polysaccharides) compared to samples treated with NaCl only.

Data in Table (2) also indicate that treating seeds only with BR has a stimulatory effect on the total soluble proteins of root with all the tested concentrations. Treating samples with saline solution only leading to a gradual decrease in protein content compared to control samples. On the otherhand, treatments of seeds with 1mg.L^{-1} BR with the tested NaCl leads to an increase the total soluble protein.

Treatment with brassinolide only at (0.1, 0.5 mg.L^{-1} concentrations) has no effect on monosaccharides while those concentrations has a stimulatory effect on disaccharides of the shoot (Table 3), but 1 ppm has a stimulatory effect on both mono and disaccharides. Shoot polysaccharides content increased in samples treated with 1mg.L^{-1} brassinolide while addition of 2 mg.L^{-1} has an inhibitory effect on all saccharide fractions. Total soluble protein of the shoot increased with increasing brassinolide concentrations. Addition of 1mg.L^{-1} BR to the saline solution leading to an increase in all saccharides and total soluble protein.

Table 1. Change in growth criteria of wheat plants root and shoot under different concentrations of brassinolide, NaCl and the combination between 1mg.L^{-1} brassinolide and NaCl concentrations

	Root			Shoot		
	Length (cm)	**Fresh weight (mg)**	**Dry weight mg**	**Length (cm)**	**Fresh weight (mg)**	**Dry weight (mg)**
Control	12.20±0.35	90.00±0.33	9.30±0.10	15.80±0.30	104.00±0.67	13.00±0.03
0.1 mg.L^{-1} Brassinolide	12.80±0.28	96.70±1.36	18.30±0.02	15.60±0.19	106.00±0.19	12.30±0.09
0.5 mg.L^{-1} Brassinolide	12.90±0.41	100.00±1.17	12.30±0.42	15.60±0.21	114.00±1.36	12.70±0.09
1.0 mg.L^{-1} Brassinolide	13.80±0.20	106.30±1.27	9.30±0.10	16.90±0.10	110.70±0.25	11.70±0.02
2.0 mg.L^{-1} Brassinolide	13.20±0.084	109.30±0.84	12.70±0.35	15.50±0.09	100.70±0.68	11.00±0.10
25 mM NaCl	11.20±0.19	90.00±0.77	7.70±0.09	14.90±0.10	96.70±0.37	12.70±0.09
50 mM NaCl	10.70±0.15	80.00±1.76	7.70±0.19	12.60±0.10	93.30±0.67	12.30±0.09
100 mM NaCl	8.60±0.09	63.30±0.77	7.30±0.09	10.20±0.03	56.70±0.39	9.00±0.10
200 mM NaCl	6.10±0.06	43.30±0.39	5.00±0.01	6.40±0.03	36.70±0.39	6.00±0.10
1.0 mg.L^{-1} Brassinolide +25 mM NaCl	14.20±0.11	102.70±0.58	10.00±0.17	16.20±0.10	116.00±0.10	11.70±0.17
1.0 mg.L^{-1} Brassinolide +50 mM NaCl	14.40±0.18	80.00±0.33	7.30±0.09	15.20±0.04	93.03±0.95	9.30±0.25
1.0 mg.L^{-1} Brassinolide +100 mM NaCl	10.50±0.23	60.70±0.20	5.70±0.10	12.30±0.05	62.70±0.67	7.70±0.09
1.0 mg.L^{-1} Brassinolide +200 mM NaCl	6.40±0.09	42.70±0.63	7.30±0.09	7.00±0.03	41.70±0.60	7.30±0.19
$LSD_{0.05}$	0.565	5.35	0.961	0.318	3.71	0.722
$LSD_{0.01}$	0.756	7.25	1.3	0.425	5.03	0.978

Table 2. Change in carbohydrate contents (Monosaccharides, disaccharides, polysaccharides) and total soluble proteins of wheat plants root (mg/gdry wt.)

