

Biochemical and Morphological Response of Common Bean (*Phaseolus vulgaris* L.) to Salinity Stress and Vitamin B₁₂

Hamed Keshavarz* and Seyed Ali Mohammad Modares Sanavy

Corresponding author: Hamed Keshavarz

ABSTRACT: The effects of saline water irrigation and cyanocobalamin (vitamin B₁₂) priming were evaluated on some growth parameters and biochemical attributes of common bean (*Phaseolus vulgaris* L.). Effects were determined by leaf area, root and shoot length, root and shoot dry matter, antioxidant enzyme activity, protein content, chlorophyll and carotenoids, lipid peroxidation and proline accumulation. The results demonstrated that application of vitamin B₁₂ provided significant protection against salinity stress compared with non treated plants. In addition, vitamin B₁₂ application showed a significant effect on morphological characteristics. According to the outcomes, antioxidant enzyme activity, lipid peroxidation, carotenoid and proline accumulation increased due to NaCl stress while protein, chlorophyll and carotenoids decreased. Seed priming with vitamin B₁₂ induced these stimulatory effects on all the measured parameters. Root protein content and leaf carotenoid content were not affected by vitamin treatment under conditions of salinity stress. These results suggest that, vitamin B₁₂ may have a critical role as an effective antioxidant and regulates osmotic balance thereby enhancing bean plant's resistance to salinity stress.

Keywords: Antioxidant activity, Common bean, Cyanocobalamin, Dry matter, Photosynthetic pigments, Plant height

Abbreviations: CAT: Catalase; Dry matter: DM; POX: Peroxidase; ROS: Reactive Oxygen Species; SOD: Superoxide dismutase

INTRODUCTION

Plants are usually exposed to different environmental stresses that limit their growth and productivity, of which salinity stress is the most severe. It has been well documented that salinity significantly inhibits plant growth (Saqib et al. 2005). An adverse effect on biochemical processes has also been reported in many species (Zhang et al. 2011). Salt stress is known to change the composition of N-containing compounds, especially proteins (Saqib et al. 2005). In addition, sodium chloride stress inhibits the uptake and transportation of K⁺ that causes an ion imbalance between Na⁺ and K⁺ causing ion toxicity and osmotic stress in plant cells (Sivritepe et al. 2005, Khan and Panda 2008). Photosynthesis, as a key metabolic pathway in plants is a process that is particularly susceptible to salt stress. It is likely that salt stress limits gaseous exchange causing a disruption to stomatal closure and limits CO₂ supply to leaves (Harris and Outlaw 1991; Fendina et al. 1994). Over reduction of the photosynthetic electron transport chain induces the generation of reactive oxygen species (ROS), such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH[•]) (Ghosh 2011). The levels of ROS are regulated by the rate at which they are generated, the rate of reaction and the potential rate of degradation on interaction with target substances such as proteins, lipids and nucleic acids. The rate of scavenging/neutralizing by enzymatic and non-enzymatic antioxidants also affects the regulation of ROS (Hasanuzzaman 2011).

Plants have developed sophisticated defense mechanisms to recognize and respond to a wide range of stresses. Plants respond to salinity by activation of numerous compounds such as various compatible solutes, polyamines and an antioxidant defense mechanism. Several enzymes are involved in the detoxification process of ROS. Superoxide converts to H_2O_2 by superoxide dismutase (SOD). Hydrogen peroxide is scavenged by catalase (CAT) and peroxidase (POX) (Bowler et al. 1992). The capacity to scavenge ROS and to reduce the damaging effect on macromolecules appears to be an important stress tolerance trait in higher plants (Sung and Hong, 2010). A close correlation between antioxidant capacity and sodium chloride tolerance has been demonstrated in numerous crops such as pea (Hernandez et al. 2000), cotton (Gossett et al. 1994) and rice (Ghosh et al. 2011).

