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[1]Department of Soil Science, Tabriz Branch, Islamic Azad University, Tabriz, Iran
[2]Agricultural and Natural Resources Research Center, Agricultural Research, Education and Extension Organization (AREEO), East Azarbaijan, Tabriz, Iran

Application of arbuscular mycorrhizal fungi and potassium nitrate improves physiological performance and glycyrrhizin production of licorice under salt stress

Rozita Davar[1], Elnaz Sabbaghtazeh[1*], Ahmad Bybordi[2], Mohammad Reza Dalalian[1], Siamak Saedi[1]

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Summary

To examine the effects of potassium nitrate fertilizer (40 and 80 kg ha^{-1}) and inoculation with arbuscular mycorrhizal fungi (25, 50, and 100 g inoculum) on the physiological performance and glycyrrhizin production of licorice plants (*Glycyrrhiza glabra* L.) under salt stress (irrigation with 4 and 8 dSm^{-1} of saline water), two field experiments were conducted in 2021 and 2022. Salinity reduced the physiological performance of plants but increased the concentration of glycyrrhizin in the roots. The application of potassium nitrate, especially at a rate of 40 kg ha^{-1}, along with mycorrhiza, resulted in increased nutrient content, antioxidative activities (catalase, peroxidase, and superoxide dismutase activities), membrane stability index, leaf relative water content, photosynthetic pigment content, glycyrrhizin production, and growth (about 45%) of licorice plants. On the other hand, the treatment with KNO_3 and mycorrhiza reduced the accumulation of sodium in plant tissues (about 16%). The application of 40 kg ha^{-1} KNO_3 with 50 g of inoculum was found to be the superior treatment for improving the performance of licorice plants under salt stress. According to the findings of this study, the use of KNO_3 in combination with arbuscular mycorrhizal fungi is a successful approach to improve plant growth and productivity under saline conditions.

Keywords: Catalase, Chlorophyll, Leaf water, Membrane stability, Plant growth, Sodium

Introduction

Salinity is an environmental factor that has detrimental effects on the growth and productivity of plants (Chandrasekaran et al., 2019; Muscolo et al., 2019). The presence of high salt levels can hinder the growth of many plant species due to the negative impacts it has on osmotic balance and nutrient uptake. The presence of salt ions in the rhizosphere creates osmotic stress and disrupts the balance of ions within plant cells. This leads to reduced absorption of important nutrients such as potassium and phosphorus, and results in the accumulation of harmful levels of sodium and chlorine inside the cells (Torabian et al., 2018). The decrease in potassium levels in plant cells can be attributed to either a deficiency of this nutrient in the rhizosphere or a reduction in its absorption by the roots due to competition with sodium ions in saline soils (Wu et al., 2018). Due to the significant role that potassium plays in modifying osmotic potential, photosynthetic activities, and cation-anion balance to mitigate the adverse effects of salt stress, it is crucial to maintain sufficient levels of potassium in saline conditions (Farhangi-Abriz and Torabian, 2017). Salinity in plants affects two metabolic processes: the production of reactive oxygen species (ROS) and hormonal signaling (Farhangi-Abriz and Torabian, 2017). Reactive oxygen species can damage biological membranes and hinder the growth and development of cells. Plants have effective defenses against salinity, such as enhancing antioxidative activity in their tissues. Although potassium nitrate and arbuscular mycorrhizal fungi can enhance a plant's ability to withstand salt stress, it is crucial to effectively manage a plant's nutrition in saline environments to ensure optimal antioxidant activity and growth (Fageria, 2016).

Potassium is a crucial nutrient that enhances plants' ability to withstand salt stress. In plants, potassium serves various important functions, including promoting root development, improving water utilization and nutrient absorption, and activating over 60 enzymes that facilitate plant growth and the transportation of carbohydrates within plant cells. (Fageria, 2016). Using potassium-containing fertilizers, such as potassium nitrate, in conjunction with plant symbiotic fungi can have significantly more positive effects compared to using these fertilizers alone when the plant is under salt stress. Mycorrhiza is a symbiotic relationship between fungi and plant roots (Rydlová et al., 2012). Arbuscular mycorrhizal fungi play a crucial role in promoting sustainable agriculture by enhancing disease management and reducing reliance on chemical fertilizers and pesticides. The presence of mycorrhizal fungi on plant roots in saline environments has been found to enhance nutrient absorption, photosynthesis, and antioxidative activity (Chandrasekaran et al., 2019). The efficacy of antioxidant enzymes like catalase and peroxidase is increased by the presence of mycorrhiza in plants growing in salty environments (Yilmaz et al., 2023). Zong et al. (2023) reported that mycorrhiza treatment increases the synthesis of osmolytes, such as proline, in plant tissues, ultimately improving salt stress tolerance. Mycorrhizal fungi affect the plant's metabolism and enhance the production of secondary metabolites, thereby influencing the potency and efficacy of medicinal plants (Rui-Ting et al., 2021). Therefore, numerous mechanisms are not only associated with enhancing plant nutrition but are also implicated in the alterations observed in plants that form symbiotic relationships with mycorrhizal fungi. This change is particularly important for identifying these mechanisms in the presence of salt stress.

Licorice (*Glycyrrhiza glabra* L.) is an herbaceous, perennial medicinal plant belonging to the Fabaceae family. Glycyrrhizin, the main compound found in the root and rhizome of the licorice plant, is a sweetener that is sweeter than sucrose. It is a significant secondary metabolite of licorice and is used in both the pharmaceutical and food industries (Abbasi and Mohammadi, 2023). Nowadays, licorice root is being marketed as a dietary supplement for various health issues, including digestive issues, coughs, and bacterial and viral infections. This particular plant possesses significant medicinal properties, and studying the effects of using saline water for irrigation could yield valuable findings. Previous research has demonstrated that the use of potassium nitrate and arbuscular mycorrhizal fungi can alleviate the negative impacts of salt stress on plants (Chandrasekaran et al., 2019). However, it is possible that combining potassium nitrate and arbuscular mycorrhiza may yield even better results compared to using them separately. The current study aimed to investigate the poten-

* Corresponding author

tial effects of applying potassium nitrate and inoculating with arbuscular mycorrhizal fungi on the physiological performance, growth, and glycyrrhizin production of licorice plants under salt stress induced by irrigation.

