



AMARANTHUS HYPOCHONDRIACUS L. CV. 'KIZLYARETS' RESPONSE TO HYDROGEN PEROXIDE, ASCORBIC, AND SUCCINIC ACIDS IN GROWTH UNDER DROUGHT CONDITIONS

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SUMMARY

The study aimed to investigate the effects of pre-sowing treatments with hydrogen peroxide (H₂O₂), ascorbic acid (AsA), and succinic acid (SuA) on morphological traits and photosynthetic pigments of the amaranth (*Amaranthus hypochondriacus* L. cv. 'Kizlyarets') under drought conditions. The study examined the content of antioxidants (amaranthine and ascorbic acid) in the amaranth leaves under normal and drought conditions. The results showed amaranth seed treatment with H₂O₂ (5 mmol) under optimal conditions increased the seedling height, leaf area, number of leaves, and above-ground weight of the amaranth seedlings compared with the control and two other treatments. However, under drought, the morphological traits and the photosynthetic pigments were lower than the control, except for amaranthine and ascorbic acid. The amaranth seed treatment with AsA (60 mg/l) and SuA (300 mg/l) improved the morphological traits and chlorophyll and carotenoid contents under normal and drought conditions. Thus, the application of AsA and SuA could promote the growth and development of amaranth plants with enhanced drought tolerance.

Keywords: Amaranth (*A. hypochondriacus* L.), drought stress, hydrogen peroxide, succinic and ascorbic acids, morphological traits, photosynthetic pigments, amaranthine

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Key findings: The results revealed applying AsA and SuA promotes the morphological traits and chlorophyll and carotenoid contents along with the general growth and development of amaranth (*A. hypochondriacus* L.) plants with enhanced drought tolerance.

INTRODUCTION

Drought has become the most severe natural disaster due to its frequency, duration, and widespread impact on crop plants. Global drought stress leads to a considerable decline in crop yields and may also surpass the impact of other yield-limiting factors. Drought, being a severe abiotic stress, disrupts the intracellular water relation, decreases seed germination inhibiting plant growth and development, disrupts photosynthesis and ion homeostasis, and boosts the production of reactive oxygen species (Wojtyla *et al.*, 2020).

Plants produce a class of secondary metabolites with a polyphenolics structure, which are biologically active compounds. These compounds emerge as a defense mechanism when the plants go through stress (Panche *et al.*, 2016). The drought stress effect on the accumulation, composition, and bioactive compound activity in various plant species is still to be fully understood. However, the plants' genetic makeup, developmental stages, exogenous compounds, and existing stress factors determine the type and concentration of bioactive compounds (Barba *et al.*, 2019). Several studies have demonstrated that drought and seed treatment with exogenous compounds can enhance the quantitative and qualitative levels of nutrients and bioactive compounds, such as flavonoids. This effect, in turn, increases the antioxidant activity of various tolerant leafy vegetables, including amaranth (Sarker *et al.*, 2017; Al-Huqail *et al.*, 2020).

Amaranth (*Amaranthus hypochondriacus* L.) can thrive in poor soils and withstand extreme weather conditions, such as low and high temperatures and limited rainfall (Nogoy *et al.*, 2021). Numerous studies have shown that at the seedling stage, water stress affects growth and development, organ formation, and seed yield of amaranth plants (Yu *et al.*, 2022; Motyleva *et al.*, 2022). Past

studies revealed major drought-induced yield loss resulted from the disruption and reduction in plant photosynthesis. In crop plants, chlorophyll is an important pigment playing a vital role in photosynthetic capacity (Polyakov *et al.*, 2023). Chlorophyll fluorescence measurements, particularly of chlorophyll a, are valuable tools for assessing the physiological state of a plant organism and its economic efficiency (Shafigullin *et al.*, 2021).