Availability of growth regulators plays a crucial role during and after seed germination and application of vitamins (as growth regulators) presents an approach to overcome any limitations (for example vitamins C and E deficiencies) of a plant's environment by improving tolerance to salinity and economic growth rate (Chen and Xiong 2005). Growth regulators have also been shown to function as potent antioxidants, particularly active in quenching free radicals (Ehrenshaft et al. 1999; Jain and Lim 2001; Osmani et al. 1999). Among these substances, the B group vitamins are known to enhance seed germination (Noggle and Wynd 1943; Kozhin and Kravtsov 1973; Burguieres et al. 2007), to promote growth of various plant species (Bonner and Bonner 1948; Aberg 1961). It is also known to be indispensable for normal growth and development of excised plant organs, particularly roots (Oertli 1987; Samiullah et al. 1988). There are various reports on the interactive effect of salinity and vitamins on protein synthesis and growth rate in some crop plants (Azooze 1990). There have also been reports on exogenous application of other vitamins such as vitamin C (Dolatadian et al. 2008; Younis et al. 2010), vitamin E (Jon and Munne Bosch 2010; Munne Bosch and Alegre 2002a) and vitamin B (Titiz et al. 2006; Barakat 2003; Burguieres et al. 2007; Chen and Xiong 2005). All these have demonstrated the capacity to increase resistance to abiotic stress and reduce levels of oxidative stress.

Vitamin B_{12} is produced by bacteria and some unicellular organisms living in the small intestines of animals and is not found in vascular plants. The active form of vitamin B_{12} is cobalamin or cyanocobalamin. Recently, the B group of vitamins has also been shown to function as a potent antioxidant, equivalent to vitamins C and E, and is particularly active in quenching singlet oxygen (Burguieres et al. 2007; Chen and Xiong 2005; Barakat 2003; Heikal et al. 2000; Azooz. 2009). These groups of vitamins are also involved in inducing or suppressing the expression of certain genes and could potentially serve as an indirect stimulator of proline biosynthesis that has been linked to the pentose phosphate pathway that is activated during stress (Shetty and Wahlqvist 2004; Shetty 1997). With relation to improving plant tolerance through stimulation of phenolic synthesis, it has been suggested that exogenously applied phenolic antioxidants are perhaps able to stimulate endogenous phenolic content in plants (Randhir and Shetty 2003; Randhir et al. 2002). This stimulation involves the concurrent activation of antioxidant enzyme metabolites that are dependent upon reductant (NADPH) produced as a product of carbon flux through the pentose phosphate pathway. It has been estimated that exogenous antioxidants that stimulate the host antioxidant enzyme response in plants may also stimulate flux through the pentose phosphate pathway, potentially leading to increased endogenous phenolic synthesis (Shetty and Wahlqvist 2004). In addition, the B group vitamins (such as B_1 , folic acid; B_2 , Riboflavin and B_6 , pyridoxine) could potentially serve as an indirect stimulator of proline biosynthesis that has been linked to pentose phosphate pathway activity during stress (Burguieres et al. 2007; Chen and Xiong 2005). Altogether, as far as we know, there are few reports assessing the effects of exogenous application of the B group vitamins (specifically vitamin B_{12}) on photosynthesis and the activity of antioxidant enzymes of common bean under salinity stress condition. Therefore, this experiment investigated the effect of vitamin B_{12} priming on alleviating oxidative stress induced by NaCl and the effects of these treatments on growth and biochemical responses such as activity of antioxidant enzymes, proteins contents, photosynthetic pigments, malondialdehyde (MDA) and proline accumulation in shoots and roots of bean plants.

MATERIALS AND METHODS

Plant growth condition and treatment

Common bean seeds were surface sterilized using sodium hypochlorite for 5 min and 96% ethanol for 30 s, and then washed several times with distilled water. The seeds were primed in various concentrations of vitamin B_{12} (0, 11, and 22 μ M) in a flask for 8 h. Finally, ten seeds of each group were sown in plastic pots (20 cm diameter) filled with 5 kg autoclaved soil mixture composed of clay, sand and farmyard manure (1:1:1, w/w/w) at the depth of 2-3 cm (total 18 pots). All pots were placed into a growth chamber property of Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran under controlled conditions (photoperiod: 16 h light and 8 h dark, temperature: 24/20 °C (day/night) and light intensity: 80,000 lux-metal halide source). The pots were watered to field capacity every other

day. After two weeks plants were thinned to five plants per pot. Four weeks after sowing (at the V6 stage), salt stress treatment was initiated and nine of the pots were subjected to salinity stress by NaCl solution (75 mM) daily over the period of 14 days under conditions as indicated above. The remaining plants, as controls were irrigated with tap water. The treatments were arranged in a complete randomized design and each treatment was replicated three times. Growth parameters and biochemical attributes were determined immediately at the end of the period. All the measurements were performed 50 days after sowing.

