

## The potential role of seed priming with ascorbic acid and nicotinamide and their interactions to enhance salt tolerance in broad bean (*Vicia faba* L.)

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### Abstract

Seed priming with vitamins is an efficient method for increasing seedlings growth and productivity under stressful conditions. This study aimed to investigate the priming of broad bean (*Vicia faba* L.) seeds using 100 mg L<sup>-1</sup> ascorbic acid and nicotinamide and to study their interactions on growth and some physiological parameters of plant under salt stress (150 mM NaCl) for 4 weeks. Salinity treatment significantly reduced growth parameters, water status, photosynthetic pigments, soluble proteins, K<sup>+</sup>, and Mg<sup>+2</sup> ions contents. On the other hand, soluble carbohydrates, free amino acids, proline, ion leakage, Na<sup>+</sup> and Cl<sup>-</sup> ions were significantly increased compared to control. However, application of the two vitamins individually or in interaction increased the photosynthetic pigments, soluble carbohydrates and proteins, proline and free amino acids content, while transpiration and ion leakage were decreased. In addition, K<sup>+</sup>/Na<sup>+</sup> ratio was increased, whereas Na<sup>+</sup>, Cl<sup>-</sup> and Na<sup>+</sup>/K<sup>+</sup> ratio were decreased. The SDS-PAGE analysis showed that salinity induced the synthesis of protein bands with molecular weights of 161, 101, 43 and 37 KDa and disappearance of 40, 28 and 17 KDa, compared to control. Application of vitamins induced the synthesis of additional protein bands with molecular weights of 28 and 17 KDa such as those appeared after treatment with vitamins C; and 56, 40 and 28 KDa under vitamin PP treatment. Moreover, three protein bands which disappeared under salinity stress were reappeared in response to vitamin treatments. The stimulatory effects of vitamin PP on the growth and other related metabolic activities were greater than that of vitamin C. However, these stimulatory effects were higher when they were applied in combination. The above results pave the way to gain the insight into the potential role of these vitamins to improve the salt tolerance of broad bean plant

**Keywords:** inorganic ions; osmotic solutes; vitamin C; vitamin PP; protein patterns.

**Abbreviations:** Vit. C\_ascorbic acid; vit. PP\_nicotinamide; RWC\_relative water content; EC%\_electrical conductivity.

### Introduction

The limited water resources in Saudi Arabia forms a major constraint for agriculture and development. In arid and semi-arid areas, salinity is one of the most pronounced problems of agricultural irrigation. In these regions, there is an urgent need to use saline water in irrigation because of limited water resources. The success of using saline water for economic crop production can be achieved using the best conditions to minimize or delay the negative effects of salinity on crop growth and productivity. Salinity is one of the environmental factors limiting soil fertility and plant production (Erdal et al., 2011; Jafar et al., 2012; Kumar et al., 2013). About 6.5% of the total land in the world is affected by salt (FAO, 2008). Increase of salinity in the soil inhibits plant to absorb soil water due to lower soil water potential, that increase the osmotic strength of the soil solution. This restricts to water absorption and causes various physiological disturbances in plant. The maintenance of plant growth and development in saline environments is associated with osmotic adjustment, synthesis of osmoprotectants and accumulation of Na<sup>+</sup> in the vacuoles (Ramani et al., 2006). Different mechanisms contribute to salt tolerance of plants through compartment of ions in vacuoles, accumulation of osmotic solutes in the cytoplasm and genetic salt resistance (Girija et al., 2002). Osmosolutes, such as proline, free amino acids and

carbohydrate compounds, could play important roles in plant salt tolerance by osmotic adjustment (Azooz, 2009; Sadak et al., 2010). Under salinity stress, osmotic adjustment is usually achieved by the uptake of inorganic ions such as Na<sup>+</sup>, Cl<sup>-</sup> and K<sup>+</sup> from the growth media (Misra and Gupta, 2006). Na<sup>+</sup> and Cl<sup>-</sup> limitation and K<sup>+</sup> accumulation in roots than shoots has been considered as a physiological trait indicator for salt tolerance in plants (Azooz et al., 2004a; Ramezani et al., 2011; Wu and Wang, 2012). High concentrations of salinity result in membrane injury and loss of permeability (Di Baccio et al., 2004; Dkhil and Denden, 2012). Broad bean (*Vicia faba* L.) is the most important legume crop and a major source of protein for both human and animal nutrition (Crepon et al., 2010). Its cultivation leads to an increase of soil N compounds (Hungria and Vargas, 2000). The effects of salinity stress in broad bean at seedling stage range from reductions in germination percentage, fresh and dry weight of shoots and roots to the uptake of various nutrient ions (Azooz and Al-Fredan, 2009; Azooz et al., 2011). The use of vitamins as antioxidants mediated salt tolerance as a selection factor as well as a driving force for improving resistance and adaptation to salt stress (Jochum et al., 2007). These vitamin supplements are known to enhance the plant activities and did not have toxic or mutagenic action (Bronzetti et al., 2001).

