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Exogenous Application of Ascorbic Acid Ameliorates Detrimental Effects of Salt Stress in Rice (MRQ74 and MR269) Seedlings

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ABSTRACT

An *in vitro* investigation was conducted to evaluate the potential role of exogenously applied ascorbic acid in alleviating the effect of sodium chloride (NaCl) in two varieties of rice, *Oryza sativa* L. (MRQ74 and MR269), that differ in salt tolerance susceptibility. Seven-day-old rice seedlings germinated on Murashige and Skooge (MS) medium were transferred to MS media containing (200 mM) NaCl and (0, 0.5, 1, 1.5 mM) ascorbic acid for 14 days. The results showed that both varieties of seedling, when exposed to salt stress, exhibited a significant reduction in N, K^+, chlorophyll and growth characteristics, fresh weight, dry weight and the lengths of shoot and root. However, the exogenous ascorbic acid in the medium led to an improvement and amelioration of the NaCl stress.

Key words: Ascorbic acid, salt stress, salinity tolerance, *in vitro*, rice (*Oryza sativa* L.)

INTRODUCTION

Abiotic and biotic stresses are considered to be a major threat to agriculture. Thus, efforts to determine what factors play important roles in plant stress tolerance are of formidable importance in raising crop productivity (Raj *et al*., 2011). Salinity stress is a major limiting factor that presents a severe challenge to food security and agriculture. The adverse influence of salinity stress is expressed on all plant levels. Plants have developed various processes that function to balance ion disequilibrium and cellular hyperosmolarity in the effort to control salt stress (Chandna *et al*., 2013). There are more than 800 million ha of cultivated areas that are influenced by salinity worldwide (Munns and Tester, 2008). About 20% of the world's arable land and approximately half of the world's irrigated lands are affected by salinity, rendering salt stress one of the most serious environmental factors that decrease the productivity of cultivated crops (Sairam and Tyagi, 2004). Salinity is a major abiotic stress that negatively affects plant growth and productivity. Salt stress may induce alterations in physiological responses and biochemical pathways (Walia *et al*., 2005). The negative effect of salinity stress comprises imbalance of the Na/K ionic ratio, cell membrane/wall damage, plasmolysis, inhibition of photosynthesis and oxidative stress, which lead to reduced yields (Tuteja, 2007; Tuteja *et al*., 2012a, b).

Rice (*Oryza sativa* L.) is a globally important food crop and feeds over half of the world's population (Sasaki, 2005; Amarawathi *et al*., 2008). It serves an important role as a staple food crop and is utilized to feed more than three billion people, providing between 50 and 80% of the daily calorie intake (Khush, 2005). The demand for rice increases with population and is expected to rise by more than 38% within 30 years, according to the United Nations (Satyanarayana, 2005). Rice is a widely consumed food crop and is grown on 160 million hectares worldwide (FAO., 2007). Asian cultured rice is an important cereal crop for world food security and also nutrition, particularly in developing countries (Vaughan *et al*., 2008). However, one of the essential constraints on rice culture is salinity. Rice (*Oryza sativa* L.) has been shown to be more salt sensitive at the seedling stage and the reproductive stage than during the vegetative growth stage and active tillering stage (Moradi and Ismail, 2007; Rao *et al*., 2008).

Ascorbic acid (vitamin C) is an important antioxidant organic compound, which is widely used in cell metabolism (Loewus and Helsper, 1982) and is necessary to synthesise hydroxyproline-containing proteins (Arrigoni *et al*., 1977). Vitamin C plays a central role in plant defence via reacting directly with singlet oxygen, hydrogen peroxide and superoxide ion (Yu, 1994) and acts as primary substrate in the cyclic pathway for the enzymatic detoxification of hydrogen peroxide; thus, it has a significant role in the activation of various biological mechanisms (Arrigoni *et al*., 1979). Sajid and Aftab (2009) studied the effect of ascorbic acid on improving the salinity tolerance of potatoes. They noted that upon exogenous application of ascorbic acid, the activities of most of the antioxidant enzymes, such as superoxide dismutase, catalase and peroxidase, increased significantly under NaCl stress conditions, thus improving the plant survival under overall environmental stress. Younis *et al*. (2010) also reported that the exogenous addition of 4 mM ascorbic acid with NaCl to the stressful media throughout the period of the experiment (12 days) induced a pronounced and statistically significant increase in the percentage resistance to salt stress and the growth of *Viciafaba* seedlings. Aly *et al*. (2012) observed in their study that the addition of 1 mM ascorbic acid to Egyptian clover (*Trifolium alexandrinum* L.) seedlings grown in NaCl medium significantly increased seed germination, carotenoid and chlorophyll contents and dry mass. In this study, we investigate the potential of ascorbic acid to reduce salinity stress in rice (*Oryza sativa* L.) seedlings grown under saline conditions in an *in vitro* system.