Sampling

For growth parameters, fourteen days after salinity stress, plants were uprooted and separated into roots and shoots, the roots were gently washed with water to remove soil. Afterwards they were dried at 105°C in a hot air oven for 72 h to calculate dry weight. To determine biochemical attributes, the roots and fourth leaves of each plant were removed. Samples were then washed, frozen in liquid nitrogen and then stored at -80° C pending biochemical analysis. Measurements were taken for chlorophyll, carotenoid and MDA contents, activities of antioxidant enzymes (SOD, POX and CAT) as well as proline and, protein contents in leaves and roots.

Assay of stress responses factor

Enzyme activity assay

Tissue samples (0.2 g) were homogenized in a mortar and pestle with 3 ml ice-cold extraction buffer (50 mM potassium phosphate, pH 7). The homogenate was centrifuged at 18,000 g for 30 min at 4°C and then the supernatant was filtered through filter paper. The supernatant fraction was used as a crude extract for assays of enzyme activity and protein content. All operations were carried out at 4°C. Enzyme activities were measured at 25°C using a spectrophotometer model Varian Cary Win UV 6000i, Australia.

Superoxide dismutase (EC 1.15.1.1) activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) according to the method cited by Giannopolitis and Ries (1977). The reaction mixture contained 200 µl 1 µM riboflavin, 200 µl 12 mM L methionine, 200 µl 50 mM Na₂CO₃ (pH 10.2) and 200 µl 75 µM NBT in 2 ml 50 mM potassium phosphate buffer (pH 7), with 200 µl crude enzyme extract in a final volume of 3 ml (with some modification). Glass test tubes containing the mixture were illuminated with a fluorescent lamp (120 W); identical tubes that were not illuminated served as blanks. After illumination for 15 min, absorbance was measured at 560 nm. A blank was run in the same way but without the enzyme. One unit of SOD was defined as the amount of enzyme activity that was able to inhibit photo reduction of NBT by 50% to purple formazan. The SOD activity of the extract was expressed as SOD units per milligram of protein.

Catalase (EC 1.11.1.6) activity was estimated by the method cited by Cakmak and Horst (1991). The reaction mixture contained 100 µl crude enzyme extract, 500 µl 10 mM H₂O₂ and 1.9 ml 50 mM potassium phosphate buffer. The decrease in absorbance at 240 nm was recorded for 1 min. Catalase activity of the extract was expressed as CAT units per milligram of protein.

Peroxidase (EC 1.11.1.7) activity was determined by the oxidation of guaiacol in the presence of H₂O₂. The increase in absorbance at 470 nm was recorded for 1 min (Ghanati et al. 2002). The reaction mixture contained 100 µl crude enzyme, 500 µl 5 mM H₂O₂, 500 µl guaiacol 28 mM and 1.9 ml 50 mM potassium phosphate buffer (pH 7). Peroxidase activity of the extract was expressed as POX units per milligram protein. Total protein content was estimated by the method cited by Bradford (1976), using bovine serum albumin as the standard.

Chlorophyll and carotenoid assay

Extraction and determination of chlorophyll and carotenoid was performed according to the method of Arnon (1949). Chlorophyll and carotenoid were extracted in 80% acetone from the leaf samples. Extracts were filtered and contents of, chlorophyll a, b, total chlorophyll and carotenoid were determined by spectrophotometry at 645, 663 and 480 nm. Chlorophyll and carotenoid contents were expressed in mg g⁻¹ fresh weight according to following equation 1.

$$\text{Chlorophyll 'a' (mg.g}^{-1}\text{)} = (0.0127) \times (A.663) - (0.00269) \times (A.645)$$

$$\text{Chlorophyll 'b' (mg.g}^{-1}\text{)} = (0.0229) \times (A.645) - (0.00468) \times (A.663)$$

$$\text{Total chlorophyll (mg.g}^{-1}\text{)} = (0.0202) \times (A.645) + (0.00802) \times (A.663)$$

$$\text{Carotenoid (mg.g}^{-1}\text{)} = A.480 + (0.114 \times A.663 - 0.638 \times A.645)$$

