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Improving Tolerance of Cotton (*Gossypium barbadense* L.) to Water Deficit by Salicylic Acid and Proline

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ABSTRACT

Amidst global climate change and the disruption of ecological equilibrium, water deficit stress emerges as one of the foremost abiotic constraints affecting cultivated crops, particularly cotton. Therefore, it is essential to study the effect of prolonging irrigation intervals on plant growth, physiological, biochemical and internal anatomical structure parameters of cotton plants *Gossypium barbadense* L. cv. Super-Giza 94. Prolonging irrigation intervals (every 30 and 45 days) decreased plant height, dry weight, leaf area, relative water content, chlorophyll pigments content or yield as well as fiber quality. On the other side, in cotton plants exposed to water deficit increased endogenous proline content. The combined application of Salicylic acid (SA) and proline effectively alleviated oxidative stress by enhancing antioxidant enzymatic activity, which was more pronounced compared to when they were applied separately. Thicker and wider stem and leaf tissues as well as xylem vessels were noticed by SA, proline and their interaction under water deficit stress conditions. The plant height, leaf area, chlorophyll pigments, relative water content, yield, and fiber quality were improved through the foliar application of SA and proline, either individually or in combination. This approach shows promise for promoting sustainable agriculture in demanding environmental conditions.

Keywords: *Gossypium barbadense* L., water deficit stress, drought tolerance inducers, antioxidant enzymes, cotton, fiber quality, chlorophyll pigments.

INTRODUCTION

Egyptian cotton (*Gossypium barbadense* L.) is internationally recognized for its exceptional quality as it possesses extra-long staple fibers. It is highly regarded as a premium cotton variety due to its superior fiber characteristics. Cotton is a significant agricultural crop cultivated across diverse regions worldwide. However, it is worth noting that cotton is highly susceptible to environmental stresses as well as drought stress. Farmers and researchers often need to employ various strategies to mitigate these environmental stresses and ensure successful cotton cultivation (Iqbal et al., 2013; Mahdi et al., 2020).

Drought stress is acknowledged as a highly damaging environmental pressure, greatly affecting the productivity of crop plants worldwide. When faced with drought stress, plants initiate different complex mechanisms to deal with unfavorable conditions. One such mechanism entails activating antioxidant defense system (Khalvandi et al., 2021). Drought increases accumulation of reactive oxygen species (ROS) in cellular organelles like chloroplasts, mitochondria or peroxisomes, harmfully impacting various processes like transpiration, photosynthesis and growth parameters (Abd El-Mageed et al., 2016). Indeed, there is a growing need to enhance water stress tolerance of field crops to

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optimize their productivity, especially given the limited availability of water resources. In recent times, there has been increased interest in exploring the potential of safe and natural antioxidants, such as salicylic acid and proline to mitigate effects of drought stress (Mahdi et al., 2020; El-Beltagi et al., 2023). Under normal conditions, plants produce ROS as by products of various metabolic processes. However, when plants are subjected to water deficit stress, their ability to regulate ROS levels becomes disrupted. This imbalance occurs because water deficiency can lead to stomatal closure, reducing the availability of carbon dioxide for photosynthesis. As a result, electron transport chain in chloroplasts becomes overexcited, leading to production of excess ROS (Sohag et al., 2020). Antioxidant enzymes are nature's defense against harmful molecules called ROS. These ROS are generated naturally during normal metabolism, but their levels can increase due to factors like water deficit stress. When ROS levels become too high, they can damage cells and tissues. Prolonging irrigation intervals significantly increased antioxidant enzymes activity like catalase (CAT), poly phenol oxidase (PPO) and peroxidase (POX).

Salicylic acid (SA) is well-known natural signaling molecule that plays significant role in plant growth, development, or stress responses. Salicylic acid is an antioxidant and is considered a phytohormone-like structure involved in various physiological processes in plants (Mahdi et al., 2020; Rharbi et al., 2023). Salicylic acid enhances plant tolerance to various biotic or abiotic stresses by altering enzymatic antioxidant activities and decreasing generation of ROS. It helps scavenge harmful free radicals generated during stress, protecting plant cells from damage. Numerous studies have indeed shown that applying SA can significantly enhance plant growth, productivity, and biochemical attributes under water stress conditions for various crops (Hafeza and Seleiman, 2017; El-Beltagi et al., 2022a,b).

Salicylic acid is involved in activation of physiological processes in plants such as stomatal regulation, nutrient uptake, chlorophyll synthesis, protein synthesis, suppression of ethylene biosynthesis and transpiration. Salicylic acid acts as a long-distance signal, triggering stomatal regulation. This helps conserve water which increases relative water content (RWC). At the same time, SA inhibits transpiration and ethylene biosynthesis (Khan et al., 2003; Shakirova et al., 2003).

Proline (Pro) is a small and non-toxic organic molecule that accumulates in plant cells under stress conditions like drought. It plays a crucial role in osmotic adjustment (osmolyte) as an adaptive mechanism of drought tolerance and detoxify ROS (Ashraf and Foolad, 2007). Proline's unusual structure also contributes to its ability to stabilize proteins and other macromolecules. His stability is essential for proper function of these molecules in various biological processes (Loutfy et al., 2022). In rice, the simultaneous use of salicylic acid (SA) and proline resulted in a more significant reduction of the negative impacts of drought stress compared to when each was applied individually. This was accomplished through heightened activity of antioxidant enzymes and the suppression of oxidative stress (Urmi et al., 2023). It has been widely reported that plants accumulate a variety of compatible solutes such as proline, as adaptive mechanism of tolerance to salinity or drought (Ashraf and Foolad, 2007; Petcu et al., 2007). It is suggested that exogenously applied of proline might have caused enhanced endogenous proline accumulation under water deficit stress, which increased antioxidant enzyme activities (Hoque et al., 2007), acted as osmolyte (Subbarao et al., 2001; Din et al., 2011) and improving metabolic functions of the cell (Mafakheri et al., 2010).

The application of salicylic acid increased the thickness of mesophyll tissues in tomato leaves under water deficit stress (Lobato et al., 2021). Similarly, exogenous proline can regulate the tissue structure of wheat flag leaves under drought stress, ultimately mitigating the impact of drought on wheat yield (Li et al., 2024). This enhancement of chlorenchyma tissues helps maintain an appropriate rate of photosynthesis (Ennajeh et al., 2010). Given the limited literature on this specific interaction, detailed anatomical studies would provide valuable insights into the structural adaptations facilitated by these compounds under water stress conditions.

The hypothesis proposes that foliar spraying salicylic acid and proline will help cotton plants better handle water deficit stress, supported by existing research on their stress-alleviating roles. Hence, the present study sought to explore the impacts of externally administered salicylic acid at a concentration of 150 mg/L and proline at 60 mg/L, along with their potential interaction, on various parameters including growth, physiological, biochemical, anatomical, yield, and yield components of cotton (*Gossypium barbadense* var. Super-Giza 94) under conditions of drought stress.

MATERIAL AND METHODS

Two field experiments were conducted at the Sakha Agricultural Research Station, Kafr El-Sheikh Governorate, specifically in Cotton Physiology Research Department and the laboratory of Agricultural Botany Department, Faculty of Agriculture, Kafrelsheikh University. Experiments aimed to investigate the response of cotton (cv. Super-Giza 94) to water deficit and improvement of growth under water deficit conditions using salicylic acid and proline treatments.

The experimental treatments consisted of twelve treatments with three replicates. The experimental design consisted of experimental plots, with each plot consisting of five rows. Each row was 4 meters long and 70 cm wide (plot area of 14 m^2). Seeds were sown on May 1 and 3 during the 2021 and 2022 seasons, respectively. There are approximately 140 plants in each plot. The treatments details are given as shown in Table 1.