Materials and methods

Experimentation

The study was conducted at the research farm of the Research and Education Center of Agriculture and Natural Resources in East Azerbaijan province, Iran (37°58'22.1'' N and 46°02'31.6'' E, 1280 meters above sea level). It followed a split-split plot arrangement using a randomized complete block design with three replications over two growing seasons (2020 and 2021). The main plots were divided into different levels of salinity: non-saline, 4 dSm^{-1}, and 8 dSm^{-1} of saline water treatments. The salinity treatments were selected based on the range of salinity levels present in the local irrigation water. Within each main plot, there were subplots with different levels of potassium nitrate (0, 40, and 80 kg ha^{-1}). Additionally, within each subplot, there were sub-subplots with different concentrations of mycorrhizal treatments: non-inoculated, 25 grams of inoculum, 50 grams of inoculum, and 100 grams of inoculum. Tabelle S1 displays the mean temperature and precipitation recorded at the research farm during the experimental period (In supplementary file). The soil and water properties at the research farm can be seen in Tab. S1 and S2, respectively.

To propagate a sufficient number of licorice plants, the rhizome cultivation technique was employed. The licorice rhizomes were divided into smaller pieces, ensuring that each piece had at least two or three sprouts, and then planted in pots. Once the seedlings were established, they were transplanted to the field in the spring. Following transplantation, the plants received treatments of potassium nitrate and mycorrhiza. The plants were grown in holes that contained mycorrhizal fungi, specifically a combination of *Glomus mosseae*, *Glomus intraradices*, and *Glomus etunicatum*, with equal amounts of each fungus. Each gram of mycorrhizal fungi contains a minimum of 100 active organisms. Once the seedlings were successfully planted in the field, the available saline water was diluted to achieve salinities of 4 and 8 dSm^{-1}. Following this, the seedlings were subjected to salt stress treatments. The field had been left uncultivated for two years before planting in the first year.

Relative water content

The relative water content of leaves (RWC) was measured during the flowering stage. Initially, 10 leaf discs of equal size were taken from the harvested leaves, and their fresh weight was recorded. These discs were then submerged in distilled water and kept in darkness at a temperature of 4 °C for 24 hours. Afterward, the discs were removed from the water and weighed again. In the next step, the leaf discs were placed in an oven set at 75 °C for 24 hours to determine their dry weight. The relative content of leaf water was determined using the following formula:

$$RWC = (F_W\text{-}D_W) / (T_W\text{-}D_W) \times 100$$

Where FW is the fresh weight, DW is the dry weight, and TW is the turgid weight (Smart and Bingham, 1974).

Proline content

The extraction of proline from leaves was conducted using the method described by Bates et al. (1973). Initially, 500 mg of fresh leaves were homogenized with 5 mL of 3% sulfosalicylic acid. To this mixture, 2 mL of glacial acetic acid and 2 mL of ninhydrin were added to a plastic tube containing 2 mL of the extracted material. The prepared specimens were then heated in a Bain Marie (BM 15 Bain Marie, Magapor SL, Spain) at 100 °C for 1.5 hours. After cooling to room temperature, the mixture was extracted with toluene, and the absorbance of the upper phase was measured at 520 nm using a SPEKOL 1500 spectrophotometer. The proline content was determined and expressed as mg g^{-1} of leaf fresh weight, using a calibration curve for pure proline.

Membrane stability index

The membrane stability index (MSI) of plant leaves was determined by measuring the electrical conductivity of substances released from leaf samples in double distilled water at temperatures of 40 °C and 100 °C. Two leaf samples, each weighing 0.2 g, were placed in separate Erlenmeyer flasks containing 20 mL of distilled water. The flasks were then placed in an oven at 40 °C for 30 minutes and at 100 °C for 15 minutes. Once the samples reached room temperature, their electrical conductivity was measured using an electrical conductivity meter (LF-90 EC). The membrane stability index (MSI) was calculated using the following equation (Petrov et al., 2018).

$$MSI = \frac{EC40}{EC100} \times 100$$

Antioxidative activities

The Bradford (1976) method was used to measure the amount of soluble protein in plant tissues. Approximately 250 mg of leaves were homogenized in a 2 mL Na-phosphate buffer and then centrifuged for 10 minutes at 12,000 g. The absorbance of the resulting supernatant was measured at 595 nm using a UV-visible spectrophotometer. Enzymes were obtained from the leaf tissues using a potassium phosphate buffer with a pH of 7.0. To determine CAT activity, changes in absorbance at 240 nm were recorded. The activities of peroxidase (POX) and superoxide dismutase (SOD) were determined using the methods described by Gueta-Dahan et al. (1997) and Giannopolitis and Ries (1977), respectively.

Photosynthetic pigments

The contents of chlorophyll and carotenoids were measured using the methods described by Arnon (1949) and Maclachlan and Zalik (1963). To accomplish this, approximately 1 gram of each sample was mixed with 4 milliliters of 80% acetone. The mixture was then centrifuged at 12,000 g for 20 minutes at a temperature of 4 °C. A portion of the liquid above the sediment was collected, and the absorbance at wavelengths of 645 and 663 nm (for chlorophylls) and 480 and 510 nm (for carotenoids) was determined using a spectrophotometer.

Nutrient content

The sodium and potassium contents were measured using the method described by Jones and Case in 1990. Licorice leaves and roots were dried and heated to 80 °C. Then, 100 mg of each sample was burned at 560 °C for 5 hours. The resulting ashes were mixed with a 10 mL solution of 1 M HCl and left at 25 °C for 24 hours. The amounts of nutrients in the plant tissues were determined using atomic absorption spectrophotometry (Shimadzu model: AA-6300, Kyoto, Japan). The phosphorus concentration was measured using the yellow method and spectrophotometric examination at 430 nm, as described by Tandon et al. in 1968.

Rhizome weight, plant biomass, and glycyrrhizin production

After subjecting the plants to salt stress for 3 months, the weight of the aboveground parts and underground stems of the plants was

measured after being exposed to a temperature of 75 °C for 48 hours. To determine the production of glycyrrhizin, approximately 1 gram of dried root tissue was ground and mixed with methanol. The mixture was then analyzed using a high-performance liquid chromatography device (HPLC) with a Sunfire C18 column (Waters, USA). The data obtained was analyzed using Millennium 32 software (ESMAEILI et al., 2020).

