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Research Article

Salt tolerance of sweet clover in response to application of ascorbic acid, proline and urea

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Abstract

Cost-effective exogenous application of some metabolites (ascorbic acid and proline) and nutrients (e.g. nitrogen) may help to minimize the harmful effects of salinity. This split-plot research was tackled to evaluate the impact of ascorbic acid (5 mM), proline (20 mM), and urea (46%N) on the alleviation of osmotic, ionic, and oxidative stresses of non-saline and 6.7 dS/m salinity in *Melilotus officinalis* according to a 2-year experiment at Urmia University. Salt stress increased the activities of superoxide dismutase, glutathione peroxidase, Phenylalanine ammonia-lyase and the amounts of malondialdehyde, peroxide hydrogen, flavonoids, sodium, potassium and calcium in leaves. The proline, potassium, calcium and relative water contents, activities of catalase and ascorbate peroxidase, plant biomass and seed yield were decreased at salinity. In salt-treated plants, foliar sprays of proline enriched the leaf cells with K^+ ions and reduced the Na^+/K^+ ratio, leading to increased relative water content. Foliar sprays of urea increased the proline content, flavonoids, relative water content, and antioxidant enzyme activities that lead to reducing oxidative damage. These findings exhibited the beneficial effects of foliar-applied urea and proline that led to considerable improvement in salt tolerance through biochemical responses.

Keywords: Flavonoids, Malondialdehyde, *Melilotus officinalis*, Osmolytes, Oxidative stress, Saline soil

Introduction

Salinity, as one of the worldwide limiting factors for crop production, can restrict several physiological aspects of the plant that are negatively affected in plants grown under salinity conditions. These harmful effects are due to the low osmotic potential of the soil solution as a result of water stress, specific ionic effects such as salt stress, nutritional imbalance, or a mixture of these factors (Isayenkov and Maathuis, 2019).

Yellow sweet clover (*Melilotus officinalis* L., Fabaceae family), an herbaceous plant with both medicinal and domestic uses, is native to Europe and Asia growing in meadows, pastures, uncultivated fields, and path margins (Burlando *et al.*, 2010). The *Melilotus* species, as the most salt-tolerant legumes, can improve sustainable agriculture in saline soils. In some countries including, Canada, Argentina, Spain, and Russia, *Melilotus* varieties grow in slightly saline regions, where common forage legume cultivation is a problem (Rogers *et al.*, 2008).

According to stress intensity, biochemical changes have occurred in the plant, including membrane disruption, reactive oxygen species (ROS) generation such as superoxide anion, hydrogen peroxide (H_2O_2) and the hydroxyl radicals ($OH^{\bullet}$), reduction of photosynthesis rate, and scavenging of antioxidants. ROS is controlled by a host of enzymatic (superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX)), and non-enzymatic (carotenoids, total phenolic, glutathione, and proline) antioxidant systems to avoid oxidative stress (Das *et al.*, 2016; Farhangi-Abriz and Ghassemi-Golezani, 2018).

To minimize the harmful effects of salinity, various strategies, including the cost-effective and efficient use of foliar spraying, are adopted to increase plant tolerance by mainly alleviating Na and Cl injuries to the plants. Vitamin C, or ascorbic acid (AA), a natural water-soluble antioxidant with several vital metabolic functions, acts as a substrate to protect the damage to several salt stress-induced ROS. In the literature, the exogenous application of ascorbic acid as a foliar spray can cause increasing in the endogenous levels (Sajid and Aftab, 2016).

Proline, a beneficial solute amino acid, protects plants as a metal chelator and an anti-oxidative defense molecule during various stresses (Hayat *et al.*, 2012). Under salinity stress, proline enhances growth-related physiological, biochemical, and anatomical

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characteristics and improves the antioxidant system defense of plants. Considering the osmotic adjustment as the most important mechanism, an exogenous foliar application of compatible osmolytes such as proline can act as an efficient approach to mitigate the damage to salt stress (Huang *et al.*, 2009; Wani *et al.*, 2016).