Amaranth is one of the pseudo-cereal food crops, and its enhanced seed production can be crucial in combating world hunger and food security (Jimoh *et al.*, 2022; Gins *et al.*, 2024a). This crop is rich in biologically active substances with antioxidant activity. The extracts of the leaves and inflorescences of *Amaranthus paniculatus* L. of the Pamyati Kovas variety are rich in betacyanins (0.2–2.1 mg/g FW), ascorbic acid (80–160 mg/g FW), and simple phenols and hydroxybenzoic acids (0.34%–0.56% DW). Moreover, they contain hydroxycinnamic acids (0.10%–0.14% DW), flavonoids (4.2%–4.5% DW), and condensed and polymer polyphenol flavonoids (0.55%–0.89% DW) (Gins *et al.*, 2021). The red-leaved amaranth variety Valentina (*Amaranthus tricolor* L.) has the most interesting antioxidant profile; namely, the total antioxidant content was 1.4 mg/g FW, amaranthine was 2.0 mg/g FW, and polyphenols were 4.8 mg/g FW. Green-leaved forms of amaranth Krepys (*Amaranthus hypochondriacus* L.), Pamyati Kovas (*Amaranthus cruentus* L.), and Kizlyarets (*Amaranthus hypochondriacus* L.) had a comparable total antioxidant content at 0.57–0.60 mg/g GAE FW and a total polyphenol content at 4.3–4.6 mg/g GAE FW (Gins *et al.*, 2024a).

Accurate prediction and assessment of drought effect on amaranth seed production is critical. Candyce *et al.* (2022) systematically examined the morphological, biochemical, and physiological (photosynthesis-related) traits in amaranth (*A. dubius* L.) for high-temperature

tolerance. Gins *et al.* (2024b) and Jamalluddin *et al.* (2021) analyzed the effect of drought and cold stress on plant growth and development, chlorophyll a fluorescence parameters, ROS markers, compatible solvents, and antioxidant activities in amaranth (*A. tricolor* L.). The dark-adapted quantum yield (Fv/Fm) could be a useful parameter for identifying drought tolerance in *A. tricolor* (Jamalluddin *et al.*, 2021). A considerable achievement has occurred in assessing and comparing the effects of average temperature, extreme heat, and drought stress on the growth of amaranth plants. However, no studies have systematically compared the drought-adaptive capacity of *A. hypochondriacus* L., the Kizlyarets variety with a green and red leaf form, which made the researchers choose this for the object of study.

The pre-sowing seed treatment is an essential approach for evaluating the productivity response traits of amaranth for developing the adaptation measures in the context of climate change and water stress conditions (Zhu *et al.*, 2021). Therefore, an urgent need to study and evaluate the effect of seed treatment is necessary on the resistance development in amaranth plants under drought stress conditions. The compounds listed below can be applicable as seed treatments that considerably provide drought resistance to the C4 plant amaranth. Hydrogen peroxide (H₂O₂) plays a crucial role, and its small doses have proven to promote plant growth by regulating growth processes, such as cell wall relaxation and cell elongation and division. Additionally, it also promotes the enhancement of morphological traits like roots, stems, and leaves in maize (Gondim *et al.*, 2011). As an oxidative signaling molecule, H₂O₂ contributes to numerous signal transduction pathways in crop plants.

H₂O₂ interaction with other signaling molecules can influence gene expression, activate signaling pathways, and regulate plant growth and stress response in potato (Lei *et al.*, 2023). Moderate H₂O₂ can activate the antioxidant defense system, increasing their resistance to adverse factors, such as drought, salinity, pests, and diseases, and helping maintain stable plant growth in maize and

cherry tomatoes (Gondim *et al.*, 2011; Ishu *et al.*, 2024; Guedes *et al.*, 2024). Ascorbic acid (AsA), also known as vitamin C, is crucial in regulating plant growth and development. It acts as an antioxidant and signaling molecule that influences various physiological processes in crop plants (Celi *et al.*, 2023). The importance of AsA in plant physiology reached highlights for its vital role in mediating gene expression and protein synthesis in tomato (Xu *et al.*, 2022; Fu *et al.*, 2023). By regulating the transcription of genes, it facilitates adaptive responses to environmental stimuli in *Cardamine hirsuta* L. (Kejariwal *et al.*, 2017). The succinic acid (SuA) influenced various physiological processes, such as seed germination, root development, photosynthesis, and stress responses in flowering kale (Kiliç, 2023).

