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Pre-treatment for heat tolerance enhancement of the Indian almond *(Pithecellobium dulce)* seedlings using ascorbic acid and potassium chloride

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Abstract

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In light of global warming, pre-treatment plants with antioxidants may reduce the damage caused by climatic changes. Indian almond seedlings were planted in pots subjected to ascorbic acid and potassium chloride alone or combined to reduce the negative impact of high field temperature. Compared with the control, all treatments improved the plant height, branch number, number of leaves, and leaf area. These treatments reduced loss in concentration of photosynthetic pigments such as chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid. Heat stress increased abscisic acid content and electrolyte leakage percentage, whereas the application of ascorbic acid alleviated this damage. Indian almond plants can better withstand high temperatures particularly using ascorbic acid treatments at 50 mg l^{-1} or treatment of ascorbic acid at 50 mg l^{-1} + potassium chloride at 250 mg l^{-1} to reduce heat stress damage.

Keywords

ascorbic acid, climate change, electrolyte leakage, heat stress, Indian almond, potassium chloride, pre-treatment

Introduction

The Indian Almond (*Pithecellobium dulce* (Roxb.)) is an important plant of tropical American origin. It belongs to the family Fabaceae and is cultivated throughout India. It is a medium-sized evergreen spiny tree. Native to the Pacific coast and the adjacent highlands of Mexico, Central America, and northern South America, it is a small to medium-sized, evergreen, spiny tree up to 18 m in height that is grown throughout the plains of India (Rao et al., 2019). The plant is famous for its edible fruits and has traditionally been consumed to treat various ailments. The fruits are legumes (pods) 10 to 13 cm long. Usually, every single pod contains 10 seeds. The pods are irregular in shape and flat, set in 1 to 3 spirals. The seeds are black and shiny, with a diameter of 1 cm suspended in the pods (Orwa et al., 2009).

The importance of the Indian almond plant lies in the diversity of its uses for medicinal purposes and as an ornamental plant, in addition to its use as windbreaks. The primary method for its propagation is seeds, helping to spread it and obtain a profitable economic return due to using currently imported seeds for breeding in local nurseries (Sukantha and Subashini, 2015). The Indian al-

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mond requires adequate care, especially during summer, when moderate temperatures are necessary. (Akubude et al., 2018).

Basrah is considered a semi-tropical region with a hot, dry climate in the summer, high soil and water salinity, and water scarcity in some seasons. High summer temperatures exceeding 48 °C in the shade in July and August determine plant growth and development (Shareef et al., 2020).

Biochemical reactions within plants are sensitive to high-temperature stress, which varies with temperature, duration of exposure, and plant type (Fragkostefanakis et al., 2015). High temperatures cause cell damage or plant death within minutes, and moderate temperatures may cause cell injury or death with prolonged exposure (Nievola et al., 2017). High temperature impairs plant growth and physiological processes and significantly reduces the productivity of many plant species (Hatfield and Prueger, 2015). High-temperature form harmful substances in plants due to disruption in the processes of photosynthesis and the ability to respiration in plants, subsequently a change in the color of leaves to yellow, inhibiting growth processes in plants and destroying chlorophyll (Jamloki et al., 2021).

The high temperatures stimulate an increase in the activity of the enzyme chlorophyllase and increase the level of abscisic acid (ABA), which accelerates the decomposition of chlorophyll (Jamloki et al., 2021). Carotenoids inhibit the formation of Singlet oxygen (O_{2-}) by quenching the triplet state of chlorophyll molecules when they appear (Ali and Alqurainy, 2006).

The proline accumulation under heat stress conditions provides plants with the energy needed for growth and endurance to stress (Liang et al., 2013). Proline, one of the chaperones, can somewhat enhance the capacity of genuine chaperones and underlines the role played by small molecules, for example, polyols, trimethylamines, and amino acids, in the protection of cells against stress (Kawagoe et al., 2018). Phenolic compounds are the most important secondary metabolites that act as natural antioxidants, free radical scavengers, inhibitors of free radical production, and catalysts for antioxidant synthesis in plants (Cosme et al., 2020). Previous studies have indicated membrane permeability as a valuable indicator of heat stress damage (Elbasyoni et al., 2017; Jia et al., 2020). Heat stress is the cause of an increase in ABA and growth inhibitors. ABA, known as one of the transduction signal components, leads to gene induction, forming proteins necessary to protect plants under stress conditions (Vishwakarma et al., 2017).