Table 1. The experimental treatments: factor A (irrigation intervals), factor B (drought tolerance inducers) and factor A x B (irrigation intervals and drought tolerance inducers combination)

Factor A	Factor B	Factor A x B
Every 15 days (I1)	• Proline at 60 mgL^{-1} (Pro) • Salicylic acid at 150 mgL^{-1} (SA) • Proline + salicylic acid combination (Pro+SA)	○ I1 + Proline at 60 mgL^{-1} (Pro) ○ I1 + Salicylic acid at 150 mgL^{-1} (SA) ○ I1 + Proline + salicylic acid combination (Pro+SA)
Every 30 days (I2)	• Proline at 60 mgL^{-1} (Pro) • Salicylic acid at 150 mgL^{-1} (SA) • Proline + salicylic acid combination (Pro+SA)	○ I2 + Proline at 60 mgL^{-1} (Pro) ○ I2 + Salicylic acid at 150 mgL^{-1} (SA) ○ I2 + Proline + salicylic acid combination (Pro+SA)
Every 45 days (I3)	• Proline at 60 mgL^{-1} (Pro) • Salicylic acid at 150 mgL^{-1} (SA) • Proline + salicylic acid combination (Pro+SA)	○ I3 + Proline at 60 mgL^{-1} (Pro) ○ I3 + Salicylic acid at 150 mgL^{-1} (SA) ○ I3 + Proline + salicylic acid combination (Pro+SA)

Fertilization followed the guidelines provided by the Cotton Research Institute (CRI). Each plot received fertilization at rate of 60 kg N/fed using urea (46.5% N) in two equal dose. Initial dose was applied post-thinning (before the first irrigation), while second dose was administered before second irrigation. Phosphorus fertilizer, in the form of calcium superphosphate (15.5% P_2O_5), was incorporated during soil preparation at a rate of 15.5 kg P_2O_5/fed. Potassium fertilizer, as potassium sulfate (48% K_2O), was applied post-thinning at a rate of 24 kg K_2O/fed. Pesticide treatments adhered to recommendations outlined by Egyptian Ministry of Agriculture for cotton cultivation. Additionally, plants were sprayed twice with salicylic acid (TECHNOGENE, 2-hydroxybenzoic acid 98%, $C_7H_6O_3$) at 150 mg/L and proline (A German Company, LANXESS, L-Proline, 99%, 147-85-3, India) at (60 mg/L), with the initial application occurring 45 days post-sowing and the subsequent application 15 days thereafter. The application was done using 1 L per plot from each used concentration.

Leaf area

Leaf area was estimated using three samples collected after 70 days from sowing using leaf area meter Model L1 – 3100.

Chlorophyll pigments measurement

Fifty days following sowing, the third leaf from the plant tip was harvested and immersed in 5 ml of N, N-dimethylformamide (DMF) for extraction. Extraction was conducted overnight at room temperature under dark conditions. Chlorophyll pigment concentrations (including both chl. a, b, and total) were then evaluated in µg/cm² using the methodology outlined by Moran (1982).

Antioxidant enzymes

After 70 days from seed sowing, the third leaf from the plant tip was collected for antioxidant enzyme activity assays. The leaf tissue was finely ground using liquid nitrogen and then extracted with ice-cold 0.1M Tris-HCl buffer (pH 7.5) containing 5% sucrose and 0.1% 2-mercaptoethanol, with a buffer volume to fresh weight ratio of 3:1. Following homogenization, the mixture was centrifuged at 10,000 g for 20 minutes at 4°C. The resulting supernatant was utilized for enzyme activity determination. It's important to note that all extraction and assay steps were conducted at 4°C to preserve enzyme stability.

Catalase (CAT, EC 1.11.1.6) activity was assessed by measuring hydrogen peroxide (H_2O_2) decomposition at 240 nm, following method of Aebi (1984). Peroxidase (POD, EC 1.11.1.7) activity was measured as per the protocol by Polle et al. (1994). Poly phenol oxidase (PPO, EC 1.14.18.1) activity was estimated following Oktay et al. (1995).

Proline determination

Proline concentration was estimated in the third collected leaf from the plant tip, using a spectrophotometer (Shimadzu UV 1601) at wavelength of 520 nm and calculated as µmol g^{-1} using standard proline according to (Bates et al., 1973).

Relative water content (RWC)

The measurement of relative water content was conducted using methodology outlined in the study by Pieczynski et al. (2013), as in the following equation.

$$RWC = (\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight}) \times 100.$$

Anatomical studies

The anatomical studies were carried out during the second season. The selected samples were taken from the third internode of stem from the apex, and the third leaf from the middle blade around the midrib after 70 days from sowing. The anatomical studies were done according to (Ruzin, 1999). The selected sections were examined microscopically to identify histological characteristics to follow changes occurring in stems of cotton plants as affected by different irrigation intervals and application of exogenous interaction between proline or salicylic acid treatment. The chosen sections were examined microscopically identify histological characteristics. Vascular conductive tissues (xylem and phloem), vascular cambium and cortex tissue thickness were measured using ImageJ software program (Fiji, http://fiji.sc/Fiji) (Nguyen et al., 2013).

Yield and yield components

At first pick (150 days from sowing), ten guarded plants were chosen at random from each plot (140 plant/plot) meticulously and labeled in order to assess the following characteristics: plant height (cm), number of fruiting branches, number of open bolls plant^{-1}, bolls weight (g), seed index (100-seed weight), lint percentage (determined by dividing weight of lint plant^{-1} by the weight of seed cotton plant^{-1} and multiplying by 100), seed cotton yield/feddan (Kentar, equivalent to 157.5 kg) and earliness index was determined based on the formula presented by Singh (2003) (calculated by dividing the seed cotton yield of the first pick by the total seed cotton yield and multiplying by 100) as well as the dry weight (g) which is measured after plant dried in electric oven at a temperature of 70^{o}C until the weight constant.

Experimental design and statistical analysis

The experimental design utilized split-plot design with three replications for each treatment. Results were pooled, and the means were taken. Analysis of variance was performed utilizing statistical Package CoState. Duncan's multiple range tests (Duncan, 1955) for the comparison of data. Statistical significance was determined at the $p \leq 0.05$ level, where means were considered significantly different. Also, a heatmap was produced to summarize the results concerning agronomic and biochemical aspects, employing the online tool ClustVis (https://biit.cs.ut.ee/clustvis/).

RESULTS AND DISCUSSION

Plant growth characteristics

The Table 2 presents the effects of irrigation intervals, as well as the application of drought tolerance inducers proline and salicylic acid, and their interaction on the growth attributes of cotton cv. Super-Giza 94, including plant height, dry weight per plant, and leaf area. During both seasons, growth traits such as plant height and dry weight per plant were notably influenced by extended irrigation intervals, ranging from 15 to 45 days. The irrigation treatments demonstrated a gradual decline in plant height, dry weight, or leaf area. The decline in growth characteristics resulting from extended intervals of irrigation could stem from water scarcity, disrupting normal physiological functions. These declines might be clarified by the considerable and permanent expansion of smaller daughter cells arising from meristematic cell divisions. Simultaneously, this could be linked to a drop in cellular moisture levels, causing a decrease in turgor pressure (Swain et al., 2014), photosynthesis, and biomass production (Farooq et al., 2017).

Applying drought tolerance inducers like proline and salicylic acid via foliar application showed promising potential in mitigating adverse effects of water deficit compared to untreated plants subjected to the same varying irrigation intervals. The use of interaction between PRO and SA not only overcomes negative effects of water deficiency, but also significantly increases growth characteristics of plants grown under different irrigation intervals conditions compared to control. In numerous plant species, heightened accumulation of proline has been noted as an indicator of stress tolerance. However, many plants, particularly under heightened stress levels, struggle to synthesize an adequate amount of these osmo-regulators (Yang et al., 2021).

Recent research indicates that externally applying proline could serve as a protective measure against drought stress (Urmi et al., 2023). Adding supplemental salicylic acid to drought-affected crops notably enhanced stomatal conductance, subsequently raising net CO_2 assimilation rate and overall plant growth (Khan et al., 2015). Furthermore, applying exogenous SA resulted in increased plant height or dry mass, while simultaneously reducing membrane lipid peroxidation in drought-stressed wheat. Additionally, Salicylic acid application led to notable reductions in ionic leakage or accumulation of toxic ions (Movahhedi-Dehnavi et al., 2019). Proline and SA can also regulate gene expression and activate stress-responsive pathways (Yang et al., 2021; Yao et al., 2021), leading to production of antioxidant enzymes and other protective molecules that mitigate oxidative damage caused by ROS accumulation (Ashraf and Foolad, 2007; Semida et al., 2017). The action of drought tolerance inducers and their interaction to increase growth parameters, eliminating the negative influences of irrigation intervals, may be due to PRO and SA contributes in promoting plant growth and osmotic adjustment under limited water availability condition (Serraj and Sinclair, 2002; Abd El-Mageed et al., 2016; El-Waraky et al., 2024).