Statical analysis

The data was analyzed using the MSTAT-C software (East Lansing, Michigan State University, USA) to conduct a combined analysis of variance. The means were compared using Duncan's multiple range test at a significance level of $p \leq 0.05$. The effect of year was considered a random factor, while other treatments (salinity, potassium nitrate, and mycorrhizal treatments) were analyzed as fixed factors. The tables were prepared based on the significance of the F test (ANOVA results are presented in Tab. S3 and S4 in the supplementary file).

Results

Leaf relative water content and proline concentration

Relative leaf water content and proline concentration were significantly affected by the interaction effect of year × salinity. Additionally, the interaction effects of salinity × potassium nitrate and salinity × mycorrhizal treatment on these traits were significant (Tab. S3).

In both years, the enhancement of salt stress led to a decrease in the water content of the leaves and an increase in the concentration of proline (Tab. 1). Under non-saline conditions, the water content of the leaves was similar across different levels of potassium nitrate. However, the application of potassium nitrate improved the water content of the leaves under salinities of 4 and 8 dSm^{-1}. The application of potassium nitrate also increased the concentration of proline in the leaves under various levels of salt stress (Tab. 2). Inoculating the plants with mycorrhiza under different levels of salt stress increased the water content of the leaves and decreased the concentration of proline, compared to the control plants. Mycorrhiza did not affect the leaf water content and proline concentration of plants under non-saline conditions (Tab. 3).

Antioxidant enzyme activity and membrane stability index

The effect of the year on peroxidase enzyme activity was significant. The activity of superoxide dismutase was significantly affected by the interaction between year and salinity. The interaction effect of salinity and potassium nitrate application was significant for the membrane stability index, as well as the activity of catalase, peroxidase, and superoxide dismutase. The effect of salinity × mycorrhizae treatment was also significant on the membrane stability index and catalase activity. The activity of peroxidase and superoxide dismutase was affected by the mycorrhizal treatment (Tab. S3).

Salt stress caused a decrease in the membrane stability index and an increase in the activity of catalase, peroxidase, and superoxide dis-

Tab. 1: The interaction effect of Year × Salinity on the relative water content (RWC), proline concentration, superoxide dismutase (SOD) activity, chlorophyll content, sodium content of shoots, and potassium to sodium ratio in licorice plants.

Year	Salinity (dSm^{-1})	RWC	Proline	SOD	Chl a	Chl b	Shoot Na^+	Root K^+/Na^+	Shoot K^+/Na^+
		%	mg g^{-1}FW	U g^{-1}FW	mg g^{-1}DW			DI	
2021	Non-saline	80.91 a	17.00 e	0.66 e	2.05 b	0.94 b	4.45 e	3.71 b	9.33 b
	4	76.05 c	45.77 c	1.69 c	1.94 c	0.80 d	12.38 c	1.46 d	3.17 d
	8	70.57 e	60.42 a	2.13 a	1.62 e	0.63 f	18.09 a	0.78 f	1.65 f
2022	Non-saline	81.19 a	16.37 f	0.69 e	2.11 a	0.97 a	4.33 e	4.05 a	9.92 a
	4	76.78 b	44.17 d	1.61 d	2.05 b	0.86 c	11.71 d	1.59 c	3.49 c
	8	71.62 d	57.24 b	2.02 b	1.77 d	0.71 e	17.27 b	0.85 e	1.81 e

Different letters indicate a significant difference at $p \leq 0.05$.
RWC: Relative water content; SOD: Superoxide dismutase; Chl: Chlorophyll; FW: Fresh weight; DW: Dry weight; DI: Dimensionless indices

Tab. 2: The interaction effect of salinity and KNO3 application on the relative water content (RWC), membrane stability index (MSI), proline content, antioxidant enzyme activity, and chlorophyll content of licorice leaves.

Salinity (dSm^{-1})	KNO_3 (kg ha^{-1})	RWC	MSI	Proline	CAT	POX	SOD	Chl a	Chl b
		%		mg g^{-1}FW	U g^{-1}FW			mg g^{-1}DW	
Non-saline	0	80.40 a	81.89 a	14.86 i	0.37 h	0.15 g	0.66 f	2.00 c	0.89 c
	40	81.50 a	80.68 b	16.55 h	0.47 g	0.29 f	0.65 f	2.07 bc	1.01 a
	80	81.25 a	81.72 a	18.64 g	0.48 g	0.37 f	0.72 f	2.16 a	0.96 b
4	0	74.82 cd	72.93 d	37.63 f	1.91 f	3.53 e	1.56 e	1.82 e	0.75 e
	40	77.57 b	75.59 c	46.60 e	2.18 e	5.25 c	1.63 d	2.14 ab	0.89 c
	80	76.85 bc	74.94 c	50.67 d	2.28 d	5.84 a	1.77 c	2.03 c	0.84 d
8	0	70.70 e	66.89 e	52.70 c	2.90 c	4.96 d	2.09 b	1.67 f	0.65 f
	40	74.44 d	72.95 d	58.51 b	3.36 a	5.87 a	2.38 a	1.92 d	0.82 d
	80	68.15 f	63.61 f	65.28 a	3.08 b	5.35 b	1.76 c	1.50 g	0.56 g

Different letters indicate a significant difference at $p \leq 0.05$.
RWC: Relative water content; MSI: Membrane stability index; CAT: Catalase; POX: Peroxidase; SOD: Superoxide dismutase; Chl: Chlorophyll; FW: Fresh weight; DW: Dry weight

mutase enzymes (Tab. 2). The activity of peroxidase was higher in the first year compared to the second year (Tab. 4). In both years, irrigation with saline water resulted in a significant increase in the activity of the superoxide dismutase enzyme compared to irrigation with non-saline water (Tab. 1). The impact of salinity on the activity of the superoxide dismutase enzyme was more pronounced in the first year than in the second year. The application of potassium nitrate at various levels of salt stress improved the membrane stability index and the activity of catalase, peroxidase, and superoxide dismutase enzymes. However, the application of 80 kg ha^{-1} potassium nitrate had a negative effect on the membrane stability index and superoxide dismutase activity under high levels of salt stress (Tab. 2). Treatment with mycorrhizal fungi increased the membrane stability index and antioxidative activities under saline conditions (Tab. 3).

Photosynthetic pigments

The carotenoid content of leaves was significantly affected by the year. The interaction effect of year × salinity and salinity × mycorrhizae on the content of chlorophylls a and b was significant. The levels of chlorophylls a and b, as well as carotenoids, were significantly influenced by the interactions between salinity and potassium nitrate (Tab. S3).