MATERIALS AND METHODS

The study was conducted in the Plant Tissue Culture Laboratory in the Department of School of Biosciences and Biotechnology, Faculty Science and Technology, University Kebangsaan Malaysia. The experimental material consisted of two rice (*Oryza sativa* L.) varieties, MRQ74 and MR269. Mature seeds were used for seedling germination. For *in vitro* establishment, rice seeds were dehusked and surface sterilized in a laminar air flow cabinet according to the method described by Zinnah *et al*. (2013). The seeds were completely washed with sterilized distilled water 3 times, then with 70% ethanol for 2-3 min. Then, the seeds were treated with 0.1% (w/v) mercuric chloride ($HgCl_2$) with the addition of a few drops of Tween-20 for 4-6 min for inner surface sterilization. In the end, the seeds were washed several times with sterilized distilled water to remove all the chemical sterilizing agents.

Sterilized seeds were germinated for 7 days in a flask containing semisolid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Three grams of gelrite (gelling agent) was added per liter of medium to prepare semi-solid media and 7-day-old seedlings were selected and transferred to culture in test tubes containing semisolid MS media supplemented with sodium

chloride (NaCl) (0 and 200 mM) and ascorbic acid (0, 0.5, 1 and 1.5 mM) for 21 days. Seedlings were cultured *in vitro*. Culture was maintained at 25±2°C under a cycle of 16 h light/8 h dark.

Data collection: To investigate the effect of ascorbic acid on the salt tolerance of two rice varieties (MRQ74 and MR269), experiments were performed varying both the NaCl (0, 200 mM) and ascorbic acid concentrations (0, 0.5, 1 and 1.5 mM) in an *in vitro* culture system used to grow rice seedlings, to study the effect of ascorbic acid pretreatment on 14-days-old rice seedlings under salinity stress. The following measurements were collected from the experimental *in vitro* system containing rice seedlings. Fourteen days after salinization, plants were randomly sampled for the observation of morphological characteristics and separated into shoot and root lengths, as well as fresh and dry weight. The measurement of chlorophyll was performed using a chlorophyll meter (SPAD-502, Japan, Special Products Analytical Division, a division of Minolta) on the leaf. The estimation of N (mmol kg^{-1} D.W. (dry weight) was performed using an Auto Analyses Model (Lachat 8000) and the Na^+ and K^+ (mmol kg^{-1} D.W (dry weight) concentrations in the acid extracts were estimated using an Atomic Absorption Spectrometer model AAS 3110. U.S. Instrument Division Norwalk, CT 06859 (USA).

Statistical analysis: All experimental data in the current study were subjected to analysis of variance (ANOVA). Significant differences among the mean values of treatments were determined using the Duncan's Multiple Range Test (DMRT) and the Least Significant Difference (LSD) was calculated at the $p \leq 0.05$ (n = 5) level. The regression relationship was determined using the SAS (Release 9.1 for Windows, SAS Institute Inc., Cary, NC, USA) data analysis software.