Table 2. Effects of irrigation intervals, proline at 60 mgL^{-1}, salicylic acid at 150 mgL^{-1} and their interaction on plant height, dry weight and leaf area of cotton cv. Super-Giza 94 during the 2021 and 2022 seasons

Treatments	Plant height (cm)		Leaf area (cm^2)		Dry weight (g)	
	2021	2022	2021	2022	2021	2022
Irrigation intervals (A)						
After 15 days (A_1)	151.08 a	152.33 a	2609.52a	2860.47a	184.95a	181.42a
After 30 days (A_2)	122.45 b	123.02 b	2490.85b	2298.14b	170.64b	166.60b
After 45 days (A_3)	119.59 c	119.50 c	1982.55c	2075.21c	156.54c	153.42c
P	.00**	.00**	.00**	.00**	.00**	.00**
Treatments (B)						
Cont. (B_1)	118.37 c	117.29 d	1706.38d	1714.63d	119.22d	114.39d
Proline (B_2)	129.79 b	131.34 c	2349.37c	2398.11c	180.24c	178.79c
Salicylic acid (B_3)	136.89 a	136.71 b	2531.25b	2903.62a	187.60b	183.64b
Proline + Salicylic acid (B_4)	139.11 a	141.14 a	2856.89a	2628.73b	195.78a	191.78a
P	.00**	.00**	.00**	.00**	.00**	.00**
Interaction:						
A_1 x B_1	137.80c	138.00d	2062.14i	1972.00i	124.04i	12.26g
A_1 x B_2	146.46b	148.29c	2694.26d	2785.14c	194.64c	191.85c
A_1 x B_3	159.67a	160.45b	2429.83e	3430.36a	207.39b	203.39b
A_1 x B_4	160.41a	162.58a	3251.88a	3254.39b	13.74a	208.23a
A_2 x B_1	110.00f	108.43i	1786.67j	1798.00j	118.93j	115.02h
A_2 x B_2	122.91e	14.73g	2217.42g	2219.45g	186.94e	182.49e
A_2 x B_3	126.99de	125.67fg	2775.26c	2698.15d	187.01e	182.58e
A_2 x B_4	130.88d	133.28e	3184.05b	2476.95f	189.68d	186.31d
A_3 x B_1	107.32f	105.46j	1270.33k	1373.89k	114.70k	105.89i
A_3 x B_2	120.00e	121.00h	2136.42h	2189.73gh	159.13h	162.04f
A_3 x B_3	125.00de	124.00g	2388.67f	2582.37e	168.40g	164.96f
A_3 x B_4	16.04de	127.55f	2134.77h	2154.85h	183.91f	180.79e
P	.02*	.00**	.00**	.00**	.00**	.00**

* and ** indicate $P<0.05$ and $P<0.01$, respectively. Means of each factor designated by the same latter are not significantly different at 5% level using Duncan's Multiple Range Test.

Relative water content (RWC) and proline content

When cotton plants are irrigated at longer intervals, such as every 30 or 45 days, it is expected that they will experience water deficit stress. One of physiological responses to water deficit stress is decrease in relative water content (Figure 1). The decrease in RWC is a common response to water deficit stress in many plant species, including cotton. The adverse effects of drought on RWC could be attributed to reduced water flow. The decrease in water flow exacerbates protoplasm dehydration, resulting in oxidative damage to chloroplasts, compromised cell membrane integrity, induced stomatal closure, and reduced concentrations of CO_2 in mesophyll cells and photosynthetic assimilates (Semida et al., 2020).

The foliar application of PRO, SA, and their combined interaction mitigated adverse effects to some extent. In contrast, prolonged irrigation intervals caused gradual rise in proline content. Proline accumulation is a common response in numerous plant species under different stress conditions, such as drought or salinity. The heightened accumulation of proline is often considered indicator of stress tolerance. While proline and other osmo-regulators lay a pivotal role in stress tolerance, some plants may struggle to synthesize an adequate amount of these compounds, especially under heightened stress levels (Bray et al., 2002; Urmi et al., 2023). Exogenous foliar application of PRO, SA and their interaction enhanced the endogenous proline content in cotton plants compared to plants irrigated every 15 days. Noreen et al. (2013) indicated that foliar applied proline may be useful strategy for promoting drought tolerance in cotton. Urmi et al. (2023) illustrated that the combined application of proline (PRO) or salicylic acid (SA) alleviated detrimental effects observed in drought-stressed rice.

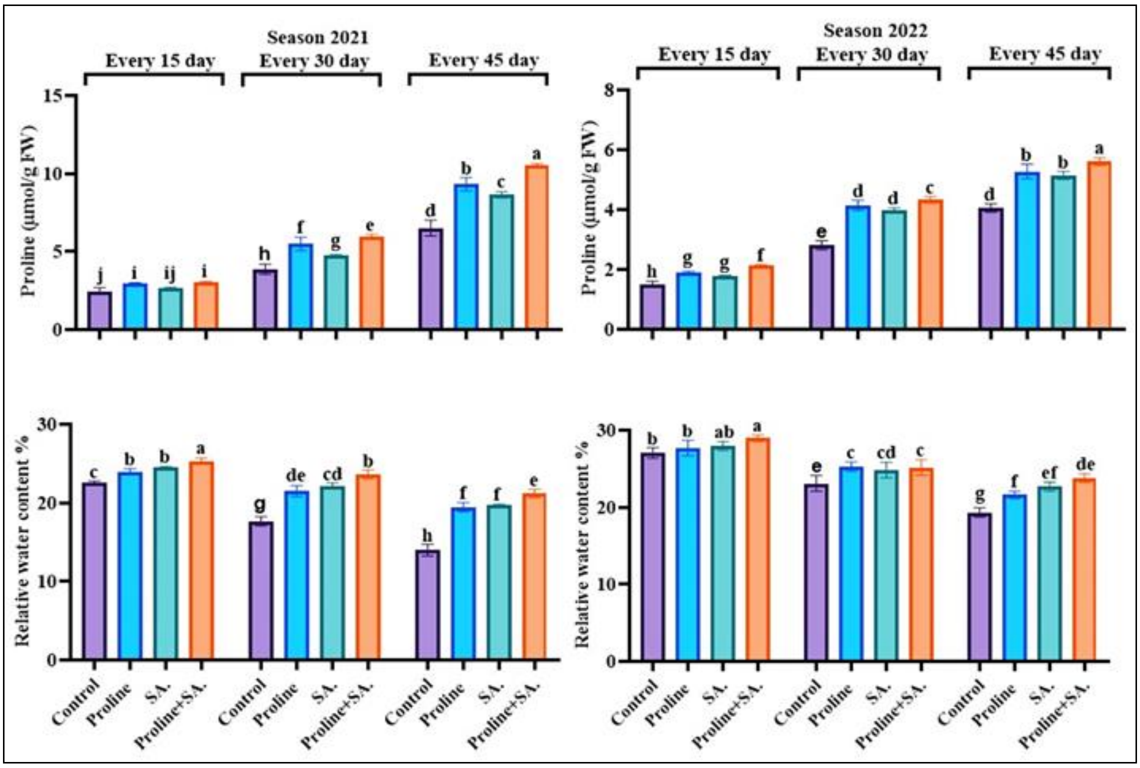

Figure 1. Effects of irrigation intervals, proline at 60 mgL^{-1}, salicylic acid at 150 mgL^{-1} and their interaction on relative water content (RWC) and proline contents in cotton plants cv. super-Giza 94 at 110 days from sowing during the 2021 and 2022 seasons, SA: Salicylic acid. Values sharing at least one identical letter were not significantly different at the $P<0.05$ level.

Chlorophyll contents

The results indicated a significant decrease in chl a, chl b, or total chlorophyll levels during both seasons due to prolonged irrigation intervals (Figure 2). Chlorophyll and carotenoids play crucial roles in photoprotection and are commonly utilized physiological indicators because they directly impact photosynthesis efficiency (Croft et al., 2017). Water stress reduces ability of mesophyll cells to use CO_2, resulting in a subsequent decline in chlorophyll content (Athar and Ashraf, 2005; Sarwar et al., 2013; Urmi et al., 2023).

