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The changes induced in the physiological, biochemical and anatomical characteristics of *Vicia faba* by the exogenous application of proline under seawater stress

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ABSTRACT

The depletion of fresh water resources leads to the utilisation of various alternative sources of water, such as seawater. In this regard, the foliar application of proline is one of the alternative shotgun approaches to increase plant stress tolerance. A pot experiment was conducted in the wire house of the National Research Centre, Dokki, Cairo, Egypt, during the winter season of 2010–2011. The experiment consisted of three concentrations of proline (0, 25 and 50 mM) and two concentrations of diluted seawater (3.13 and 6.25 dS m^{-1}), whereas control plants were irrigated with tap water (0.23 dS m^{-1}). Diluted seawater caused significant reductions in growth parameters, photosynthetic pigments, some mineral contents (P, K, Ca^{+2}), the K^+:Na^+ ratio and the level of total carbohydrates. In contrast, N, Na^+, and Cl^- contents, osmoprotectants (soluble carbohydrates, total phenolic concentrations, free amino acids, proline), and activities of antioxidant enzymes (peroxidase and polyphenol oxidase) significantly increased with an increasing salinity level compared with control plants. The foliar application of 25 mM proline caused significant increases in growth parameters, photosynthetic pigments, N, P, K^+, and Ca^{+2} %, the K^+:Na^+ ratio, total carbohydrates, and soluble carbohydrates, accompanied by significant decreases in Na^+, Cl^-, phenolic contents, free amino acids, proline, and the activities of antioxidant enzymes compared with the control. In addition, 25 mM proline minimised the deleterious effect of salinity on the anatomical structure of the faba bean stem and leaf. The proline treatment at 50 mM was as essentially toxic to faba bean plants as to that of salinity stress. This toxicity was apparent by the reduction of growth parameters, photosynthetic pigments, N, P, K^+, and Ca^{+2}, K^+:Na^+ ratio and significant increases in Na^+ and Cl^- concentrations. Therefore, the exogenous application of proline at a concentration of 25 mM partially alleviated the toxicity of diluted seawater on faba bean plants, whereas the 50 mM proline treatment was toxic.

1. Introduction

Abiotic stresses are considered the most important factors for yield reductions in agricultural crops. It is estimated that yield losses in agricultural crops due to different abiotic stresses include 15% due to low temperature, 17% due to drought, 20% due to salinity, 40% due to high temperature, and 8% due to other environmental factors (Ashraf et al., 2008).

Water is an essential factor during the entire life of plant growth, from seed germination to the final growth stage. With increasing aridity, in conjunction with a fast increase in human population, water will become a scarce commodity soon, particularly in third-world countries. Hence, the depletion of fresh water resources has led to the utilisation of various alternative sources of water. The main problem in using different sources of water arises from salinity hazards.

Salinity stress limits plant growth by adversely affecting various physiological and biochemical processes, such as photosynthesis, antioxidant phenomena, nitrogen metabolism, ion homeostasis, and osmolyte accumulation (Ashraf, 2004). Thus, salinity exerts its undesirable effects through osmotic inhibition and ionic toxicity and by disturbing the uptake and translocation of nutritional ions (Misra and Dwivedi, 2004).

The rate of plant growth depends on several important events, such as cell division, cell enlargement and cell differentiation, as well as genetic, morphological, physiological, and ecological events and their complex interactions, which are severely affected by abiotic stress (Taize and Zeiger, 2006). When plants are exposed to harsh conditions

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(e.g., salinity stress), plants exhibit certain adaptive features, which may be morphological, anatomical, physiological, or biochemical in nature to minimise the deleterious effects of unfavourable environmental conditions (Sakamoto and Murata, 2002) and to help plants to sustain and thrive under stress conditions. In this regard, plants perceive stress through their roots and send signals to change their metabolism for the activation/synthesis of defence mechanisms in different parts of the plant (Siopongco et al., 2008).

Plants must maintain their internal water potential below that of the soil to maintain turgor and water uptake for growth. This maintenance requires an increase in osmotica, either by the uptake of soil solutes or by the synthesis of compatible solutes (Tester and Davenport, 2003). These compatible organic solutes are of low molecular weight and are highly soluble compounds that are usually nontoxic at high cellular concentrations and that do not interfere with the plant's metabolism, even at molar concentrations (Alonso et al., 2001). The accumulation of such compatible osmolytes allows additional water to be taken up from the environment, thus buffering the immediate effect of water shortage within the plant (Nanjo et al., 1999). Furthermore, because some of these solutes also protect cellular components from dehydration injury during stress, these solutes are commonly called osmoprotectants. The osmoprotectants synthesised in plants in response to adverse environmental conditions include a variety of proteins and amino acids (such as proline) and carbohydrates (Ashraf, 2010). Normally, these osmoprotectants protect plants from different abiotic stresses in several ways, including their role in adjusting cellular osmosis, scavenging reactive oxygen species (ROS), protecting cellular membranes, and stabilising proteins/enzymes and enzyme activities (Gill and Tuteja, 2010).

The generation of reactive oxygen species (ROS) is a common phenomenon in plants under normal growth conditions. However, their production increases under adverse environmental conditions, including salinity. The production of these ROS under stress conditions is highly dangerous because ROS impair the normal functions of cells due to their oxidative reaction with membrane proteins, lipids, and deoxyribonucleic acid, as well as the inactivation of enzymes (Ashraf, 2009). The detoxification of ROS in plant cells can be categorised as enzymatic and non-enzymatic in almost all plants. The non-enzymatic antioxidants include ascorbic acid, tocopherols, flavonoids, phenolics and carotenoids. The important anti-oxidant enzymes include peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (APX) (Ashraf, 2010; Ali and Ashraf, 2011).

The use of osmoprotectants as seed priming or as a foliar spray can be an economically viable strategy to enhance stress tolerance under adverse environmental conditions (Ali et al., 2007; Ashraf and Foolad, 2007; Ali and Ashraf, 2011). Proline is one of the compatible osmolytes induced by salinity stress in plants. Several functions are proposed for the accumulation of proline in tissues exposed to salinity stress: C and N reserves for growth after stress relief (Hayashi et al., 2000), the stabilisation of proteins and membranes (Mansour, 1998), the protection of macromolecules from denaturation (Hamilton and Heckathorn, 2001), osmoprotection (Kishor et al., 1995), free radical scavenging (Chen and Dickman, 2005), anti-oxidation (Hoque et al., 2007), and as a readily available source of energy and reducing power (Stewart et al., 1974).