The following study evaluated the role of H₂O (the control), H₂O₂, AsA, and SuA in improving the morphological, physiological, and biochemical characteristics of amaranth seedlings under drought. Exogenous application of H₂O₂, AsA, and SuA has been shown to affect plant growth and development (Nurnaeimah *et al.*, 2020; MacDonald *et al.*, 2022; Kiliç, 2023). Efforts have progressed to study how seed treatments with these substances induce drought tolerance in amaranth seedlings and find out the most effective seed treatments.

MATERIALS AND METHODS

Seed treatment

The promising studies commenced in the Laboratory of Physiology, Biochemistry, Introduction, and Functional Products at the Federal State Budgetary Scientific Institution Federal Scientific Vegetable Center, Russia. The experiment layout had a randomized complete block design with five replicates. The seeds of amaranth (*A. hypochondriacus* L. cv. 'Kizlyarets') (registered in 2004 by the Russian Federation) came from the aforementioned Federal Scientific Vegetable Center. The cultivar Kizlyarets seemed to be able to withstand drought conditions. The treatment

solutions used were distilled water (as the control), hydrogen peroxide (H₂O₂, 5 mmol), ascorbic acid (AsA, 60 mg/l), and succinic acid (SuA, 300 mg/l). The selection of all the solution concentrations occurred in preliminary experiments. The amaranth seeds, evenly placed on moistened double-layer filter paper, became sealed in a box (9 cm × 9 cm × 3 cm) and kept at 25 °C for six hours. Thereafter, the immediate washing of the remaining treatment solution from the surface of the seeds used distilled water. In the amaranth seeds, the excess moisture attained absorption with blotting paper, with the seeds air-dried under natural conditions until obtaining the initial seed mass.

Experimental procedure

After acquiring the initial seed mass, sowing of 10 soaked seeds of amaranth from each treatment followed in plastic pots (9 cm diameter and 10 cm depth), with each one containing 200 g of organic soil. At the beginning of the experiment, both treatments entailed ordinary water treatment to ensure normal growth of the amaranth seedlings. The seeds received tap water irrigation once a day for three days, then twice a week at 60% field capacity, as grown under natural environmental conditions (16/8 h, 25 °C/15 °C ± 2 days/night, 65% relative humidity) for 20 days. Consequently, a drought treatment followed (50% of the irrigation rate of the normal group) for the drought group, keeping the total weight constant. The watering intervals remained at twice per week. The incubation continued until the end of the experiment (40 days). The amaranth seedlings underwent processing, with aerial parts weighed to obtain morphological traits, and the amaranth leaves collected at 0.2 g and 10 g for subsequent analysis.

Biomass measurement

Amaranth cv. Kizlyarets incurred measurements for above-ground mass, plant height, leaf area, and the number of true leaves.

Photosynthetic pigments and amaranthine

The use of the spectrophotometric method helped determine the photosynthetic pigment content. Calculating the chlorophyll a, b, the total chlorophyll, and the carotenoid contents followed the formulas below (Gins *et al.*, 2024a).

$$\text{Chlorophyll a (mg/g)} = 0.001(13.36A_{664.2} - 5.19A_{648.6})V/m$$

$$\text{Chlorophyll b (mg/g)} = 0.001(27.43A_{648.6} - 8.12A_{664.2})V/m$$

$$\text{Chlorophyll a+b (mg/g)} = \text{Chlorophyll a} + \text{Chlorophyll b}$$

$$\text{Carotenoids (mg/g)} = 0.001(4.785A_{470} + 3.657A_{664.2} - 12.76A_{648.6})V/m$$

Where:

A₄₇₀, A_{648.6}, and A_{664.2} = the absorption at 470, 648.6, and 664.2 nm, respectively, and the thickness of the cuvette was 1 cm;

V = the volume of the extract (ethanol 96%) in ml; and

m = the fresh weight of the sample in g.