RESULTS

The results presented in Table 1 and Fig. 1 (a, b and c) showed a reduction in the growth parameters of *in vitro* seedlings of the two rice varieties tested with sodium chloride (NaCl) compared to the control treatment and illustrated that there was a significant difference between the treatments with NaCl and ascorbic acid in length, fresh weight and dry weight of seedlings of both rice varieties. Increasing the NaCl concentration in the culture medium from 0-200 Mm clearly decreased the growth of seedlings of the two rice varieties, but the addition of ascorbic acid to culture medium containing NaCl showed a significant improving effect on the growth of the seedlings. The interaction between the two factors, NaCl and ascorbic acid, showed that the

Table 1: Growth region whether shoot or root of MRQ74 and MR269 rice seedlings are as affected by 200 mM NaCl supplemented with ascorbic acid (AsA) (0, 0.5, 1 and 1.5 mM)

		Length (cm)		Fresh weight (mg)		Dry weight (mg)	
Varieties	Treatments	Shoot	Root	Shoot	Root	Shoot	Root
MRQ74	Control	14.04±0.27^{a}	5.31±0.21^{a}	94.56±1.37^{a}	15.28±0.27^{b}	10.62±0.19^{a}	4.01±0.01^{b}
	NaCl 200	9.20±0.35^{f}	3.07±0.16^{d}	50.25±2.05^{f}	9.47±0.24^{g}	5.92±0.13^{de}	2.91±0.09^{c}
	NaCl 200+AsA 0.5	11.08±0.69^{d}	3.62±0.25^{c}	58.93±1.71^{d}	10.60±0.49^{f}	6.55±0.09^{c}	3.27±0.20^{c}
	NaCl 200+AsA 1	12.59±0.52^{c}	3.86±0.11^{bc}	65.12±0.96^{c}	11.40±0.00^{e}	7.18±0.45^{b}	3.71±0.27^{b}
	NaCl 200+AsA 1.5	10.24±0.48^{e}	3.56±0.15^{c}	58.45±1.81^{d}	10.35±0.56^{f}	6.07±0.27^{cd}	3.19±0.17^{c}
MR269	Control	14.83±0.40^{b}	4.12±0.6b	89.88±0.84^{b}	26.60±0.87^{a}	10.20±0.29^{a}	4.65±0.40^{a}
	NaCl 200	8.13±0.42^{g}	1.92±0.19^{f}	47.12±0.20^{g}	8.30±0.337^{h}	5.01±0.21^{f}	2.90±0.10^{c}
	NaCl 200+AsA 0.5	10.50±0.46^{de}	2.03±0.09^{f}	55.38±1.33^{e}	11.60±0.40^{e}	5.53±0.25^{e}	3.15±0.18^{c}
	NaCl 200+AsA 1	12.67±0.43^{c}	3.01±0.20^{d}	60.25±1.28^{d}	14.40±0.39^{c}	7.05±0.47^{b}	3.73±0.26^{b}
	NaCl 200+AsA 1.5	12.14±0.45^{c}	2.57±0.14^{e}	59.21±0.87^{d}	13.08±0.26^{d}	5.85±0.35^{de}	3.23±0.34^{c}

Means±SD, n = 5. Mean values in each column followed by different letters are significantly different $p \leq 0.05$ (n = 5) level according to the Duncan's multiple range test