The levels of carotenoids in the leaves were higher in the second year compared to the first year (Tab. 4). Salinity had a negative effect on the chlorophyll content in both years of the experiment (Tab. 1). Mild salt stress (4 dSm^{-1}) increased the carotenoid content in licorice leaves, but severe salt stress (8 dSm^{-1}) decreased it. The application of potassium nitrate at a rate of 40 kg ha^{-1} increased both chlorophyll and carotenoid content under various salt stress levels (Tab. 5). However, application of 80 kg ha^{-1} potassium nitrate only increased the content of photosynthetic pigments under mild salt stress. Under high salt stress, the application of 80 kg ha^{-1} potassium nitrate resulted in a decrease in the content of photosynthetic pigments in the licorice plant (Tab. 2 and 5). Inoculation with mycorrhiza at various doses increased the concentration of chlorophyll a and b at all salinity levels (Tab. 3).

Nutrient content

The year had a significant effect on the levels of potassium and phosphorus in both the roots and shoots, as well as the sodium content in the roots. The interaction effect of year × salinity on the sodium content of roots and the potassium-to-sodium ratio of roots and shoots was significant. The interaction effect of salinity and potassium nitrate application significantly affected the potassium content of the root, the sodium content of the root and shoot, and the potassium-to-sodium ratio of the shoot. The interaction effect of salinity × mycorrhizae on the concentrations of sodium and phosphorus in the roots and shoots was significant. The triple interaction effect of salinity stress, potassium nitrate, and mycorrhizae on the shoot potassium concentration and potassium to sodium ratio of licorice root was significant. The mycorrhizal treatment significantly affected the potassium content of the roots and the potassium-to-sodium ratio of the shoots (Tab. S4).

In the second year, there was a significant increase in potassium concentrations in both the roots and shoots compared to the first year. However, the concentrations of sodium in roots and phosphorus in both roots and shoots decreased (Tab. 4). Salinity caused an increase in sodium concentration in roots and shoots, as well as a decrease

Tab. 3: The interaction effect of salinity and mycorrhizae application on the relative water content (RWC), membrane stability index (MSI), proline concentration, catalase (CAT) activity, chlorophyll content, root and shoot sodium and phosphorus contents, and glycyrrhizin production in licorice plants.

Salinity (dSm^{-1})	Mycorrhizae (g)	RWC	MSI	Proline	CAT	Chl a	Chl b	Root Na^+	Shoot Na^+	Root P	Shoot P	Glycyrrhizin
		%	%	mg g^{-1}FW	U g^{-1}FW				mg g^{-1}DW			
Non-saline	0	81.00 a	81.30 a	17.47 f	0.42 f	2.07 ab	0.84 cd	7.26 g	4.51 f	1.36 h	1.08 ef	17.65 h
	25	80.92 a	81.40 a	17.03 f	0.41 f	2.05 ab	0.98 a	6.93 g	4.48 f	2.83 d	1.59 c	38.47 d
	50	81.22 a	81.05 a	16.18 f	0.44 f	2.08 a	0.99 a	6.73 g	4.41 f	3.49 b	1.91 ab	49.93 b
	100	81.07 a	81.97 a	16.05 f	0.48 f	2.11 a	1.01 a	6.55 g	4.16 f	3.74 a	2.07 a	51.4 ab
4	0	74.45 c	71.93 c	52.27 c	1.90 e	1.84 cd	0.72 e	18.53 d	13.35 d	1.2 h	0.98 f	29.33 f
	25	76.58 b	74.20 b	46.20 d	2.10 d	1.95 bc	0.81 d	16.80 e	12.58 d	2.47 e	1.34 d	43.03 c
	50	77.18 b	75.75 b	41.18 e	2.23 d	2.10 a	0.88 bc	15.30 f	11.18 e	3.16 c	1.6 c	51.75 ab
	100	77.45 b	76.05 b	40.22 e	2.26 d	2.10 a	0.91 b	14.98 f	11.07 e	3.23 c	1.76 bc	53.02 a
8	0	68.48 e	64.63 f	67.55 a	2.87 c	1.58 f	0.61 g	24.68 a	19.58 a	0.89 i	0.75 g	22.75 g
	25	70.92 d	67.23 e	60.22 b	3.08 b	1.67 ef	0.65 f	23.27 b	18.03 b	1.6 g	1.01 f	30.35 f
	50	72.42 d	69.55 d	54.03 c	3.23 ab	1.75 de	0.72 e	21.52 c	16.65 c	2.06 f	1.2 de	35.78 e
	100	72.57 d	69.85 cd	53.52 c	3.27 a	1.80 d	0.73 e	21.27 c	16.47 c	2.26 ef	1.36 d	38.23 d

Different letters indicate a significant difference at $p \leq 0.05$.
RWC: Relative water content; MSI: Membrane stability index; CAT: Catalase; Chl: Chlorophyll; FW: Fresh weight; DW: Dry weight

Tab. 4: The effect of year on some parameters of licorice plants.

Year	POX	Carotenoid	Root K^+	Shoot K^+	Root Na^+	Root P	Shoot P	Shoot dry weigh	Rhizome dry weight	Glycyrrhizin
	U g^{-1}FW	mg g^{-1}DW								
2021	3.56 a	0.96 b	20.95 b	34.81 b	15.63 a	2.41 a	1.42 a	13.03 b	9.98 b	39.38 a
2022	3.46 b	0.98 a	21.95 a	36.11 a	15.01 b	2.32 b	1.36 b	13.53 a	10.34 a	37.56 b

Different letters indicate a significant difference at $p \leq 0.05$.
DW: Dry weight; FW: Fresh weight

in potassium content in roots and the potassium-to-sodium ratio in shoots (Tab. 1). When potassium nitrate was added to the soil, there was an increase in the concentration of potassium in the roots and an increase in the potassium-to-sodium ratio in the shoots. Additionally, there was a decrease in the concentration of sodium in both the roots and shoots (Tab. 5). In non-saline conditions, there was no significant difference in sodium concentration between plants with mycorrhizal and plants without mycorrhizal associations. However, mycorrhiza inoculation resulted in a significant reduction in sodium concentrations in plant tissues under low and severe salinity conditions. Salt stress led to a decrease in phosphorus content in plant tissues. Mycorrhizal treatments increased the concentration of phosphorus in plant tissues (Tab. 3). The application of potassium nitrate at various levels of salt stress and mycorrhizal treatments resulted in an increase in the potassium concentration in shoots and the potassium-to-sodium ratio in roots. Overall, mycorrhizal treatments improved the potassium content in plant tissues (Tab. 6).