The exogenous application of proline is known to induce abiotic stress tolerance in various plant species (Ali et al., 2007, 2008; Ashraf and Foolad, 2007; Abdelhamid et al., 2013). Ali et al. (2007) reported that the exogenous application of 30 mM proline at all growth stages of maize was found to be most effective in inducing drought tolerance, enhancing the biomass production, and increasing the photosynthetic rate, stomatal conductance, and internal CO_2 concentration. In another study of maize, Ali et al. (2008) reported that the exogenous application of proline enhanced the nutrient uptake in roots and shoots under water deficit conditions and correlated the results with an enhanced plant transpiration rate. In contrast, the effect of proline is dependent on its concentration, as mentioned by Ashraf and Foolad (2007), because an excessive amount of free proline has negative or side effects on cell growth or on protein functions. The over-accumulation of intracellular proline significantly represses several genes involved in the synthesis of other amino acids or normal morphogenesis in *Arabidopsis* plants (Nanjo et al., 2003). Proline overaccumulation at a concentration as low as 100 mM suppresses the activity of the major chloroplastic enzyme ribulose 1,5-bis-phosphate carboxylase in higher plants (Sivakumar et al., 1998); therefore, intracellular proline must be present at an appropriate level to confer stress tolerance. The effectiveness of proline applied as a foliar spray depends on the type of species, plant developmental stage, time of application and on the concentration (Ashraf and Foolad, 2007). Therefore, it is necessary to determine the optimal concentrations of exogenously applied proline that can provide beneficial effects in economically important crop plants, such as *Vicia faba*, when exposed to abiotic stress.

The faba bean (*V. faba* L.) plant is one of the most important crops in Egypt due to its high nutritive value in terms of both energy and protein contents. Therefore, increasing faba bean production is one of the most important targets of agricultural policy in Egypt.

This study aimed to measure the potential effects of the exogenous application of proline on some physiological, biochemical and anatomical parameters at the vegetative growth stage of faba bean plants irrigated with diluted seawater.

2. Materials and methods

2.1. Plant materials and growth conditions

A pot experiment was conducted in a wire-house at the National Research Centre, Dokki, Cairo, Egypt (30°20′ N; 31°53′ E) from 6 December 2010 to 10 February 2011. During this period, daytime temperatures ranged from 14.5 to 30.2 °C, with an average of 23.2 ± 3.8 °C. Night temperatures ranged from 8.0 to 17.6 °C, with an average of 12.4 ± 1.8 °C. The daily relative humidity averaged 57.7 ± 9.6% and ranged from 38.1 to 78.7%.

Seeds of faba bean (*V. faba* L.; cv. Giza 843) were obtained from the Agricultural Research Centre, Ministry of Agriculture and Land Reclamation, Egypt. Healthy faba bean seeds (n = 10) were selected for uniformity by choosing those seeds of equal size and of identical colour. The selected seeds were washed in distilled water, sterilised in 1% (v/v) sodium hypochlorite for approx. 2 min, washed thoroughly again in distilled water, and left to dry at room temperature (25 °C) for approx. 1 h.

Ten uniform, air-dried faba bean seeds were sown along a centre row in each plastic pot (30 cm in diameter) at a depth of 30 mm, in approx. 7.0 kg of clay soil. To reduce compaction and to improve drainage, the soil was mixed with yellow sand in a proportion of 3:1 (v:v).

A granular commercial *Rhizobium leguminosarum* (obtained from the Biofertilizer Inoculum Production Unit, Department of Microbiology, Soils, Water and Environment Research Institute, Agricultural Research Centre, Giza, Egypt) was incorporated into the top 30 mm of soil in each pot with the seeds at the time of sowing. Granular ammonium sulphate [20.5 (w/w) % N] was applied at a rate of 40 kg N ha^{-1}, and single superphosphate [15% P_2O_5] was added at a rate of 60 kg P_2O_5 ha^{-1} to each pot. These N and P fertilisers were mixed into the soil in each pot immediately before sowing.

The experiment was arranged in a factorial arrangement, with three levels of seawater (S0, S1, or S2) and three levels of proline (P0, P1, or P2). Four replicates were used. To induce salt stress, seawater was dissolved in fresh water, and the plants were watered with an equal volume of 0.23, 3.13 and 6.25 dS m^{-1} 3 weeks after sowing (treatments S0, S1, and S2, respectively). Saline water was prepared by mixing fresh water (0.23 dS m^{-1}) with seawater (51.2 dS m^{-1}) to achieve salinity levels of 3.13 and 6.25 dS m^{-1}. Electrical conductivity (EC), pH,

and the concentration of cations and anions in the irrigation water and in the soil used in the pot experiment are shown in Table 1. The soil water capacity was estimated by saturating the soil in each pot with water and weighing the soil after the soil had drained for 48 h. The water capacity of the soil in each pot was 0.36 kg kg^{-1}. Soil water contents were maintained at approx. 90% of the pot water capacity. The level of soil moisture was controlled by weighing each pot, and any loss of water was supplemented daily.

Ten days after sowing (DAS), faba bean seedlings were thinned to four seedlings per pot and irrigated with equal volumes of tap water until 15 DAS. Starting from 16 DAS, plants were sprayed three times, with a 7 day interval between each spraying and with three levels of proline (i.e., 0, 25 or 50 mM), which were considered P0, P1 and P2, respectively. The control treatment was sprayed with distilled water only. Simultaneously, plants were irrigated with either tap water (0.23 dS m^{-1}) or differently diluted seawater (3.13 and 6.25 dS m^{-1}) for the remainder of the experiment.

At the end of the experiment, 65 DAS, the aboveground portion of each plant was carefully removed from the pot and separated into leaves and stems. Two fresh leaves per plant were washed with distilled water to remove any surface dust and used to determine the concentration of photosynthetic pigments and the activities of two anti-oxidant enzymes [polyphenol-oxidase (PPO) and peroxidase (POX)]. Plant organs (leaves, stems) were oven-dried for 72 h at 70 °C, and their dry weights (DWs) were recorded. The dried leaves were ground into a powder and kept in a desiccator to determine their concentrations of phenolic compounds, total free amino acids, proline, total soluble carbohydrates (TSCs), total carbohydrates (TCs), total nitrogen (N), phosphorus (P), potassium (K^+), calcium (Ca^{2+}), sodium (Na^+), and chloride (Cl^-).