	Monosaccharides	Disaccharides	Polysaccharides	Total soluble protein
Control	390.60± 4.45	101.50±7.06	9.00±0.08	34.49±0.69
0.1 mg.L^{-1} Brassinolide	296.10±1.47	62.78±0.59	10.00±0.00	52.42±0.06
0.5 mg.L^{-1} Brassinolide	338.90±0.37	122.60±4.19	12.20±0.10	65.53±1.13
1.0 mg.L^{-1} Brassinolide	395.4±0.08	203.90±2.50	13.60±0.04	62.08±0.01
2.0 mg.L^{-1} Brassinolide	247.10±0.64	178.90±0.05	11.40±0.02	53.80±0.41
25 mM NaCl	327.40±3.96	71.55±1.92	7.30±0.09	23.45±0.77
50 mM NaCl	261.90±2.56	46.52±0.86	6.60±0.00	35.85±0.55
100 mM NaCl	308.80±4.69	40.00±2.62	6.60±0.02	30.35±0.37
200 mM NaCl	304.00±0.06	12.70±0.04	8.20±0.01	22.07±0.00
1.0 mg.L^{-1} Brassinolide +25 mM NaCl	440.20±0.32	157.70±0.93	7.20±0.01	45.53±1.24
1.0 mg.L^{-1} Brassinolide +50 mM NaCl	383.20±2.22	98.20±2.22	8.80±0.02	36.56±1.45
1.0mg.L^{-1} Brassinolide +100 mM NaCl	319.90±0.95	44.90±0.95	8.50±0.03	39.32±1.31
1.0 mg.L^{-1} Brassinolide +200 mM NaCl	329.40±0.02	93.30±0.74	10.00±0.16	61.39±0.76
$LSD_{0.05}$	14.63	15.01	0.374	4.49
$LSD_{0.01}$	19.83	20.34	0.507	6.08

Table 3. Change in carbohydrate contents (Monosaccharides, disaccharides, polysaccharides) and total soluble proteins of wheat plants shoot (mg/g dry wt.)

Treatment	Monosaccharides	Disaccharides	Polysaccharides	Total soluble protein
Control	326.20±3.48	109.90±3.87	9.20±0.32	37.25±0.28
0.1 mg.L^{-1} Brassinolide	323.70±5.53	99.70±5.95	9.30±0.09	98.64±5.12
0.5 mg.L^{-1} Brassinolide	316.70±0.00	150.90±0.00	11.20±0.00	81.39±1.22
1.0mg.L^{-1} Brassinolide	490.90±0.05	155.90±0.50	12.60±0.02	118.64±0.55
2.0 mg.L^{-1} Brassinolide	272.40±0.02	95.10±0.01	5.60±0.02	135.19±0.01
25 mM NaCl	321.50±2.70	69.50±1.35	8.70±0.09	36.56±0.62
50 mM NaCl	329.40±0.63	31.50±0.94	7.30±0.21	38.63±0.02
100 mM NaCl	316.70±0.04	55.54±0.03	8.70±0.01	35.87±0.00
200 mM NaCl	253.90±5.15	37.00±0.48	7.40±0.02	33.11±0.01
1.0 mg.L^{-1} Brassinolide +25 mM NaCl	312.00±0.48	105.10±0.82	11.20±0.21	121.40±1.10
1.0mg.L^{-1} Brassinolide +50 mM NaCl	289.80±1.11	81.60±2.00	11.00±0.10	86.91±0.41
1.0mg.L^{-1} Brassinolide +100 mM NaCl	418.00±0.00	77.50±3.83	13.00±0.10	133.81±0.86
1.0 mg.L^{-1} Brassinolide +200 mM NaCl	319.90±0.95	65.80±3.25	15.40±0.14	157.96±1.59
$LSD_{0.05}$	14.39	14.72	0.803	9.19
$LSD_{0.01}$	19.5	19.95	1.09	12.45