Plant biomass and glycyrrhizin production

The effects of year and salinity × potassium nitrate application, on shoot biomass, rhizome weight, and glycyrrhizin production were significant. The production of glycyrrhizin was significantly affected by the interaction effects of salinity × mycorrhizal and potassium nitrate application × mycorrhizal treatments. The effect of mycorrhizal treatment on shoot and rhizome dry weights was significant (Tab. S4).

In 2022, the shoot and rhizome dry weights were higher compared to 2021. However, the highest level of glycyrrhizin production was observed in 2021 (Tab. 4). The application of potassium nitrate increased the shoot and rhizome dry weight, as well as glycyrrhizin production, under different levels of saline and non-saline conditions. In most cases, the application of 40 kg ha^{-1} potassium nitrate was the most effective treatment for increasing mass and glycyrrhizin production (Tab. 5). The use of mycorrhizal treatments also increased glycyrrhizin production under different levels of salt stress, with more significant improvements observed under mild salt stress (Tab. 3). Applying mycorrhiza at all doses increased the dry weights of shoots and rhizomes, with the highest weights observed when using 50 or 100 grams of mycorrhizal inoculum (Tab. 7). The combined use of mycorrhiza with potassium nitrate application had a more favorable effect on increasing glycyrrhizin production compared to using these treatments individually (Fig. 1).

Tab. 6: Triple interaction effect of salinity, KNO3, and mycorrhizae application on shoot potassium content and the potassium to sodium ratio of roots.

Salinity (dSm^{-1})	KNO_3 (kg ha^{-1})	Mycorrhizae (g)	Shoot K^+ mg g^{-1}DW	Root K^+/Na^+ DI
Non-saline	0	0	33.25 lmno	2.17 kl
		25	34.40 lm	2.22 k
		50	33.55 lmn	2.61 hi
		100	34.80 kl	2.52 ij
	40	0	38.65 hi	3.28 f
		25	39.90 gh	3.59 e
		50	41.15 efg	3.69 e
		100	41.70 ef	3.96 d
	80	0	47.50 b	5.21 c
		25	48.95 b	5.67 b
		50	51.30 a	5.69 b
		100	50.80 a	5.94 a
4	0	0	28.65 s	0.61 tuv
		25	30.45 qr	0.74 stu
		50	31.80 opq	0.84 rst
		100	32.35 nop	0.91 qrs
	40	0	33.60 lmn	1.01 pqr
		25	35.00 kl	1.2 op
		50	37.15 ij	1.47 mn
		100	36.45 jk	1.44 mn
	80	0	40.20 fgh	1.95 l
		25	42.10 de	2.33 jk
		50	43.45 cd	2.82 gh
		100	44.25 c	3.01 g
8	0	0	22.45 w	0.33 w
		25	24.15 v	0.44 vw
		50	27.45 st	0.57 uvw
		100	28.30 st	0.62 tuv
	40	0	25.25 uv	0.53 uvw
		25	26.75 tu	0.62 tuv
		50	29.10 rs	0.76 stu
		100	28.50 s	0.71 stu
	80	0	31.45 pq	1.09 pq
		25	32.70 mnop	1.23 nop
		50	34.25 lm	1.38 mno
		100	34.85 kl	1.49 m

Different letters indicate a significant difference at $p \leq 0.05$.
DW: Dry weight; DI: Dimensionless indices

Tab. 5: The interaction effect of salinity and KNO_3 application on the carotenoid content of leaves, potassium content of roots, sodium content of roots and shoots, potassium to sodium ratio in shoots, shoot and rhizome dry weights, and glycyrrhizin production in licorice plants.

Salinity (dSm^{-1})	KNO_3 (kg ha^{-1})	Carotenoids	Root K+	Root Na+	Shoot Na+	Shoot K+/Na+	Shoot dry weight	Rhizome dry weight	Glycyrrhizin
		mg g^{-1}DW				DI	mg g^{-1}DW		
Non-saline	0	0.93 e	17.70 f	7.45 g	4.68 f	7.35 c	16.42 c	12.40 d	34.06 f
	40	1.02 d	25.29 c	6.98 g	4.36 fg	9.38 b	19.63 a	13.93 a	41.64 d
	80	1.06 c	34.63 a	6.17 h	4.13 g	12.15 a	19.13 b	13.25 b	42.39 c
4	0	1.06 c	15.65 g	20.51 c	14.52 c	2.18 f	11.18 e	10.05 f	39.20 e
	40	1.31 a	21.50 d	17.13 d	12.73 d	2.86 e	16.20 c	12.93 c	49.60 a
	80	1.23 b	28.39 b	11.57 f	8.88 e	4.95 d	14.27 d	11.57 e	44.05 b
8	0	0.65 f	13.66 h	28.09 a	20.73 a	1.26 h	7.47 g	5.85 h	29.20 h
	40	0.93 e	15.71 g	24.01 b	18.24 b	1.52 g	9.42 f	7.00 g	34.41 f
	80	0.54 g	20.54 e	15.95 e	14.09 c	2.40 f	5.28 h	4.50 i	31.73 g

Different letters indicate a significant difference at $p \leq 0.05$.
DW: Dry weight; DI: Dimensionless indices

Tab. 7: The effect of mycorrhizae application on the activities of POX and SOD, potassium content in roots, potassium to sodium ratio in shoots, and the dry weights of shoots and rhizomes in licorice plants.