As expected, application of PRO, SA significantly increased chlorophyll pigments contents. The most positive impacts of chlorophyll content were observed by the interaction between PRO and SA treatment during both seasons. A close association between exogenous application of PRO and SA and chlorophyll pigments content and growth parameters under water stress conditions has been observed. Exogenous application of PRO and SA has been shown to play a protective role in maintaining chloroplast and thylakoid structures (Farooq et al., 2017; Aldesuquy et al., 2018) and from photoinhibition (Hare and Cress, 1997) under drought stress conditions. Salicylic acid and proline both play roles in preserving chlorophyll content and leaf turgor, while also improving stomatal conductance, all of which are linked to drought tolerance (Kumar et al., 2016; Panda et al., 2021).

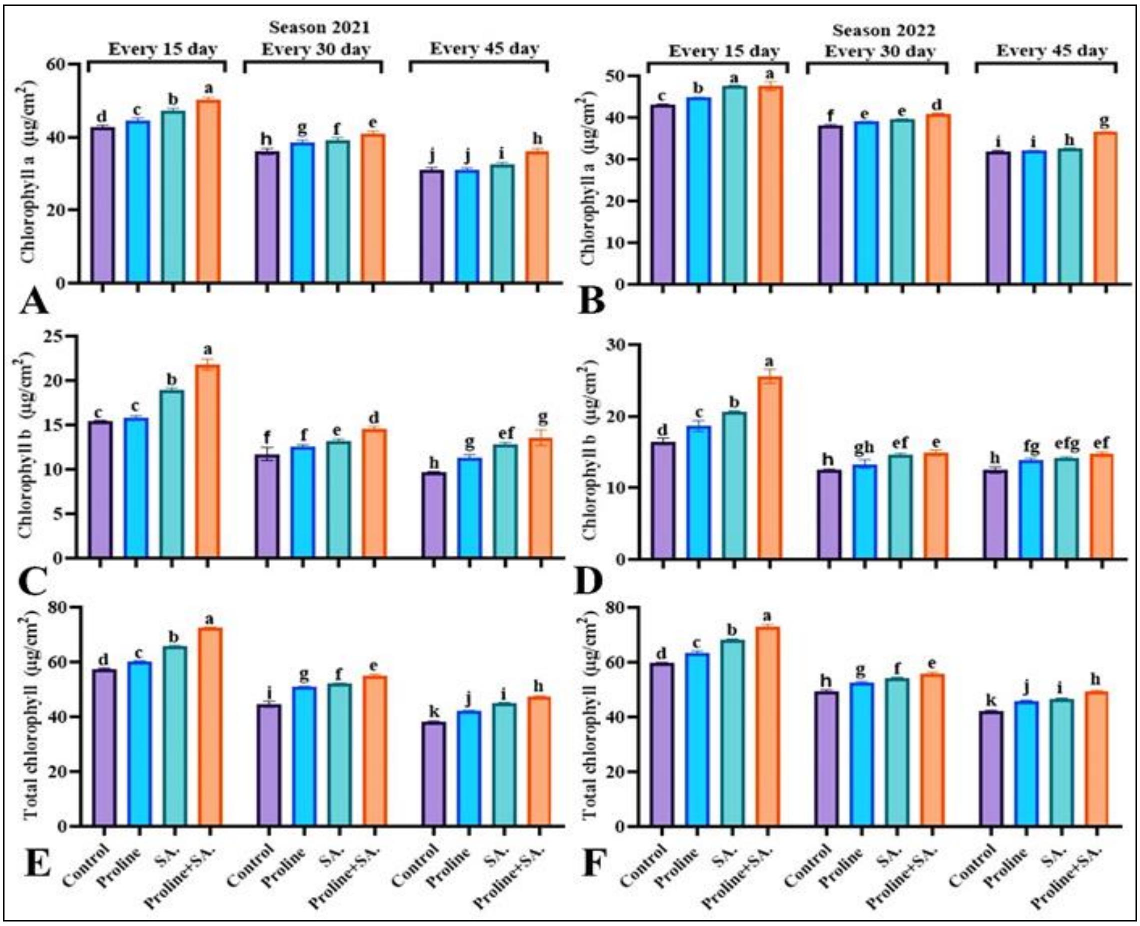

Figure 2. Effects of irrigation intervals, proline at 60 mgL^{-1}, salicylic acid at 150 mgL^{-1} and their interaction on chlorophyll content (Chl. a Chl. b and total chl.) in cotton cv. super-Giza 94 at 110 days from sowing during 2021 and 2022 seasons, SA: Salicylic acid. Values sharing at least one identical letter were not significantly different at the P<0.05 level.

Antioxidant enzymes

The data presented in Figure 3 indicate that activity. The results indicated that activities of antioxidant enzymes, such as CAT, POX, and PPO, were increased with increasing irrigation intervals. This suggests that the plants experiencing longer intervals between irrigation showed an upregulation of antioxidant defense mechanisms (Pontes et al., 2024). Furthermore, the foliar application of Pro and SA, whether applied separately or in combination, resulted in an increase in antioxidant enzymes. CAT (catalase) is an enzyme that contains heme and functions to mitigate the overproduction of hydrogen peroxide (H_2O_2) during oxidative stress by converting H_2O_2 into water (H_2O) and oxygen (O_2) (Waszczak et al., 2018). Peroxidase not only scavenges ROS but also generates related compounds such as lignin, guaiacol, and pyrogallol. These compounds serve as electron donors for scavenging hydrogen peroxide (H_2O_2) both intra- and extracellularly (Mishra and Panda, 2017).

Catalase and POX are very important H_2O_2 scavenging enzymes (Willekens et al., 1997). Many studies have shown that drought stress often leads to elevated CAT, POX and PPO levels in plants. This is thought to be a protective mechanism against ROS generated by drought stress. Thipyapong et al. (2007) indicated that increased specific activity of PPO under drought stress is likely intertwined with the considerable synthesis of total soluble phenols (TSPs). Overall, the higher CAT, POX and PPO activities may suggest the higher efficiency of cotton plants under drought. External application of SA can mitigate the harmful impacts caused by water deficit by regulating the levels of reactive oxygen species (ROS) and enhancing the activity of antioxidant enzymes (Lobato et al., 2021). The interaction between proline and salicylic acid treatment significantly increased CAT, POX and PPO activities under all irrigation intervals. The best results were achieved by the interaction between proline and salicylic acid under irrigation intervals. Increasing antioxidant enzymes by drought tolerance inducers alleviate injuries from ROS under water deficit stress (Salama et al., 2009; Agami et al., 2019; Shalaby et al., 2023; El-Beltagi et al., 2024a,b). In rice, the joint application of salicylic acid (SA) and proline exhibited a more pronounced alleviation of the adverse effects of drought stress compared to their individual application. This was achieved by increasing the activity of antioxidant enzymes, and suppressing oxidative stress (El-Beltagi et al., 2022c; Urmi et al., 2023).