The present data (Figs. 1 and 2) indicate that the seeds treated only with BR have a gradual increase in both amylase and protease activities reaching its maximum with samples treated with 1 mg.L^{-1} brassinolide, while samples treated only with saline solution show a gradual decrease in both the tested enzymes activities compared to control samples. Addition of 1mgL^{-1} brassinolide to the different tested saline concentrations leading to an increase in the measured activities of both amylase (Fig. 1) and protease (Fig. 2). The increase in amylase activity was positively correlated with the concentration of both mono- and disaccharides (r = 0.6) while it was negatively correlated with polysaccharide concentration (r = - 0.513). On the other hand the protease activity was positively correlated with protein concentration (data not shown).

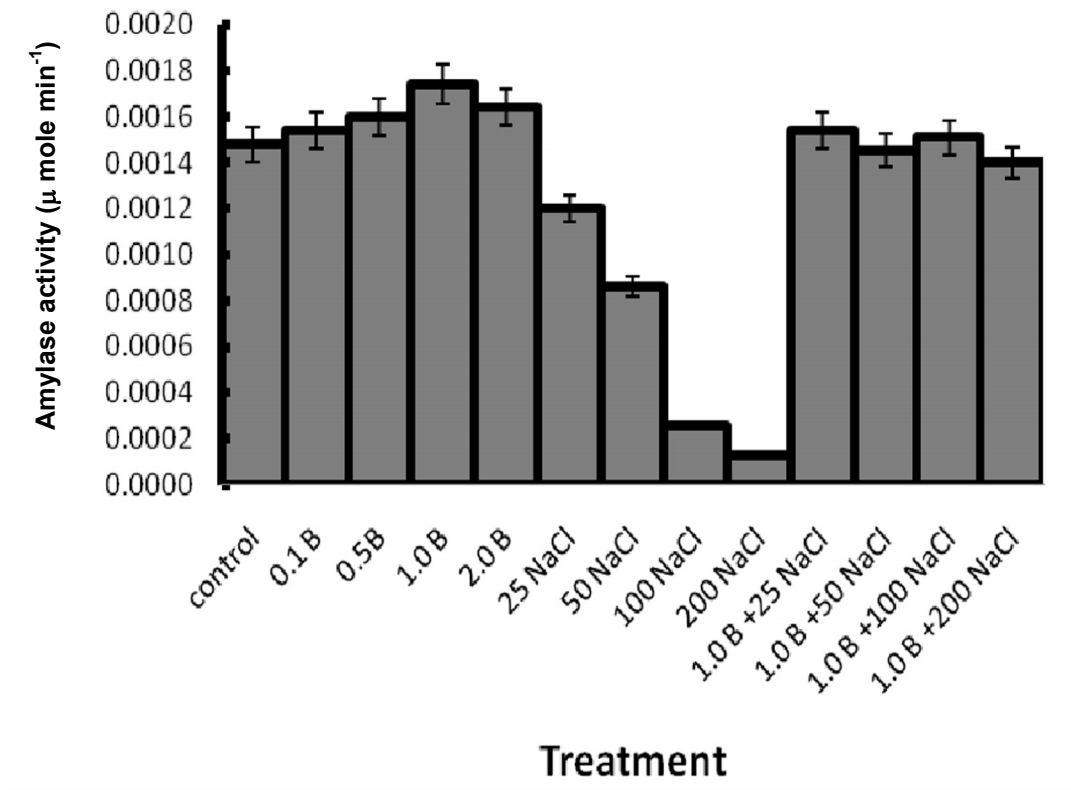

Fig. 1. Effect of different concentrations of brassinolide, NaCl and the combination between 1mg.L^{-1} brassinolide and NaCl concentrations on amylase enzyme activity (μ mole/min.)