They act as coenzyme systems and have a role in the metabolic regulation. Nicotinamide and ascorbic acid as antioxidants vitamins have positive roles in plant growth and development. Nicotinamide is a water-soluble vitamin and is part of the vitamin B group. It's a stress-associated compound that induces and regulates secondary metabolic accumulation and/or the manifestation of defense metabolism in plants (Berglund and Ohlsson, 1995). It might be a link between various types of stress which leads to plant defensive gene transcription (Berglund, 1994). Ascorbic acid is an abundant antioxidant small molecule in plants that plays an important role in photosynthesis and defense system against oxidative stress (Athar et al., 2008; Azooz and Al-Fredan, 2009). It also affects many physiological processes including the regulation of growth, differentiations and metabolism of plants under saline conditions and increasing physiological availability of water and nutrient (Barakat, 2003; Khan, 2011). Azzedine et al. (2011) reported that the application of ascorbic acid was effective to mitigate the adverse effect of salt stress on plant growth due to increased leaf area, improved chlorophyll and carotenoids contents and enhanced proline accumulation. Seeds presoaking in the two vitamins could be exploited to ensure better growth, particularly under stress conditions (Azooz and Al-Fredan., 2009; Sadak et al., 2010; Yazdanpanah et al., 2011). The role of vitamins in modifying the salt-stress induced changes in osmoprotectant contents was also investigated by some investigators (Heikal et al., 2000; Dolatabadian et al., 2008; Fercha et al., 2011). Vitamins are also involved in the regulation of gene expression during adaptive responses to abiotic and biotic stresses (Barakat, 2003; Azooz, 2004; Azooz and Al-Fredan, 2009; Ekmekçi and Karaman, 2012). It has been shown that vitamins increased the amount of the original proteins which were already present in vitamin-untreated plants, as well as, induced the appearance of additional new bands.

Many studies have been performed to assess the positive effect of vitamins in enhancing salt tolerance and modulating salinity stress, when they are applied individually. However, currently very little is known about the effect of antioxidant vitamins when they applied in combination (interaction). Therefore, this work aimed to investigate the potential role of 100 mg L<sup>-1</sup> of ascorbic acid or nicotinamide solution, individually or in combination (interaction) for enhancing salt tolerance of broad bean plant grown under NaCl salinity to evaluate whether any application is more effective in ameliorating the drastic effect of salinity stress, and the possible role played by these vitamins, in regulating salt-induced changes in growth, some relevant metabolic activities and protein patterns of broad bean plant.