The unique characteristics of urea, including rapid absorption by leaves and transport essentially to the young shoots, low toxicity, and high solubility in both soil and water, are the cause for its application as a nitrogen fertilizer source. Foliar urea application, with its uncharged nature and transmission through the cuticle, provides an alternative strategy for reducing the risk of nitrogen over-fertilization and directly affects nitrogen metabolism in saline soils and, consequently, amino acids synthesis. Foliar urea maintained NO_3^- and reduced Na^+ concentrations in the leaves under mild salinity conditions (Ruan and Gerendas, 2015).

The worldwide problem of salinity can be mitigated by altering the yield-related biochemical responses of the crop through effective amendments such as fertilizers and phytochemicals. Based on the successful role of foliar spraying in decreasing the harmful effects of salt stress on different plants, the present study was conducted to evaluate yellow sweet clover plants by foliar application of ascorbic acid, proline, and urea in non-saline and saline conditions.

Materials and methods

Experimental conditions: This 2-year (2017-2018) field experiment was executed at Urmia University (37° 39′ 24.82″ N latitude, 44° 58′ 12.42″ E longitude, 1338 m altitude). For each year, the experiment was arranged in a split-plot design with three replications. Treatments were soil salinity (0.9 dS/m – as none-saline and 6.7 dS/m as saline soil) as main plots, and foliar application including proline (20 mM, 2.3 g/L, Xi'an DN Biology Co., Ltd in Bali, Indonesia), ascorbic acid (5 mM, 0.9 g/L, Green Agri Solution, Umbergaon, India), urea (46% N, 10 g/L) and control (water spraying) as subplots. The seeds (*Melilotus officinalis*, biennial species; Norgold cultivar) provided by Agricultural Research, Education and Extension Organization (AREEO), were sown at a depth of 3 cm in plots of 250 by 200 cm size, with plant spacing of 30-by-2 cm on April 5, 2017. The main properties of experimental saline and non-saline soil are presented in Table 1. The foliar treatments were sprayed on plant leaves on June 5, 2017 and 2018 (60 days after sowing, DAS) and were repeated twice more on July 5 and August 5 (full bloom and 30 days after full bloom).

Plant biomass and seed yield: At the full flowering stage on August 23 (135 days after sowing, DAS) and September 6 (150 DAS) for saline and non-saline conditions, respectively, all the plants from 1 m^2 of each experimental plot were harvested, and the aerial parts of the plants as biomass (forage yield) were oven-dried at 80 °C for 48 hours and expressed in kg/ha. Seeds were harvested (from 1 m2) and weighed (with 15% moisture content) on September 23 (165 DAS) and October 7 (180 DAS) for saline and non-saline soil, respectively.

Cation analysis: Leave samples were burned in an electric furnace at 600 °C for 5 hours and the ground ingredients were digested in 2 M HNO_3 for 24 hours at 25 °C. Then, the samples were kept for 1 hour on a hot plate at 120 °C. The digested samples were filled with 50 ml of double-distilled water and tested for sodium, potassium, and calcium contents (mg/g dry weight) by atomic absorption spectrometry (Shimadzu model: AA-6200, Japan) (Gao *et al.*, 2016).

Relative water content (RWC): Fresh leaf samples (0.2 g) were incubated in 50 ml of distilled water for 4 hours and the turgid weights of the leaf samples were measured. Then the leaf samples were dried at 70 °C for 48 hours. The dry weights of the samples were taken, and the relative water content was determined according to the equation:

$$\text{RWC}\ (\%) = \frac{(\text{Fresh weight} - \text{Dry weight})}{(\text{Turgid weight} - \text{Dry weight})} \times 100$$

Biochemical parameters: Fresh leaf samples were collected on August 15, 2017, and 2018 and stored at -80°C. These samples were applied for the analysis of biochemical parameters.