2.2. Biochemical studies

Chlorophyll *a*, chlorophyll *b* and carotenoids were determined using a spectrophotometry method according to Metzner et al. (1965). For enzyme extracts and assays, fresh leaf tissues were frozen and then ground in a 4 mL solution containing 50 mM phosphate buffer (pH 7.0) and 1% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at 15 000 ×*g* for 30 min at 4 °C, and the supernatant was collected and used for enzyme assays. Polyphenol-oxidase (PPO, EC 1.10.3.1) activity was assayed using the method of Soliva et al. (2001). Peroxidase (POD, EC 1.11.1.7) activity was assayed according to Kumar and Khan (1982). Proline was estimated according to Bates et al. (1973). Total free amino acids were determined according to Muting and Kaiser (1963). The total phenolic compound concentration was determined using the Folin–Ciocalteu method (Zhang and Wang, 2001). A calibration curve of gallic acid was prepared, and the results were expressed as mg GAE (gallic acid). The phenol–sulphuric acid method was used for the determination of total carbohydrates (TCs) (Smith et al., 1956). Total soluble carbohydrates (TSC) were determined according to Yemm and Willis (1954). The N, P, K^+, Ca^{2+}, Na^+, and Cl^- contents of oven-dried faba bean leaves (70 °C for 72 h) were determined according to Chapman and Pratt (1978).

2.3. Anatomical studies

A comparative microscopic examination was performed on plant material for treatments that showed a remarkable response. The tested material included the main stem at its median portion and the lamina of the first leaflet blade of the compound leaf, which developed on the median portion of the main stem of normal *V. faba* plants and those plants that were grown under salinity stress of 6.25 dS m^{-1}, as well as those plants affected by foliar spraying with 25 mM proline and those plants receiving the combined treatment of salinity and proline. Specimens were collected from plants aged 65 days, killed and fixed for at least 48 h. in F.A.A. (10 ml formalin, 5 ml glacial acetic acid and 85 ml ethyl alcohol 70%). The selected materials were washed in 50% ethyl alcohol, dehydrated in a normal butyl alcohol series, embedded in paraffin wax of melting point 56 °C, sectioned to a thickness of 20 μm, double stained with safranin-light green, cleared in xylene and mounted in Canada balsam (Nassar and El-Sahhar, 1998). Sections were examined to detect histological manifestations of noticeable responses resulting from the mentioned treatments and photomicrographed.

2.4. Statistical analysis of the data

All data were subjected to an analysis of variance (ANOVA) for a randomised complete block design, after testing for the homogeneity of error variances according to the procedure outlined by Gomez and Gomez (1984). Statistically significant differences between means were compared at $P \leq 0.05$ using Duncan's multiple range test.

3. Results and discussion

The changes induced in the physiological, biochemical and anatomical structures of faba bean by the exogenous application of proline (0, 25, 50 mM, considered P0, P1 and P2, respectively) under seawater salinity (0.23, 3.13 and 6.25 dS m^{-1} considered S0, S1 and S2, respectively) are shown in Tables 2, 3, 4, 5, 6, and 7 and in Figs. 1 and 2.

3.1. Changes in some growth parameters

Table 2 shows the effects of proline on plant height, leaf number per plant, plant fresh and dry weights of faba bean plants grown under seawater salinity. Both salinity levels (S1 and S2) caused significant reductions in plant height, leaf number per plant, and in fresh and dry weights of plants compared with control plants irrigated with tap water (S0 = 0.23 dS m^{-1}). The plant dry weight was significantly reduced by 19.4% and 37.7% due to irrigation with diluted sea water (S1 and S2), respectively, compared with control plants (S0). Cicek and Cakirlar (2002) mentioned that salinity stress restricts the ability of plant cells to take up water and some mineral nutrients dissolved in the culture medium and reduces plant growth. The negative impact of salinity on plant growth has also been reported in several other plant species, as reported by Nessim et al. (2008), Bekheta et al. (2009), Abdelhamid et al. (2010), Doğan (2011); Abd El-Samad et al. (2011), Hossain et al. (2011), and Abdelhamid et al. (2013). In contrast, the proline application at

Table 1
Electrical conductivity (EC), pH, and concentration of cations and anions of the irrigation water and the soil used in the pot experiment.

	EC (dS m^{-1})	pH	Cations (meq l^{-1})				Anions (meq l^{-1})			
			Ca^{2+}	Mg^{2+}	Na^+	K^+	HCO_3^-	CO_3^{2-}	SO_4^{2-}	Cl^-
Water										
Tap water	0.23	7.35	1.00	0.50	2.40	0.20	0.10	0.00	1.30	2.70
Sea water	51.2	7.76	43.20	15.12	454.57	1.51	6.05	0.00	76.36	432.00
Soil										
Sandy	0.14	8.11	2.60	2.40	1.31	0.21	1.13	0.00	4.22	0.70
Clay	1.40	7.59	5.60	1.90	5.90	0.37	1.50	0.00	6.77	5.50

Table 2
Effect of proline on plant height, leaf number per plant, plant fresh weight, and plant dry weight of faba bean grown under seawater salinity.

Treatment	Plant height (cm)	Leaf number per plant	FW (g plant^{-1})	DW (g plant^{-1})
Seawater				
S0	58.2a[†]	10.7a	17.22a	2.52a
S1	53.9b	9.7b	13.60b	2.03b
S2	48.4c	9.4b	10.57c	1.57c
Proline				
P0	52.4b	9.8b	13.69b	2.06b
P1	55.8a	10.8a	15.70a	2.32a
P2	52.3b	9.9b	12.00c	1.75c

Measurements were made 65 days after sowing (DAS). S0 (0.23 dS m^{-1}); S1 (3.13 dS m^{-1}); S2 (6.25 dS m^{-1}); P0 (0 mM proline); P1 (25 mM proline); P2 (50 mM proline).
[†]Mean values (n = 4) in the same column for each trait followed by the same lowercase letter are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

25 mM (P1) caused significant increases in all growth parameters under investigation compared with control plants (P0). The plant dry weight significantly increased by 12.6%. The increase in plant biomass due to the exogenous application of compatible osmolytes may be attributed to an active role of these osmolytes in plant osmotic adjustment, which, in turn, enhanced water uptake and improved the growth of plants. In various plant species grown under stress conditions, exogenously applied proline exerting positive effects on plant growth might be due to its role as a nutrient, as well as its role as an osmoprotectant (Ali et al., 2007; Yan et al., 2011). Special attention must be given to the highest proline level (50 mM) because this level caused significant reductions in fresh and dry weights of faba bean plants compared with control plants (P0), by 12.3 and 15.0%, respectively. Nanjo et al. (2003) mentioned that the exogenous proline application at high concentrations may be harmful and cause the retardation of plant growth.