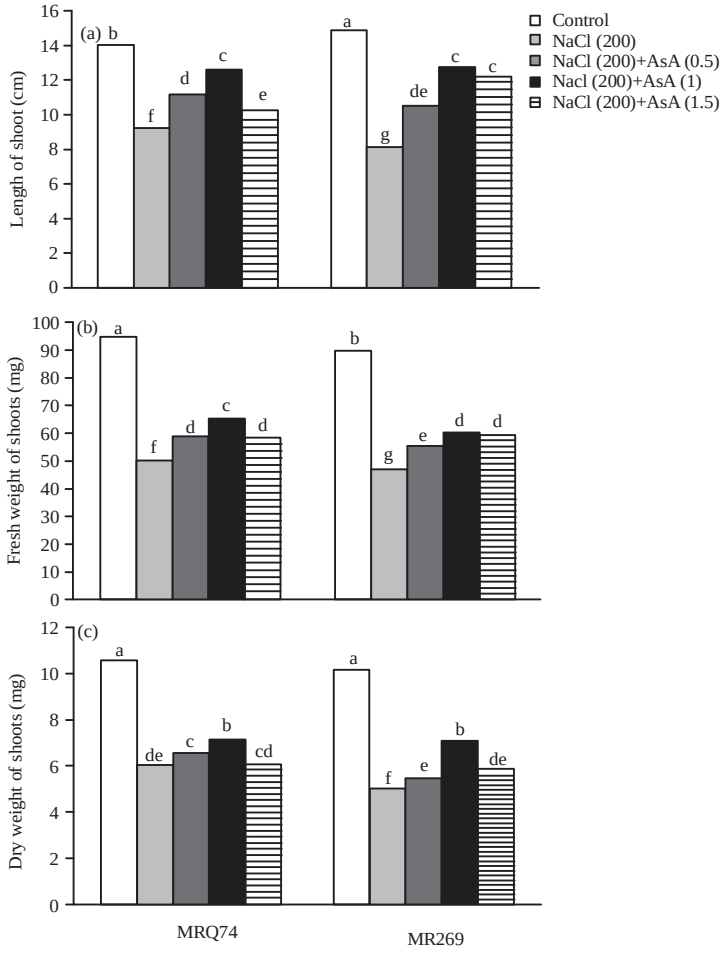

Fig. 1(a-c): Effect of NaCl at (0 and 200 mM) and ascorbic acid (AsA) (0, 0.5, 1 and 1.5 mM) on (a) Length of shoot, (b) Fresh weight of shoot and (c) Dry weight of shoot of MRQ74 and MR269 rice seedlings

maximum growth length and fresh and dry weight of seedlings was observed for 1 mM ascorbic acid under NaCl stress compared to other treatments.

The data for the length, fresh weight and dry weight of roots are shown in Table 1 and Fig. 2a-c. It is clear from the data that the medium, as well as NaCl pretreatment, had an increasing effect on rooting that had suffered morphological damage and the effects of sodium toxicity on all root parameters were decreased by excluding sodium from the roots in both varieties. Treatment with ascorbic acid decreased the effects of salt stress on the roots and gave the highest value of roots for both varieties compared to the NaCl treatments. Treatment with 1 mM ascorbic acid and 200 mM NaCl had an increasing effect on the roots, which were significantly higher in length, fresh weights and dry weights of root for both varieties compared to plants grown under stress alone.

Increasing NaCl concentration resulted in a significant decrease in the amounts of N and K^+ in shoots and the minimum values were found in the shoots subjected to 200 mM NaCl, as shown in Table 2 and Fig. 3a-b. The exogenous addition of ascorbic acid to the culture medium improved both N and K^+ in the presence of NaCl. The level of ascorbic acid increased the amounts of N and K^+ in the shoots. When the highest concentration of NaCl, 200 mM, was applied to the medium