The method of Bates *et al.* (1973), based on reaction between proline and ninhydrin was used to measure proline content. The total flavonoid content was determined according to the aluminum chloride colorimetric method described by Zhishen *et al.* (1999). The absorbance was spectrophotometrically determined at 510 nm.

The concentration of total phenolics was assessed by the Folin-Ciocalteu method according to the procedures described by Kim *et al.* (2006). Finally, absorbance was reported by using a spectrophotometer at 760 nm. The total phenolic content was expressed through a calibration curve with Gallic acid.

For enzyme extraction, 0.25 g of frozen leaves were homogenized in 2 ml of sodium phosphate buffer (pH=7) containing 0.2 mM ethylene-diamine tetra acetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15000 g for 20 min at 4 °C. The supernatant was applied for the measurement of antioxidant enzyme activities. The activity of superoxide dismutase (SOD) at 560 nm (Dhindsa and Matowe, 1981), catalase (CAT) at 240 nm (Bergmeyer, 1974), glutathione peroxidase (phGPX) at 340 nm (Flohe and Gunzler, 1984), and ascorbate peroxidase (APX) at 290 nm (Nakano and Asada, 1981) were measured.

Phenylalanine ammonia-lyase (PAL) was measured by the Zucker (1965) method. The radical-scavenging activity of 2,2-diphenyl-1-picrylhydrazyl was determined by the slightly modified method of Islam *et al.* (2019).

DPPH radical−scavenging activity (%)= (A - B)/A ×100

A = absorbance of DPPH radical + methanol; B = absorbance of DPPH radical + leaf extract as the sample.

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Table 1. Soil properties for saline and non-saline soil samples

	Silt Clay Sand	OM (%)	pH	EC (dS/m)	N (%)	Available P (mg/kg)	Available K	Na
Non-saline soil	32 41 27	1.6	7.6	0.9	0.09	13	187	13
Saline soil	36 40 25	1.03	7.4	6.7	0.07	10	185	165

OM: Organic matter, EC: Electrical Conductivity, N, P, K, and Na: nitrogen, phosphorus, potassium and sodium, respectively.

Malondialdehyde was measured by the level of oxidation of membrane polyunsaturated fatty acids. The concentration of H_2O_2 was analyzed by homogenizing about 0.5 g of plant tissues in 5 ml of 0.1% trichloro-acetic acid at 4 °C and centrifuging at 12,000 g for 15 min. Then 0.5 ml of the supernatant was added to the 0.5 ml of potassium phosphate buffer (pH 7.0) plus 1 ml of potassium iodide (1 M), and the absorbance was recorded at 390 nm (Velikova *et al.*, 2000).

Statistical analysis: The analysis of variance for the two-year data was performed using the GLM procedure (SAS 9.1.3, SAS Institute Inc., Cary, NC, USA) as combined over years according to the experimental design. The mean values were compared with Duncan's Multiple Range tests at $P \leq 0.05$.

Results and discussion

Plant biomass and seed yield: The effects of salt stress and foliar spraying on plant biomass and seed yield of sweet clover were significant ($P \leq 0.01$). The means comparison indicated an 18% and 7% reduction in biomass and seed yield, respectively. In non-saline conditions, urea showed the highest yields (5753 kg/ha for biomass and 327 kg/ha for seed). Though, ascorbic acid had no advantage in both saline and non-saline conditions (Table 2). The accumulation of extra carbon following the nutrient stress leads to the greater production of carbon-based secondary metabolites and their precursors (Yongke *et al.*, 2005). In non-saline conditions, urea and subsequently proline greatly increased seed and biological yield. In general, exogenous urea and proline improved biomass and seed yield by alleviating adverse effects of salinity, while foliar-applied AA plants did not show additive effect on the yield (Table 2).