3.2. Changes in photosynthetic pigments

The effects of proline on photosynthetic pigments (in mg g^{-1} FW) in the leaves of faba bean plants grown under seawater salinity are shown in Table 3. Of many different biochemical attributes, leaf chlorophyll is the most important feature that reflects the health status of plants and that is related to the plant water availability and to the nutrition level. The irrigation of faba bean plants with diluted sea water (3.13 or 6.25 dS m^{-1}) caused significant and gradual decreases in chlorophyll *a*, chlorophyll *b*, carotenoids and total photosynthetic pigments in leaves as the salinity level increased compared with those plants irrigated with tap water (S0). The total pigments decreased by 6.7% and 15.6% due to S1 and S2, respectively, compared with control plants. Deleterious effects of salinity stress on leaf chlorophyll contents have been reported in several crops, such as chickpea (Mafakheri et al., 2009), peanut (Hossain et al., 2011), soybean (Doğan, 2011), maize (Abd El-Samad et al., 2011; Awad et al., 2012), common bean (Abdelhamid et al., 2013), and faba bean (Bekheta et al., 2009; Abd El-Samad et al., 2011; Taie et al., 2013). The reduction in chlorophyll content in most stressed plants may be due to the disorganisation of thylakoid membranes, with more degradation than synthesis of chlorophyll via the formation of proteolytic enzymes, such as chlorophyllase, which is responsible for degrading chlorophyll, as well as damaging the photosynthetic apparatus (Rong-hua et al., 2006), reducing the plant photosynthetic rate (Mafakheri et al., 2009), and inhibiting accumulated ions (Jaleel et al., 2008). The application of 25 mM proline caused significant increases in all components of photosynthetic pigments compared with control plants (P0). The increase in chlorophyll *a* was 9.4%, in chlorophyll *b* was 6.3%, in carotenoids was 4.2% and in total pigment was 8.3%. Ali et al. (2007) mentioned that the exogenous application of 30 mM proline increased photosynthetic pigments of maize plants grown under water deficit stress. Despite the positive effects of applied proline for inducing tolerance, some reports concerning the inhibitory effects of proline are also available (Yamada et al., 2005; Ashraf and Foolad, 2007). However, the application of 50 mM proline caused significant decreases in all components of photosynthetic pigments. Sivakumar et al. (1998) demonstrated a negative effect of an exogenous application of 100 mM proline in the level of Rubisco activity in three plant species. Moreover, millimolar concentrations of proline have been demonstrated to cause disruptive effects on chloroplast and mitochondria membranes in *Arabidopsis* because of increased levels of reactive oxygen intermediates (Hare et al., 2002).

Table 3
Effect of proline on photosynthetic pigments (mg g^{-1} FW) in the leaves of faba bean grown under seawater salinity.

Treatments	Chlorophyll *a*	Chlorophyll *b*	Carotenoids	Total pigments
Seawater				
S0	1.426a[†]	0.497a	0.191a	2.113a
S1	1.353b	0.454b	0.165b	1.972b
S2	1.214c	0.428c	0.142c	1.784c
Proline				
P0	1.324b[1]	0.458b	0.166b	1.947b
P1	1.448a	0.487a	0.173a	2.108a
P2	1.221c	0.434c	0.158c	1.813c

Measurements were made 65 days after sowing (DAS). S0 (0.23 dS m^{-1}); S1 (3.13 dS m^{-1}); S2 (6.25 dS m^{-1}); P0 (0 mM proline); P1 (25 mM proline); P2 (50 mM proline).
[†]Mean values (n = 4) in the same column for each trait followed by the same lower case letter are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

3.3. Changes in mineral concentrations

Table 4 shows the effects of proline on N, P, K^+, Ca^{2+}, and Na^+ concentrations, which are expressed in % of DW, and Cl^- concentration, which is expressed in ppm in the leaves of faba bean plants grown under seawater salinity. The availability of nutrients in the soil decreases under stress conditions due to decreased solubility. The precipitation of salts alters physiological processes, including low absorption and the uptake of nutrients in plants grown under such conditions (Garg, 2003). Moreover, plant species and cultivars within a species differ in absorbing nutrients from soil and in transporting these nutrients to the root and then from the root to the shoot under stress conditions (Garg, 2003; Gunes et al., 2006; Ali et al., 2008). Table 4 shows that the salinity levels (S1 or S2) caused significant and gradual reductions

Table 4
Effect of proline on total nitrogen (N), phosphorus (P), potassium (K^+), calcium (Ca^{2+}), sodium (Na^+), expressed in % of DW and chloride (Cl^-) expressed in ppm in the leaves of faba bean grown under seawater salinity.

Treatments	N	P	K	Ca	Na	Cl (ppm)	K:Na ratio
	(%)						
Seawater							
S0	1.91c[†]	0.36a	2.39a	3.04a	0.12c	2.03c	19.92a
S1	2.12b	0.27b	2.03b	2.72b	0.20b	2.94b	10.15b
S2	2.42a	0.24c	1.79c	2.52c	0.29a	3.62a	6.17c
Proline							
P0	2.15b[1]	0.29b	2.06b	2.76b	0.20b	2.87b	10.30b
P1	2.24a	0.32a	2.20a	2.84a	0.17c	2.70c	12.94a
P2	2.06c	0.25c	1.95c	2.68c	0.24a	3.02a	8.12c

Measurements were made 65 days after sowing (DAS). S0 (0.23 dS m^{-1}); S1 (3.13 dS m^{-1}); S2 (6.25 dS m^{-1}); P0 (0 mM proline); P1 (25 mM proline); P2 (50 mM proline).
[†]Mean values (n = 4) in the same column for each trait followed by the same lowercase letter are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