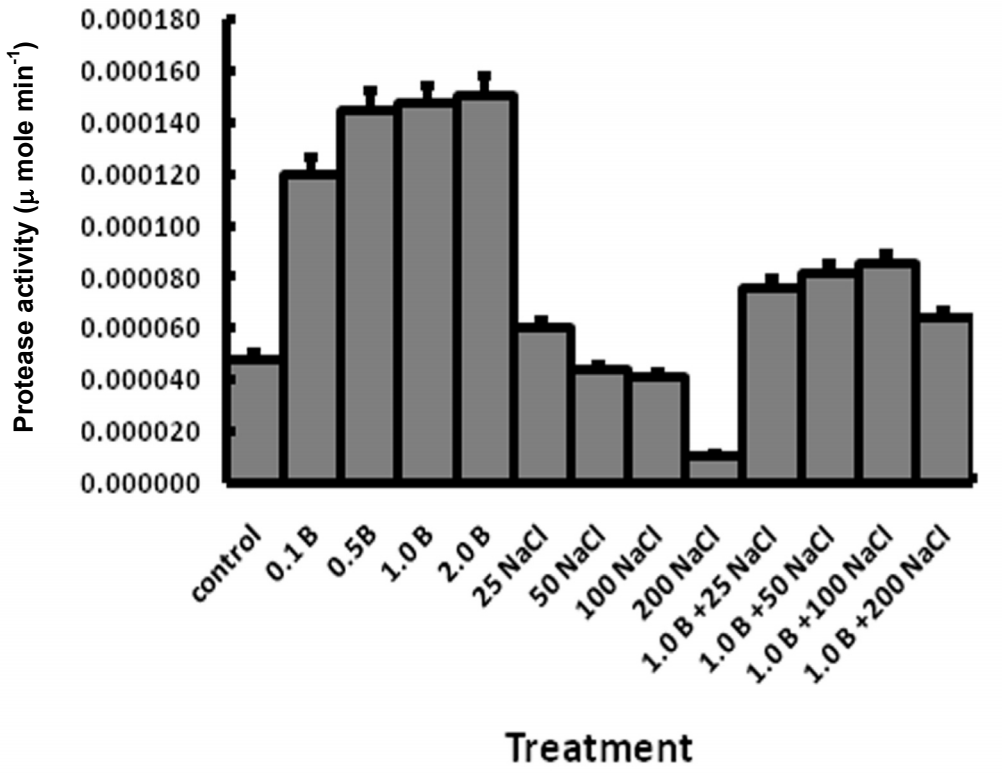

Fig. 2. Effect of different concentrations of brassinolide, NaCl and the combination between 1mg.L^{-1}brassinolide and NaCl concentrations on protease enzyme activity (μ mole/min.)

4. DISCUSSION

Enhancing plant's ability to resist environmental stresses such as drought, salinity and heat is a main goal for all scientists. Plant growth regulators and related compounds have shown beneficial functions on the enhancement of plant growth performance and great potential to help realize the above mentioned goal. In the present study, susceptibility of wheat plants to high concentration of NaCl is demonstrated by, growth reduction and loss of fresh mass as shown in our results (Table 1). These effects are probably due to an excessive increase and translocation of Na+ and Cl- ions to the leaf tissue, which cause alterations in the osmotic potentialin accordance to El-Khallal et al. [19]. Yasseen et al. [25] demonstrated that NaCl inhibits growth by reducing both cell division and cell enlargement; such results are also in agreement with those of Hathout [26] who found that salinity above 800 ppm reduced plant height, fresh and dry weights.

Application of brassinolide markedly increased all the measured growth parameters of wheat plants in comparison with stressed or non-stressed controls which may be due to promotion of cell elongation and division. Nakamura et al. [27] explained the enhancement in plant growth by tracheary element differentiation, delaying of abscission, enhancement of gravity-induced bending, promotion of ethylene biosynthesis, and enhancement of stress resistance. Also Daszkowska-Golec [28] postulated that seed application with BR is sufficient to reduce

the impact of salt stress on growth by restored pigment levels, increased nitrate reductase, nucleic acids and proteins.