The amaranthine content calculation used the following formula:

$$\text{Amaranthine (mg/g)} = A_{536}(MW)V(DF)/\epsilon Lm$$

Where:

A₅₃₆ is the absorbance at 536 nm (λ_{max}), V is the total extract volume, DF is the dilution factor, L is the path length of the cuvette, and m is the fresh weight of the sample in g. The molecular weight (MW) and molar extinction coefficient (ε) of amaranthine were 726.6 and 5.66 × 10⁴ M⁻¹cm⁻¹, respectively (Gins *et al.* 2024b).

Ascorbic acid determination

Ascorbic acid measurement employed Dzhos *et al.*'s (2024) methodology with some modifications. In measuring the ascorbic acid content, sample macerating comprised 1% HCl and 1% C₂H₂O₄. An aliquot from the sample underwent titration with Tillman's reagent (2,6-dichlorophenol indophenol) until a pale pink endpoint was visible, which persisted for

15–20 s (Dzhos *et al.*, 2024). When measuring ascorbic acid using Tillman's reagent, it determines the reduced form of ascorbic acid.

Statistical analysis

All the recorded data's analysis engaged the Microsoft Excel 2016 software package. The reported data represent the means of at least five replicates $\pm$ standard errors (SEs). The data, as analyzed, used the analysis of variance (ANOVA), followed by Duncan's Multiple Range Test at $p \leq 0.05$ using SPSS for Windows. Means with one or more identical letters in common do not differ significantly from each other.

RESULTS AND DISCUSSION

Seed treatment effect on amaranth biomass

The present results detailed that the seed treatment with appropriate concentrations of H_2O_2 , AsA, and SuA considerably improved the morphological traits of amaranth seedlings under normal conditions (Figure 1). Compared with the control treatment under normal conditions, the amaranth seed treatment with H_2O_2 , AsA, and SuA enhanced the seedlings' height by 68%, 82%, and 103%; the leaf area by 93%, 22%, and 27%; the number of true leaves by 34%, 19%, and 16%; and enhanced the above-ground biomass weight of amaranth by 324%, 102%, and 178%, respectively (Figure 2). Several studies showed H_2O_2 , AsA, and SuA have a positive impact on the growth and development of various crop plants (Yao *et al.*, 2021; Saavedra *et al.*, 2022; Tania *et al.*, 2022; Zahid *et al.*, 2023).

After a 20-day drought stress experiment, various physiological parameters of the amaranth seedlings in the experimental group declined to varying degrees (Figure 3). Among them, drought control reduced the plant height in amaranth by an average of 27%, the leaf area by 48%, the number of true leaves by 13%, and the above-ground biomass weight by 29% compared with the control

(Figure 2). Drought, as one of the most severe abiotic stress factors, has tremendously affected the growth and development and biochemical traits of the amaranth seedlings (Motyleva *et al.*, 2022).

However, under drought conditions, the pre-sowing treatments with three compounds (H_2O_2 , AsA, and SuA) had varied effects on the morphological characteristics of amaranth seedlings in the fight against drought. The amaranth seed treatment with H_2O_2 showed primary manifestations of a decrease in the seedling plant height by 7%, a decline in the leaf area by 54%, and a reduction in the number of true leaves by 21%. Moreover, it lessened above-ground biomass weight by 90% compared with the control under drought conditions. Based on the results, one can conclude that the pre-sowing seed treatment of amaranth with low concentrations of H_2O_2 has a positive effect on the amaranth growth under normal conditions; however, it does not help the amaranth plants in reducing drought stress under drought conditions (Figures 2 and 3). The application of H_2O_2 showed a considerable positive effect by improving the growth and development of maize (Gondim *et al.*, 2011) and cherry tomatoes (Guedes *et al.*, 2024).