Mycorrhizae (g)	POX	SOD	Root K^+	Shoot K^+/Na^+	Shoot dry weight	Rhizome dry weight
	U g^{-1}FW		mg g^{-1}DW	DI	mg g^{-1}DW	
0	3.35 b	1.36 c	19.73 c	4.40 b	12.19 c	9.53 c
25	3.48 ab	1.43 b	20.97 b	4.68 b	13.04 b	9.98 bc
50	3.60 a	1.53 a	22.51 a	5.18 a	13.74 a	10.46 ab
100	3.62 a	1.55 a	22.61 a	5.32 a	14.16 a	10.68 a

Different letters indicate a significant difference at $p \leq 0.05$.
POX: Peroxidase; SOD: Superoxide dismutase; FW: Fresh weight; DW: Dry weight; DI: Dimensionless indices

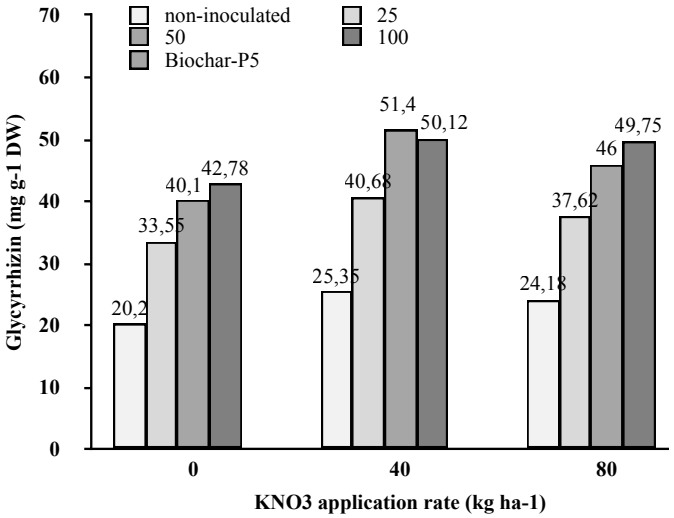

Fig. 1: The interaction effect of KNO_3 application and mycorrhizal treatments on the production of glycyrrhizin in licorice roots.
Different letters in each column indicate a significant difference at $p \leq 0.05$.

Discussion

The decrease in leaf-relative water content and the increase in proline concentration in licorice plants under salt stress are caused by reduced water absorption and the toxic effects of sodium ion accumulation. Proline accumulation is a metabolic response of plants to water scarcity and salinity stress. The high level of proline under salt stress is beneficial for plants because it contributes to osmotic potential and regulates leaf osmotic balance. Additionally, proline helps protect proteins, and enzymes, and maintain membrane stability under various conditions (Trovato et al., 2019). The increase in proline accumulation under salt stress is attributed to the enhanced activity of the proline synthetase enzyme (Guan et al., 2020). The application of potassium nitrate further promotes the accumulation of proline under salt stress by enhancing the activity of ornithine aminotransferase in the ornithine pathway (Hervieu et al., 1995). Potassium itself acts as a mineral osmotic regulator in plants, enhancing water absorption during periods of environmental stress (Farhangi-Abriz and Ghassemi-Golezani, 2022). The use of mycorrhiza can also enhance water uptake in plants by altering root morphology and anatomy (Saxena et al., 2022). Other studies have reported improved water absorption in plants through mycorrhizal treatments (Chandrasekaran et al., 2019; Saxena et al., 2022). In the second year, the leaf relative water content, photosynthetic pigment content, potassium ion levels, potassium to sodium ion ratio, and dry weight of shoots and rhizomes were higher compared to the first year. This could be the result of increased rainfall and lower temperatures, which are conducive to plant growth this year.

The MSI is a valuable tool for assessing damage to the plant cell wall. A decrease in MSI during salt stress may be caused by an overproduction of ROS. These ROS can be harmful to the cell membrane and eventually lead to cell death. Plants have a defense system that includes antioxidant enzymes, which help protect against oxidative damage (Jamshidi Zinab et al., 2022). These enzymes protect membranes, proteins, and macromolecules from damage caused by reactive oxygen species, enabling plants to tolerate adverse conditions such as salinity. Therefore, there is a direct correlation between a plant's antioxidant capacity and its ability to tolerate stress. The application of potassium nitrate under salt stress can improve plant tissue's MSI by enhancing antioxidative activities. Potassium nitrate enhances the activity of antioxidant enzymes, such as CAT, POX, and SOD, which aid in the detoxification of harmful ROS and safeguard the integrity and permeability of plasma membranes against oxidative damage. Mycorrhizal treatments can also improve antioxidative activities under salt stress by enhancing nutrient uptake and water availability to plants, thereby improving cell MSI. The activity of CAT, POX, and SOD, which are metalloenzymes, depends on the availability of micronutrients (Fageria, 2016).

The presence of sodium ions in leaf tissues can disrupt the structure of chloroplasts and break down chlorophyll, leading to a decrease in photosynthetic pigments during salt stress. This degradation of chlorophyll is caused by an increase in the activity of the chlorophyllase enzyme under conditions of salt stress (Hundare et al., 2022). Chloroplasts are highly susceptible to chlorine, and research has shown that chlorine toxicity is a significant contributor to the degradation of chlorophyll (Hameed et al., 2021). The application of potassium nitrate, especially at a rate of 40 kg ha^{-1}, can enhance the content of photosynthetic pigments by increasing the potassium levels in the leaves. Potassium helps in the synthesis of chlorophyll by preventing a significant decrease in the production of 5-aminolevulinic acid, which occurs under salt stress (Wu et al., 2021). When mycorrhizal treatment is applied to plants exposed to salt stress, the improvement in chlorophyll content can be attributed to two mechanisms. Firstly, mycorrhiza stores sodium in its tissues, preventing its transfer to the plant, and also increases the ratio of potassium to sodium in the rhizosphere and plant roots. Secondly, mycorrhiza enhances the absorption of nutrients such as potassium and magnesium, which are crucial for chlorophyll synthesis in plants (Evelin et al., 2019).

Plants experience a decrease in potassium uptake when they are exposed to high levels of salt stress. This is because sodium and potassium ions have similar hydration radii, which leads to competition for absorption and disrupts the activity of proteins responsible for transporting these ions (Farhangi-Abriz and Ghassemi-Golezani, 2016). Additionally, sodium depolarizes the cell membrane by entering the apoplastic space and displacing calcium. This depolarization reduces the selective absorption of ions and blocks potassium absorption channels, causing an increase in potassium leakage through potassium release channels. However, applying potassium nitrate at varying salinity levels can mitigate the competitive impact of sodium on potassium absorption. This is achieved by increasing the concentration of potassium in the rhizosphere, which enhances the accumulation of potassium ions and raises the potassium-to-sodium ratio. Mycorrhizal symbiosis can also enhance potassium absorption by activating potassium channels in host plants (Zhang et al., 2017). Furthermore, in saline soils, mycorrhizal fungi produce a glycoprotein called glomalin, which binds to the soil and reduces the solubility and uptake of sodium (Hammer and Rillig, 2011).