in P, K^+, and Ca^{+2} concentrations, as well as in the K^+:Na^+ ratio, accompanied by gradual and significant increases in N, Na^+ and Cl^- concentrations compared with control plants (S0). Notably, the K^+:Na^+ ratio decreased by 49.0% and 68.9% due to S1 and S2, respectively. These results are similar to those results reported by Nessim et al. (2008), Doğan (2011), Abdelhamid et al. (2010), and Abd El-Samad et al. (2011) from different plant species. Garg (2003) observed that, under abiotic stress, an increase in N uptake occurs with concomitant decreases in P uptake. Generally, the decrease in nutrient uptake under stress conditions occurs due to a reduction in the transpiration rate (Ali et al., 2008). The effect of salinity on plant growth may be more related to the Na^+:K^+ ratio of the plant tissue than to the absolute Na^+ concentrations. Thus, the cultivars that have an ability to minimise this ratio may be more salt tolerant than those cultivars with lower K^+ concentrations (Lingle et al., 2000). A high sodium to potassium ratio due to the accumulation of high amounts of sodium ions inactivates enzymes and affects metabolic processes in plants (Booth and Beardall, 1991). According to Weimberg (1987), high levels of Na^+ inhibit K^+ uptake, thereby causing an increase in the Na^+:K^+ ratio. The deleterious effects of Na^+ seem to be related to the structural and functional integrity of membranes (Kurth et al., 1986). In contrast, 25 mM proline caused significant increases in the concentrations of N, P, K^+, and Ca^{+2}, as well as in the K^+:Na^+ ratio, compared with P0. However, 50 mM proline resulted in an opposite trend, where the K^+:Na^+ ratio decreased by 21.2%. Special attention must be given to the Na^+ and Cl^- concentrations, which significantly decreased under the effect of 25 mM proline, whereas these concentrations significantly increased at 50 mM proline. These results are similar to those results reported by Nessim et al. (2008) concerning corn and by Abd El-Samad et al. (2011) concerning maize and broad bean plants. The role of different compatible solutes in plant tolerance to drought stress is significant because these solutes regulate a multitude of metabolic processes, including ion transport (Ali et al., 2008). In addition, the application of the 30 mM proline concentration was more effective compared with the other levels in up-regulating ion transport and in increasing the transpiration rate (Ali et al., 2007).

3.4. Changes in some osmoprotectant and antioxidant enzyme activities

Table 5 shows the effect of proline on some osmoprotectants [total carbohydrate (%), soluble carbohydrate (%), total phenolic concentration (mg g^{-1} DW), free amino acid (mg g^{-1} DW), and proline concentration (mg g^{-1} DW)], and antioxidant enzyme activities [peroxidase (units g^{-1} FW) and polyphenol-oxidase (units g^{-1} FW)] in the leaves of faba bean plants grown under seawater salinity.

3.4.1. Changes in soluble and total carbohydrate concentrations

Seawater salinity levels (S1 and S2) resulted in a reduction in total carbohydrates of faba bean leaves, accompanied by significant increases in soluble carbohydrates relative to S0. Meanwhile, proline levels (P1 or P2) resulted in increases in both parameters compared with P0 (Table 5). The spraying of 25 mM proline gave the highest values. Faba bean plants irrigated with either tap water or diluted seawater and treated with two proline levels showed increases in total and soluble carbohydrates compared with corresponding controls. Unfavourable conditions enhance plants to increase their respiration rates as a prerequisite to produce ATP to activate stressed cells and osmotic soluble substances, which reduce the cell osmotic potential, thus increasing cell water uptake (Khalil et al., 2012). Soluble carbohydrates are a major category of organic compatible solutes that play a key role in alleviating salinity stress either via osmotic adjustment or by conferring some desiccation resistance to plant cells (Hassanein et al., 2009). Soluble carbohydrates increase by salinity, as reported by Khattab (2007) and Hassanein et al. (2009). In contrast, the exogenous proline application might counteract the negative effects of high salinity on carbohydrate metabolism, which, consequently, could promote the entire plant growth, as noted by Nessim et al. (2008) and by Abd El-Samad et al. (2011). This observation could be due to the role of proline in minimising the adverse effects of salinity, which are associated with the decrease in both the Na^+ content and Cl^- concentrations and with increases in K^+ concentrations in faba bean leaves.

3.4.2. Changes in total phenolic concentrations

Higher plants manifest a unique capability to synthesise non-enzymatic secondary metabolites, such as phenolics, which have an anti-oxidative role in scavenging ROS (Reddy et al., 2004). The synthesis and release of phenolic compounds are induced by various biotic and abiotic stress factors. Table 5 shows that total phenolic concentrations were significantly increased under seawater salinity stress compared with their corresponding controls (S0). In this regard, phenolic contents protect cells from potential oxidative damage, increase the stability of cell membranes (Burguieres et al., 2006), and mitigate salinity stress

Table 5
Effect of proline on some osmoprotectants and antioxidant enzymes in the leaves of faba bean grown under seawater salinity.

Treatment		Total carbohydrate (%)	Soluble carbohydrate (%)	Phenolic content (mg g^{-1} DW)	Free amino acids (mg g^{-1} DW)	Proline (mg g^{-1} DW)	Peroxidase (enzyme activity g^{-1} FW)	Polyphenol-oxidase (enzyme activity g^{-1} FW)
Seawater								
S0		17.44a†	6.06c	19.22c	11.78c	0.61c	11.52c	8.18c
S1		17.23a	6.50b	21.62b	17.27b	0.74b	13.41b	10.37b
S2		15.07b	7.29a	22.28a	19.83a	0.79a	15.60a	13.30a
Proline								
P0		15.31c†	6.33b	21.77a	16.61a	0.74a	14.05b	11.23a
P1		17.88a	6.92a	21.19b	15.98b	0.72a	12.02c	9.57b
P2		16.55b	6.60ab	20.16c	16.28ab	0.68b	14.46a	11.06a
Seawater x proline								
S0	P0	16.6cd†	5.76e	18.76f	11.24e	0.57a	9.78 h	6.39 g
S0	P1	18.4a	6.28c–e	19.26e	11.75de	0.66a	11.07 g	8.75f
S0	P2	17.3b	6.15de	19.63e	12.34d	0.61a	13.73d	9.42e
S1	P0	16.0d	5.98e	22.84b	18.41b	0.81a	14.37c	11.47c
S1	P1	18.3a	6.83bc	21.81c	16.62c	0.73a	11.89f	9.00ef
S1	P2	17.4b	6.68b–d	20.21d	16.76c	0.69a	13.97cd	10.65d
S2	P0	13.3f	7.25ab	23.71a	20.18a	0.85a	18.01a	15.84a
S2	P1	16.9bc	7.67a	22.50b	19.57a	0.78a	13.11e	10.95 cd
S2	P2	15.0e	6.96b	20.64d	19.75a	0.74a	15.68b	13.12b

Measurements were made 65 days after sowing (DAS). S0 (0.23 dS m^{-1}); S1 (3.13 dS m^{-1}); S2 (6.25 dS m^{-1}); P0 (0 mm proline); P1 (25 mM proline); P2 (50 mM proline).
†Mean values (n = 4) in the same column for each trait followed by the same lower-case letter are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

injuries (Khattab, 2007). The accumulation of phenolic compounds in response to abiotic stress would be attributed to the activation of phenylalanine ammonia lyase (PAL) (Rivero et al., 2001). The foliar spraying of proline (P1 or P2) resulted in significant decreases in the total phenolic concentration compared with the control (P0). Moreover, faba bean plants treated with an exogenous application of proline under seawater salinity showed significant decreases in phenolic concentrations compared with controls. These reductions may be due to their oxidation by antioxidant enzymes, which withdrew phenols as their substrate (Khattab, 2007), and may be due to the decline in their biosynthesis and the activation of their degradation.