Plant treatment with antioxidants under external conditions provides additional factors that improve plant tolerance to unfavourable environmental conditions and protect biochemical processes (Shareef et al., 2020). Therefore, it is necessary to apply technologies to increase the heat tolerance of the Indian almond plant, especially in the Middle East, which is witnessing increases in temperature during spring and summer. One of the simplest ways to reach this goal is the exogenous application of some osmotic-modifying compounds, such as potassium, or antioxidants, such as ascorbic acid.

Ascorbic acid has important functions in plant development, hormone signaling, the cell cycle, and the cellular redox system. This substance is a significant plant metabolite that protects plants against many environmental stresses, such as high temperature and salinity (Farooq et al., 2013). The high ascorbic acid content in plant chloroplasts indicates its essential role in the photosynthetic system (Chen et al., 2017).

Potassium is an element that modulates the osmotic pressure in plant cells (osmoticum) to relieve various stresses (Shahid et al., 2019). Potassium-induced regulation of plant water relations reduces heat sensitivity, while potassium availability under stress enhances photosystem II quantum yield, enzymatic activities, and chlorophyll biosynthesis. Similarly, the availability of potassium under stress conditions improves the plant's ability to fix carbon (carboxylation) by regulating the synthesis of RuBisCO and sucrose synthase (Zahoor et al., 2017). Potassium in homeostasis enhances enzyme synthesis, relieves oxidative stress, and improves signal transduction during stress (Souto et al., 2018). Furthermore, the plant can retain chlorophyll and sucrose translocation for a relatively long time in a sufficient potassium supply (Chrysargyris et al., 2017; Shareef, 2019).

In light of this knowledge, pre-treatment plants with the above substances may reduce their damage under climate change. In this study, a tropical plant of Indian almond was grown in a subtropical environment. Subtropical regions suffer from high temperatures and little or no rain in the summer. We tested the potential of reducing the negative impact of high field temperature in Indian almond plants by using ascorbic acid and potassium chloride alone or in combination with a foliar spray.

Materials and methods

Seeds of Indian almonds (*Pithecellobium dulce* (Roxb.)), obtained from Aljouri Agricultural materials (UAE), were grown in a local nursery in 20 × 25 cm pots. On 1 September 2020, fifty-four seedlings were transferred to plant at the wooden canopy of the Department of Horticulture and Landscaping - College of Agriculture - University of Basrah (30°33'47.3"N 47°44'38.4"E). Then, large plastic pots with a diameter of 30 cm were washed well with water, sterilized with formalin, and filled with autoclaved growth medium; (bet moss: soil (1:1) of electrical conductivity (EC) 2 dS m^{-1}, irrigation water of 1.2 dS m^{-1}). One plant was transplanted into one pot and let grow until reaching 30–40 cm in height. All the experimental plants were fertilized with the compound fertilizer NPK (20-20-20) from 1 February 2021 once per month at a rate 1g pot^{-1}. The pots were taken from the wooden canopy and put in the field on 10 February 2021. The treatments were conducted monthly on 9 March, 9 April, and 9 May 2021 as a foliar spray. Tween 20 was added at 1 ml l^{-1} as a diffuser to reduce the surface tension of the leaves. It was sprayed in the early morning using polyethylene bags to prevent contamination from other treatments. The following nine treatments were applied with 250 ml plant^{-1} solution: 1) Control (spray with distilled Water), 2) AsA 50 mg l^{-1}, 3) AsA 100 mg l^{-1}, 4) KCl 250 mg l^{-1}, 5) KCl 500 mg l^{-1}, 6) AsA 50 mg l^{-1} + KCl 250 mg l^{-1}, 7) AsA 50 mg l^{-1} + KCl 500 mg l^{-1}, 8) AsA 100 mg l^{-1} + KCl 250 mg l^{-1}, 9) AsA 100 mg l^{-1} + KCl 500 mg l^{-1}. After 60 days of the end of the treatments (spraying), the leaves were collected for morphological and biochemical assay from every treatment. In June and July, the minimum temperature was 27 and 28 °C, the maximum was 44 and 46 °C, the relative humidity was 30 and 27%, and the light intensity was 1,740 and 1,750 µmol m^{-2} s^{-1}, respectively.