The decrease in phosphorus levels in licorice plants, when exposed to salt stress, is caused by the presence of high concentrations of anions such as chlorine and sulfate in the irrigation water. These anions

compete with phosphorus to pass through the ion channels in the cell membrane of plant roots. The increased absorption of phosphorus due to mycorrhizal treatments may be linked to improved root performance, as well as increased activity of acid phosphatases and oxalates (DE SOUZA CAMPOS et al., 2021). Ultimately, this leads to improved availability of phosphorus for the plants.

Salinity decreases the weight of licorice shoots and rhizomes. The presence of salt ions in the rhizosphere creates stress in plant tissues, which leads to reduced plant growth. The decrease in chlorophyll content in leaves may be responsible for this reduction. However, the application of potassium nitrate can improve shoot dry weight by providing sufficient potassium and nitrogen for crucial physiological processes such as photosynthesis. The availability of potassium promotes root expansion and ultimately increases the weight of the rhizome. Additionally, potassium nitrate can improve the potassium-to-sodium ratio under salt stress, thereby enhancing plant growth, especially root growth. Mycorrhizal treatments can also enhance the growth of licorice under salt stress by improving nutrient absorption. This is achieved through increased water and nutrient uptake, as well as an increase in the rate of photosynthesis (CHANDRASEKARAN et al., 2019). The development of the root system is influenced by various factors, including the hormonal balance of the plant. Mycorrhizal fungi stimulate the production of auxin, which promotes root development (CHEN et al., 2022).

Glycyrrhizin, a terpenoid secondary metabolite, is produced in licorice plants as a defense mechanism against salinity stress. This production mechanism is similar to that of other plants under similar conditions. Secondary metabolite production increases in response to salinity stress. Glycyrrhizin possesses antioxidative properties that aid in the reduction of oxidative damage (SHIRAZI et al., 2019). The genes *CYP88D6* and *CYP72A154* play a crucial role in glycyrrhizin biosynthesis in licorice rhizomes, and their expression is significantly increased under salt stress (SEKI et al., 2011).

The ratio of NH_4/NO_3 in the soil affects the concentration of terpenoid compounds in plants treated with nitrogen-based fertilizers. Changes in this ratio have been found to affect the composition of terpenoid compounds in medicinal plants (SALONER and BERNSTEIN, 2022). Therefore, the increase in nitrate levels in the rhizosphere could be a possible reason for the enhanced response of licorice glycyrrhizin to potassium nitrate fertilizer. Additionally, mycorrhizal inoculation has been observed to increase the content of glycyrrhizin in licorice rhizomes. This symbiotic relationship benefits terpenoid biosynthesis by increasing the phosphorus content in plant tissues. Phosphorus improves the accumulation of pyrophosphate compounds, including important precursors of terpenoid biosynthesis, such as isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which contain high-energy phosphate bonds (VERMA and SHUKLA, 2015; WELLING et al., 2016).

Conclusions

Salt stress, caused by the presence of sodium ions in irrigation water, has been found to negatively impact licorice plants. This stress reduces the relative water content of leaves, the membrane stability index, photosynthetic pigments, and disrupts the ionic balance. As a result, the growth and productivity of licorice plants significantly decrease. However, the application of potassium nitrate and mycorrhizae improved the growth and productivity of licorice plants under salt stress. This was achieved by enhancing nutrient uptake, increasing the concentration of photosynthetic pigments, and promoting antioxidative activities. Additionally, the KNO_3 and mycorrhizal treatments reduced the plants' sodium absorption. The combined application of potassium nitrate, particularly at a rate of 40 kg ha^{-1}, and mycorrhizal inoculation at a rate of 50 g of inoculum, had a greater positive effect on plant performance compared to applying these treatments individually. The study demonstrated that the use of potassium nitrate fertilizer with mycorrhizae can enhance the growth and production of glycyrrhizin in licorice plants under saline conditions. Molecular investigations have the potential to be valuable for upcoming studies. Further research can investigate the impact of these treatments on the growth and productivity of medicinal plants under various environmental stresses, including exposure to heavy metals.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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ORCIDS

Rozita Davar https://orcid.org/0009-0000-5406-2811
Elnaz Sabbaghtazeh https://orcid.org/0000-0002-7141-5268
Ahmad Bybordi https://orcid.org/0000-0003-3474-3956
Mohammad Reza Dalalian https://orcid.org/0000-0002-4566-7496
Siamak Saedi https://orcid.org/0009-0003-1142-0422

Address of the corresponding author:
Elnaz Sabbaghtazeh; Department of Soil Science, Tabriz Branch, Islamic Azad University, Tabriz, Iran
E-mail: elnazsabbagh@iaut.ac.ir

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Tab. S1: Soil and weather parameters of the experimental site during the growing seasons

Soil	**2021**	**2022**	**Weather**	**2021**	**2022**
Texture	Sandy clay loam	Sandy clay loam	**Temperature (°C)**		
pH	7.7	7.7	April	11	12.3
EC (dSm^{-1})	3.8	4.2	May	15.3	19
OM (%)	0.88	0.88	June	11.3	23.8
Total N (%)	0.08	0.09	July	26.8	27.7
P ($mg\ kg^{-1}$)	11.11	13.5	August	28.8	27.1
K ($mg\ kg^{-1}$)	82	96	September	16.6	24.5
Mg ($mg\ kg^{-1}$)	44.7	33.6	**Rainfall (mm)**		
Cu ($mg\ kg^{-1}$)	0.21	0.31	April	12.4	18.8
Zn ($mg\ kg^{-1}$)	0.42	0.33	May	33.6	33.3
Fe ($mg\ kg^{-1}$)	5.3	6.7	June	0.5	4.9
Mn ($mg\ kg^{-1}$)	15.4	13.1	July	0.4	15
CEC ($cmol\ kg^{-1}$)	17.5	18.1	August	1	21.1
			September	0.5	1.1

EC: Electrical conductivity; OM: Organic mature; CEC: Cation exchange capacity

Tab. S2: The analysis of the water used for irrigating plants on the farm.