Induction in growth parameters of in the present study after brassinolide addition might be related to the induction of assimilating area, photosynthetic pigments and protein biosynthesis which consequently delayed leaf senescence which is induced by salt stress as stated by Takeuchi et al [29] Moreover, Vardhini and Rao [30] who found that brassinosteroids increased shoot fresh weight of tomato plant. Tanaka et al. [31] reported that brassinosteroids play a role in promoting germination, controlling cell elongation and cell division of Arabidopsis.

Vardhini and Rao [30] found that different types of brassinosteroids increased shoot length, root length, and shoot fresh weight, number of fruits per plant and total weight of tomato fruit per plant. Lu et al. [32] reported that brassinolide at 0.005 mg/L promoted shoot growth and increased the shoot height of *Sportina patens* cultured callus. In the present study, application of 1mg.L^{-1} brassinolide leading to increased content of mono, di and polysaccharide of both root and shoots of wheat plant can be explained on the basis that BR has positive effect on root growth under salt stress. The total soluble and reducing sugar contents induced by higher amylase activity in the present study were sufficient to promotethe root growth under salt stress. These results are in agreement with those ofSariam[33] who noted that application of brassinolide increased photosynthesis as reflected in the higher levels of carbohydrate fractions of wheat plant. Total chlorophyll, soluble protein, sugars and catalase activity were found to be the maximum in the brassinolide treated plants [17,34]. El-Khallal et al. [19] stated that treatment of maize plants with brassinolide greatly alleviate the harmful effect of salt stress by increasing levels of antioxidant enzymes, endogenous growth hormones and induced the appearance of salt defense proteins. The present data stated that protein content increased by brassinolide application even under NaCl stress, being also higher than control conditions, which is consistent with Ozdemir et al. [6]. In the present study, both the activity of amylase and protease were decreased in salt treated samples, while the addition of brassinolide to salt treated samples leading to a significant increase in both the tested enzymes (Figs. 1 and 2). Bera et al. [17] found that, seed germination, seedling growth and hydrolytic enzymes (amylase and protease) associated with seedling development were adversely affected by NaCl salt stress. Reduction in DNA, RNA and soluble proteins and increase in peroxidase and free proline in rice seedling were observed with increasing levels of salt stress. Brassinolide, a steroidal component of plant origin was found to counter the adverse effect of salt stress. Ameliorative effects of brassinolide were associated with increase in the levels of nucleic acids and soluble proteins under salt stress. Also El-Khallal et al. [19] and Alyemeni et al. [35] stated that application of brassinolide increased maize resistance against salt stress by enhancing growth, metabolic activities and productivity of grain yield).Our results were partially consistent with the hypothesis that NaCl-induced inhibition of early seedling growth which might be mediated through mobilization of endosperm reserves as explained by Prakash and Prathapasenan [36]. Plant hormones are considered as key regulators to seed germinationand development. Brassinolide, a hormone like compound, may have the same effect as GA3 which is well known to induce the synthesis of amylase and hydrolysis of starch in seeds as stated by Palmiano and Juliano [37]. In our study, the observation that highest concentrations of brassinolide (2mg.L^{-1}) hasa negative effects even at non-saline conditionis in consistent with Ashraf et al. [38] who found that higher concentration has an inhibitory effect in plant growth by affecting physiological processes, thus, decreasing fresh and dry shoot weights and saccharides fractions and protein contents.

5. CONCLUSION

Wheat growth was negatively affected by the increase in NaCl. Treatment of wheat plant with different concentrations of brassinolide (0.1, 0.5,1 and 2mg.L^{-1}) caused an improvement in wheat growth reaching its maximum value with 1mg.L^{-1}, the experimental results provide evidence for enhancing the stress resistance of wheat plant using 1mg.L^{-1} brassinolide also build a basis for recovering plants growing under salinity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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