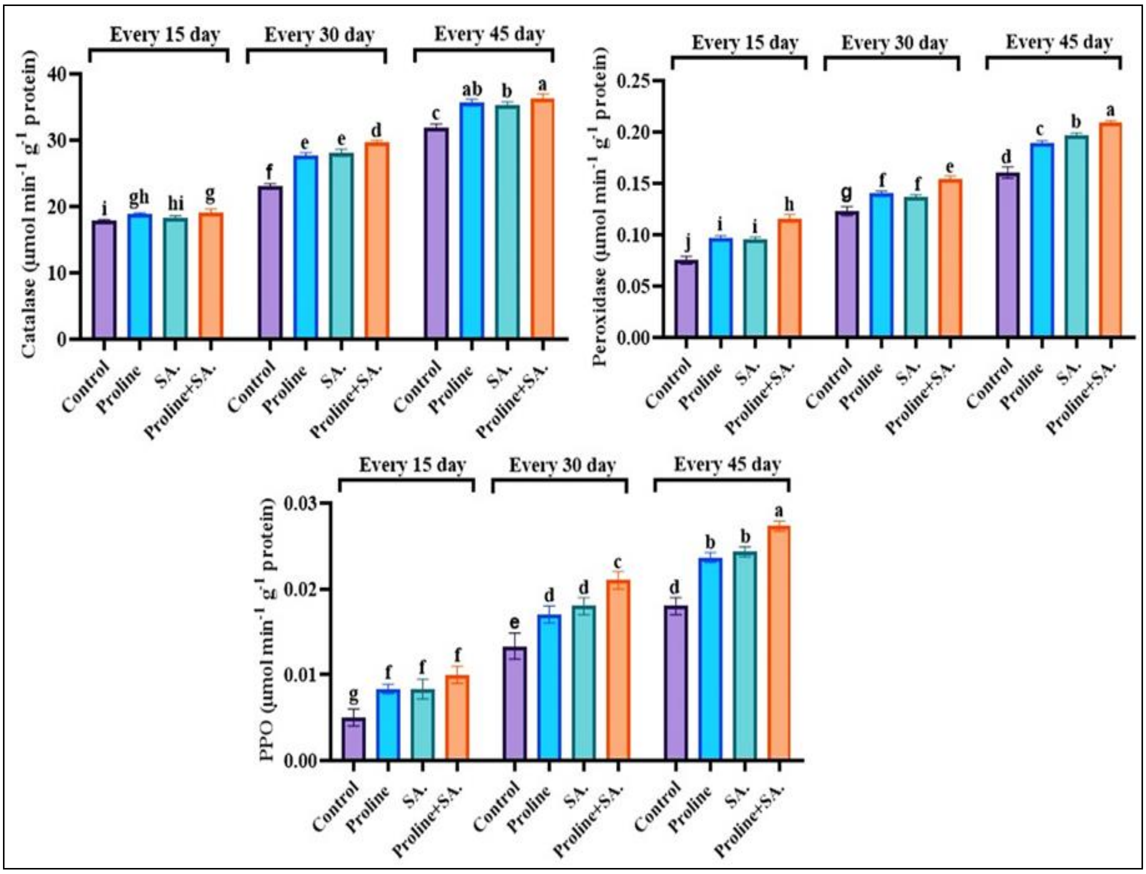

Figure 3. Effects of irrigation intervals, proline at 60 mgL^{-1}, salicylic acid at 150 mgL^{-1} and their interaction on catalase (CAT), Peroxidase (POX) and polyphenoloxidase (PPO) activity in cotton cv. super-Giza 94 at 110 days from sowing during 2021 and 2022 seasons, SA: Salicylic acid. Values sharing at least one identical letter were not significantly different at the P<0.05 level.

Anatomical traits

From the date presented in Figure 4 and Table 3 there is asignificant reduction in stem tissues thickness (xylem, phloem, vascular cambium and cortex) were recorded gradually with prolonging irrigation intervals. The observed reduction in tissue thickness of xylem, phloem, vascular cambium, and cortex with prolonged irrigation intervals indicates a potential physiological response of plant to water stress. Xylem and phloem tissues are essential for transporting water, nutrients, and sugars throughout the plant, while the vascular cambium is responsible for secondary growth in stems. Under normal conditions, the vascular cambium undergoes active cell division, resulting in the formation of secondary xylem towards the inside and secondary phloem towards the outside. However, under drought stress, the activity of the vascular cambium may be significantly reduced or even temporarily cease altogether. This reduction in vascular cambium activity is an adaptive response of the plant to conserve water and energy resources during periods of water scarcity. Several factors contribute to the reduction in vascular cambium activity under drought stress including water availability, resource allocation, hormonal regulation as well as reduction in photosynthesis (Li and Jansen, 2017). From the obtained results it has been shown that, the best resuls in plant growth, relative water content %, endogenous proline content, antioxidant enzymes, chlorophyll pigments as well as yield were achieved by the interaction between proline at 60 mgL^{-1} and salicylic acid at 150 mgL^{-1} under all irrigation intervals. Proline and salicylic acid interactions significantly increased these anatomical parameters under all irrigation intervals in compared with irrigation intervals treatments.

Cotton leaf anatomy, including palisade and spongy tissues notably increased with longer irrigation intervals, mirroring a common trend. On the other hand, vascular tissues thickness and xylem vessels diameter were decreased with increasing in irrigation intervals. Drought stress induces dehydration in mesophyll cells by closing stomata, leading to damage in photosynthetic organs (Zagoto and Violita, 2019). Mesophyll thickness decreases under drought stress, possibly due to reduced cell expansion or cell death, especially in severe drought (Salsinha et al., 2021). Prolonged irrigation intervals induce water stress, prompting plants to adopt adaptive strategies to conserve water and sustain essential physiological functions. This may involve reducing growth and development of certain tissues to minimize water loss via transpiration (Yavas et al., 2024). With increasing water deficit stress, mesophyll cell arrangement shifted from loose to tight, cells shrank, and water loss occurred. Severe drought stress further reduced intercellular spaces, cell volume, and improved leaf fixation post-water loss, resulting in more intact leaf slices. These findings are consistent with those reported by Liu et al. (2021) on *Phedimus aizoon* L.

Foliar application of proline and SA interaction improved these parameters under all irrigation treatments (Figure 5 and Table 4). Spongy tissue cells were obviously compressed in plants which irrigated every 45 days. The narrow xylem vessels were formed in cotton stems and leaves under water deficit stress condtions. Proline and SA are two compounds that play significant roles in plant responses to diverse environmental stresses, including drought stress (Agami et al., 2019). Lobato et al. (2021) showed that SA allievated the adverse effeects of water deficit stress, which led to increase the thickness of palisade and spongy tissues of tomato leaves.

The combined application of proline (Pro) and salicylic acid (SA) may enhance plant tolerance to drought by regulating various physiological and biochemical processes, including the development of thicker stem and leaf tissues and enhanced conductive tissues. This combination promotes the development of thicker palisade and spongy tissues in plants, crucial for photosynthesis. Under water deficit stress, plants undergo adaptations to conserve water and survive, which are reflected in both their external appearance and internal structure. Research by Bai et al. (2019) demonstrated that drought-tolerant apple cultivars develop longer palisade tissue and thicker spongy parenchyma tissue under water deficit stress conditions. Similarly, Yin et al. (2017) observed thicker xylem tissue in drought-stressed rapeseed plants compared to well-irrigated plants. Increasing leaf chlorenchyma tissue (palisade and spongy tissues) under water deficit stress may aid plants in adapting to insufficient water supply while maintaining an appropriate rate of photosynthesis (Ennajeh et al., 2010). The foliar application of proline and SA interaction improves internal anatomical parameters, possibly by enhancing water and mineral uptake and mitigating injuries from ROS under water deficit stress (Agami et al., 2019). The enhancement of stem and leaf anatomical parameters in cotton resulting from the application of SA or proline indicates increased translocation of absorbed water and nutrients into cells, facilitating their utilization in various metabolic processes (Agami et al., 2019).

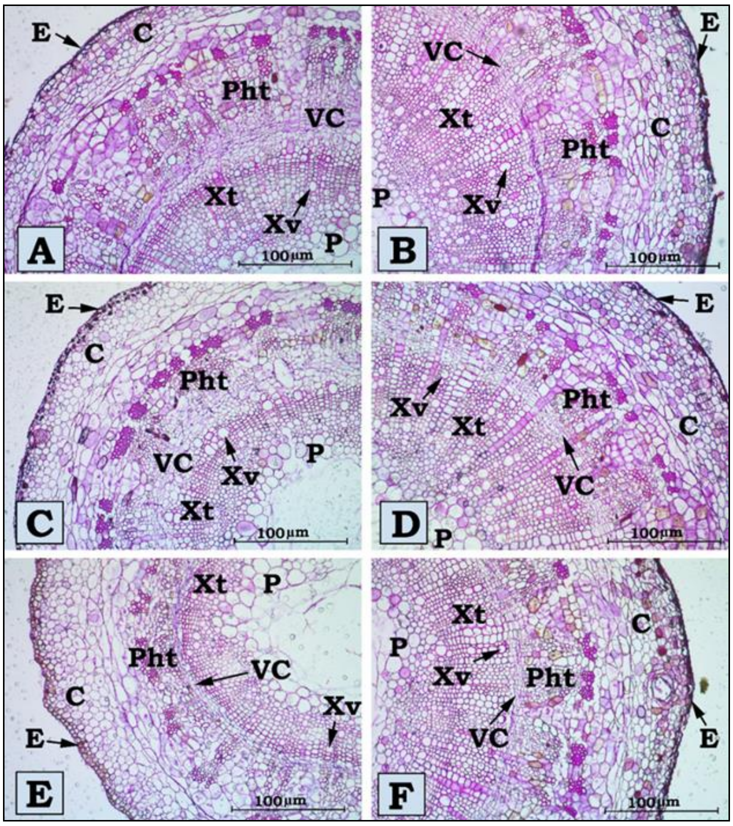

Figure 4. Transverse sections of cotton stem plants illustrating the anatomical changes resulting from water deficit stress (A, C and E) and proline and salicylic acid interaction in plants irrigated every 15 days (B), every 30 days (D) and every 45 days (F), epidermis tissue (E), cortex tissue (C), phloem tissue (Pht), vascular cambium (VC), xylem tissue (Xt), xylem vessel (Xv) and pith tissue (P)