Growth parameters

Plant height was measured using metric tape from the soil surface to the top of the plant. The number of lateral branches of each plant was calculated, and the means were recorded. The number of leaves on the main stem and the lateral branches was calculated, and the average was taken multiplied by the number of branches to get the total number of leaves of the plant. The total leaf area was measured using the ImageJ program according to Easlon and Bloom (2014), and after taking ten leaves for each replicate, they were placed in a scanner. The readings representing the leaf area of the plant were taken according to the following equation:
The leaf area of a plant (cm^2) = the average leaf area (cm^2) × the number of leaves.

Photosynthetic pigments concentration

Leaf pigments were isolated from leaves using the method of Lichtenthaler and Wellburn (1983). Leaf tissue of 0.2 g was ground in 10 ml acetone (80%) and centrifuged at 2,000 rpm for five min. Then, the absorbance of the supernatant at 645, 663, 534, and 470 nm was determined, and pigment concentration was calculated.

Soluble carbohydrates concentration

The phenol-sulphuric acid technique assessed the carbohydrate, following Yemm and Willis (1954). Approximately 100 mg fresh leaf sample was homogenized in 5 mL 2.5N HCl and put into a boiling water bath for 3 h. The cooled rough homogenate was balanced and centrifuged at 10,000 rpm for 10 minutes. To 100 µl supernatant, 100 µl phenol [5% (v/v)] and 500 µl of sulfuric acid [96% (v/v)] were added. Then, the absorbance of the reaction mixture at 490 nm was defined.

Free proline concentration

The proline content was assessed by Bates et al. (1973). About 0.5 g of leaf material was homogenized with 5 mL of 3% sulfosalicylic acid. After filtration 3 mL of filtrate was mixed with ninhydrin reagent and glacial acetic acid, 3 mL each. The reaction mixture was incubated at 95 °C for an hour and cooled. A chromophore was shaped by adding 4 mL toluene to the cooled mixture. The absorbance of the chromophore was measured at 520 nm utilizing a UV-VIS spectrophotometer.

Total phenolics concentration

The Folin-Ciocalteu technique was applied (Waterman and Mole, 1994). Twenty-five µL supernatant (500 µg mL^{-1}) was mixed with 25 µL of (1:1) Folin-Ciocalteu reagent and 100 µL of 7.5% sodium bicarbonate solution and incubated at room temperature for 2 hours in the dark. Then, absorbance at 765 nm was recorded using a UV-VIS spectrophotometer.

Ascorbic acid concentration

Ascorbic acid (AsA) was measured according to Luwe et al. (1993) procedure. Leaf samples (0.5 g) were homogenized with 10 ml of 6% trichloroacetic acid. The supernatant was mixed with 2 ml of 2% dinitrophenylhydrazine (pH 5) and one drop of 10% thiourea (in 70% ethanol). This mixture was boiled for 15 min in a water bath, and after cooling at room temperature, 5 ml of 80% (v/v) H_2SO_4 was mixed into the mixture at 0 °C. Absorbance at 265 nm was determined.

Abscisic acid concentration

One gram of leaf tissue was homogenized in 70% methanol at 4 °C, filtered under a vacuum, using Whatman filter paper (No. 1). The pH of the aqueous stage was changed to 8, utilizing a 0.2 M phosphate buffer. The aqueous stage was diluted twice utilizing methanol. A rotary evaporator eliminated the methanol stage, and pH of the aqueous stage was tuned to 2.5, using 1 N HCl. ABA estimated by the injection of the concentrate into a turnaround stage HPLC on a switch stage C12 column in an isocratic elution mode using a convenient stage including $(CH_3)_2CO$: H2O (26:74) with 30 mmol phosphoric acids according to Tang et al. (2011). PH was changed to 4 using 1 N sodium hydroxide. The transition rate was 0.6 ml min^{-1}, and the elution of abscisic acid was seen at 270 nm at 25 ºC.