Equation 1

Malondialdehyde assay

The level of lipid peroxidation was analyzed in terms of malondialdehyde (MDA) contents reacting to thiobarbituric acid (TBA) reactive substance using the method cited by De Vos et al. (1991). Samples were homogenized in an aqueous solution of TBA (10 % w/v) and then a 1 ml aliquot of an appropriately diluted sample was added to a test tube with an equal volume of thiobarbituric acid (TCA) solution containing 25% (w/v) TCA then mixtures were heated at boiling water (95 °C) for 25 min. The amount of MDA was determined from the absorbance of the supernatant at 532 and 600 nm. MDA content was determined using the extinction coefficient of MDA ($\epsilon=155 \mu\text{M}^{-1}\text{cm}^{-1}$).

Proline assay

Proline content was determined according to the method cited by Bates et al. (1973), which was modified as follows. Samples of both root and leaves (0.2 g) were homogenized in a mortar and pestle with 3 ml sulphosalicylic acid (3% w/v) and then the homogenate was centrifuged at 18,000 g for 15 min. Then 2 ml of the supernatant was transferred to a fresh test tube in which 2 ml glacial acetic acid and 2 ml freshly prepared acid ninhydrin solution (3% w/v ninhydrin in 60% v/v 6 M phosphoric acid) were added. Tubes were incubated in a water bath for 1 h at 100 °C. After cooling the tubes in ice, 4 ml of toluene was added and mixed on a vortex mixer and the upper (toluene) phase decanted in to a glass cuvette and its absorbance was measured at 520 nm in a spectrophotometer. The proline content was calculated from a proline standard curve and was expressed as mg g⁻¹ per fresh weight.

Statistical analysis

All data were analyzed using SAS software (SAS Institute Inc. 2002). Each treatment was analyzed in three replications. Analyses of variance (ANOVA) were performed to test for difference between salinity, vitamin B₁₂ and their interaction. When ANOVA showed significant effects, Duncan’s multiple range tests were applied to compare the means at ($P \leq 0.05$) (Steel and Torrie 1980).

RESULTS AND DISCUSSION

Results

The analysis of variance revealed that the effect of salinity stress was significant on all studied traits except for shoot length (Table 1). In addition, seed priming with vitamin significantly affect growth parameters and biochemical attributes, however shoot length, carotenoid content, root protein content, root malondialdehyde content and superoxide dismutase activity either in roots or shoots were not affected (Table 1). Interaction between salinity and vitamin was significant on leaf area, total chlorophyll content, leaf protein content, leaf and root proline content as well as leaf and root malondialdehyde content, catalase activity in leaves, peroxidase activity in roots and shoots and finally superoxide dismutase activity in the roots (Table 1).

Table 1: Analysis of variance on growth parameters and biochemical attributes

| SOV | d.f | Shoot length | Root length | Shoot dry weight | Root dry weight | Leaf area | Total chlorophyll | Carotenoids | Protein (leaf) | Protein (root) | Proline (leaf) |
|-------------|-----|--------------|-------------|------------------|-----------------|-----------|-------------------|-------------|----------------|----------------|----------------|
| Salinity | 1 | ns | * | ** | ** | ** | ** | * | ** | ** | ** |
| Vitamin | 2 | ns | ** | * | ** | ** | ** | ns | ** | ns | ** |
| Interaction | 2 | ns | ns | ns | ns | ** | ** | ns | ** | ns | ** |
| Error | 12 | 4.84 | 6.53 | 0.05 | 0.01 | 640.16 | 0.00 | 0.00 | 0.02 | 0.03 | 0.00 |
| C.V (%) | | 16.86 | 13.69 | 15.50 | 16.41 | 10.83 | 3.96 | 6.14 | 3.25 | 5.35 | 8.81 |

*, ** and ns significant at 0.05, 0.01 probability level and no significant, respectively