## Results and Discussion

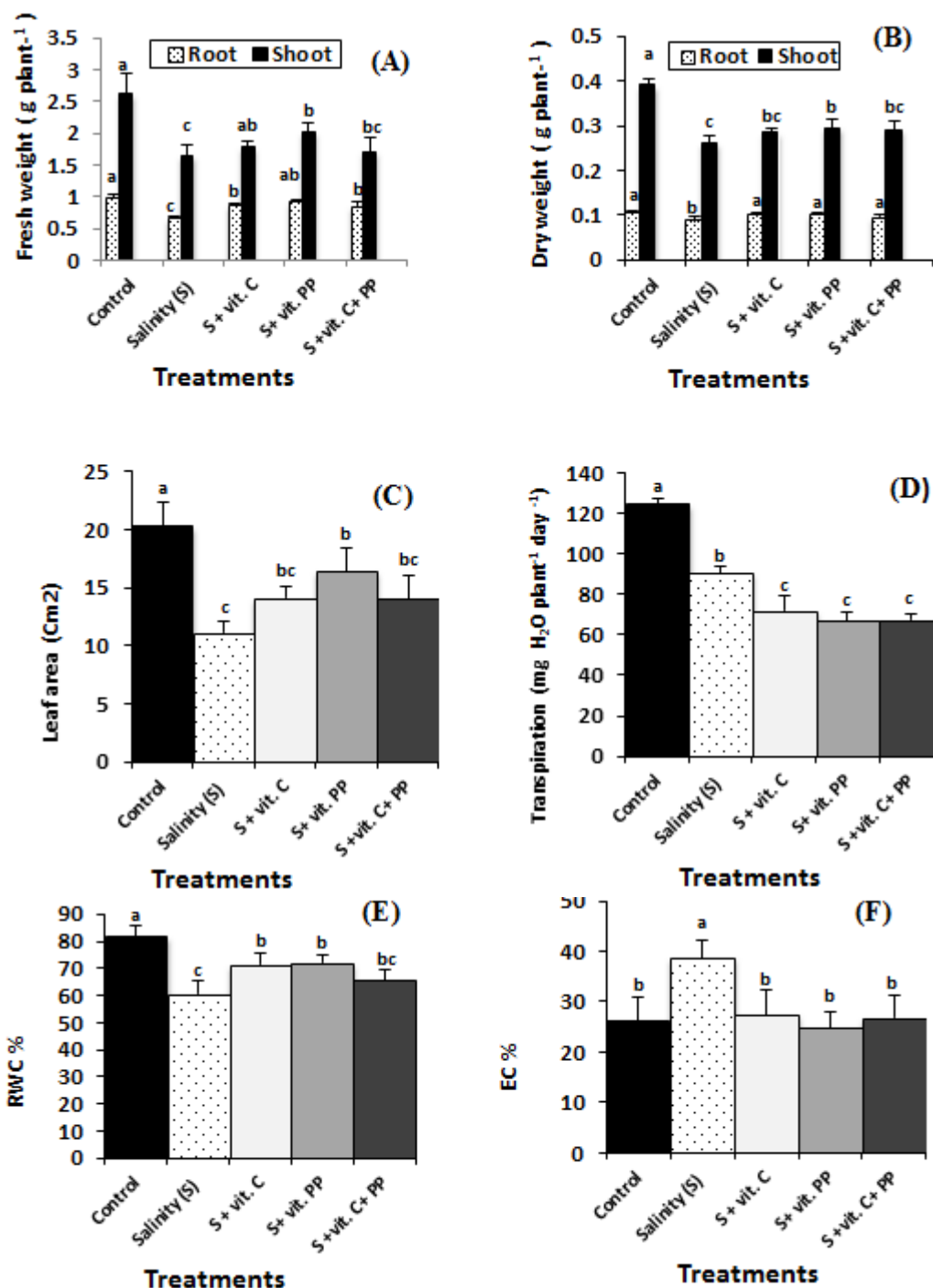
### Growth parameters

Growth performances of *Vicia faba* L. plants were estimated by growth parameters (fresh and dry weights of roots and shoots and leaf area) and water status (transpiration and leaves relative water content). The effect of salinity and vitamins treatment on growth parameters of *Vicia faba* L. (Fig. 1A-F) showed that these plants exhibited a significant reduction ( $p \leq 0.05$ ) in their growth parameters and water status in response to 150 mM NaCl, while membrane permeability as EC% (Fig. 1F), was markedly increased, compared to control plants (0.0 mM NaCl). The reduction percentage in dry weight was higher (31%) in roots than shoots (24%). Similar reduction in growth performance were found in some plants under saline conditions (Ates and

Tekeli, 2007; Azooz, 2009; Ekmekçi and Karaman, 2012; Kaya et al., 2013). This might be attributed to the toxic effect of salinity or increased crucial osmotic pressure, at which the broad bean plants would not be able to absorb water due to osmotic effect and decrease in some physiological activities. The higher reduction in roots dry weight than shoots as response to salinity stress may be due to the ability of these plants to limit Na<sup>+</sup> and Cl<sup>-</sup> transport into the shoots. This is critically important for the maintenance of high growth rates, and protection of metabolic processes in cells elongation from the toxic effects of Na<sup>+</sup> (Razmjoo et al., 2008), leading to the better growth of shoots than roots. Kaya et al. (2003) reported that, growth of roots in *Carthamus tinctorius* L. plant was more sensitive and adversely affected as compared to shoots growth under salinity conditions. Vitamin treatments had stimulation effects on such parameters, either when they applied individually or in interaction. In contrast, transpiration rate and membrane permeability (Fig. 1 D and F, respectively) were decreased, compared to Vitamins-untreated plants. These results are in agreement with those obtained by others (Azooz, 2004; Athar et al., 2008; Bassuony et al., 2008; Azooz, 2009; Erdal et al., 2011; Ekmekçi and Karaman, 2012). They indicated that, vitamins could accelerate cell division and cell enlargement and induce improvement of membrane integrity, which may have contributed in reducing ion leakage, and consequently improving growth. Increasing of leaves relative water content (RWC) and reduction in transpiration rate indicated that these vitamins probably reflect the efficiency of water uptake and utilization or reduction of water loss, which consequently causes increase in leaf water potential. Hence, it could be concluded that the beneficial effect of vitamins on growth parameters of *Vicia faba* L. plants has been related to the efficiency of their water uptake and utilization. Many studies have reported that vitamins, when used with optimal concentration, exhibited beneficial effect on growth and yield of some crop plants grown under saline conditions (Azooz, 2004; Khan et al., 2006; Bassuony et al., 2008; Ekmekçi and Karaman, 2012). However, these vitamins at critical level could behave as antagonistic action in affecting plant growth. These reports explain and support our results, in respect to the observed antagonistic effect of these vitamins on growth parameters of broad bean plants, when they were applied in combination.

### Photosynthetic pigments

Salinity treatments showed a statistically significant decrease ( $p \leq 0.05$ ) on photosynthetic pigments viz., chl. a, b, carotenoids and total chlorophyll content of *Vicia faba* L. leaves, compared to control. Total chlorophyll content was decreased about 42% less than control (Fig. 2). The negative effects of salinity stress on photosynthetic pigments could be due to the inhibition of chlorophyll biosynthesis or increase of its degradation by chlorophyllase, which is more active under salinity stress (Khan et al., 2006; Ziaf et al., 2009; Akça and Samsunlu, 2012). Furthermore, an oxidative stress could happen due to salinity stress leading to deterioration in chloroplast structure, and consequently decrease in chlorophyll content. The inhibitory effect of salinity stress on photosynthetic pigments was partially or completely ameliorated, as a result of vitamin treatments. The ameliorative effect of vit. PP was higher than vit. C; however, their effects were lower when they were applied in combination. These results reinforce the results obtained by others (Khan et al., 2006; Beltagi, 2008; Azooz and Al-



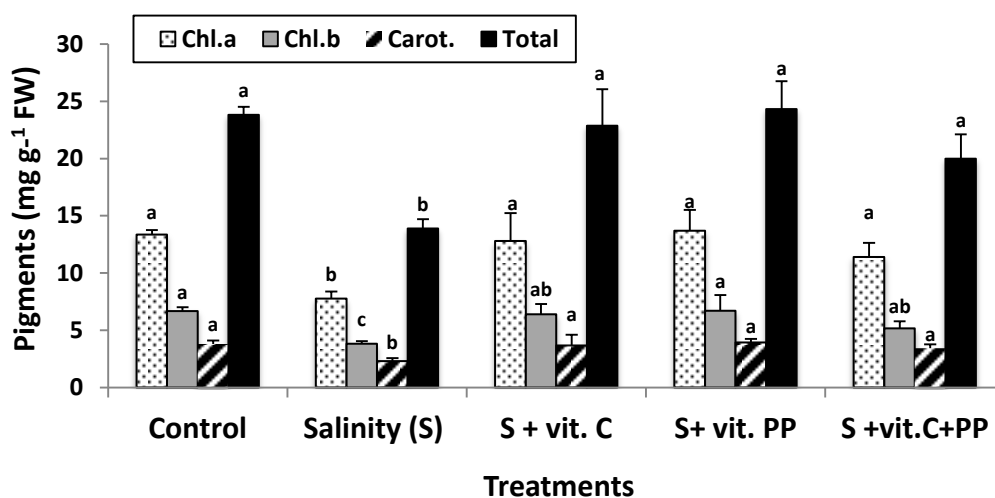
**Fig 1.** Fresh weight (A), dry weight (B), leaf area (C), transpiration (D), leaves relative water content (E) and ion leakage (F) of *Vicia faba* L., in response to salinity (150 mM) and vit.C or/and vit. pp (100 mgL<sup>-1</sup>). Vertical bars represent  $\pm$  SD of three replicates ( $n=3$ ). Bars carrying different letters are significantly different at  $P \leq 0.05$  between the control, salinity and vitamins treated-plants.