Electrolyte leakage

Leaf segments of one gram were submerged into 12 ml distilled water at 27 ºC. The first conductivity of the eluate (C1) was determined after getting a thermometer test of 27 ºC along with a conductivity meter. The second conductivity (C2) was defined after 15 min autoclaving and cooling of the system to 26 °C. Finally, C1/C2 ratio was calculated (Shanahan et al., 1990).

Potassium concentration

After fresh leaf samples were dried at 70 °C to the constant weight, Cresser and Parsons (1979) method was applied. Samples were mineralized in concentrated sulphuric acid and submitted to potassium concentration analysis using atomic absorption spectrophotometer.

Statistical analysis

The experiment was conducted as a randomized block complete design of nine treatments. Each one consisted of four replications. Obtained data were submitted to the analysis of variance (ANOVA) utilizing SPSS variation 20.0 (SPSS, Chicago, IL), and the means were isolated utilizing the Duncan test at the 0.05 confidence level.

Results

Growth parameters

Significant changes in growth parameters in Indian almond seedlings subjected to AsA and KCl alone or combined under high field temperatures are shown in Table 1. Applying K and AsA improved plant height, branch number, leaves number, and leaf area. Plants treated with 50 mg l^{-1} AsA dominated in height, and those treated with 50 mg l^{-1} AsA + 250 mg l^{-1} KCl in the leaf number. Combination of AsA and KCl in all concentrations – except for the highest – caused the largest (6.5 fold) increase in total leaf area compared to the control.

Physiological parameters

Each treatment alleviated the loss of photosynthetic pigments caused by heat (Table 2). The largest values were obtained in treatments combining AsA and KCl.

There was a significant increase in proline content under heat stress, whereas total soluble carbohydrate, phenolic, and ascorbic acid contents decreased (Fig. 1). AsA treatments mostly reduced the proline content and stimulated the phenol concentration. The highest concentrations of soluble sugars were found in AsA-treated plants and those treated with the combination of AsA and KCl at the lowest concentrations. Leaf ascorbate concentration rose with its concentration in the spray, but when combined with KCl, it was slightly reduced.

Leaf abscisic acid level grew with heat exposure (Fig. 2), but treatments reduced its accumulation similarly (about one-third). Electrolyte leakage observed in control was almost half reduced in all treatments except for pure KCl ones. AsA a slightly stimulated accumulation of potassium in leaves, KCl treatment, doubled its concentration, and combinations of both substances brought intermediate results.

Table 1. Effect of exogenous application of AsA and KCl alone or combined on plant height, number of branches, number of leaves, and leaf area in Indian almond seedlings under high field

Treatments (mg l^{-1})	Plant height (cm)	Number of branches	Number of leaves (Leaf plant^{-1})	Leaf area (cm^2 plant^{-1})
Control	63.25 ± 3.30 d	2.50 ± .57 b	156.75 ± 156.75 c	168.50 ± 35.85 c
AsA 50	94.25 ± 4.19 a	5.00 ± 0.05 a	191.25 ± 61.69 c	256.46 ± 82.73 bc
AsA 100	92.50 ± 2.38 ab	4.50 ± 1.00 a	299.50 ± 67.95 b	454.34 ± 103.08 b
KCl 250	91.00 ± 2.58 ab	4.25 ± 0.95 a	319.75 ± 71.39 ab	385.93 ± 86.17 bc
KCl 500	89.75 ± 4.78 ab	4.50 ± 0.57 a	343.75 ± 45.71 ab	464.40 ± 61.75 b
AsA 50 + KCl 250	79.50 ± 2.64 c	4.50 ± 0.57 a	414.75 ± 50.16 a	1094.94 ± 132.44 a
AsA 50 + KCl 500	78.25 ± 3.40 c	4.00 ± 1.15 a	386.50 ± 114.05 ab	1163.75 ± 343.42 a
AsA 100 + KCl 250	93.00 ± 6.48 ab	3.75 ± 0.95 a	358.75 ± 84.87 ab	972.21 ± 230.00 a
AsA 100 + KCl 500	87.50 ± 2.08 b	4.50 ± 1.00 a	167.50 ± 35.64 c	456.45 ± 110.84 b

Means of 4 replications ± SD. Using Duncan's multiple range test, means with different letters are different at $p \leq 0.05$.