Table 1. Continued

| SOV | d. f | Proline (root) | Malondialdehyde (leaf) | Malondialdehyde (root) | Catalase (leaf) | Catalase (root) | Peroxidase (leaf) | Peroxidase (root) | Superoxide dismutase (leaf) | Superoxide dismutase (root) |
|-------------|------|----------------|------------------------|------------------------|-----------------|-----------------|-------------------|-------------------|-----------------------------|-----------------------------|
| Salinity | 1 | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| Vitamin | 2 | ** | ** | ns | ** | ** | ** | ** | ns | ns |
| Interaction | 2 | ** | ** | ** | * | ns | ** | ** | ns | ** |
| Error | 12 | 0.11 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.01 |
| C.V (%) | | 13.98 | 7.02 | 8.10 | 4.20 | 9.75 | 8.10 | 8.37 | 4.44 | 7.84 |

*, ** and ns significant at 0.05, 0.01 probability level and no significant, respectively

Comparison of means indicated that salinity stress significantly reduced root length, shoot and root dry weight and root protein content (Table 2). By contrast, carotenoids content, catalase activity in roots and superoxide dismutase activity in leaves increased due to salinity stress (Table 2). On the other hand, although there was no significant difference between vitamin concentrations (11 and 22 μM), seed priming with vitamin B₁₂ increased root length, shoot and root dry weight and catalase activity in roots compared with control treatment (Table 3).

Table 2. Main effect of salinity on growth parameters and biochemical attributes

| Salinity | Root length | Shoot weight | dry weight | Root weight | dry weight | Carotenoids | Protein (root) | Catalase (root) | Superoxide (leaf) | dismutase |
|----------|-------------|--------------|------------|-------------|------------|-------------|----------------|-----------------|-------------------|-----------|
| 0 mM | 20.16a | 2.28a | | 1.03a | | 0.32b | 3.63a | 0.03b | 0.96b | |
| 75 mM | 17.14b | 0.61b | | 0.42b | | 0.68a | 3.28b | 0.05a | 1.27a | |

Values within the each column and followed by the same letter are not different at P < 0.05 by an ANOVA protected Duncan's Multiple Range Test

Table 3. Main effect of vitamin on growth parameters and biochemical attributes

| Vitamin | Root length | Shoot dry weight | Root dry weight | Catalase (root) |
|------------------|-------------|------------------|-----------------|-----------------|
| 0 μM | 13.46b | 1.31b | 0.56b | 0.03b |
| 11 μM | 21.66a | 1.36b | 0.76a | 0.051a |
| 22 μM | 20.83a | 1.66a | 0.85a | 0.05a |

Values within the each column and followed by the same letter are not different at P < 0.05 by an ANOVA protected Duncan's Multiple Range Test

The results indicated that under non-stress conditions seed priming with vitamin B₁₂ (11 or 12 μM) would increase leaf area in common bean. Similar results were observed when plants were irrigated with saline water. In other words, seed priming with 22 μM vitamin B₁₂ increased leaf area in stressed plants compared with control treatment (Table 4).

Table 4. Interaction between salinity and vitamin on growth parameters and some biochemical attributes

| Salinity | Vitamin | Leaf area | Total chlorophyll | Protein (leaf) | Proline (leaf) | Proline (root) | Malondialdehyde (leaf) | Malondialdehyde (root) | Catalase (leaf) | Peroxidase (leaf) | Peroxidase (root) | Superoxide dismutase (root) |
|----------|------------------|-----------|-------------------|----------------|----------------|----------------|------------------------|------------------------|-----------------|-------------------|-------------------|-----------------------------|
| 0 mM | 0 μM | 284.00b | 2.77b | 5.50a | 0.63c | 1.45c | 0.60d | 0.40c | 0.33d | 0.42d | 1.50c | 1.32c |
| | 11 μM | 440.00a | 2.84b | 5.56a | 0.65c | 1.90c | 0.63cd | 0.42c | 0.46c | 0.49d | 1.52c | 1.17c |
| | 22 μM | 458.00a | 3.06a | 5.58a | 0.72c | 1.63c | 0.70cd | 0.47c | 0.50c | 0.47d | 1.46c | 1.13c |
| 75 mM | 0 μM | 52.00d | 1.19d | 3.34c | 1.04b | 2.63b | 1.69a | 0.73a | 0.74b | 0.96c | 2.74b | 1.64b |
| | 11 μM | 46.00d | 2.47c | 4.45b | 1.15b | 2.87b | 1.12b | 0.74a | 0.79a | 1.37b | 3.58a | 1.74b |
| | 22 μM | 121.00c | 2.49c | 5.30a | 2.17a | 3.96a | 0.74c | 0.60b | 0.82a | 1.51a | 3.74a | 2.09a |