Table 3. Anatomical differences of cotton stems cv. Super-Giza 94 as affected by prolonging irrigation intervals, every 15 days, 30 days and 45 days in combination with drought tolerance inducers (proline at 60 mgL^{-1} and salicylic acid at 150 mgL^{-1}) interaction

Treatments	Thickness (µm)				
	Xylem	Phloem	Cambium	Cortex	Xylem vess. diameter
Irrigation intervals (A)					
After 15 days (A_1)	78.67a	67.73a	17.69b	65.93c	9.27a
After 30 days (A_2)	59.26b	52.25b	24.27a	73.19b	8.46a
After 45 days (A_3)	53.01c	47.10c	13.14c	80.79a	6.88b
P	.00**	.00**	.00**	.00**	.00**
Treatments (B)					
Cont. (B_1)	44.58b	50.33b	18.59a	68.71b	6.87b
Proline + Salicylic acid (B_4)	82.71a	61.06a	18.14a	77.89a	9.53a
P	.00**	.00**	NS	.00**	.00**
Interaction:					
A_1 x B_1	62.56c	70.72a	17.03c	53.37c	8.19cd
A_1 x B_2	94.79a	64.74ab	18.35bc	78.49ab	10.33a
A_2 x B_1	36.31d	45.14c	27.61a	72.35b	7.36d
A_2 x B_2	82.21b	59.37b	20.94b	74.02ab	9.56ab
A_3 x B_1	34.87d	35.13d	11.15d	80.41ab	5.07e
A_3 x B_2	71.14bc	59.07b	15.14c	81.16a	8.69bc
P	.02*	.00**	.00**	.00**	.02*

*, ** and NS indicate P<0.05, P<0.01, and nonsignificant, respectively. Means of each factor designated by the same latter are not significantly different at 5% level using Duncan's Multiple Range Test.

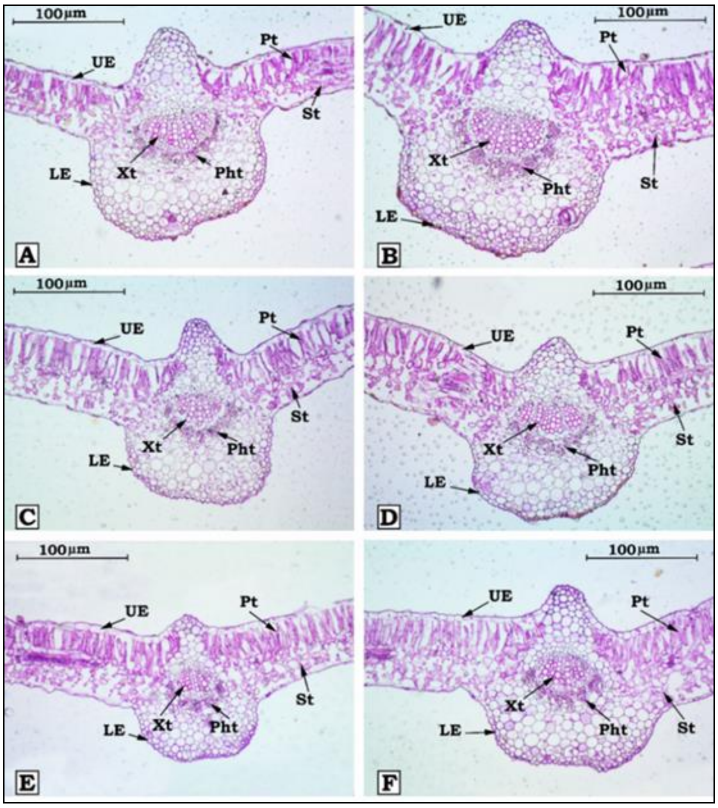

Figure 5. Transverse sections of cotton leaf plants illustrating the anatomical changes resulting from water deficit stress: (A, C and E) and proline and salicylic acid interaction in plants irrigated every 15 days (B), every 30 days (D) and every 45 days (F): upper epidermis tissue (UE), Palisade tissue (Pt), spongy tissue (St), phloem tissue (Pht), xylem tissue (Xt), lower epidermis tissue (LE)

Table 4. Anatomical differences of cotton leaves cv. Super-Giza 94 as affected by prolonging irrigation intervals, every 15 days, 30 days and 45 days in combination with drought tolerance inducers (proline at 60 mgL^{-1} and salicylic acid at 150 mgL^{-1}) interaction

Treatments	Thickness (µm)							Xylem vess. diameter
	Lamina	Mid rib	Xylem	phloem	Palisade	Spongy	VB width	
Irrigation intervals (A)								
After 15 days (A_1)	65.04b	200.72a	30.92a	14.98a	21.77b	23.19c	82.97a	4.65a
After 30 days (A_2)	68.79ab	166.57b	24.48b	12.63ab	26.43a	27.03b	68.72b	3.83b
After 45 days (A_3)	72.88a	148.29c	21.84c	10.51b	27.73a	30.20a	58.88c	3.73b
P	.03*	.00**	.00**	NS	.00**	.00**	.00**	.00**
Treatments (B)								
Cont. (B_1)	61.16b	162.49b	23.27b	10.79b	22.59b	22.84b	63.31b	3.53b
Proline + Salicylic acid (B_4)	76.65a	181.23a	28.22a	14.61a	28.03a	31.43a	77.07a	4.61a
P	.00**	.00**	.00**	.00**	.00**	.00**	.00**	.00**
Interaction:								
A_1 x B_1	50.40e	188.96	30.02ab	12.43b	13.74b	18.93c	76.66b	3.92bc
A_1 x B_2	79.68a	212.47a	31.83a	17.53a	29.80a	29.45b	89.27a	3.39a
A_2 x B_1	64.28d	167.26c	22.22d	8.78c	26.17a	24.52b	63.57d	3.56cd
A_2 x B_2	73.29bc	165.89c	26.75bc	12.23b	26.69a	29.53b	73.87bc	4.09bc
A_3 x B_1	68.79cd	131.25d	17.58e	11.19bc	27.86a	25.08b	49.68e	3.12d
A_3 x B_2	76.97ab	165.33c	26.08c	14.08b	27.60a	35.33a	68.09cd	4.34b
P	.00**	.00**	NS	NS	.00**	NS	NS	.02*

*, ** and NS indicate P<0.05, P<0.01, and nonsignificant, respectively. Means of each factor designated by the same latter are not significantly different at 5% level using Duncan's Multiple Range Test.

Yield and yield components

Results of seed index, boll weight or seed yield as affected by irrigation intervals, application of irrigation intervals, drought tolerance inducers or their interaction illustrated in Table 5. Gradually reduction in seed index, boll weight and seed cotton yield with prolonging irrigation intervals. Insufficient soil water content during critical growth phases, such as squaring, flowering, and fruit setting, can result in several adverse effects on plant development (Saleem et al., 2015). These effects include a decline in number of fruiting branches, a decrease in number of seeds per boll, diminished seed cotton yield, and other related yield attributes (Rehman et al., 2021). Water deficit stress during the flowering stage can lead to decrease in overall plant biomass, fewer bolls per plant, reduced seed weight, and lower seed cotton yield per plant. This is primarily attributed to diminishing trends in photosynthesis caused by the water stress. These outcomes align with previous findings that link decreased cotton seed yield to a higher number of cotton boll abortions. Moisture deficits during reproductive growth can lead to increased boll abortions, consequently reducing boll production and ultimately impacting lint yield (Rehman et al., 2021).