The negative effects of salt on plant growth are related to a reduction in the osmotic potential of growing media, specific ion toxicity, and nutrient imbalance (Lu *et al.*, 2023). Excess ions in the soil water lower the solute potential (ψs) and thereby the total water potential (ψw) of the soil. To maintain water uptake and turgor under such conditions, plants need to keep their internal water potential (ψw) below that of the soil (Atta *et al.*, 2023). Increased accumulation of osmolytes helps plants lower their water potential to facilitate water uptake from saline soils (Zhao *et al.*, 2021). Changes in water relations, transpiration, nutritional imbalances, stomatal conductance, and oxidative damage due to salt stress all contribute to a drop in yield (Atta *et al.*, 2023). The expression of genes involved in proline biosynthesis is activated by salinity stress, which subsequently leads to the production and accumulation of proline in plant cells. Therefore, exogenous application of proline significantly reduced the inhibitory effects of salinity and improved plant growth and yield under salt stress conditions (Balasubramaniam *et al.*, 2023). Leaf ascorbic acid is significantly increased in exogenous application and it was increasing trend by higher concentrations. Ascorbic acid had potential to mitigate the adverse effects of salinity by reducing oxidative injury in agricultural crops especially lettuce by enhancing enzyme (e.g. superoxide dismutase, catalase and peroxidase) content and activity (Naz *et al.*, 2024). As the soil salt stress increased, the plant stems and fruits demonstrated a gradual enhancement in their N uptake and regulatory capabilities, albeit with a slight decrease observed in the leaves. These findings emphasize that soil salt stress diminishes nitrogen uptake and transport, potentially exacerbating nitrogen pollution in the absence of optimized nitrogen fertilization (Heng *et al.*, 2024).

Na^+, K^+, Na^+/K^+ and Ca^{+2} contents: There was a significant interaction of "salinity × foliar spraying" on the ion contents of plant leaves ($P \leq 0.01$). The Na^+, K^+, and Ca^{2+} concentrations in leaves were increased under salinity conditions. Foliar sprays of both proline and ascorbic acid were able to reduce the Na^+ content under salinity but had a more limited effect on this cation concentration under non-saline conditions. Proline application demonstrated a more significant effect on the reduction of Na^+ in comparison with ascorbic acid. Exogenous proline application noticeably improved the K^+ content under saline and non-saline conditions. Treatment of plants with urea, proline and ascorbic acid under non-saline conditions increased the Ca^{2+} content by 15, 15 and 13 percent, respectively, but treatments respectively reduced the Ca^{2+} content in saline soil by 18, 7, and 19 percent. The reduction of Ca^{2+} was more evident with the ascorbic acid application (Table 2). The Na^+/K^+ ratio increased with salt stress. Foliar sprays of proline caused a reduction in this ratio under saline soil.

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Table 2. The effects of salinity and foliar spraying treatments on traits of *Melilotus*

Salinity	Foliar treatments	Biomass (kg/ha)	Seed yield	K^+ (mg/g DW)	Na^+ (%)	Na^+/K^+ (%)	Ca^{+2} (mg/g DW)
Non-saline	Control	4765±1129^{c}	291±95^{c}	22.39±5.72^{d}	0.02± 0^{e}	0.0083±0.0019^{e}	2.21±0.66^{d}
	Urea	5753 ±1085^{a}	327 ±94^{a}	18.02 ±2.87^{e}	0.02 ±0^{e}	0.0121±0.0022^{d}	2.6 ± 0.83^{b}
	Proline	5342 ±899^{b}	306 ±93^{b}	25.26 ±6.39^{c}	0.03 ±0^{d}	0.0103±0.0028^{de}	2.62± 0.76^{b}
	Ascorbic acid	4790 ±1138^{c}	300 ±93b^{c}	23.32±8.84^{d}	0.028±0.004^{d}	0.0118±0.0028^{d}	2.56 ± 0.66^{b}
Saline	Control	3897 ±1396^{d}	272 ±94^{d}	26.98±1.88^{b}	0.052 ±0.017^{ab}	0.0192±0.0071^{b}	2.81 ± 0.67^{a}
	Urea	3970 ±1444^{d}	275 ±93^{d}	25.22 ±1.57^{c}	0.058 ±0.013^{a}	0.0233±0.0060^{a}	2.29 ± 0.66^{c}
	Proline	3813 ±927^{d}	264 ±94^{d}	28.93 ±6.21^{a}	0.043 ±0.010^{c}	0.0150±0.0064^{c}	2.61 ± 0.77^{b}
	Ascorbic acid	3425 ±1093^{e}	244 ±92^{e}	23.28±1.83^{d}	0.048 ±0.014^{bc}	0.0211±0.0051^{b}	2.25 ± 0.79^{d}
F test		7.53**	9.84**	42.25**	12.18**	7.00**	143.63**