Table 2. Effect of exogenous application of AsA and KCl alone or combined on leaf chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid content in Indian almond seedlings under high field temperature

Treatments (mg l^{-1})	Chlorophyll *a* (mg g^{-1})	Chlorophyll *b* (mg g^{-1})	Total chlorophyll (mg g^{-1})	Carotenoid (mg g^{-1})
Control	2.63 ± 0.14 f	3.41 ± 0.05 c	6.04 ± 0.09 d	0.017 ± 0.00 e
AsA 50	3.67 ± 0.20 bc	3.90 ± 0.07 bc	7.57 ± 0.13 c	0.019 ± 0.00013 d
AsA 100	3.29 ± 0.35 de	4.04 ± 0.46 ab	7.33 ± 0.25 c	0.021 ± 0.00106 c
KCl 250	3.10 ± 0.24 e	4.11 ± 0.08 ab	7.21 ± 0.15 c	0.019 ± 0.00000 d
KCl 500	3.50 ± 0.27 cd	3.74 ± 0.34 bc	7.25 ± 0.11 c	0.020 ± 0.00125 cd
AsA 50 + KCl 250	3.71 ± 0.14 bc	4.54 ± 0.26 a	8.26 ± 0.25 ab	0.023 ± 0.00085 b
AsA 50 + KCl 500	4.12 ± 0.25 a	4.17 ± 0.58 ab	8.30 ± 0.38 ab	0.026 ± 0.00097 a
AsA 100 + KCl 250	3.74 ± 0.11 bc	4.31 ± 0.33 ab	8.05 ± 0.40 b	0.023 ± 0.00106 b
AsA 100 + KCl 500	3.89 ± 0.29 ab	4.58 ± 0.52 a	8.48 ± 0.23 a	0.025 ± 0.00106 a

Means of 4 replications ± SD. Using Duncan's multiple range test, means with different letters are different at $p \leq 0.05$.