Table 5. Effects of irrigation intervals, proline at 60 mgL^{-1}, salicylic acid at 150 mgL^{-1} and their interaction on seed index, boll weight and seed yield of cotton cv. Super-Giza 94 during the 2021 and 2022 seasons

Treatments	Seed index		Boll weight		Seed cotton yield	
	2021	2022	2021	2022	2021	2022
Irrigation intervals (A)						
After 15 days (A_1)	10.63 a	10.47 a	2.38 a	2.41a aa	8.56 a	8.96 a
After 30 days (A_2)	10.08 b	10.17 b	2.29 b	2.30 b	6.63 b	6.74 b
After 45 days (A_3)	10.32 c	8.43 c	2.13 c	2.17 c	6.23 c	5.8 7 c
P	.00**	.00**	.00**	.00**	.00**	.00**
Treatments (B)						
Cont. (B_1)	8.70 d	8.73 d	2.17 c	2.21 c	6.57 b	6.77 d
Proline (B_2)	9.51 c	9.55 c	2.28 b	2.30 b	7.02 ab	7.13 c
Salicylic (B_3)	9.86 b	9.88 b	2.28 b	2.31 b	7.50 a	7.28 b
Proline + Salicylic acid (B_4)	10.64 a	10.59 a	2.34 a	2.35 a	7.74 a	7.57 a
P	.00**	.00**	.00**	.00**	.03*	.00**
Interaction:						
A_1 x B_1	9.19 f	9.11 e	2.33 d	2.36 cd	8.31 abc	8.45 d
A_1 x B_2	9.86 e	9.90 d	2.36 c	2.38 c	8.72 ab	8.85 c
A_1 x B_3	11.20 b	10.89 b	2.40 b	2.43 b	7.92 bcd	9.07 b
A_1 x B_4	12.26 a	11.98 a	2.46 a	2.48 a	9.32 a	9.47 a
A_2 x B_1	9.06 f	9.05 e	2.20 f	2.22 fg	6.14 fg	6.44 g
A_2 x B_2	10.41 cd	10.46 c	2.30 e	2.33 de	6.53 efg	6.63 g
A_2 x B_3	10.13 de	10.37 c	2.30 e	2.00 e	6.77 def	6.83 f
A_2 x B_4	10.74 c	10.78 b	2.36 c	2.34 cde	7.09 cdef	7.07 e
A_3 x B_1	7.86 g	8.03 g	2.00 h	2.05 h	5.26 g	5.44 j
A_3 x B_2	8.26 g	8.30 f	2.16 g	2.19 g	5.81 fg	5.93 i
A_3 x B_3	8.26 g	8.38 f	2.16 g	2.20 g	7.83 bcde	5.96 i
A_3 x B_4	8.91 f	9.03 e	2.20 f	2.25 f	6.02 fg	6.18 h
P	.00**	.00**	.00**	.00**	.01*	.02*

* and ** indicate P<0.05 and P<0.01, respectively. Means of each factor designated by the same latter are not significantly different at 5% level using Duncan's Multiple Range Test.

Foliar application of drought tolerance inducers (proline and salicylic acid), along with their interaction, mitigated the adverse effects on seed index, boll weight, or seed cotton yield under various irrigation intervals. These adverse effects were significantly reduced with prolonged irrigation intervals. On the other hand, the lint percentage significantly increased. These inducers can improve water-use efficiency, enhance osmotic adjustment, nutrient uptake and assimilation and protect cell membranes, which can support better cell division and expansion during boll development (Farooq et al., 2017; Loutfy et al., 2022; Rharbi et al., 2023).

Concerning the number of fruiting branches and open bolls per plant percentage of lint and earliness Data in Table 6 revealed that there was significant reduction in all these previous parameters gradually with prolonging irrigation intervals. Water deficit stress during sensitive growth, flowering, or fruiting stages, may affect yield and yield components (Eid et al., 2022). Water deficit stress disrupts crucial plant processes like photosynthesis and nutrient uptake, ultimately leading to stunted growth and decreased crop yields. When cotton plants experience water deficit stress, their growth slows down and prioritizes survival over fiber production. This leads to shorter and weaker fibers than plants grown under optimal conditions (Başalh and Ünay, 2006). Enhancement of these yield traits by application of water deficit tolerance inducers were linked to plant growth stimulation, chlorophyll pigment contents, osmolyte concentration and activities of ROS scavenging enzymes, including CAT, POX or PPO (Zivcak et al., 2016; Urmi et al., 2023).

Table 6. Effects of irrigation intervals, proline at 60 mgL^{-1}, salicylic acid at 150 mgL^{-1} and their interaction on number of fruiting branches/plant, number of open bolls/plants, lint% and earliness % of cotton cv. super-Giza 94 during the 2021 and 2022 seasons

Treatments	No. of fruiting branches / plant		No. of open bolls/plant		Lint %		Earliness %	
	2021	2022	2021	2022	2021	2022	2021	2022
Irrigation intervals (A)								
After 15 days (A_1)	14.81a	15.12a	16.17a	16.25a	41.64a	40.31c	64.30a	64.78a
After 30 days (A_2)	12.64b	12.68b	12.65b	12.74b	41.47b	41.13b	54.21b	55.06b
After 45 days (A_3)	11.34c	11.70c	11.77c	11.85c	40.69c	41.46a	45.36c	47.99c
P	.00**	.00**	.00**	.00**	.00**	.00**	.00**	.00**
Treatments (B)								
Cont. (B_1)	11.50c	11.67c	13.12d	13.21d	41.42a	41.21a	51.35c	52.34d
Proline (B_2)	12.82b	13.08b	13.42c	13.52c	41.34a	41.01ab	54.44b	55.61c
Salicylic (B_3)	13.48a	13.62ab	13.66b	13.75b	41.18b	40.90ab	56.46a	56.51b
Proline + Salicylic (B_4)	13.91a	14.30a	13.91a	13.99a	41.12b	40.74b	57.58a	59.33a
P	.00**	.00**	.00**	.00**	.00**	.00**	.00**	.00**
Interaction:								
A_1 x B_1	13.00c	13.55cd	15.63d	15.68c	40.88e	40.67cd	13.55abc	60.01d
A_1 x B_2	14.36b	14.63bc	16.18c	16.28b	40.78e	40.36de	14.63ab	64.27c
A_1 x B_3	15.83a	16.00ab	16.27b	16.33b	40.62f	40.21de	16.00a	65.07b
A_1 x B_4	16.06a	16.28a	16.58a	16.72a	40.46g	40.00e	16.28a	69.78a
A_2 x B_1	11.33ef	11.00f	12.22h	12.31g	41.60b	41.30ab	11.00cd	52.59h
A_2 x B_2	12.73cd	13.00de	12.32g	12.45f	41.56b	41.18abc	13.00bcd	54.14g
A_2 x B_3	13.06c	13.00de	12.89f	13.01e	41.40cd	41.07b	13.00bcd	55.78f
A_2 x B_4	13.42bc	13.72cd	13.16e	13.22d	41.32d	40.98bc	13.72abc	57.73e
A_3 x B_1	10.18f	10.46f	11.52k	11.63k	41.78a	41.66a	10.46d	44.42k
A_3 x B_2	11.37ef	11.60ef	11.77j	11.82j	41.87ab	41.50ab	11.60cd	48.43
A_3 x B_3	11.50de	11.85ef	11.81j	11.92i	41.53bc	41.43ab	11.85bcd	48.67j
A_3 x B_4	12.26cde	12.91de	11.98i	12.02h	41.59b	41.24ab	12.91bcd	50.47i
P	.03*	NS	.00**	.00**	NS	NS	.03*	.00**

*, ** and NS indicate P<0.05, P<001, and nonsignificant, respectively. Means of each factor designated by the same latter are not significantly different at 5% level using Duncan's Multiple Range Test.

Figure 6 summarizes the detrimental impacts of three different irrigation intervals (every 15, 30 and 45 days), affecting various parameters such as vegetative growth, chlorophyll pigment content, antioxidant enzymes, water and nutrients uptake, seed cotton yield, as well as anatomical differences. Simultaneously, water deficit stress induced an increase in reactive oxygen species (ROS) content. However, the application of salicylic acid and proline, either alone or in combination via foliar spray, ameliorated the negative effects of water deficit stress on all examined parameters and reduced ROS levels.