Fredan, 2009). They concluded that, chlorophyll content of plants treated with vitamins was increased due to the protection effect of these vitamins. Dolatabadian et al. (2008) reported that, salt stress leads to an increase in free radicals in chloroplasts and destruction of chlorophyll molecules by reactive oxygen species, while ascorbic acid can detoxify and neutralize the reactive oxygen species by prevention of free radicals activity, leading to increase in chlorophyll content of vitamin- treated plants. Hassanein et al. (2009) suggested that these vitamins may interfere with the protection of chloroplasts and their membrane against NaCl toxicity and;

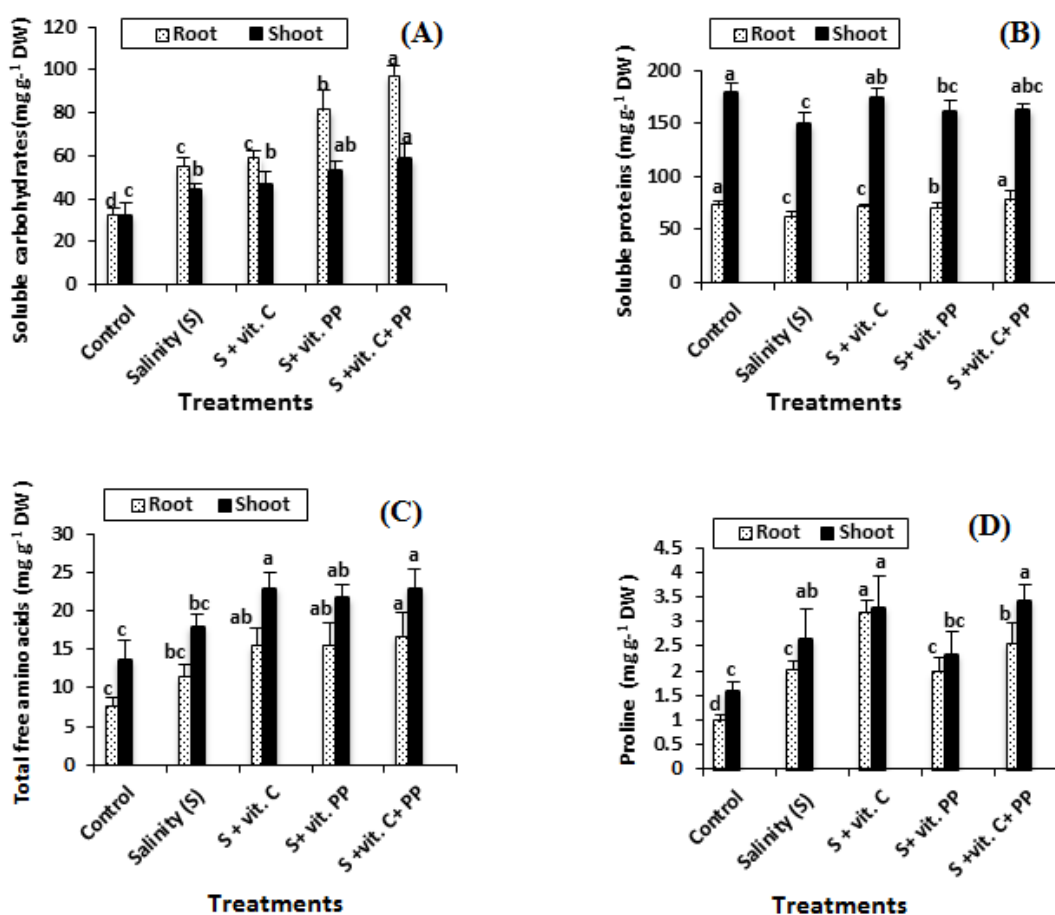
thus, maintaining their integrity. Azzedine et al. (2011) reported that, application of vitamin C was effective to mitigate the adverse effect of salt stress on plant growth due to increased leaf area, improved chlorophyll and carotenoids contents.

#### Organic solutes

Organic solutes such as soluble carbohydrates, soluble proteins, total free amino acids and proline have been shown involved in osmotic regulation in plant, playing an important



**Fig 2.** The content ( $\text{mg g}^{-1}\text{FW}$ ) of chl. *a*, chl. *b*, carotenoids and total pigments in *Vicia faba* L. leaves, in response to salinity (150 mM) and vit. C or/and vit. PP ( $100 \text{ mg L}^{-1}$ ). Vertical bars represent  $\pm$  SD of three replicates ( $n=3$ ). Bars carrying different letters are significantly different at  $P \leq 0.05$  between the control, salinity and vitamins treated-plants.