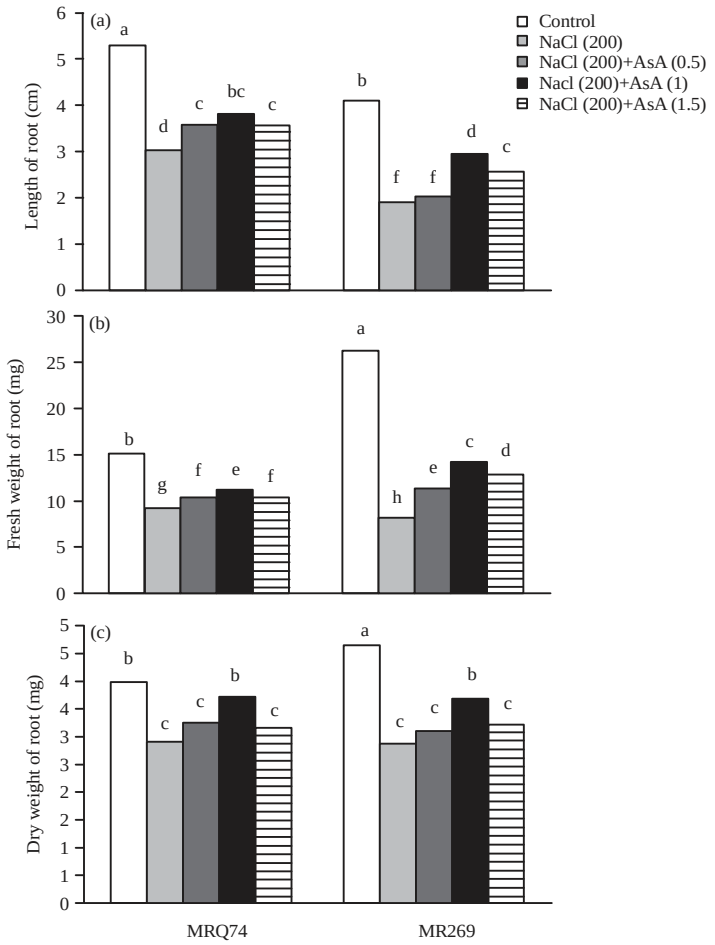

Fig. 2(a-c): Effect of NaCl at (0 and 200 mM) and ascorbic acid (AsA) (0, 0.5, 1 and 1.5 mM) on (a) Length of shoot, (b) Fresh weight of shoot and (c) Dry weight of shoot MRQ74 and MR269 rice seedlings

Table 2: N, K^+ Na^+, Na/K ratio and Chlorophyll parameters of MRQ74 and MR269rice seedlings are as affected by 200 mM NaCl supplemented with ascorbic acid(AsA) (0, 0.5, 1 and 1.5 mM)

Varieties	Treatments	N (mmol kg^{-1}) DW	K^+ (mmol kg^{-1}) DW	Na^+(mmol kg^{-1}) DW	Na/K ratio	Chlorophyll content
MRQ74	Control	3328.47±50.55^{b}	670.29±12.92^{a}	82.79±5.10^{h}	0.12±0.01^{e}	25.48±0.51^{a}
	NaCl 200	2511.66±59.77^{e}	303.48±16.21^{f}	532.42±6.27^{d}	1.76±011^{b}	18.45±0.55^{d}
	NaCl 200+AsA 0.5	2770.13±62.72^{d}	332.38±16.84^{def}	472.82±8.30^{e}	1.42±0.05^{c}	20.25±0.34^{c}
	NaCl 200+AsA 1	3005.47±60.54^{c}	431.18±11.37^{c}	404.40±7.88^{g}	0.94±0.01^{d}	23.28±0.76^{b}
	NaCl 200+AsA 1.5	2713.94±57.47^{d}	358.49±13.69^{d}	479.92±7.91^{e}	1.34±0.03^{c}	20.18±1.03^{c}
MR269	Control	3456.20±43.44^{a}	587.77±16.50^{b}	80.33±8.94^{h}	0.14±0.02^{e}	26.30±0.28^{a}
	NaCl 200	2377.77±80.38^{f}	324.19±20.42^{ef}	640.07±7.53^{a}	1.98±0.14^{a}	10.87±0.91^{g}
	NaCl 200+AsA 0.5	2713.20±60.23^{d}	327.66±21.97^{ef}	571.91±7.91^{c}	1.75±0.10^{b}	13.70±0.87^{f}
	NaCl 200+AsA 1	3070.55±71.01^{c}	442.40±13.03^{c}	440.41±10.03^{f}	1.00±0.02^{d}	15.21±0.37^{e}
	NaCl 200+AsA 1.5	2511.66±62.24^{e}	350.96±13.59^{de}	623.47±9.70^{b}	1.78±0.06^{b}	12.71±0.46^{f}

Mean±SD, n = 5. Mean values in each column followed by different letters are significantly different $p \leq 0.05$ (n = 5) level according to the Duncan's multiple range test, DW: Dry weight

along with ascorbic acid (0-1.5 mM), the highest amounts of N and K^+ were observed when 1 mM ascorbic acid was added to the medium, compared with the control treatment (without ascorbic acid). The MRQ74 exhibited higher amounts of N and K^+ with or without NaCl compared to MR269.