Furthermore, the application of AsA and SuA considerably alleviated the negative effects of drought stress on the amaranth seedlings versus the control under drought conditions. The main manifestation made with the amaranth seed treatment with AsA and SuA was the increase of plant height by 67% and 75%; the leaf area by 85% and 102%; the number of true leaves by 18% and 25%; and the above-ground biomass weight enhanced by 41% and 75% under drought stress conditions compared with the control (Figure 2). Based on these results, a conclusion can be that the amaranth seed treatment with AsA and SuA could improve the morphological traits under drought conditions. Past studies revealed AsA (150 mg/l) proved effective in improving drought tolerance in wheat (Al-Kazzaz, 2023). As a regulator of redox processes, AsA maintains the balance of reactive oxygen species in plant cells, preventing oxidative



Figure 1. General view of 40-day amaranth plants with pre-sowing seed treatments (from left to right: control, 5 mmol H_2O_2 , 60 mg/l AsA, and 300 mg/l SuA) under normal conditions (16/8 hours, 25 °C/15 °C $\pm$ 2 day/night, 65% relative humidity).

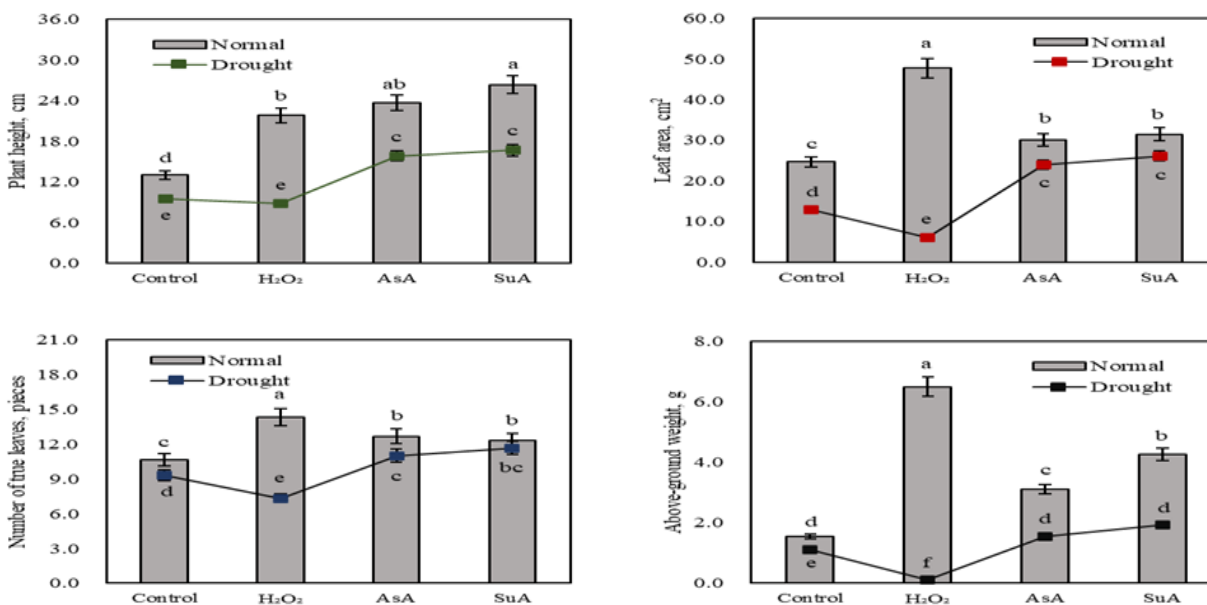


Figure 2. Effect of pre-sowing seed treatment with different preparations (control: H_2O , H_2O_2 : 5 mmol, AsA: 60 mg/l, and SuA: 300 mg/l) on morphological traits of amaranth plants under normal and drought conditions. Bars are means $\pm$ SE.



Figure 3. General view of 40-day amaranth plants with pre-sowing seed treatment under normal (two pots on the left) and drought (two pots on the right) conditions (A: control, B: 5 mmol H_2O_2 , C: 60 mg/l AsA, and D: 300 mg/l SuA).