**Fig 3.** Soluble carbohydrates (A), soluble proteins (B), total free amino acids (C) and proline (D) in root and shoot of *Vicia faba* L., in response to salinity (150 mM) and vit. C or/and vit. pp ( $100 \text{ mg L}^{-1}$ ). Vertical bars represent  $\pm$  SD of three replicates ( $n=3$ ). Bars carrying different letters are significantly different at  $P \leq 0.05$  between the control, salinity and vitamins treated-plants.

role in tolerance of plant to salinity stress (Bartels and Sunkar, 2005), and may use as a protective strategy to alleviate Na<sup>+</sup> toxicity (Chen et al., 2002). In the current study, salinity stress caused a significant increase ( $p \leq 0.05$ ) of soluble carbohydrates, total free amino acids and proline, while soluble proteins were decreased in both roots and shoots of broad bean plants, compared to un-salinized control plants (Fig. 3A-D). The increase in soluble carbohydrates due to salinity stress has been reported in other plants (Ramezani et al., 2011). The reduction of protein was previously recorded by Bassuony et al. (2008) and Sadak et al. (2010). They concluded that, the reduction of protein under salinity stress was suppressed by the accumulation of total amino -N and proline. The accumulation of proline and amino acids in the cytoplasm plays an important role in the osmotic balance of plants and are good indicators of salinity tolerance (Azooz, 2002; Azooz et al., 2004b; Ramezani et al., 2011; Uiddin et al., 2012). These conclusions are confirmed with the results of this study. The increased proline content in broad bean suggests an excellent mechanism to decrease the osmotic potential in this plant. This supports the assumption that proline accumulation is a part of physiological response of plant to intense stress (Ain-Lhout et al., 2001). It is noteworthy that the accumulation of proline and other free amino acids were coincided with a decrease in soluble proteins, suggesting that this accumulation seems to be at the expense of soluble proteins reduction under salinity stress. This conclusion is in agreement with Roy-Macauley et al. (1992) which showed, accumulation of free amino acids in plants under salt stress are often attributed to alterations in biosynthesis and degradation processes of amino acids and proteins. The higher content of soluble carbohydrates in roots than shoots was accompanied by the higher reduction observed in growth of roots than shoots. This indicates that soluble carbohydrates have proved to be helpful in osmoregulation rather than growth, playing an important role in improving salt tolerance of broad bean plant either under salinity alone or in combination with vitamins.

The positive effect of NaCl on free amino acids and proline content has significantly magnified in response to vitamin treatments, except in case of proline in salinized plants-treated with vit. PP, where the opposite trend was observed. These results confirm the earlier findings (Azooz et al., 2002; Azooz, 2004; Fercha et al., 2011). The effect of vitamins on proline content suggests that these vitamins probably improve growth of stressed plants. Azzedine et al. (2011) reported that application of vitamin C was effective to mitigate the adverse effect of salt stress on plant growth of durum wheat due to increased leaf area and enhanced proline accumulation. There is surprising situation in the content of proline. Most vitamins tend to increase the proline content, but an opposite trend was observed regarding the vit. PP in the present study. This may be attributed to the better growth observed in plants treated with this vitamin, suggesting that the inhibitory effect of salinity stress on broad bean was alleviated by vit. PP, through inhibiting proline synthesis and/or enhancing the biosynthesis of other free amino acids and their incorporation into protein (Barakat, 2003; Azooz, 2004). Dolatabadion et al. (2008) revealed that salt stress increased proline content, and application of vitamins scavenged ROS and prevented the biosynthesis of extra proline in canola plant.

### **Inorganic ions**

Salinity treatments caused a considerable increase of Na<sup>+</sup> and Cl<sup>-</sup> (Fig. 4A and E, respectively), and decrease of K<sup>+</sup> and

Mg<sup>2+</sup> (Fig. 4B and D, respectively). However, Ca<sup>2+</sup> content (Fig. 4C) was not affected as compared with non-salinized plants. This leading to a significantly increase of Na<sup>+</sup>/K<sup>+</sup> and decreased of K<sup>+</sup>/Na<sup>+</sup> ratio (Fig. 4F and G, respectively). The ratio of Na<sup>+</sup>/K<sup>+</sup> was higher in roots than shoots, whereas an opposite trend was observed in K<sup>+</sup>/Na<sup>+</sup> ratio. The accumulation of Na<sup>+</sup> and Cl<sup>-</sup> ions might be involved in the osmotic adjustment (Agarwal and Pandey, 2004).