Different letters in each column indicate significant difference by Duncan test at $P \leq 0.05$. *, ** significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. DW: dry weight; K^+, Na^+, and Ca^{+2}: potassium, sodium, and calcium, respectively. The values are the means of three replicates ± standard error.

Regardless of salinity levels, urea improved the ratio of Na^+/K^+, while ascorbic acid had no significant effect on this ratio (Table 2). The relationship between K^+ content and salinity may vary from one species to another. An increase in K^+ content under saline conditions (Table 2) indicated that salt-tolerant *Melilotus* species showed a low amount of Na^+ in contrast to higher K^+ concentrations in their leaves (Rogers *et al.*, 2008). Since most soils have adequate amounts of K^+, a plant with a high uptake of selective potassium at high salinity absorbs more K^+ than Na^+. Therefore, maintaining adequate potassium concentration in the saline condition is essential for plant survival. The observed decrease in the Na^+ content following proline and ascorbic acid application can be related to the changes in the Na^+ uptake and translocation to the shoots. Spraying with either proline or ascorbic acid restricted Na^+ uptake and enhanced the uptake of K^+ and Ca^{2+} in non-saline conditions (Table 2). Plants' control of the Na^+ accumulation in the aerial parts can be described by reducing sodium ion loading in roots and exporting from roots to the aerial parts (Liu *et al.*, 2012). Foliar application of proline under salt stress enhanced the leaf K^+ concentration. Proline excludes maximum Na^+ from the leaves and maintains the plant's osmotic potential, playing a vital role in the osmotic adjustment (Ahmed *et al.*, 2011). Furthermore, proline caused reduced K^+ efflux, and ionic homeostasis was maintained by enhancing the H^+ ATPase activity (Cuin and Shabala, 2007). According to the increase of K^+ and decreasing Na^+ content by exogenous proline under salt stress, a meaningful reduction of the ratio of Na^+/K^+ in *Melilotus* leaves was expected (Table 2).

Proline: A similar condition of no proline accumulation in the salt-tolerant species has been observed in the present study (Table 3) (Huang *et al.*, 2009; Al Hassan *et al.*, 2016). Proline was significantly affected by salinity and foliar spraying ($P \leq 0.01$), causing a reduction in proline content in saline soil (Table 4). In saline conditions, foliar applications significantly increased leaf proline content. In this way, the maximum content was obtained in urea-treated plants (Table 4). Osmo-regulators could perform as mechanisms concerning keeping cell water potential in plants under salinity stress (Abdelaal *et al.*, 2018).

Proline accumulation and concomitant synthesis of different osmolytes in salt-treated plants have been recently reported. According to reports, it is recognized that each species utilizes preferentially one particular compound for osmotic balance (Al Hassan *et al.*, 2016; Hayat *et al.*, 2012; Wani *et al.*, 2016). Despite the reduction of proline content at salinity, spraying with urea, ascorbic acid, and proline improved proline content (Table 4). Therefore, the promotion of salt stress tolerance can be attributed to exogenous proline application, not the endogenous accumulation of proline (Huang *et al.*, 2009). Leaf proline concentration also indicated an increasing trend with ascorbic acid application under salinity stress (Table 4). The application of ascorbic acid scavenges ROS by biosynthesis of extra proline (Dolatabadian *et al.*, 2008). Moreover, treatment with urea, as the most nitrogenous compound, had a stronger effect on proline content as an amino acid (Garde-Cerdan *et al.*, 2014). Foliar spraying, by increasing the soluble carbohydrates and proline contents of leaves, can be responsible for adjusting the water content of plant cells (Table 4).