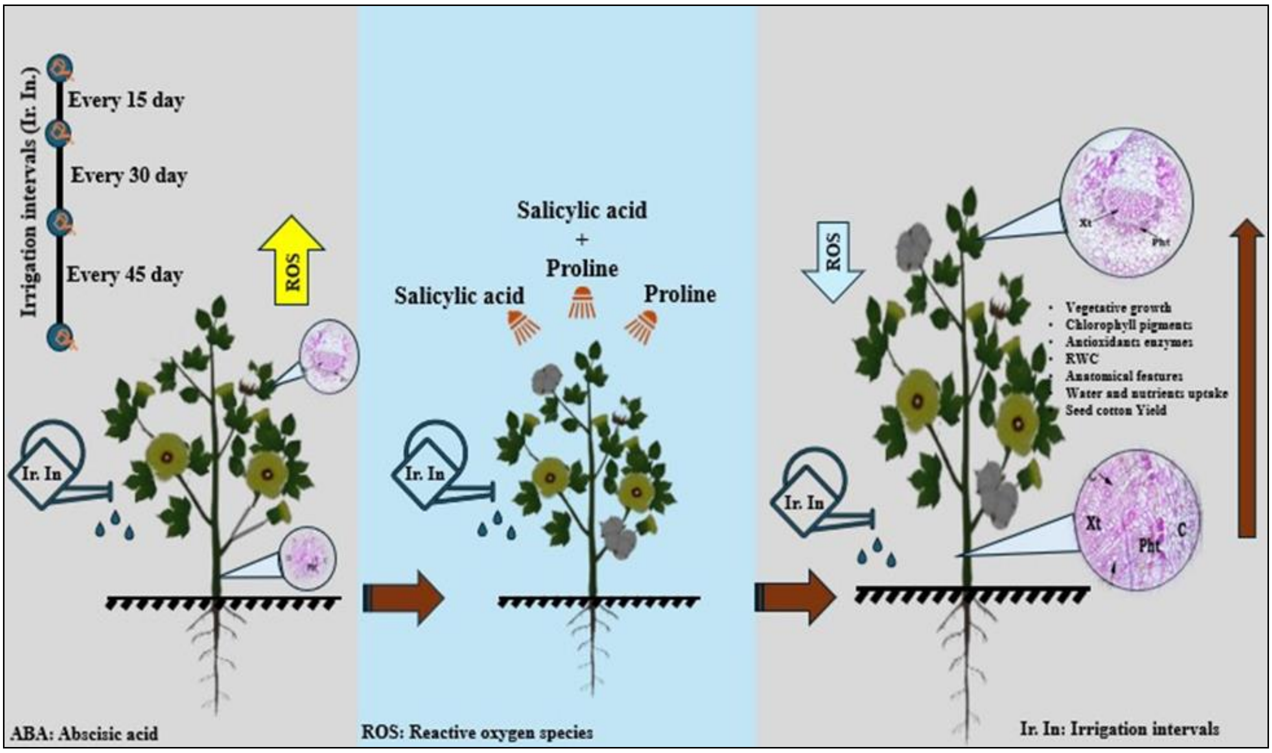

Figure 6. A graphical abstract outlining the adverse effects of different irrigation intervals (every 15, 30 and 45 days) and the mitigation of these negative impacts through the application of salicylic acid, proline, whether applied individually or in combination via foliar spray

Comparison of responses across treatments

A comparative heatmap analysis was carried out to investigate the parameters measured in this study (Figure 7). These parameters included dry weight, plant height, earliness, lint percentage, yield (K/Feed), open bolls, number of branches, endogenous proline content, RWC, activity of antioxidant enzymes (CAT, POX, or PPO), as well as chlorophyll content (Chl. a, b, and total chlorophyll). Also, the leaf anatomical parameters included thickness of lamina, leaf midrib, xylem, phloem, palisade, spongy as well as midrib vascular bundle width. Stem anatomical parameters included thickness of cortex, xylem, phloem, xylem vessels diameters as well as vascular bundle (Figure 8). The analysis unveiled distinct categorizations of plant growth, physiological, and biochemical responses under drought stress conditions, both with or without treatments of either proline or salicylic acid alone or in combination. Particularly noteworthy was the significantly higher alleviation of drought stress observed in plants treated with combined Pro and SA.

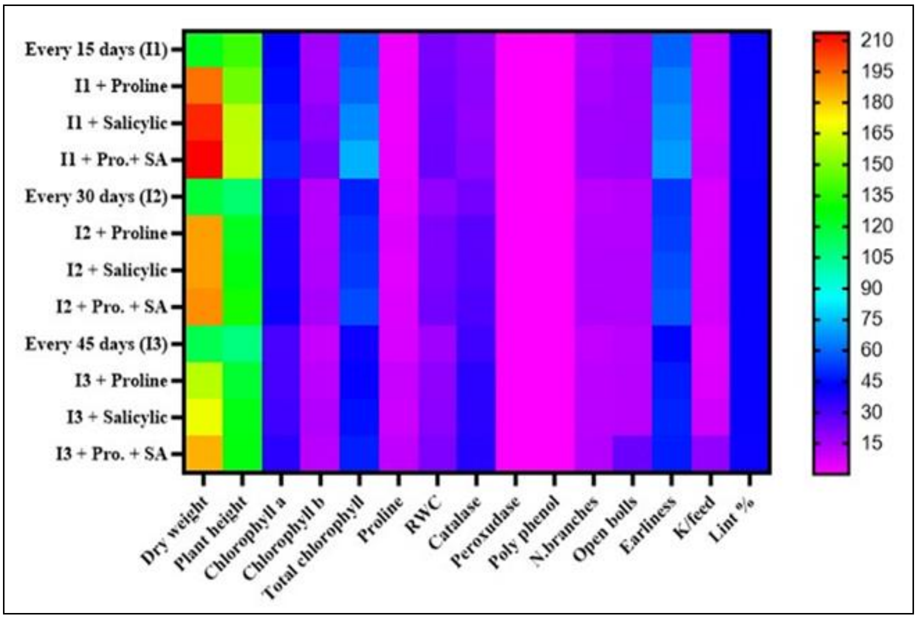

Figure 7. Heatmap analysis of the growth, biochemical, physiological and yield attributes in cotton plants unstressed and stressed (Three different irrigation intervals) treated with proline, salicylic acid, and their interaction

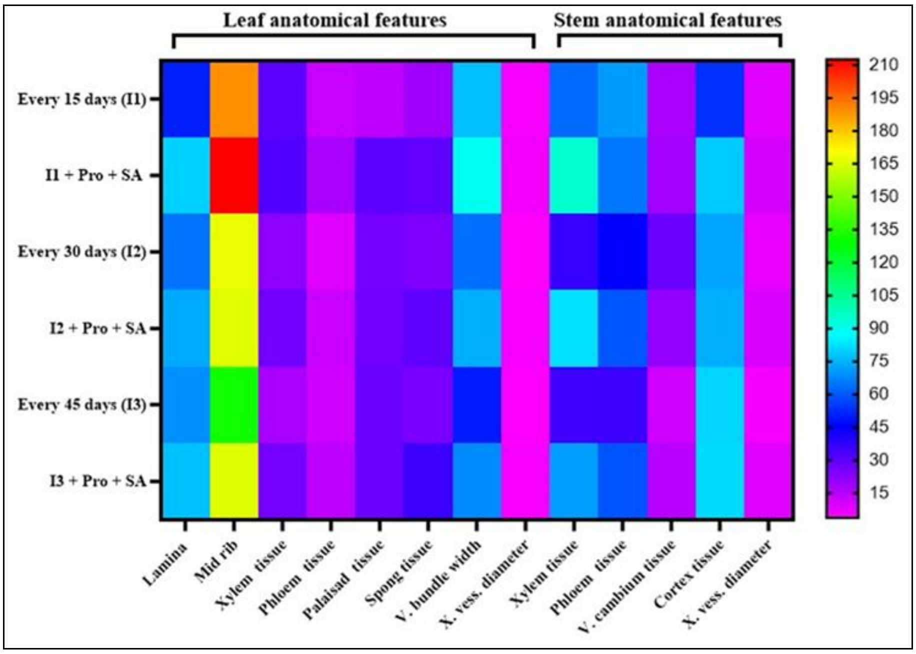

Figure 8. Heatmap analysis of leaf and stem anatomical parameters in cotton plants unstressed and stressed (Three different irrigation intervals) treated with proline, salicylic acid, and their interaction

CONCLUSIONS

Our results established that the plant growth, relative water content, chlorophyll content, internal anatomical traits yield, and yield component were reduced under water deficit stress conditions. Foliar application of salicylic acid (150 mgL^{-1}) and proline (60 mgL^{-1}) mitigated these adverse effects on cotton plants grown under water deficit stress conditions. Salicylic acid and proline interaction treatment was the best treatment on improvement the most traits in all irrigation intervals treatments.

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