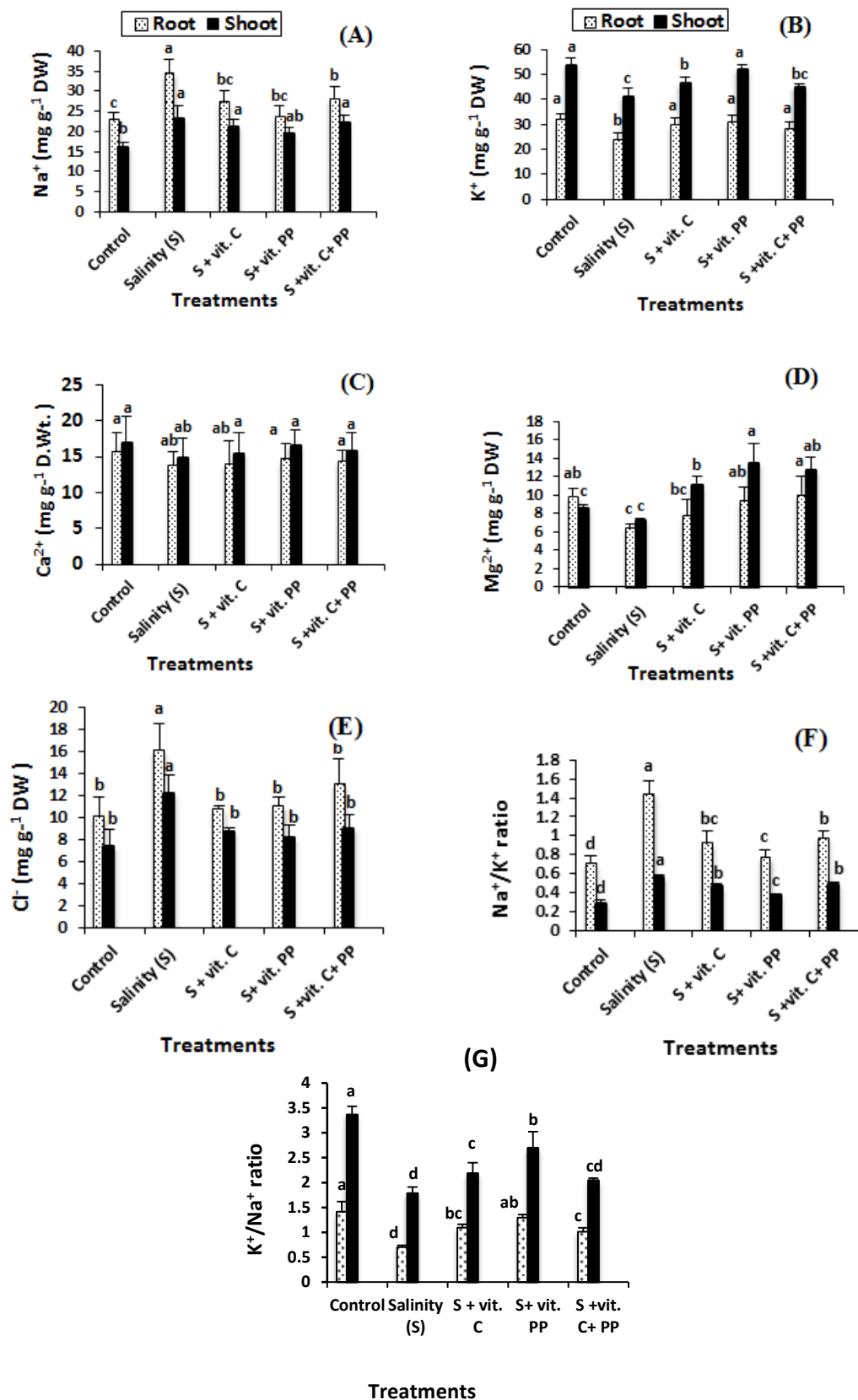
Munns and Tester (2008) concluded that, plants may respond to low water potential induced by salt stress by accumulation of some organic solutes and inorganic ions. The accumulation of Na<sup>+</sup> and Cl<sup>-</sup> was higher in roots than those of shoots. This may be due to low transport rate of Na<sup>+</sup> and Cl<sup>-</sup> to shoots, which is one of the important adaptive components of plant to salt stress (Munns, 2002; Azooz et al., 2011). The promotion of Na<sup>+</sup> uptake by salinity was accompanied by a corresponding decline in K<sup>+</sup> concentration. This, in turn, leads to increased Na<sup>+</sup> uptake at the expense of K<sup>+</sup> and consequently increased of Na<sup>+</sup>/K<sup>+</sup> ratio (Azooz et al., 2004a; Azooz, 2009; Kaya et al., 2013). The results in the present study revealed that, Na<sup>+</sup>/K<sup>+</sup> ratio in roots was higher than 1 in plants subjected to salinity stress, while it was less than 1 in vitamin-treated plants. The opposite trend was observed in K<sup>+</sup>/Na<sup>+</sup> ratio. These results revealed that the stimulatory effect of vitamins was associated with a lower Na<sup>+</sup>/K<sup>+</sup> and a higher K<sup>+</sup>/Na<sup>+</sup> ratio. This interaction between relative K<sup>+</sup> and Na<sup>+</sup> concentration has been considered a key factor in improving salt tolerance in plants treated with vitamins.

It can be concluded that the reduction in growth of broad bean plants, at least in part, relates to ionic disorders induced by salinity stress. However, vitamins improve the salt tolerance of vitamin-treated plants. The reduction in Mg<sup>2+</sup> uptake under salt stress conditions might be due to the suppressive effect of Na<sup>+</sup> on its uptake. Application of vitamins individually or in combination, caused a reduction of Na<sup>+</sup> and increase of K<sup>+</sup> and Mg<sup>2+</sup> contents, while Ca<sup>2+</sup> remained mostly unchanged, compared to control plants. It is worthy to note that similar trends were observed in the chlorophyll contents in broad bean leaves in relation to Mg<sup>2+</sup> content. The antagonistic relations between Na<sup>+</sup> and K<sup>+</sup> may be taken as an indication of the role played by vitamins in modifying K<sup>+</sup>/Na<sup>+</sup> selectivity and increase of K<sup>+</sup> and Mg<sup>2+</sup> under salt stress (Wu and Wang, 2012). This could play an important action in osmoregulation, increasing the efficiency of water absorption and utilization under salt stress, leading to increase of broad bean plant - salt tolerance.