Na^+	$Ca^{2+} + Mg^{2+}$	SO_4^{2-}	Cl^-	HCO_3^-	TDS	pH	EC
meq L^{-1}					mg L^{-1}		dSm^{-1}
40	34.4	20.5	55.4	7.6	37.5	7.4	8.5

TDS: Total dissolved solids; EC: Electrical conductivity

Tab. S3: Significance test of variation sources (mean square) for some studied parameters of licorice plants

Treatments	RWC	MSI	Proline	CAT	POX	SOD	Chl a	Chl b	Carotenoids
Year	25.42**	9.75	175.50**	0.20	0.50*	0.14**	0.60**	0.18**	0.02*
Replication (Year)	0.009	9.82	44.20**	0.13	0.31	0.23**	0.01**	0.002*	0.01*
Salinity	1786.37**	3335.87**	33220.64**	131.67**	573.28**	37.11**	2.91**	1.40**	4.44**
Year × Salinity	2.61*	0.80	29.62**	0.01	0.16	0.09**	0.04**	0.01**	0.003
Error a	0.32	3.82	0.96	0.04	0.09	0.01	0.002	0.001	0.003
KNO3 application	147.22**	184.46**	1737.12**	1.54**	21.56**	0.40**	0.83**	0.41**	0.76**
Year × KNO3 application	0.13	0.004	0.007	0.001	0.001	0.001	0.001	0.001	0.001
Salinity × KNO3 application	74.76**	205.42**	184.56**	0.36**	9.17**	1.09**	0.49**	0.10**	0.33**
Year × Salinity × KNO3 application	0.08	0.08	0.01	0.001	0.001	0.001	0.001	0.001	0.001
Error b	12.12	1.35	6.43	0.02	0.02	0.012	0.01	0.003	0.003
Mycorrhiza	65.77**	118.31**	976.07**	0.81**	0.83**	0.42**	0.34**	0.28**	0.01
Year × Mycorrhiza	0.293	0.19	0.06	0.001	0.001	0.001	0.001	0.001	0.001
Salinity × Mycorrhizae	16.20*	26.95*	178.78**	0.13*	0.20	0.009	0.06*	0.009*	0.009
Year × Salinity × Mycorrhizae	0.30	0.03	0.03	0.001	0.001	0.001	0.001	0.001	0.001
KNO3 × Mycorrhiza	0.60	2.84	7.13	0.02	0.011	0.008	0.009	0.009	0.007
Year × KNO3 × Mycorrhiza	0.34	0.04	0.027	0.001	0.001	0.001	0.001	0.001	0.001
Salinity × KNO3 application× Mycorrhizae	1.00	3.95	9.22	0.007	0.01	0.006	0.01	0.001	0.002
Year × Salinity × KNO3 application × Mycorrhizae	0.07	0.07	0.04	0.001	0.001	0.001	0.001	0.001	0.001
Error c	5.82	10.97	23.98	0.06	0.13	0.02	0.029	0.004	0.01
CV (%)	3.17	4.44	12.20	13.18	10.25	11.12	8.80	7.85	10.14

** and *: significant at 1% and 5% probability levels, respectively.

RWC: Relative water content; MSI: Membrane stability index; CAT: Catalase; POX: Peroxidase; SOD: Superoxide dismutase; Chl a: Chlorophyll a; Chl b: Chlorophyll b

Tab. S4: Significance test of variation sources (mean square) for some studied parameters of licorice plants

Treatments	K-Root	K-Shoot	Na-Root	Na-Shoot	K/Na Root	K/Na Shoot	P-Root	P-Shoot	Shoot DW	Rhizome DW	Glycyrrhizin
Year	53.70**	92.87**	21.82**	15.68**	1.79**	6.85**	0.47**	0.23*	13.50*	6.82*	178.21**
Replication (Year)	6.63**	14.26**	0.69	0.97	0.003	0.1	0.86**	0.28**	14.42*	10.78**	10.48
Salinity	1542.98**	2877.57**	4564**	3202**	184.97**	1255**	25.23**	0.20**	2125**	1086**	2856.90**
Year × Salinity	0.16	0.31	1.49	2.40*	0.33**	0.86**	0.001	0.001	0.26	0.04	2.41
Error a	0.34	0.26	0.66	0.43	0.01	0.04	0.003	0.03	2.31	1.16	5.34
KNO3 application	2690.60**	2514.04**	1027.21**	337.60**	70.02**	156.85**	0.27	0.21	209.40**	69.77**	1120.28**
Year × KNO3 application	0.26	0.25	0.66	0.23	0.07	0.22	0.001	0.001	0.02	0.007	0.99
Salinity × KNO3 application	154.43**	95.75**	193.56**	66.54**	9.15**	20.46**	0.26	0.218	46.96**	15.73**	100.86**
Year × Salinity × KNO3 application	0.03	0.02	0.10	0.06	0.008	0.02	0.001	0.002	0.04	0.007	0.13
Error b	1.78	0.18	0.89	0.62	0.03	0.15	0.11	0.09	0.27	0.11	0.55
Mycorrhiza	101.68**	137.35**	74.67**	42.80**	2.58**	10.05**	40.87**	6.51**	40.02**	13.99**	6655.08**
Year × Mycorrhiza	0.05	0.004	0.08	0.02	0.02	0.06	0.001	0.001	0.001	0.001	4.46
Salinity × Mycorrhizae	5.61	3.58	10.42*	8.74**	0.11**	0.65	1.09**	0.14*	0.51	0.07	354.12**
Year × Salinity × Mycorrhizae	0.006	0.01	0.007	0.002	0.003	0.01	0.001	0.001	0.00	0.001	0.21
KNO3 × Mycorrhiza	0.52	1.05	4.07	2.17	0.14**	1.39	0.01	0.01	0.39	0.25	41.27**
Year × KNO3 × Mycorrhiza	0.002	0.004	0.005	0.003	0.001	0.002	0.001	0.001	0.001	0.001	0.09
Salinity × KNO3 application× Mycorrhizae	2.90	3.48*	0.91	0.84	0.08*	0.89	0.03	0.01	0.37	0.06	9.17
Year × Salinity × KNO3 application × Mycorrhizae	0.1	0.01	0.005	0.007	0.001	0.007	0.001	0.001	0.001	0.001	0.02
Error c	4.84	1.85	3.73	2.75	0.03	0.91	0.13	0.06	1.60	1.56	8.23
CV (%)	10.26	3.84	12.62	14.59	9.28	19.47	15.33	18.55	9.55	12.29	7.46

** and *: significant at 1% and 5% probability levels, respectively.
DW: Dry weight