Concerning the effect of ascorbic acid, the results in Table 2 and Fig. 3c-d reveal significant effects of NaCl and ascorbic acid on both Na^+ and the Na/K ratio. Furthermore, the

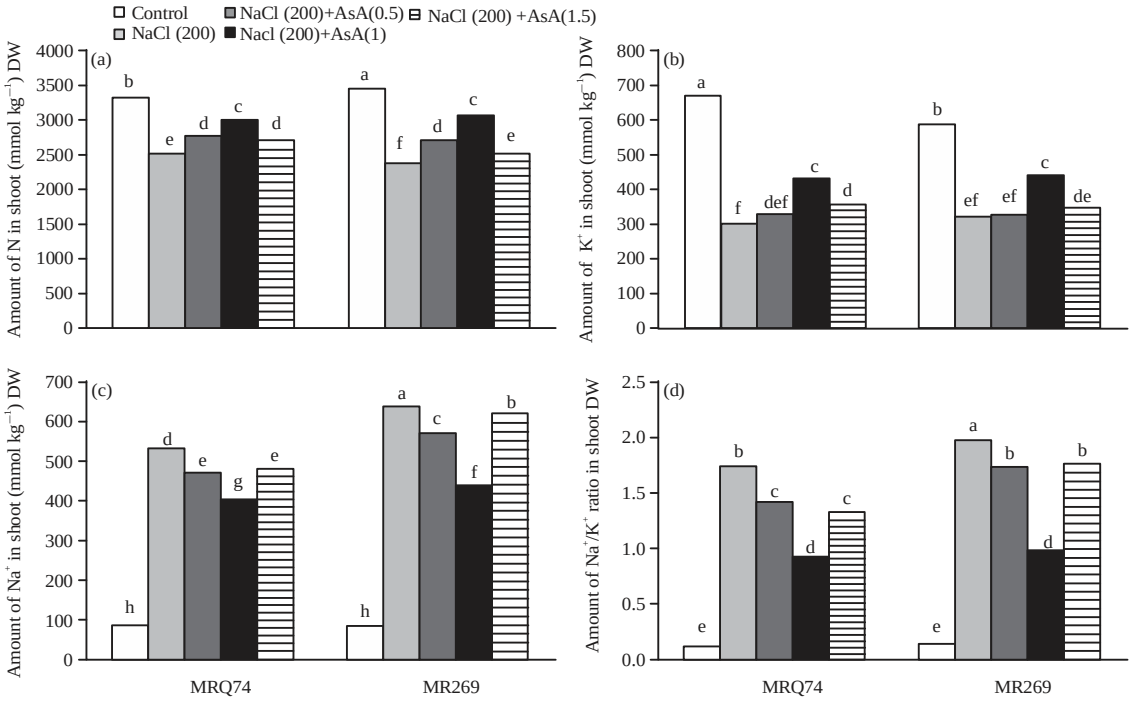

Fig. 3(a-d): Effect of NaCl at (0, 200 mM) and ascorbic acid (AsA) (0, 0.5, 1 and 1.5 mM) on amount of N, K^+,Na^+ and Na^+/, $K^+$of MRQ74 and MR269 rice seedlings

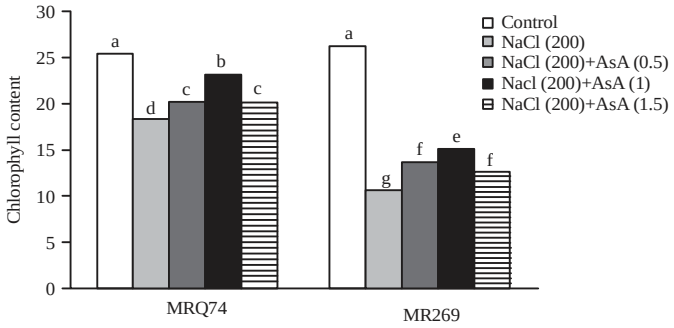

Fig. 4: Effect of NaCl at (0, 200 mM) and ascorbic acid (AsA) (0, 0.5, 1 and 1.5 mM) on chlorophyll of MRQ74 and MR269 rice seedlings

maximum values of both Na^+ and the Na/K ratio were recorded in the seedlings pre-treated with 200 mM NaCl. The interaction between the two factors in a medium containing NaCl and ascorbic acid significantly decreased the level of Na^+ and the Na/K ratio in the seedlings. The MR269 showed higher values of Na^+ and the Na/K ratio with or without NaCl compared to MRQ74.