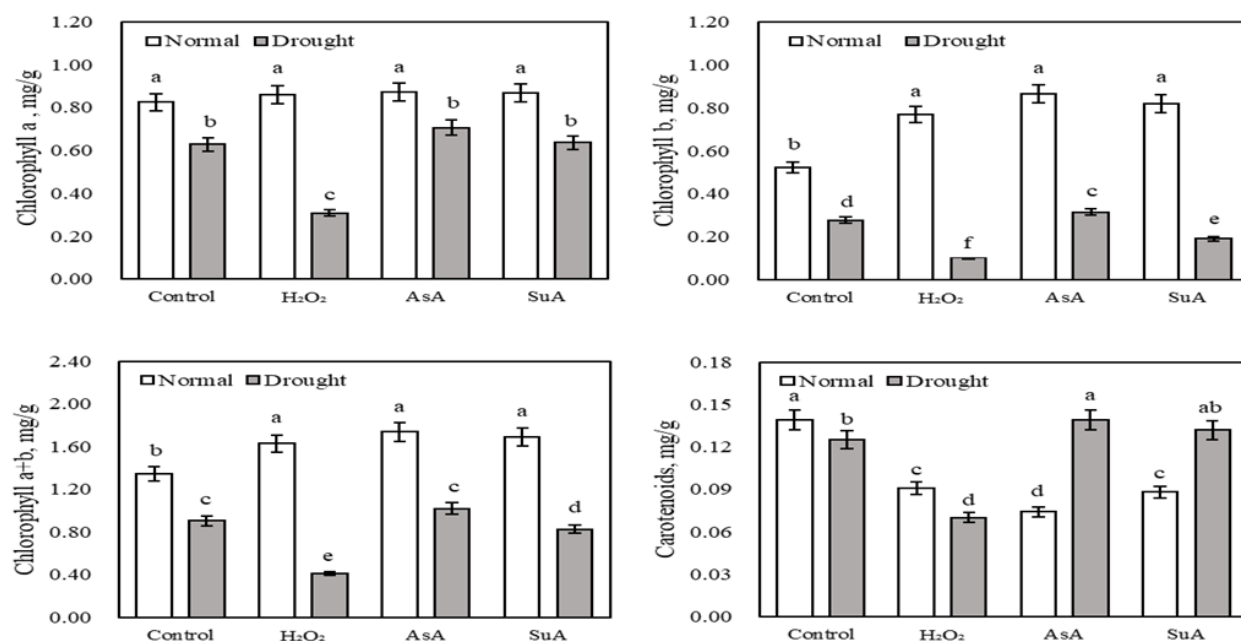


Figure 4. Effect of pre-sowing seed treatment with different preparations (control: H₂O, H₂O₂: 5 mmol, AsA: 60 mg/l, and SuA: 300 mg/l) on photosynthetic pigments in amaranth plants under normal and drought conditions. Bars are means $\pm$ SE.

damage and promoting the growth and development of *Amaranthus tricolor* (Gins *et al.*, 2024b). By treating the maize seeds with SuA, it enhanced the drought tolerance, as well as significantly improved the seed germination, seedling growth, moisture content, chlorophyll content, and antioxidant enzyme activity (Choudhary and Kumar, 2015).

Seed treatment effect on photosynthetic pigments

In the amaranth leaves, the analysis of chlorophylls and carotenoids revealed drought stress and pre-sowing seed treatments correlated with certain variations in these traits (Figure 4). Compared with the control group, the seed treatment with H₂O₂, AsA, and SuA resulted in a significant increase in chlorophyll content in amaranth leaves under normal conditions. Notably, the chlorophyll b content rose by 47%, 66%, and 57%, respectively. However, with all three seed treatments, the carotenoid content decreased versus the control in the amaranth leaves. Reports stated ascorbic acid can mitigate abiotic stresses and

play a positive role in managing the physiological and biochemical parameters of the plants (Celi *et al.*, 2023).