3.4.3. Changes in free amino acid concentrations

Free amino acid concentrations significantly increased by increasing the salinity level, whereas proline treatments decreased these concentrations compared with controls S0 and P0, respectively (Table 5). Amino acids have been reported to accumulate in higher plants under salinity stress (El-Bassiouny and Bekheta, 2005; Khattab, 2007). Moreover, faba bean plants irrigated with diluted sea water and subjected to the exogenous application with two proline levels showed marked decreases in free amino acids compared with controls. Nanjo et al. (2003) showed that proline treatments significantly repressed several genes involved in the synthesis of other amino acids in *Arabidopsis* plants.

3.4.4. Changes in proline concentration

Table 5 shows that salinity levels (S1 and S2) caused significant increases in the proline content of faba bean leaves with increasing salinity levels compared with control plants (S0). In contrast, proline treatments showed an opposite trend. However, faba bean plants subjected to the exogenous application of proline under seawater salinity stress showed non-significant changes in proline concentrations compared with controls. Proline accumulates in larger amounts than other amino acids in salt stressed plants (Yamada et al., 2005; Bekheta et al., 2009; Abd El-Samad et al., 2011; Taie et al., 2013; Abdelhamid et al., 2013). Its accumulation in plants could be due to de novo synthesis, to decreased degradation, or both and depends on the type of species and the extent of the stress (Kavi-Kishor et al., 2005). The accumulation of proline in plant tissues in response to different abiotic stresses may play an important role against oxidative damages caused by ROS due to its action as a single oxygen quencher (Alia et al., 2001), participating in cellular osmotic adjustment (Yamada et al., 2005), buffering the cellular redox potential, stabilising the membrane and protein 3D structure, and protecting the subcellular structures, such as mitochondria and chloroplasts, under adverse environmental conditions (Kavi-Kishor et al., 2005; Ashraf and Foolad, 2007), as well as participating in the induction of stress responsive genes (Chinnusamy et al., 2005). In contrast, Abd El-Samad et al. (2011) and Abdelhamid et al. (2013) mentioned that the spraying of proline under all salinisation levels was accompanied by decreases in the proline concentration compared with control values. These reductions may be explained by proline degradation mechanisms that are induced after stress recovery, and this process leads to the generation of reducing equivalents (Hare et al., 1998). Moreover, proline degradation can provide carbon, nitrogen and energy sources. Upon oxidation, one proline molecule can release 30 ATPs (Kavi-Kishor et al., 2005).

3.4.5. Changes in peroxidase and polyphenol-oxidase enzyme activities

Table 5 shows the impact of the proline application on the changes induced in antioxidant enzyme activities (peroxidase and polyphenol-oxidase expressed in enzyme activity g^{-1} FW) in faba bean leaves grown under seawater salinity. Anti-oxidative enzymes are the first response mechanism against environmental stresses; thus, their activity profiles are important in the evaluation of tolerance mechanisms. Polyphenol-oxidase (PPO) and peroxidase (POD) activities in faba bean leaf tissues showed gradual and significant increases with increasing salinity levels (Table 5). Similar results were reported by El-Bassiouny and Bekheta (2005) and Khattab (2007). Bian and Jiang (2009) reported an enhancement in stress tolerance due to increased activities and to the over-production of these antioxidant enzymes. The increases in the activities of anti-oxidative enzymes under salt stress could be considered indicative of the increased production of ROS and of a build-up of a protective mechanism to reduce oxidative damage triggered by stress experienced by plants, as mentioned by Dolatabadian and Jouneghani (2009). The activities and amount of these antioxidant enzymes under stress conditions differ among plant species and even within cultivars of the same species, as well as the development and the metabolic state of the plant and the duration and intensity of the stress (Reddy et al., 2004; Doğan, 2011). Dionisio-Sese and Tobita (1998) observed that the salt sensitive cultivars exhibited an increase in peroxidase activity under high salinisation. However, the salt tolerant rice cultivar showed only a slight decrease in peroxidase activities. While working with different wheat cultivars, Nikolaeva et al. (2010) reported a sharp increase in the activities of different antioxidant enzymes at early growth stages under water deficit conditions and then decreases at later stages. In tolerant plants, POD activity was found to be higher to protect plants against oxidative stresses (Sreenivasulu et al., 1999). The enhancement of POD activity by salinity has also been observed in rice (Lee et al., 2001). Meanwhile, plants irrigated with diluted sea water (S1 and S2) and treated with two proline levels (P1 and P2) showed significant decreases in both enzymes relative to the corresponding control. Notably, the decrease was more pronounced due to 25 mM proline compared with 50 mM proline.

3.5. Changes in the anatomical structure of faba bean plants

As inferred earlier throughout the study on vegetative growth, increasing salinity levels (3.13 and 6.25 dS m^{-1}) decreased all of the studied growth parameters (plant height, number of leaves per plant, fresh and dry weights of faba bean plants). In contrast, foliar spraying with 25 mM proline increased all investigated growth parameters. This result may justify a further study concerning the internal structure of the main stem and the leaves of normal faba bean plants, those plants grown under salinity stress, and of those plants sprayed with proline grown either under tap water irrigation or under salinity stress. Microscopic characteristics were examined through specimens of the median internode of the main stem and its corresponding leaf from plants aged 65 days. This result clearly highlights the effect of the studied treatments on the microscopic characteristics of these organs.

3.5.1. Anatomical structure of the main stem

Microscopic measurements of certain histological characteristics in transverse sections through the median internode of the main stem of faba bean plants grown under salinity stress of 6.25 dS m^{-1} and affected by foliar spraying with 25 mM proline are provided in Table 6. Likewise, microphotographs illustrating these treatments and the untreated plants are shown in transverse sections in Fig. 1. Salinity with concentrations of 6.25 dS m^{-1} decreased the internode diameter by 25% compared with the control. This decrease in the internode diameter was primarily due to a decrease in the thickness of the stem wall and in the diameter of the hollow pith. The reductions compared with the control were 26.2% and 23.9%, respectively. The decrease observed in the stem wall thickness as a result of salinity stress could be attributed to the decrements induced in all included tissues. The thicknesses of the epidermis, cortex, fibre tissue, phloem tissue, xylem tissue and parenchymatous area of the pith were decreased in treated plants compared with the control by 4.0, 19.8, 16.5, 16.1, 30.3 and 31.9%, respectively. Likewise, the mean value of the vessel diameter in treated plants decreased compared with the control by 23.4%. In conclusion, salinity stress at 6.25 dS m^{-1} caused considerable thinned stems of faba bean plants by decreasing the internode diameter due to a decrease in thickness of the stem wall and in the diameter of the hollow pith. The

Table 6
Effect of proline (25 mM) on measurements (indicated in microns) of histological characters in transverse sections through the median portion of the main stem of faba bean grown under seawater salinity (6.25 dS m^{-1}).