Values within the each column and followed by the same letter are not different at P < 0.05 by an ANOVA protected Duncan's Multiple Range Test

The highest chlorophyll content was observed in plants which were grown from the primed seeds in 22 μM vitamin B₁₂ solution and irrigated with fresh water (Table 4). Seed soaking with 11 and 22 μM vitamin B₁₂ increased chlorophyll content in salt stressed plants compared with control treatment, however there was no significant difference between concentrations (Table 4).

Leaf protein content was not affected by vitamin treatments under normal condition; however, increase in vitamin concentration was parallel with increase in leaf protein content in salt stressed plants (Table 4).

Significant accumulation of proline was detected in both leaves and roots once bean seeds were soaked in vitamin solution. The highest proline content was obtained from plants grown from the primed seeds in 22 μM vitamin B₁₂ solution and irrigated with saline water (Table 4).

According to the results, malondialdehyde content, as an important index of lipid peroxidation, decreased in leaves and roots on account of vitamin B₁₂ in salt stressed plants (Table 4). Under normal condition, malondialdehyde content was not affected by vitamin B₁₂, in other words, there was no significant difference between treatments in terms of malondialdehyde content (Table 4).

The results demonstrated that catalase activity in leaves was promoted due to vitamin B₁₂ application in both normal and salt stress conditions (Table 4). It should be noted that there was no significant difference between vitamin concentrations (Table 4).

Peroxidase activity in leaves and roots increased with increasing vitamin concentration in plants grown under salt stress conditions (Table 4). On the contrary, under normal conditions, there was no significant difference between treatments (Table 4). Similar results were found in case of superoxide dismutase activity in bean roots (Table 4).

Discussion

Seed priming is a common technique involving the direct application of plant growth regulators to seeds that is known to improve stress tolerance and increase overall plant growth (Taylor and Harman 1990; Burguieres et al. 2007). In this study, salt stress caused a significant reduction in most growth parameters. It might be attributed to the inhibitory effect of salinity on cell division and cell expansion by ABA which was stimulated by salinity (Nabil and Coudret 1995) or to the osmotic effect of salinity which leads to disturbance in the water balance of stressed plants resulting in a retarded growth rate (Chaparzadeh 2004; Hojati 2011). The decrease in shoot dry matter by salinity could be ascribed to a decrease in photosynthesis output as indicated by a decrease in photosynthesis pigments in salt stressed plants. Application of vitamin B₁₂ by seed priming had an ameliorative effect as well as a growth promoting effect under both non saline and saline conditions and caused significant increase in growth parameters in salt stressed plants. By contrast, vitamin B₁₂ could partially mitigate or completely alleviate the adverse effects of salinity. Thus, we can conclude that vitamin B₁₂ improves water uptake efficiency and protects chlorophyll and photosynthesis. Moreover, this vitamin may act as a growth inducer, which can play a role in mitigating the inhibitory effects of sodium chloride on processes related to growth through enhancing cell enlargement or stimulating cell division (Barakat 2003). These results can be related to earlier studies which observed that exogenous application of vitamins could promote and counteract stress induced factors on growth inhibition in a range of crop species (Shi et al. 2002; El Bassiony 2005; Dolatabadian 2008). The B group vitamins are known to promote root growth in various plant species (Bonner and Bonner 1948; Ansari et al. 1990) and the beneficial effect of vitamin application has also been reported for various crops in other researches (Oertli, 1987; Zavenyagina and Bukin, 1969, Samiullah et al., 1988). In this work, plants raised from vitamin B₁₂ treated seeds, showed increased root growth, which could have contributed to increased nutrients uptake. This hypothesis is supported in other studies. For example high N and P contents have been reported in *Mentha piperita* (Dimitrova-Russeva and Lilova, 1969) and *Vigna radiata* (Gopala Rao and Raghava Reddy 1985) treated with the B group vitamins.