Relative water content: The interaction of salinity and foliar spraying treatment was significant ($P \leq 0.01$) for the leaf-relative water content. Salinity significantly decreased RWC content. The RWC of *Melilotus* leaves was not statistically changed by foliar treatment of urea on non-saline soil, but proline and ascorbic acid increased this trait. Relative water content was improved under salinity by foliar-applied treatments that exogenous application of urea and proline showed more effect on RWC increment, compared with ascorbic acid (Table 4). Akinroluyo *et al.* (2019) reported that salinity stress reduced the relative water content of *Lolium multiflorum*.

Antioxidant activities and lipid peroxidation: The interaction of "salinity × foliar spraying" was significant for the antioxidant enzymes (SOD, phGPX, PAL, DPPH) activities ($P \leq 0.05$) and MDA and H_2O_2 of *Melilotus* leaves ($P \leq 0.01$) (Table 4 and Table 5). Catalase and ascorbate peroxidase activities were only

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Table 3. The effects of salinity on catalase and ascorbate peroxidase in *Melilotus*

Salinity	Catalase	Ascorbate peroxidase
	(U/g FW)	
Non-saline	4.04[a]	1.15[a]
saline	3.75[b]	1.05[b]
F test	6.61[*]	5.68[*]

Different letters in each column indicate significant difference by Duncan test at P ≤ 0.05. *, ** significant at P ≤ 0.05 and P ≤ 0.01, respectively. The values are the means of three replicates ± standard error.

Table 4. The effects of salinity and foliar spraying treatments on physiological traits of *Melilotus*

Salinity	Foliar treatments	Proline (mg/g FW)	RWC (%)	SOD	phGPX	PAL	DPPH (µg/g FW)
				(U/g FW)			
Non-saline	Control	12.4 ±0.9[c]	54±2.55[b]	1.89±0.50[ab]	0.86 ± 0.20[bcd]	10.55±1.28[b]	31.19±1.39[c]
	Urea	13.7± 2.5[a]	53±3.01[b]	2.07± 0.27[a]	1.09±0.33[abcd]	13.69± 0.96[a]	36.97 ±2.80[a]
	Proline	10.9 ± 2.0[e]	64±3.47[a]	2.05 ±0.23[a]	1.08±0.21[abcd]	11.41± 2.20[b]	36.97±2.82[a]
	Ascorbic acid	10.2± 1.2[f]	61±2.87[a]	1.95 ±0.29[ab]	0.77±0.28[d]	11.31± 0.99[b]	35.05 ±2.67[ab]
Saline	Control	10.6 ± 1.6[ef]	38±3.18[d]	1.98 ± 0.32[ab]	0.99±0.18[abcd]	11.10± 0.97[b]	34.82±4.00[ab]
	Urea	13.1 ± 1.2[b]	52±2.54[b]	2.08 ±0.28[a]	1.18±0.19[a]	11.65± 2.39[b]	37.12 ±3.04[a]
	Proline	11.7 ± 1.7[d]	58±2.77[b]	1.69 ±0.32[b]	1.17±0.41[a]	11.61± 1.75[b]	33.5 ±3.91[abc]
	Ascorbic acid	11.9 ±1.8[cd]	48±1.95[c]	1.36 ±0.15[c]	0.85±0.16[cd]	10.4± 0.76[b]	32±2.59[bc]
F test		5.22[**]	17.12[**]	4.07[*]	4.71[*]	2.80[*]	4.28[*]

Different letters in each column indicate significant difference by Duncan test at P ≤ 0.05. *, ** significant at P ≤ 0.05 and P ≤ 0.01, respectively. TSS: total soluble sugars, RWC: relative water content, SOD: superoxide dismutase, phGPX: glutathione peroxidase, DPPH: diphenylpicrylhydrazyl, PAL: phenylalanine ammonia-lyase. The values are the means of three replicates ± standard error.