Histological characters	Treatments						
	S0	S2	± % to S0	P1	± % to S0	S2 + P1	± % to S0
Stem diameter	4650	3487	−25.0	5241	+12.7	4259	−8.4
Stem wall thickness	982	725	−26.2	1063	+8.3	906	−7.7
Epidermis thickness	25	24	−4.0	28	+12.0	26	+4.0
Cortex thickness	116	93	−19.8	112	−3.5	102	−12.1
Fibre tissue thickness	97	81	−16.5	123	+26.8	103	+6.2
Phloem tissue thickness	87	73	−16.1	91	+4.6	84	−3.5
Xylem tissue thickness	231	161	−30.3	242	+4.8	241	+4.3
Vessel diameter	47	36	−23.4	48	+2.1	50	+6.4
Parenchymatous pith thickness	429	292	−31.9	467	+8.9	352	−17.9
Hollow pith diameter	2684	2042	−23.9	3115	+16.1	2446	−8.9

Measurements were made 65 days after sowing (DAS). S0 (0.23 dS m^{-1}); S2 (6.25 dS m^{-1}); P1 (25 mM proline). Means of three sections from three specimens.

decrease in the stem wall thickness was accompanied by decrements in all included tissues. These results are generally in harmony with those results reported by Reda et al. (2000) for leucaena plants and by Reda (2007) for coffee senna plants. The foliar application with 25 mM proline induced an increase in the internode diameter by 12.7% compared with the control. This increment in the internode diameter was primarily due to the prominent increase in the thickness of the stem wall and in the diameter of the hollow pith by 8.3 and 16.1% over the control, respectively. Clearly, the increase that was observed in the stem wall thickness could be attributed to the increments induced in the thickness of all included tissues, except that of the cortex, which was decreased by 3.5% compared with the control. The increments due to the proline effect were 12.0, 26.8, 4.6, 4.8 and 8.9% compared with the control for the thickness of the epidermis, fibre tissue, phloem tissue, xylem tissue and parenchymatous area of the pith, respectively. Notably, the mean value of the vessel diameter also increased by 2.1% compared with the control. The data presented in Table 6 and microphotographs shown in Fig. 1 reveal that proline treatment enhanced all histological characteristics of salinity stressed stems of faba bean plants, and this result suggests that proline treatment recovered the harmful effects of salinity on the stem anatomy of faba bean plants. The stem diameter decreased by 8.4% compared with the control. Such a decrement in the stem diameter could be attributed to the decrease induced in the stem wall thickness and in the hollow pith diameter by 7.7 and 8.9% compared with the control, respectively. The decrease in the stem wall thickness could be primarily attributed to the decrements induced in the thickness of the cortex, phloem tissue and parenchymatous area of the pith by 12.1, 3.5 and 17.9% compared with the control, respectively. Other included tissues showed increments in this respect. The thickness of the epidermis, fibre tissue and xylem tissue increased compared with the control by 4.0, 6.2 and 4.3%, respectively. In addition, the mean diameter of the vessel increased by 6.4% compared with the control.

3.5.2. Anatomical structure of the leaf

Certain microscopic characteristics in transverse sections through the first leaflet blade of the compound leaf that developed on the median portion of the main stem of faba bean plants grown under salinity stress and that were affected by foliar spraying with proline were followed up in the form of counts and measurements provided in Table 7. These characteristics in control and treated plants are further shown as microphotographs illustrated in Fig. 2. Salinity stress of 6.25 dS m^{-1} reduced the thickness of both the midvein and lamina of the leaflet blade by 16.7% and 17.6% less, respectively, than those thicknesses of the control. The thinner leaflets induced by salinity stress could be attributed to the decrease induced in the thickness of both the palisade and spongy tissues, as well as in the dimensions of midvein bundles. The decrements compared with the control were 30.3, 13.6, 14.6 and 15.8% for the palisade tissue, spongy tissue, length of midvein bundles and width of midvein bundles, respectively. Additionally, the number of the vessels/midvein bundles decreased by 24.1% compared with the control. Moreover, the mean diameter of the vessel for leaves of stressed plants decreased by 8.8% compared with the control. The obtained results are generally in agreement with those results reported by Wignarajak et al. (1975) for beans, by Reda et al. (2000) for leucaena, and by Boghdady (2009) for mung bean. The foliar application with 25 mM proline increased the thickness of both the midvein and lamina of leaflet blades of faba bean plants by 6.3 and 10.3% compared with the control, respectively. The increase in the lamina thickness was accompanied by 4.9 and 17.3% increases in the thickness of the palisade tissue and of the spongy tissue compared with the control, respectively. Likewise, the main vascular bundle of the midvein increased in size because of the proline treatment. The increment was primarily due to the increase in the length by 4.2% and in width by 26.9% compared with the control. Moreover, the average number of vessels/midvein bundles increased by 6.9% over the control. However, the mean value of the vessel

Table 7
Effect of proline (25 mM) on counts and measurements in microns of histological characters in transverse sections through the first leaflet blade of the compound leaf developed on the median portion of the main stem of faba bean grown under seawater salinity (6.25 dS m^{-1}).

Histological characters	Treatments						
	S0	S2	± % to S0	P1	± % to S0	S2 + P1	± % to S0
Thickness of the midvein	862	718	−16.7	916	+6.3	821	−4.8
Thickness of the lamina	427	362	−17.6	471	+10.3	466	+9.1
Thickness of the palisade tissue	142	99	−30.3	149	+4.9	161	+13.4
Thickness of the spongy tissue	214	185	−13.6	251	+17.3	235	+9.8
Dimensions of the midvein bundle							
Length	336	287	−14.6	350	+4.2	295	−12.2
Width	171	144	−15.8	217	+26.9	154	−9.9
No. of vessels/midvein bundle	29	22	−24.1	31	+6.9	26	−10.3
Vessel diameter	34	31	−8.8	33	−2.9	32	−5.9

Measurements were made 65 days after sowing (DAS). S0 (0.23 dS m^{-1}); S2 (6.25 dS m^{-1}); P1 (25 mM proline). Means of three sections from three specimens.
Seawater + 25 mM proline.