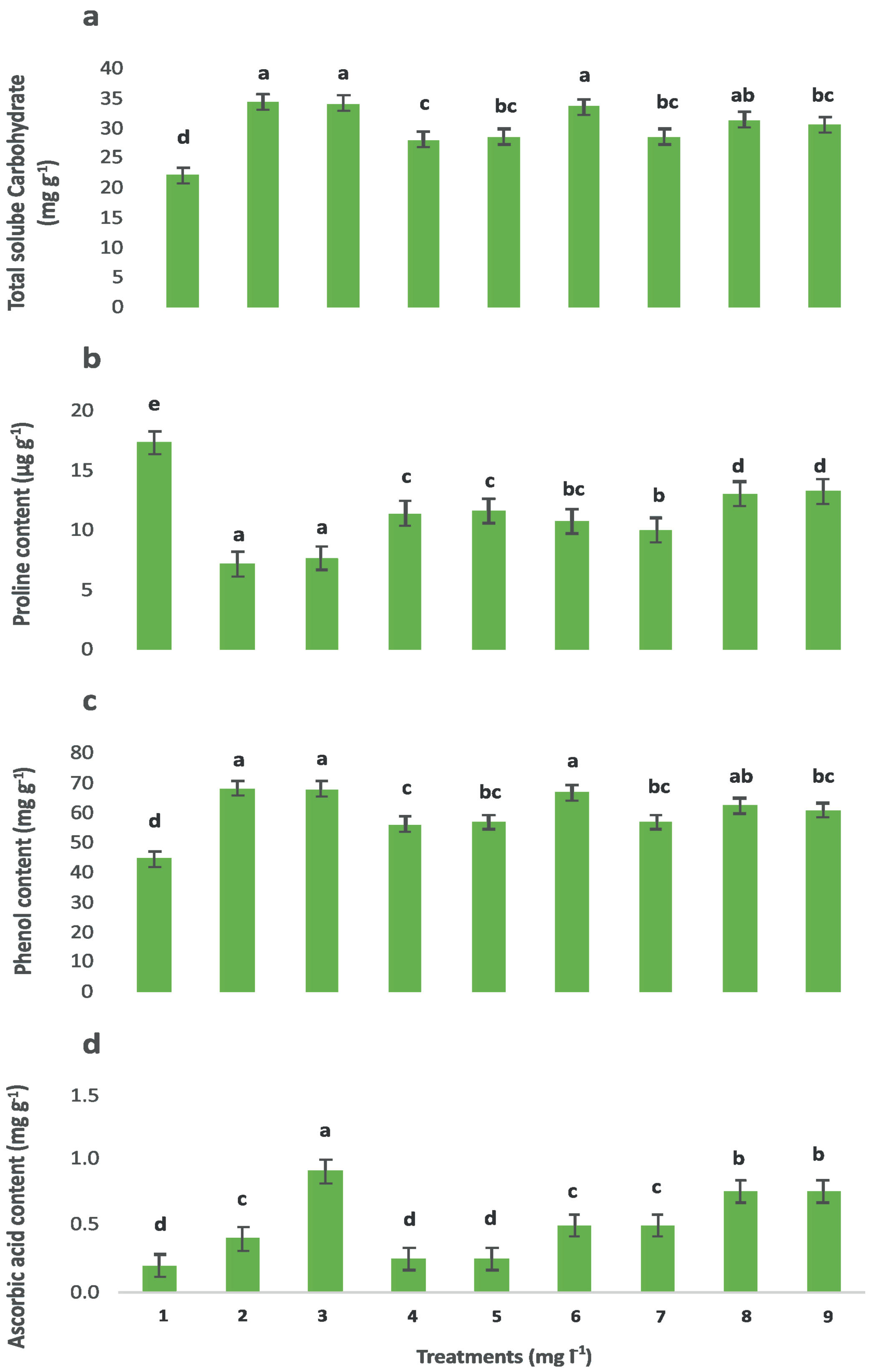

Fig. 1. Effect of exogenous application of AsA and KCl alone or combined on total soluble carbohydrate (a), proline content (b), phenol content (c), and ascorbic acid (d) content in Indian almond seedlings under high field temperature in July. Treatment numbers are described as follows: 1) Control, 2) AsA 50, 3) AsA 100, 4) KCl 250, 5) KCl 500, 6) AsA 50 + KCl 250, 7) AsA 50 + KCl 500, 8) AsA 100 + KCl 250, 9) AsA 100 + KCl 500. Data are means of four replicates ± SD. Different values within the columns show significant differences among treatments at $P \leq 0.05$ confidence level.