Table 5. The effects of salinity and foliar spraying treatments on malondialdehyde, peroxide hydrogen, flavonoid, and phenolic of *Melilotus*

Salinity	Foliar treatments	MDA	H_2O_2	Flavonoid	Phenolic
		(mmol/g FW)		(mg/g FW)	
Non-saline	Control	8.14± 1.08[c]	5.03±0.47[c]	13.03±0.75[b]	9.31±1.31[ab]
	Urea	7.56±2.12 [c]	5.53± 0.48[b]	15.56 ±0.82[a]	9.89±2.99[a]
	Proline	8.66±0.98 [bc]	4.7± 0.47[d]	15.71± 1.73[a]	7.98± 0.83[d]
	Ascorbic acid	8.51±0.85 [bc]	6.22± 0.41[a]	14.48 ± 2.38[ab]	8.37 ±1.06[cd]
Saline	Control	9.5 ±2.26[ab]	5.52 ±1.06[b]	14.52± 2.29[ab]	9.00 ±1.63[bc]
	Urea	8.02±1.33[c]	5.42± 0.56[b]	15.56± 1.92[a]	8.89±1.77[bc]
	Proline	8.2± 0.63[c]	4.69 ±0.52[d]	13.29 ± 3.00[b]	8.64 ±1.65[bcd]
	Ascorbic acid	10.24±1.06[a]	5.05 ±0.35[c]	13.56± 0.55[b]	9.22 ±1.15[abc]
F test		5.27[**]	7.30[**]	6.06[**]	5.16[**]

Different letters in each column indicate significant difference by Duncan test at P ≤ 0.05. *, ** significant at P ≤ 0.05 and P ≤ 0.01, respectively. MDA: malondialdehyde, and H_2O_2: peroxide hydrogen. The values are the means of three replicates ± standard error.

influenced by salinity (P ≤ 0.05) (Table 3). There was a significant increase in SOD, DPPH, and phGPX activities against the reduction of CAT and APX (Table 3 and Table 4) under saline soil. PAL activity was not statistically changed by salinity (Table 4). Urea and proline spraying were more effective in increasing SOD, DPPH, and phGPX activities under saline and non-saline conditions. PAL activity was only significantly enhanced by urea in non-saline soil.

In saline soil, the concentrations of MDA and H_2O_2 were enhanced in leaves. Treatments under non-salinity had no significant effect on MDA content. Foliar application of urea and proline reduced MDA and H_2O_2 content under salinity (Table 5). The imposition of salinity stress reduces membrane stability which is correlated with ROS accumulation in terms of H_2O_2 content and lipid peroxidation as MDA content (Hasanuzzaman *et al.*, 2014). Rising MDA and H_2O_2 a common effects of salt stress as oxidative damage (Table 3). On the contrary, salt-treated plants supplemented with exogenous treatments, especially urea and proline showed lower MDA and H_2O_2 contents (Table 3) which were due to their higher antioxidant defense system (Hasanuzzaman *et al.*, 2014; Das *et al.*, 2016).

Salt stress-induced generation of ROS and enhanced activities of many antioxidant enzymes under salinity have been reported in many plant species. Salinity led to a marked rise in the activities of SOD and phGPX (Table 3). However, CAT and APX activities were reduced in salinity (Table 3). At the salt stress, urea and proline enhanced the activities of two phGPX and SOD