Salinity is known to cause damage to cellular components (such as oxidized DNA, degraded oxidized proteins, and oxidized lipids) deteriorating the health of plants (Ghosh et al. 2011). The enzymatic antioxidant apparatus is an important biochemical system to intercept and neutralize the effects of destructive oxygen free radicals produced by cellular metabolism during stresses. Similarly, increased SOD, POX and CAT activity have been correlated to salinity tolerance (Agarwal and Shaheen 2007). Additionally, vitamins are known as positive participate in antioxidant systems. Antioxidant enzymes plays important roles such as scavenging of ROS and modulation of gene expression (Osmani et al. 1999; Daub and Ehrenshaft 2000; Schneider et al. 2000; Wrenger et al. 2005; Huq et al. 2007; Spinneker et al. 2007). Thereupon, stimulation of antioxidant enzyme activity can be regarded an important technique in cellular defense system against free radicals and this treatment effectively initiates physiological processes in plants' antioxidant systems. Based on these results, treated seed with vitamin B could make available free phenolic compounds for free radical scavenging that would explain the observed rise in antioxidant activity that vitamin treatment has a possible protective role against ROS, acting as an antioxidant system and scavenging free radicals. Enhanced SOD activity could potentially increase oxidative stress due to increased production of H₂O₂. This clearly indicates that any H₂O₂ that was formed from SOD activity was used by catalase and peroxidase. If the activity of POX and CAT is not matched by an increase in SOD activity it could potentially increase oxidative stress due to increased production of H₂O₂ (Babitha et al. 2002). Changes in CAT activity due to salt stress have been reported by Dolatabadian and co-workers (2008) as well as in this study.

The results of the current study demonstrated that treated plants with saline water and vitamin B₁₂ had higher average for antioxidant activity compared with control plants. It is reasonable to assume that exogenous application of vitamin B₁₂ may be able to induce endogenous phenolic compounds in bean plants. This increase in phenolic compounds was consistent with increase in antioxidant response of seedlings to stress treatments (Burguieres et al. 2007). Vitamin B₁₂ may lead to an increase in phenolic compounds and provide the energy required to operate antioxidant activity for protecting the plants against the oxidative stress induced by salinity. On the basis of all aforementioned reports, as well as data recorded in this study, it can be hypothesized that vitamin B₁₂ could potentially act as an indirect stimulator of antioxidant enzyme activity and an increase in antioxidative activity in plants

subjected to salinity shows that vitamin application can ameliorate the reactive oxygen species. Earlier studies have shown that exogenous application of vitamin B promotes phenolic biosynthesis thus quenching any stress induced growth inhibition due to oxidative stress. In a short term experiment, folic acid treatment increased phenolic compounds in pea tissue and the highest total phenolic compounds were found in pea plants germinated from primed seeds with 50 μM vitamin folic acid (Burguieres et al. 2007).

Furthermore, plants treated with the B group vitamins demonstrated the induction of new protein bands and indicated that B vitamins stimulate the effect of the protein component of plants. It has been reported that proteins in plants increase tolerance to environmental stresses (Hoyos and Zhang 2000; Patharkar and Cushman 2000). Besides, many of these proteins protect a cell against the adverse effects of stress conditions. Salinity treatment was found to induce the disappearance of a protein band and this band that had disappeared in salinized plants returned when those seedlings were treated with vitamin B6 (Barakat 2003). The protein content of bean plants increased by seed priming with a vitamin B₁₂ application and this effect was more pronounced at 22 μM vitamin B₁₂ under saline conditions, whereas under non saline conditions there was no effect of vitamin B₁₂ application on protein of root and leaf tissue. Maybe, low level of protein in treated plants with NaCl related to oxidative damage that was mediated by the degradation of proteins (Noctor and foye 1998).

Moreover, changes in protein synthesis under salt stress might be due to changes in the efficiency of mRNA translation (Hala and El Bassiouny 2005). The expression of proteins during salt stress is related to the adaptation process of plants to salinity (Dell'Aquila and Spada, 1992 and 1993). Barakat et al. (2003) reported that those wheat cultivars that are salt tolerant appear to be the least affected by salinity in terms of growth and protein patterns. Treatments of seeds with this vitamin induced the appearance of some new protein bands in seedlings of the three wheat cultivars. The induction of new bands indicates that the B group vitamins have a profound effect on changes in the protein component of plants. Therefore, we suggest that vitamin B₁₂, by activation of the antioxidant system and neutralization of free radicals prevents the destruction of protein that serves to increase leaf protein so, an increase in protein content in plants treated with vitamin B₁₂ is acceptable.