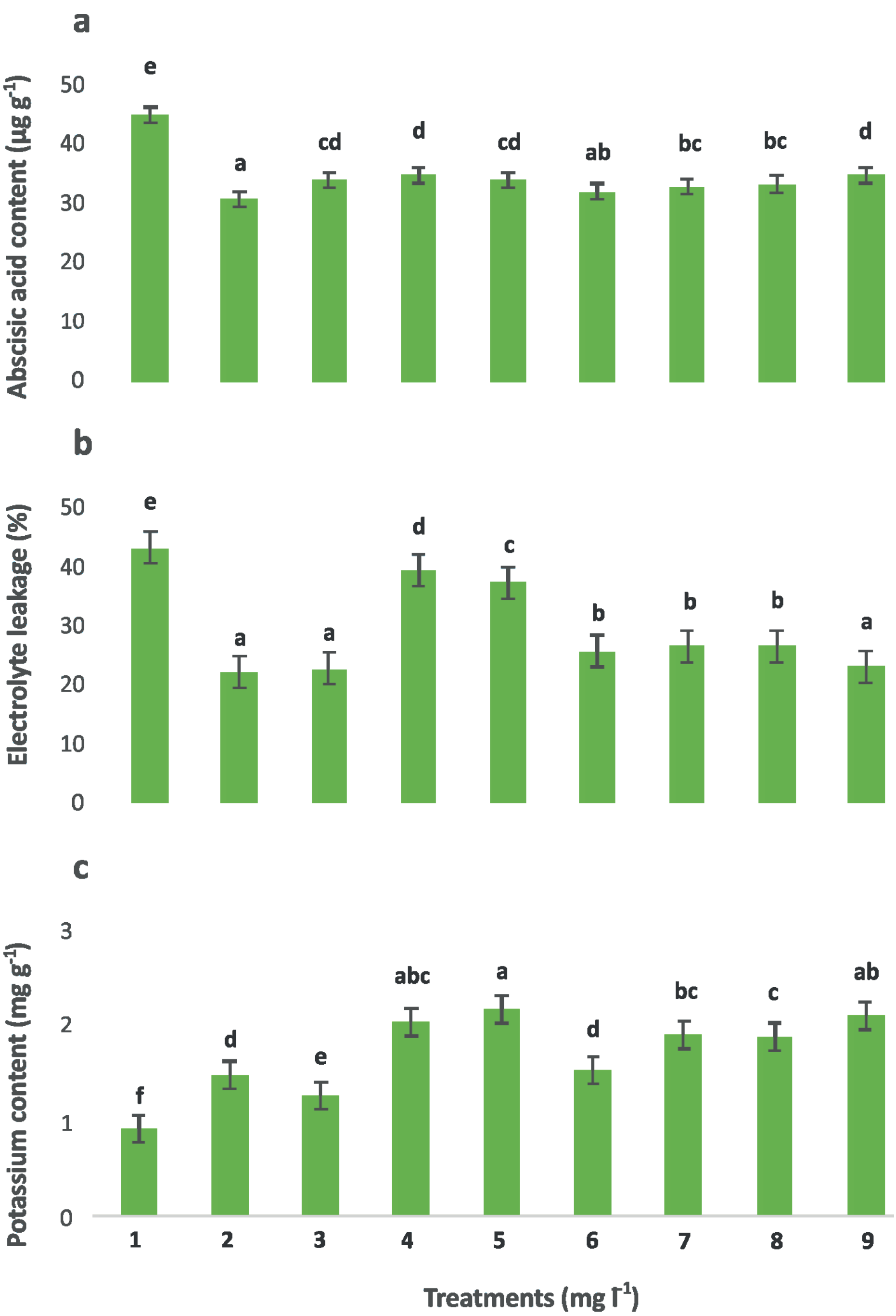

Fig. 2. Effect of exogenous application of AsA and KCl alone or combined on total abscisic acid content (a), Electrolyte leakage (%) (b), and Potassium content (c) in Indian almond seedlings under high field temperature in July. Treatment numbers are described as 1) Control, 2) AsA 50, 3) AsA 100, 4) KCl 250, 5) KCl 500, 6) AsA 50 + KCl 250, 7) AsA 50 + KCl 500, 8) AsA 100 + KCl 250, 9) AsA 100 + KCl 500. Data are means of four replicates ± SD. Different values within the columns show significant differences among treatments at $P \leq 0.05$ confidence level.

Discussion

Higher temperatures in the summer caused a decrease in growth parameters, photosynthetic pigments, and more metabolic compounds, whereas proline and abscisic acid (ABA) increased (Figs 1, 2). Water evaporates greatly from plants, causing dryness in plants. The high temperatures increase the activity of the enzyme chlorophyllase, destroy chlorophyll, and increase the level of ABA, which accelerates the decomposition of chlorophyll (Jamloki et al., 2021).

The effect of heat stress was reversed due to the foliar spray with antioxidants, as ascorbic acid is one of the non-enzymatic antioxidants that remove active oxygen radicals (ROS), which reduces the breakdown of chlorophyll pigment under stress conditions (Akram et al., 2017). The role of AsA in increasing plant growth is attributed to the increase in cell size and division rate, as well as the stimulation of photosynthesis and carbohydrate metabolism (Alabdulla et al., 2020). The effect of ascorbic acid on growth parameters agrees with many studies showing that AsA has a role in encouraging growth and reducing the harmful effect of environmental stresses, such as Faisal et al. (2014) on broad bean; Atta Ullah et al. (2016) on barley plants.