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in both saline and non-saline conditions (Table 3). Exogenous urea mitigates the harmful effects of salt stress more than exogenous proline by increasing these antioxidant enzyme activities. Catalase activity was not affected by the application of exogenous treatments. Results revealed that the effect of the exogenous urea and proline application on antioxidant enzyme activities is not predictable in salinized plants. Accordingly, the response of the APX to the foliar treatments was contrary to SOD and phGPX activities (Huang *et al.*, 2009; Hasanuzzaman *et al.*, 2014). Foliar-applied ascorbic acid had no useful effect on the improvement of enzymatic antioxidants (Table 4). This result is confirmed by the evidence that major detoxification of ROS produced during photosynthesis has interfered with the scavenging mechanism of phGPX, CAT, and SOD. Also, ascorbate and glutathione as cellular redox buffers can scavenge ROS. In addition, such a mechanism may be important in salt stress which decreases stomata conductance (Dolatabadian *et al.*, 2009). Exogenous ascorbic acid decreases the activity of these enzymes may be by the elimination of free radicals (Dolatabadian *et al.*, 2008). Moreover, nitrogen availability significantly affected the activity of PAL (Table 4). Phenylalanine ammonia-lyase plays an important role in the synthesis of secondary metabolites. In the present study, the significant effect of urea application in the PAL activity demonstrates the direct impact of nitrogen on the accumulation of secondary metabolites (Ahanger *et al.*, 2019). DPPH radical scavenging activity as a measure of non-enzymatic antioxidant activity increased significantly under salinity. Under saline conditions, the DPPH activity was strongly enhanced in urea-applied plants (Table 4). It is noteworthy that the DPPH assay mostly measures the activity of the water-soluble antioxidants. High DPPH activity in *M. officinalis* extract under exogenous urea might be due to the high accumulation of some components, such as flavonoids and phenolics, in the plant (Ibrahim *et al.*, 2011).

Total phenolics and flavonoids: The interaction effect of "salinity×foliar spraying" was significant on the phenolic and flavonoid content of leaves ($P \leq 0.01$). Flavonoid and phenolic content were not changed by salinity. In non-saline conditions, flavonoid content was significantly improved by foliar application of urea and proline, but these treatments had no significant effect on flavonoid under saline conditions. Foliar application (urea, proline, and ascorbic acid) did not show a considerable effect on total phenolic content under salinity, while exogenous AA and proline reduced the phenolic content of leaves in non-saline conditions (Table 5). Non-enzymatic antioxidants like phenols and flavonoids have a protective role in avoiding ROS generation. These solutes protect plants against oxidative damage and allow them to maintain turgor pressure, a requirement for maintaining stomata aperture and gas exchange. Phenolic levels were similar in control and saline conditions, while flavonoids increased significantly in parallel with salinity (Table 5). Phenols and flavonoids had a maximum concentration in urea-supplemented plants in well-watered as well as stressed conditions (Table 5). Nitrogen availability presented a beneficial influence on the synthesis of secondary metabolites such as flavonoids and phenols (Ahanger *et al.*, 2019). This may be supported by the carbon-nutrient balance hypothesis.

Conclusion

This study provides a better understanding of the effect of salinity on the biochemical aspects of *Melilotus officinalis*. Salinity drastically influences the growth and yield of plants. Increasing oxidative and osmotic damages caused by salinity reduced proline, relative water content, potassium and calcium ions, plant biomass, and seed yield. Proline foliar application decreased the Na^+/K^+ ratio, resulting in a reduction in Na^+ entry into the leaf cells. In this regard, proline spraying mitigated oxidative and osmotic damages by improving enzyme activities, proline, and water content and decreasing the H_2O_2 content of plants. The improvement of nitrogen and amino acids by foliar urea application has been efficient through increasing the proline content, enzyme activities (SOD, phGPX, PAL, DPPH), and, however, the synthesis of secondary metabolites (e.g., flavonoids and phenolic compounds). This suggests *Melilotus officinalis*, as a legume, can serve as green manure in a saline agricultural system. Salinity is caused by reducing the biomass more than the seed yield. Despite the non-significant effect of urea, proline, and ascorbic acid applications in saline conditions, urea showed the highest yields (5753 kg/ha for biomass and 327 kg/ha for seed), followed by proline spraying under non-salinity stress. Ascorbic acid did not improve the general performance of plants.

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Declaration of interest statement

The authors have no conflicts of interest to declare.

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