In the seed treatment with H₂O₂, the chlorophyll and carotenoid contents in the amaranth leaves under drought conditions were approximately 0.5 times lower than the control. However, by treating with AsA under drought conditions, an increase appeared in chlorophyll a, b, and carotenoids (12%, 14%, and 11%, respectively) compared with the control in the amaranth leaves. However, the chlorophyll and carotenoid contents in the amaranth leaves treated with SuA did not differ significantly ($p \leq 0.05$) from the control group under drought conditions. As a multifaceted regulator of plant growth, AsA contributed to photosynthesis, cell division, hormone biosynthesis, and stress response in plants (Viviani *et al.*, 2021). Additionally, AsA functions as a signaling molecule in plant signaling pathways, interacting with other phytohormones, such as abscisic acid, ethylene, and auxins, to coordinate the growth and development processes.

Seed treatment effect on amaranthine and ascorbic acid

In the amaranth plants, the amaranthine pigment derived from ketones and quinones is a secondary metabolite, belonging to the betacyanins subtype of bio-pigments in the betalain family and is distinct from anthocyanins (Gins *et al.*, 2024b). Amaranthine has various physiological and medicinal functions, including the prevention of cardiovascular diseases and protection of vision, as well as antioxidant, anti-aging, and anti-inflammatory effects (Sarker and Oba, 2019).

The seed treatment with H₂O₂ considerably increased the amaranthine content (by 5%) compared with the control in amaranth plant leaves under normal conditions (Figure 5). However, the amaranth seeds treated with AsA and SuA showed a lower content of amaranthine than the control under optimal conditions. Under drought conditions, the seeds treated with H₂O₂, AsA, and SuA boosted the amaranthine content in the leaves of *A. hypochondriacus* L. cv. Kizlyarets by 9%, 88%, and 76%, respectively, versus the control. The H₂O₂, AsA, and SuA treatments increase the amaranthine content in amaranth leaves under drought through different mechanisms (such as activating defense responses, regulating energy metabolism, and providing synthetic precursors) to help plants cope with adversity (Gill and Tuteja, 2010;

Miura and Tada, 2014; Choudhary and Kumar, 2015).

Under normal conditions, the amaranth seed treatment with H₂O₂ increased the ascorbic acid content in the amaranth leaves by 21% compared with the control (Figure 6). However, the seed treatment with AsA and SuA reduced the ascorbic acid content in the amaranth leaves by 20% and 34%, respectively. Under drought conditions, the amaranth seed treatment with H₂O₂, AsA, and SuA enhanced the ascorbic acid content in amaranth leaves by 48%, 72%, and 61%, respectively, against the control. The increase in ascorbic acid content in amaranth leaves may activate and enhance the antioxidant defense system through H₂O₂, AsA, and SuA. Likewise, they could regulate gene expression and antioxidant enzyme activity, provide energy and precursor substances, and help plants cope with oxidative stress caused by drought and maintain cell homeostasis (Smirnov, 2018).

The amaranth seed treatment with H₂O₂ increased the antioxidant content in the amaranth leaves, specifically the content of amaranthine and ascorbic acid, compared with the control under normal and drought conditions. The results showed H₂O₂ treatment could be a viable treatment for increasing the antioxidant levels in amaranth leaves. Mittler *et al.* (2004) reported the role of reactive oxygen species and their impact on the antioxidant levels, such as ascorbic acid, in

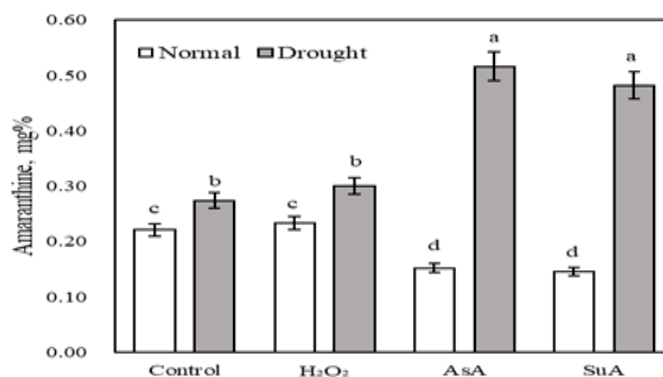


Figure 5. Effect of pre-sowing seed treatment with different preparations (control: H₂O, H₂O₂: 5 mmol, AsA: 60 mg/l, and SuA: 300 mg/l) on amaranthine content in amaranth plants under normal and drought conditions. Bars are means $\pm$ SE.