The effects of NaCl and ascorbic acid on the chlorophyll of *in vitro* seedlings of two rice varieties are as shown in Table 2 and Fig. 4. The results indicated that chlorophyll showed a highly significant difference compared to the control treatment, but NaCl treatment accelerated the loss of chlorophyll more strongly in MR269 than in MRQ74. Chlorophyll decreased with increasing NaCl concentration and 200 mM NaCl was more toxic compared to the control. MRQ74 was less affected than MR269 by NaCl concentration compared with the control, but exogenous ascorbic acid in the

medium increased chlorophyll to a level that generally higher than in the control. Ascorbic acid also improved the chlorophyll of the seedlings under NaCl stress and the values in most cases were significantly higher than in the seedlings under stress alone. The most efficient treatment was 1 mM ascorbic acid, which resulted in the highest chlorophyll content.

DISCUSSION

Salt stress is an important factor in decreasing plant growth and productivity. The impact of salinity on plants can depend on many different responses: Nutritional imbalance caused by the interference of saline ions with essential nutrients in absorption and translocation processes, dehydration of the cells through the reduction in water potential, production and accumulation of Reactive Oxygen Species (ROS) during salinity stress and toxicity due to the increased accumulation of Cl^- and Na^+ in the cytoplasm (Arab and Ehsanpour, 2006).

A study by Szarka *et al*. (2012)observed that salinity stress is strongly interconnected to the overproduction of Reactive Oxygen Species (ROS) in plants, which are extremely toxic and reactive and cause damage to DNA, proteins, carbohydrates and lipids, ultimately resulting in oxidative stress. The salt stress-induced accumulation of ROS is counteracted by enzymatic antioxidant systems and non-enzymatic low molecular weight metabolites, such as α-tocopherol, glutathione and ascorbate. These mentioned low molecular weight antioxidants can enhance the activities of scavenging ROS and chelating metal ions. Therefore, in plant cells, this triad of low molecular weight antioxidants (α-tocopherol, glutathione and ascorbate) forms an important part of the abiotic stress response.

The results of this investigation reveal that seedling growth decreased upon exposure to increasing levels of NaCl stress. An increase in the tolerance to salt stress was shown by improved growth characteristics and the content of photosynthetic pigments. The decrease in growth characteristics in plants subjected to salt stress is often associated with a reduction in photosynthetic pigments and a reduction in chlorophyll due to NaCl stress (Agami, 2014). Similar results have also been reported in rice (Prajuabmon *et al*., 2009).

Ascorbic acid increased chlorophyll content seedlings both with and without NaCl stress(Dolatabadian and Jouneghani, 2009), which is consistent with Agami (2014), Arab and Ehsanpour (2006), Aly *et al*. (2012), Khan *et al*. (2010) and Woodward and Bennett (2005). Prajuabmon *et al*. (2009) reported that salt stress due to increasing NaCl levels (0-200 mM) led to marked decreases in the growth, length and fresh and dry weight of shoots and roots of *in vitro* seedlings of rice cultivars. The Na^+/K^+ ratio in the plants was dramatically increased.

These effects could be attributed to the effect of exogenous ascorbic acid on various physiological processes, including the organization of growth, metabolism and differentiation of plants under NaCl stress and increasing physiological availability of nutrients and water (Barakat, 2003). Ascorbic acid currently holds a significant position in plant physiology, mainly due to its possession of cellular reductant and antioxidant properties and its diverse functions in plant development and growth and in the regulation of a broad spectrum of plant cellular mechanisms against salt stresses (Khan *et al*., 2011). Additionally, ascorbic acid protects metabolic processes against H_2O_2 and toxic derivatives of oxygen which affect various enzyme activities and decreases the damage caused through oxidative processes by functioning in synergy with other stabilizing membranes and antioxidants (Pourcel *et al*., 2007; Shao *et al*., 2008).