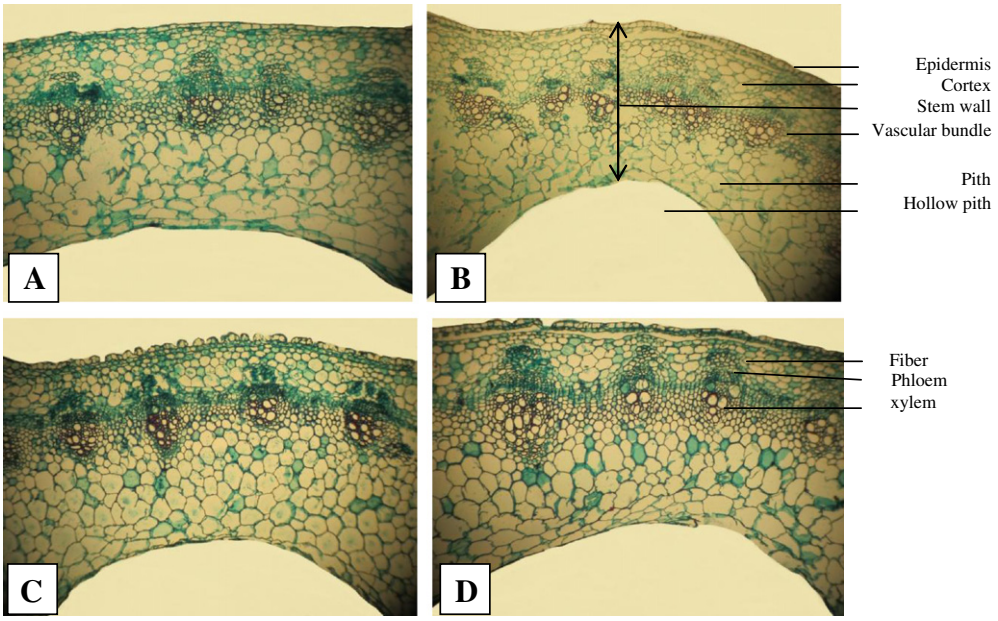

Fig. 1. Changes in cross sections of the stem anatomy of faba bean plants grown under seawater stress and exogenous application of proline (×68). A: tap water without proline (0.23 dS m^{-1}); B: 6.25 dS m^{-1} seawater; C: tap water (0.23 dS m^{-1}) + 25 mM proline; D: 6.25 dS m^{-1} seawater + 25 mM proline.

diameter decreased by 2.9% compared with the control. These results are generally in harmony with those results reported by Boghdady (2009) for mung bean and by Hussein et al. (2012) for *Jatropha*. These results also indicated that proline treatment enhanced most of the histological characteristics of leaflets of stressed plants, and this result suggests that the foliar application with 25 mM proline had the ability to minimise the deleterious effect of salinity on the anatomical structure of faba bean leaves. It is clear that the midvein thickness decreased by 4.8% compared with that of the control. Likewise, the length of the midvein bundles, the width of the midvein bundles, the number of vessels/midvein bundles and the mean vessel diameter decreased below the control values by 12.2, 9.9, 10.3 and 5.9%, respectively. In contrast, the thickness of the lamina increased by 9.1% compared with that of the control primarily due to the increase observed in the thickness of the palisade tissue by 13.4% and in the thickness of the spongy tissue by 9.8% compared with the control. The obtained results are in agreement with those results reported by Boghdady (2009) for mung bean.

4. Conclusions

The foliar application of 25 mM proline alleviated seawater induced reductions in growth parameters, photosynthetic pigments, mineral contents and total carbohydrates in faba bean. Salinity stress at 6.25 dS m^{-1} reduced the thickness of both leaflet blades and stem walls, whereas the foliar application with 25 mM proline counteracted such effects. It can be concluded that the exogenous application of

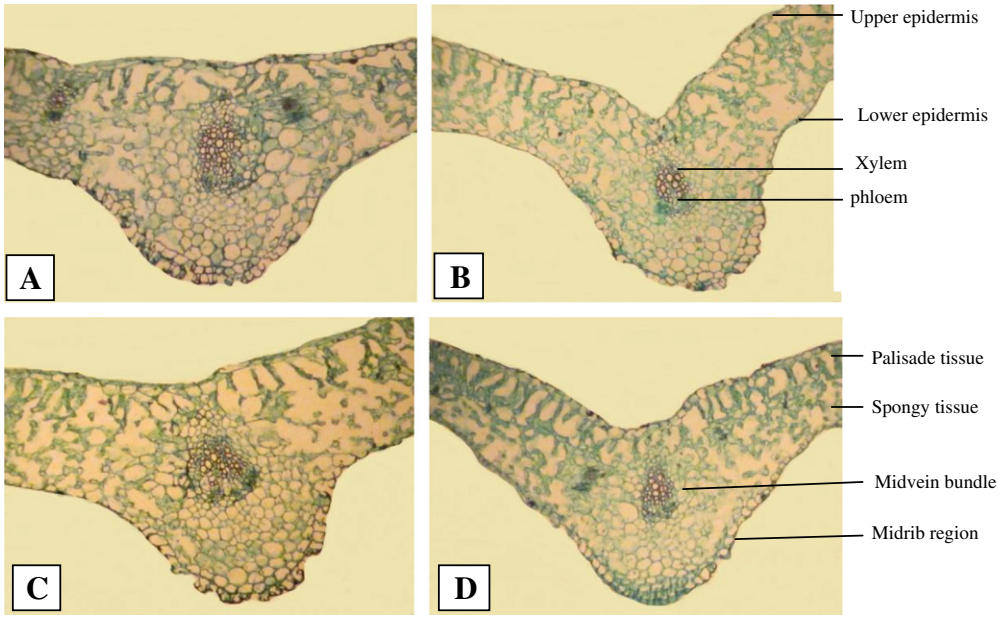

Fig. 2. Changes in transverse section of the leaflet blade of faba bean plants grown under seawater salinity and exogenous application of proline (×68). A: tap water without proline (0.23 dS m^{-1}); B: 6.25 dS m^{-1} seawater; C: tap water (0.23 dS m^{-1}) + 25 mM proline; D: 6.25 dS m^{-1} seawater + 25 mM proline.

proline at a concentration of 25 mM partially alleviated the toxicity of diluted seawater on faba bean plants, whereas the 50 mM proline treatment was toxic.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sajb.2014.03.002.

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