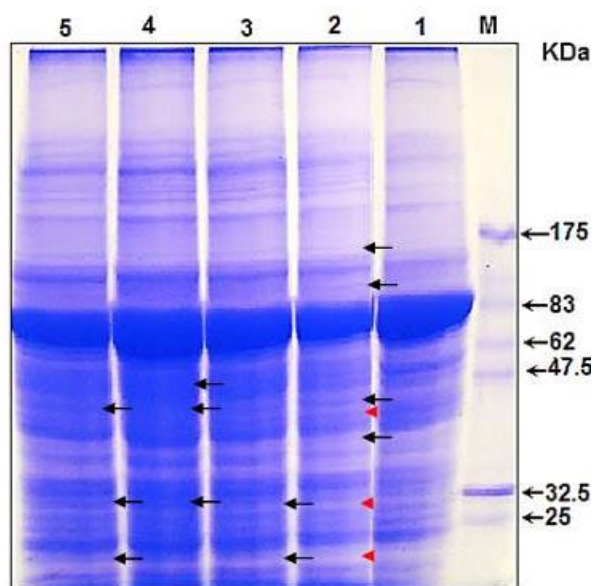
The maintenance of Ca<sup>2+</sup> level in vitamin-treated plants could induce reduction of K<sup>+</sup>, since the presence of Ca<sup>2+</sup> seems to be necessary for K<sup>+</sup>/Na<sup>+</sup> selectivity. Nicotinamide was more effective than ascorbic acid. Their effective was higher when they were applied individually than in combinations. The results indicated that, vitamins could regulate K<sup>+</sup>/Na<sup>+</sup> homeostasis in broad bean plants by enhancing the selectivity for K<sup>+</sup> over Na<sup>+</sup> in shoots. The influence of vitamins on the uptake mechanism of ions may be related to its effect on membrane permeability and rate of ion entry through the membrane.

### **SDS-PAGE protein patterns**

Alteration in protein expression in germinated seeds of *Vicia faba* L., under 150 mM NaCl, alone or in combination with vitamins (compared with control) was analyzed by SDS-PAGE as illustrated in Fig. 5. Salinity treatments induced the



**Fig 4.** Na<sup>+</sup> (A), K<sup>+</sup> (B), Ca<sup>2+</sup>(C), Mg<sup>2+</sup> (D) , Cl<sup>-</sup> (E), ratio of Na<sup>+</sup>/K<sup>+</sup>(F) and K<sup>+</sup>/Na<sup>+</sup> (G) in root and shoot of *Vicia faba* L., in response to salinity (150 mM) and vit.C or/and vit. pp (100 mg L<sup>-1</sup>). Vertical bars represent ± SD of three replicates (n=3). Bars carrying different letters are significantly different at P ≤ 0.05 between the control, salinity and vitamins treated-plants.



**Fig 5.** SDS-PAGE protein profile of *Vicia faba* L. seedlings germinated under 150 mM NaCl and primed in vit.C or vit.PP individually or in interaction. Newly appeared bands are indicated by arrows (↔) and disappeared bands are indicated by arrows (◄). M: Protein marker. Lane (1): Control (0.0 mM NaCl), Lane (2): Salinity (150 mM NaCl), Lane (3): Salinity + vit. C, Lane (4): Salinity + vit. PP, Lane (5): Salinity + vit.C + vit. PP

appearance of 4 protein bands with molecular weights of 161, 101, 43, and 37 KDa, and disappearance of 3 protein bands with molecular weights of 40, 28 and 17 KDa as compared with control (Fig. 5, Lane 2). Application of vit. C and vit. PP individually allowed the synthesis of additional two (28 and 17 KDa), and three (56, 40 and 28 KDa) new protein bands, respectively. It is interesting to note that 3 protein bands with molecular weights of 40, 28 and 17 KDa, which disappeared under salinity stress are reappeared in response to vitamins treatment (Fig. 5 Lanes 3,4 and 5).

These results were supported by previous studies in other plant species (Azooz, 2004; Beltagi, 2008; Azooz and Al-Fredan, 2009). These results suggest the involvement of these polypeptides for osmotic adjustment and improved the salt tolerance of *Vicia faba* L. plant. The polypeptides that were disappeared during salinity stress might be compensated by others. The expression of new protein bands and the significant increase in the intensity of some original bands, indicate that the applied vitamins have a stimulatory effect on the quantitative and qualitative changes in protein biosynthesis. These new protein bands may be due to *de novo* synthesis of new proteins in germinated seedlings under 150 mM NaCl or in combination with vitamins, as reported by other investigators (Barakat, 2003; Azooz, 2004; Bassuony et al., 2008; Azooz and Al-Fredan, 2009). They reported that, vitamin treatments induced alterations in the enzymes related to protein metabolism. These enzymes might act as activators of protein synthesis that appeared in seedlings treated with salinity or/and vitamins. This may play an inductive role in triggering a special defense system helping these seedlings to improve their salt tolerance and consequently their growth and productivity.

## Materials and Methods

### Plant growth and treatments

Homogenous broad bean (*Vicia faba* L.) seeds were divided into 5 groups: The first and second groups were soaked in distilled water, the third and fourth groups were soaked

separately in 100 mg L<sup>-1</sup> freshly prepared solution of ascorbic acid and nicotinamide, respectively, while the fifth group was soaked in a mixture of 100 mg L<sup>-1</sup> of both the two mentioned vitamins for 10 hours, and then air-dried for 48 h. Seeds were surface sterilized with 0.1% Hg Cl<sub>2</sub> for 3 minutes and washed three times with sterilized distilled water. The sterilized seeds were sown in plastic pots containing 2 kg dried soil. The first group was supplemented with appropriate amount (at field capacity) of water (control), while the other four groups were supplemented with 150 mM NaCl. All pots were kept in a growth chamber maintained at 25/20°C day/night (12 h) temperature cycles and light intensity of 110 mol m<sup>-2</sup> s<sup>-1</sup>, during the experimental period, and irrigated with normal water as required. After complete seed germination, five uniform seedlings were left in each pot. The plants were watered with normal water to the field capacity by weighing the pots every two days and left to grow until the end of experiment (4 weeks after sowing). Three replicates from each group were prepared. Four days after complete germination, some of seedlings from each group were harvested; frozen with liquid nitrogen and stored for protein electrophoresis.