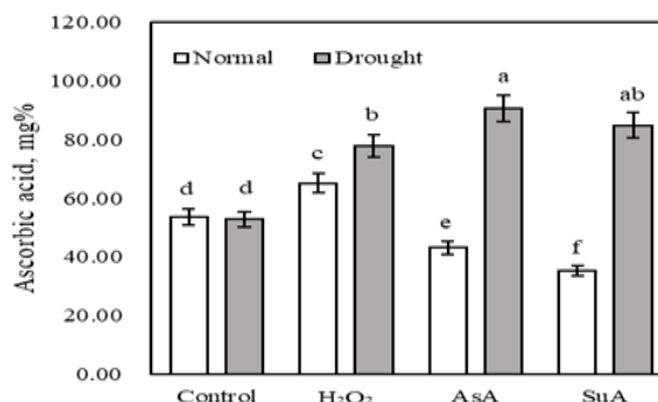


Figure 6. Effect of pre-sowing seed treatment with different preparations (control: H₂O, H₂O₂: 5 mmol, AsA: 60 mg/l, and SuA: 300 mg/l) on ascorbic acid in amaranth plants under normal and drought conditions. Bars are means $\pm$ SE.

plants. As a form of abiotic stress, a low concentration of H₂O₂ stimulates the synthesis of amaranthine in amaranth plants, improving its functions and increasing its ability to withstand stress (Tania *et al.*, 2022). The H₂O₂ acts as a signaling molecule involved in adaptive signaling, triggering tolerance against various abiotic stresses at low concentrations (Almeida *et al.*, 2005).

The amaranth seeds' treatment with AsA and SuA had varying effects on the antioxidant content in the amaranth leaves under normal and drought conditions. Under drought conditions, the content of amaranthine and ascorbic acid in the leaves increased by 60%, while it decreased by 20% under normal conditions. Additionally, an elevated content of the antioxidants in amaranth plants can enhance their tolerance to drought stress. The AsA regulates plant growth under drought conditions. As an antioxidant, AsA controls plant growth during drought conditions by scavenging free radicals, reducing cell oxidative damage, and maintaining cellular homeostasis. The AsA also adjusts the osmotic pressure balance of cells, helping plants maintain water balance during drought conditions. It regulates cellular water absorption and release, reduces water loss, and helps plants to cope with drought conditions. Previous studies described the mechanisms by which ascorbic acid regulates plant development under drought conditions

(Noctor and Foyer, 2016; Jiang and Zhang, 2001).

Furthermore, AsA serves as a signaling molecule to regulate various plant adaptive responses under drought stress. It interacts with other signaling molecules, affects plant gene expression, regulates the metabolic pathways, and coordinates plant growth and development under drought stress conditions. The SuA has been evident to stimulate root growth in plants. It enhances the activity of nutrient transporters in root cells, facilitating the uptake of minerals, such as nitrogen, phosphorus, and potassium, from the soil. Kiliç has reported about SuA being a plant growth regulator, highlighting its mechanisms of action, interactions with plant hormones, and its positive role in enhancing plant growth and stress tolerance (Kiliç, 2023).

CONCLUSIONS

The findings demonstrated that under normal conditions, the amaranth (*A. hypochondriacus* L.) cv. Kizlyarets seedlings treated with H₂O₂, had the largest leaf area, the highest number of true leaves, and maximum above-ground biomass weight. Moreover, such seedlings with H₂O₂ treatment provided higher levels of chlorophyll, amaranthine, and ascorbic acid than the control and other two treatments; however, these parameters decreased under

drought conditions. Regarding the amaranth seeds' treatment with ascorbic and succinic acids, the morphological traits and the chlorophyll and carotenoid contents considerably improved under normal and drought conditions. Therefore, the application of ascorbic and succinic acids can promote growth and development, improve drought tolerance, and potentially reduce the negative effects of drought stress on amaranth seedlings.

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