The growth stimulating effects of ascorbic acid may be related to its antioxidative function, as demonstrated by diminished H_2O_2, lipid peroxidation and superoxide radical production and higher contents of chlorophyll and antioxidant enzymes (phenoloxidase and peroxidase) (Midan and Sorial, 2011).

Ascorbic acid is beneficial to the chloroplast system in stressed plants, mitigating damage from a wide range of oxidative stress radicals, which decompose the structure of very important molecules in the cell, such as chlorophylls. Thus, ascorbic acid exists naturally at a high level in the chloroplast. A study by Smirnoff (2000) found that ascorbic acid has been detected in the majority of plant cell types, apoplasts and organelles under physiological conditions; ascorbic acid exists mostly in the reduced form, making up 90% of the ascorbate pool in chloroplasts and leaves. Its intracellular concentration can build up to the millimolar range, e.g., 20-30 mM in the chloroplast stroma and approximately 20 mM in the cytosol (Noctor and Foyer, 1998). This finding was in agreement with El-Ghamriny *et al*. (1999), Farahat *et al*. (2007), Nahed *et al*. (2009), Kumar *et al*. (2011) and Midan and Sorial (2011) as well as Aly *et al*. (2012) in *Trifolium alexandrinum* L. When the medium was supplemented with ascorbic acid, despite the concentration levels, the activity of acid phosphatase was improved and increased. It has been well documented that salt stress increases acid phosphatase activity (Arab and Ehsanpour, 2006). Furthermore, the defence of plants against salinity stress by an exogenous source of ascorbic acid is believed to result indirectly from its influence on K^+ uptake which plays an important role in several metabolic processes, such as photosynthesis and thus the creation of starch. Moreover, the interaction between ascorbic acid and salt levels reduced Na^+ % and uptake in leaves (Abd El-Aziz *et al*., 2006). The exogenous ascorbic acid in the medium decreased Na^+% and uptake in shoots. Hence, the use of exogenous ascorbic acid, as shown in the growth medium with NaCl and ascorbic acid could be recommended to overcome the destructive effects of salt stress. These results are consistent with the results of Agami (2014) in barley, where ascorbic acid was used to increase the inhibition of oxidative damage in plant cells through the development of enzymatic antioxidants responsible for organizing the ROS through NaCl stress. The increased efficiency of the antioxidant system at least partially protected the photosynthetic mechanism, increasing the salt tolerance of the plant.

The application exogenous ascorbic acid alleviated the stress caused by NaCl and improved the parameters mentioned above in all vegetative and root growth. Barakat (2003) observed that ascorbic acid affects several physiological processes, leading to the regulation of growth, metabolism and differentiations of plants under saline environments and the increasing physiological availability of nutrients and water. All of these factors tend to increase the salt tolerance of the plant. These results were consistent with the results of Agami (2014) in barley (*Hordeum vulgare* L.), Aly *et al*. (2012) in *Trifolium alexandrinum* L., Arab and Ehsanpour (2006) in alfalfa (*Medicago sativa* L.) and Younis *et al*. (2010) in *Viciafaba*.

CONCLUSION

Overall, this report shows that treating the rice (*Oryza sativa* L.) varieties MRQ74 and MR269 with 1 mM ascorbic acid substantially influences several metabolic processes, leading to increased capacity for seedling survival and growth through balancing or changes in the Na^+ and K^+ levels as well as the intracellular content of ascorbic acid as well, the protection of plant against salinity stress by using of ascorbic acid is possibly to be caused as indirectly as a result of its effect on N and K^+ uptake and also increase amount of chlorophyll which plays an essential role in several metabolic processes for example photosynthesis process and then formation of starch, Thus, it may be concluded that exogenously applied ascorbic acid has low external osmotic potential and ion toxicity and may be effective in amelioration of the adverse effects of salt stress. The results indicate that the salt tolerance level was increased by exogenous ascorbic acid.

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