Chlorophyll content in stressed plants decreased which might be attributed to toxic action of NaCl on pigments and membrane permeability that damage the chloroplast membrane system and photosynthetic reaction centers. In such a situation, chlorophyll needs to be degraded quickly to avoid cellular damage with a down regulation of its biosynthetic pathways and hydrolysis of chlorophyll by enzymes such as chlorophyllase. Inhibition of chlorophyll biosynthesis has been reported in other plants under salt stress (Ghosh 2011, Dolatabadian et al. 2008). Application of vitamin B₁₂ could alleviate the inhibitory effect of salinity stress on photosynthesis pigments. The results of this study are in agreement with Dolatabadian et al. (2008) finding that treating canola with ascorbic acid (vitamin C) mitigated the detrimental effect of salinity on photosynthesis pigments. Similarly, research by Hala and El Bassiouny (2005) reported an increase in chlorophyll content in response to nicotinamide treatment. Application of vitamin B₁₂ in most cases, did not only alleviate the inhibitory effect of salinity stress on the biosynthesis of photosynthetic pigments, but also induced a significant stimulatory effect greater than that observed in corresponding controls, a response which may contribute directly to the effectiveness on photosynthetic apparatus and completely alleviate the inhibitory effect of salt stress. It seems that application of vitamin B₁₂ activated the antioxidant system and alleviated the adverse effects of salt stress. However, the same positive effect of 100 ppm (73.3 μM) pyridoxine was observed on the growth of two broad bean lines when seeds were soaked in vitamin B6 solution in the presence of NaCl and led to increased photosynthesis pigments of beans plants (Heikal et al. 2000). In view of these reports, as well as data reported here, it is evident that plant response depends on specific concentrations of the B group of vitamins. In addition, vitamin B₁₂ may interfere with the protection of chloroplasts and their membrane against NaCl toxicity, thus maintaining their integrity.

The lipid peroxidation level as indicated by MDA accumulation, increased significantly under salt stress, this suggested that oxidative damage due to salinization is not prevented by antioxidant enzymes in this study, while the content of MDA significantly decreased as a result of pretreatment of vitamin B₁₂. These findings are in agreement with Azooz (2009) observing that exogenous application of vitamin B₂ promoted the antioxidant system and counteracted the lipid peroxidation due to abiotic stresses. These decreases might be related to the induction of antioxidant responses, thus it could be suggested that treatment with vitamin might increase enzymes activity which made the plant resistant to adverse effect of salinity and led to a decline in the level of MDA.

Our results showed notable changes in proline concentration. Salinity led to increase in proline content compared with control treatment. It has been shown that application of vitamin B₂ correlated with an increase in proline content in seedling under salinity stress (Azooz 2009). Furthermore, these results are in agreement with Burguieres et al. (2007) who found that treated plants with vitamin B₁ inhibited adverse effect of high concentrations of osmotic stress by increasing proline. This suggests that it contributed to osmotic adjustment and cell water status adjustment, so it

can play a key role in alleviating salinity stress. The increasing proline content in plants treated with vitamin B₁₂ indicates that stimulation of pentose phosphate pathway may be linked to proline synthesis and phenolic enhancement during salt stress so plants employed proline as a form of osmoprotection (Shetty 1997). From these studies, it was also clear that proline accumulation in response to salinity treatment were correlated to phenolic content and antioxidant activity as reflected by the free radical scavenging assay and the antioxidant enzyme response.

CONCLUSION

The results of this work showed that an application of vitamin B₁₂ can increase the survival capacity of bean plants under salinity condition and is this associated with the response that stimulates the activity of free radical scavenging; and this enhanced antioxidant response probably occurred through the stimulation of phenolic linked antioxidant. Therefore it can be recommended that priming seeds with vitamin B₁₂ has a significant biochemical implication through improved antioxidant response and can reduce the harmful effects of ROS to improve plant resistance.

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