Farouk (2011) indicated that the increase in antioxidants and the decrease in hydrogen peroxide (H_2O_2) delay leaf aging. The antioxidants protect the chloroplast, preventing the breakdown of chlorophyll by scavenging ROS. In our research, the effect of heat stress was reversed due to the foliar spray with K and AsA. The increase in growth parameters when treated with potassium may be due to its positive role in improving the overall physiological activities within the plant cells, the most important of which is improving the effectiveness of photosynthesis and the composition of cell organelles (Aly et al., 2015), as well as improving hormonal balance by increasing growth-promoting hormones and reducing growth-inhibiting hormones (Hasanuzzaman et al., 2018). The reason for the increase in the leaf area can be attributed to the increase in the potassium concentration to the fact that the abundance of this nutrient for the plant in sufficient quantities is necessary for growth, specifically concerning its role in cell division, as well as improving the performance of plant hormones such as auxins and gibberellins, which directly enter into the expansion and elongation of cells, which increases leaf area (Shareef, 2019). This result agrees with Al-Furtuse et al. (2019) obtained in cowpea plants (*Vigna sinensis* L.).

The decrease in total soluble carbohydrates in control due to the effects of high temperature and exposure of seedlings to thermal quiescence programming is represented by a decrease in the leaf area and the total chlorophyll content (Tables 1, 2). It reduces the activity of the enzymes responsible for carbon dioxide reduction, especially the RuBisCO enzyme and RuBP Carboxylase (Kahrizi et al., 2012). The increase in the total soluble carbohydrates in the leaves, when sprayed with ascorbic acid and potassium, is due to these two compounds' role in increasing the efficiency of photosynthesis, and the leaf area exposed to light led to an increase in the carbohydrates of leaves. Our findings agree with those of Abd El-Aziz et al. (2009), studying gladiolus plants (*Gladiolus grandflorum* L.).

The increase in plant pigments when treated with potassium may be due to its effect on increasing the absorption of some elements, including iron and magnesium, which play an essential role in building chlorophyll, or it may be due to the role potassium plays in increasing the activity of the ATPase enzyme in plasma membranes and tonoplast (Hasanuzzaman et al., 2018).

The increased proline in plants exposed to stress may represent an adaptation to such conditions. In addition, proline protects the organelles from harmful effects such as oxidizing factors and contributes to the destruction of free radicals (Kishor et al., 2015). The treatment with the antioxidants ascorbic acid and potassium led to a modification of the proline content in the leaves. It can be attributed to their role in relieving heat stress in plants. Our findings agree with the work of Farooq et al. (2013) in wheat (*Triticum aestivum* L.).

The increase in total phenols by treatment with ascorbic acid and potassium is due to the critical role of this antioxidant in reducing respiration (Cosme et al., 2020). Increased phenols indicate plant adaptation to extreme environmental conditions by scavenging ROS (Ismael et al., 2022). Our findings agree with Abd El-Aziz et al. (2009) in gladiolus plants.

The decrease in plant growth at 49/31°C was associated with the emergence of stress damages reflected in decreased membrane stability, indicating membrane damage. It is probably due to a loss of the ability to rapidly and completely reorganize cell membranes (Shareef et al., 2020). Our results also show that heat stress decreases the membrane stability (electrolyte leakage rises), but treatment with ascorbic acid and potassium stabilized them significantly. The higher membrane stability index resulting from ascorbic acid and potassium is related to the antioxidant responses that protect the plant from oxidative damage and to the higher ionic content and induced activities of antioxidant enzymes (Da Silva et al., 2021). Our results agree with the findings of Dwivedi et al. (2018) in wheat.

Conclusion

Pre-adaptation of the Indian almond plants to high temperatures during the summer in semi-tropical regions is possible by ascorbate and/or potassium spray. Good results can be achieved by the application of AsA at 50 mg l^{-1} or a combination of ascorbic acid at 50 mg l^{-1} and potassium chloride at 250 mg l^{-1}.

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