### Determination of growth parameters

Plants of all groups were harvested, and split up into roots and shoots. The freshly harvested roots and shoots were rinsed with deionized water and blotted on paper towels before being weighed (fresh weight). To determine dry weight, the freshly roots and shoots were dried, in an aerated oven at 80°C until constant weight. The oven-dried samples were grinded into fine powder for later analysis.

Transpiration rate was estimated as described by Bozcuk (1975). Leaf area was estimated by using Digital Planimeter (Placom KP-90). Leaf relative water content (RWC%) was determined according to Smart (1974). Leaf material was weighed (0.5 g) to determine fresh weight and placed in double-distilled water for 4 h and then turgid weight was recorded. Finally, the samples were dried in an oven at 65°C for 48 h and the dry weights were recorded. RWC was

calculated as:  $RWC\% = [(Fresh\ weight - Dry\ weight)/(Turgid\ weight - Dry\ weight)] \times 100$ .

#### **Electric conductivity**

Ion leakage was determined as electrical conductivity (EC%) according to Yan et al. (1996). Leaf samples were cut into discs of uniform size and placed in 10 ml of double-distilled water at 40°C for 30 min, and its conductivity recorded (C1) using conductivity meter (Jenway 470 portable conductivity meter). Then it was kept in a boiling water bath (100°C) for 15 min and its conductivity also recorded (C2). The percentage of electrolyte leakage was calculated according to this formula:  $EC\ (\%) = (C1/C2) \times 100$ . Where C1 and C2 are the electrolyte conductivities measured before and after boiling, respectively.

#### **Determination of photosynthetic pigments**

Photosynthetic pigments (chl. *a* and chl. *b*) and total carotenoid contents in leaves were estimated in 80% acetone extracts according to Lichtenthaler and Wellburn (1983). Leaf material (0.1 g each) of young fully expanded leaves of three plants from each replicate were used for pigment extraction. The pigments extract was measured against a blank of pure 85% acetone at wave-lengths of 663, 644 and 452.5 nm using the spectrophotometrically.

#### **Determination of organic solutes**

Soluble carbohydrate content was determined by anthrone sulphuric acid method according to Badour (1959). Soluble protein content was determined according to the method of Bradford, (1976). Proline was determined according to the procedures described by Bates et al. (1973). Total free amino acids were extracted from plant tissues and determined according to the method of Lee and Takahashi (1966).

#### **Determination of inorganic ions:**

The content of inorganic ions ( $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Na^+$ ,  $K^+$  and  $Cl^-$ ) in plant roots and shoots were determined. Dry samples of roots and shoots systems were ground into a fine powder, and the inorganic ions were extracted. The content of  $Na^+$  and  $K^+$  were determined by flame photometry according to Williams and Twine (1960). The concentrations of calcium and magnesium were determined by atomic absorption. Chloride was determined by titration with 0.1 N  $AgNO_3$  according to Johnson and Ulrich (1959). The ratios of  $Na^+/K^+$  and  $K^+/Na^+$  were made to evaluate salt tolerance regulated by salinity or vitamin treatments.

#### **Sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) protein patterns:**

One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was applied according to the procedure of Laemmli (1970) with some modifications to investigate the changes of protein patterns in broad bean seedlings. The frozen tissue (0.5 g) was homogenized in 2 mL of SDS-PAGE extraction buffer [50 mM Tris-HCl (pH 6.8), 20 mM dithiothreitol (DTT), 0.5 mM  $MgCl_2$ , 0.004% EDTA and 10% sucrose] and boiled for 4 min. Thereafter, it was centrifuged at 13,000 g for 20 min at 4°C. The supernatants were used for electrophoresis.

#### **Gel staining**

After electrophoresis, protein bands in the gel were visualized by a Coomassie Brilliant Blue R-250 (CB) with Bismarck Brown R (BBR) according to Choi et al. (1996).

#### **Gel analysis and digitization**

The protein bands in the gel were analyzed and digitized using UN-SCAN-IT gel 6.1 software for Windows (Silk Scientific).

#### **Statistical analysis**

The data were statistically analyzed by one-way ANOVA analysis of variance using SPSS program. Values in the figures indicate the mean values  $\pm$ SD based on three independent determinations ( $n=3$ ), and the Least Significant Difference (LSD) was used to test the differences between treatments; and  $p \leq 0.05$  was considered statistically significant.

#### **Conclusion**

This investigation showed that salinity causes a reduction in plant growth parameters and water status. Seed priming with ascorbic acid and nicotinamide individually or in combination/interaction enhanced salt tolerance of *Vicia faba* L. plants. This was accompanied by increase in photosynthetic pigments, decrease of ion leakage,  $Na^+$  and  $Cl^-$  content and greater accumulation of osmotically soluble carbohydrates, protein, total free amino acids and proline. Furthermore, vitamins caused a modification in  $Na^+/K^+$  and  $K^+/Na^+$  selectivity, and increased  $K^+$  and  $Mg^{+2}$  contents. These mechanisms had a cumulative effect in protecting membranes and protection of plant from the negative effects of salt stress. The stimulatory effect of vit. PP on growth and the other related metabolic activities was higher than vit. C. The stimulatory effects of these vitamins were individually higher than that, when they were applied in combination. So, it can be suggested that, *Vicia faba* L. plant can grow better under salt stress along with seed priming in 100 mg L<sup>-1</sup> ascorbic acid or nicotinamide, when they applied individually rather than in combination/interaction.

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