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ARTICLE

Effects of Water Stress on the Morpho-Physiological Characteristics of *Gossypium hirsutum* (Cotton) and *Abutilon theophrasti* (Velvetleaf)

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ABSTRACT: The effects of water stress on the morphophysiological and biochemical characteristics of cotton (*Gossypium hirsutum* L.) and velvetleaf (*Abutilon theophrasti*) were investigated in a pot experiment under greenhouse conditions. Both species were exposed to four irrigation levels: no stress (1000 mL H_2O), mild stress (800 mL H_2O), moderate stress (600 mL H_2O), and severe stress (400 mL H_2O), with treatments applied every 2 days over a period of eight weeks. The results demonstrated the negative impacts of water stress, especially under moderate and severe conditions. Specifically, there was a reduction in height and dry weight in both species, with cotton plants exhibiting greater reductions, ranging from 9–38.6% in height and 40.1–62.1% in dry weight. Compared to the control, velvetleaf responded to water stress more mildly, with reductions in height and dry weight ranging from 3–19.3% and 7–44.3%, respectively. A 2.5- and 2-fold reduction in the root dry weight was also recorded for cotton and velvetleaf, respectively. Chlorophyll content in severely stressed cotton plants (400 mL) decreased by ~45% compared to initial values, while velvetleaf maintained relatively stable chlorophyll concentrations across all irrigation treatments. Notably, severely stressed velvetleaf plants resorted to leaf abscission at the fourth week after treatment (WAT). Finally, an increase in proline and total phenolic content was observed as a response to water stress. Specifically, proline increased by ~505% in cotton (WAT 6) and ~567% in velvetleaf (WAT 4). Total phenolics peaked at WAT 3, with increases of ~145% and ~120% in cotton and velvetleaf, respectively, before subsequently declining by ~75% in cotton and ~58% in velvetleaf.

KEYWORDS: Abiotic stress; antioxidants; chlorophyll; total phenolics; proline; biomass; growth stage

1 Introduction

Cotton (*Gossypium* spp.) represents one of the world's most significant row crops, comprising the primary raw material for the global textile industry with an annual economic impact exceeding € 500 billion [1]. Among the four commercially cultivated species, *Gossypium hirsutum* L. dominates global production, accounting for over 90% of the world's cotton fiber production [2]. In 2019, global cotton cultivation exceeded 20 million hectares, yielding approximately 25 million tons of seeds [1]. In Greece, cotton is one of the most economically significant arable crops, with fiber quality being influenced by both environmental and regional conditions [3]. Cotton cultivation relies heavily on consistent water availability throughout its growth cycle, requiring approximately 6–7 million liters of water per hectare, depending on rainfall and soil characteristics [1,4]. The crop thrives under optimal temperature conditions ranging from 24 to 32°C, with irrigation management constituting one of the most critical agricultural practices, especially in arid and semi-arid regions, where water availability is inherently limited [3].

However, the sustainability of cotton production faces numerous challenges stemming from climate change, mostly because of alterations in seasonal temperature, precipitation patterns, and water availability [5–7]. The increasing frequency and intensity of drought events pose a considerable threat to cotton cultivation worldwide. Water stress induces significant morphological alterations in cotton plants, including reductions in plant height, above-ground biomass, leaf area index, and node number [8–10]. Yield losses under water-stress conditions can be substantial, with sensitive growth stages such as flowering and boll formation being particularly vulnerable [11,12]. As drought phenomena are projected to become more frequent and severe under future climate scenarios, understanding how cotton and associated weed species respond to water scarcity is increasingly critical for the sustainability of cotton-based agricultural systems [13].

The challenges posed by drought are further exacerbated by biotic stresses, and particularly weed competition. Weeds represent a major constraint to cotton productivity, competing for essential resources including water, nutrients, light, and space [14]. Yield losses attributable to weeds can range from 40% to 85%, with the extent of damage depending on weed species composition, density, duration of competition, and crop developmental stage [15]. Among the most problematic weed species in cotton systems is velvetleaf (*Abutilon theophrasti* Medik.), an annual summer broadleaf weed of the Malvaceae family [14,16]. Velvetleaf is widely distributed across all the cotton-growing regions worldwide. It presents unique management challenges due to its prolific seed production (700 to 28,000 seeds per plant) and exceptional seed longevity, with viability in soil exceeding 50 years [17]. Under adequate water availability, velvetleaf exhibits rapid and vigorous growth, enabling it to effectively outcompete cotton during critical early growth stages [16]. Mausbach et al. (2022) [16] reported that even under moderate water stress, velvetleaf plants reached heights of 108 to 123 cm and achieved 50% of their maximum height within just six weeks after emergence, highlighting its remarkable growth capacity even under suboptimal conditions. Furthermore, velvetleaf demonstrates considerable adaptability to water-limited environments. Karkanis et al. (2011) [18] observed that root growth in velvetleaf is largely unaffected by water stress, while the plant resorts to early leaf abscission and accelerated seed maturation as survival strategies under severe water stress. This combination of competitive vigor under favorable conditions and physiological resilience under stress constitutes velvetleaf, a particularly challenging weed in cotton systems facing increasing drought pressure due to climate change [15,17].

At the physiological level, water stress triggers extensive biochemical and metabolic alterations in plant tissues. In cotton, water deficit significantly reduces photosynthetic rate, transpiration, stomatal conductance, and chlorophyll content [10,19]. Chlorophyll *a* and b, hold a fundamental role in the photosynthetic capacity of plants. Under water stress, cotton plants typically exhibit reductions in chlorophyll *a*, chlorophyll b, and total chlorophyll concentrations, thereby reducing cotton plants' photosynthetic efficiency [20,21]. These reductions occur primarily through two mechanisms: inhibition of chlorophyll biosynthesis and acceleration of chlorophyll degradation driven by enhanced reactive oxygen species (ROS) generation [21,22]. Stomatal closure under water deficit further limits CO_2 assimilation, creating a feedback loop that compounds chlorophyll loss and reduces overall photosynthetic capacity [19]. The extent of chlorophyll degradation varies among cultivars and species, with drought-tolerant genotypes generally exhibiting greater chlorophyll stability under water deficit conditions [21,22]. Comparatively, the chlorophyll responses of broadleaf weeds, such as velvetleaf, to water stress have received considerably less attention, representing a gap in our understanding of how weed species maintain photosynthetic function under stress conditions. Environmental stresses, including drought, stimulate the production of ROS within plant cells, leading to oxidative stress when ROS accumulation exceeds antioxidant defenses [23].

Elevated ROS levels can damage cellular components through oxidation of photosynthetic pigments and degradation of membrane lipids, proteins, and nucleic acids [22]. To counteract oxidative damage, plants have evolved sophisticated enzymatic and non-enzymatic antioxidant systems, among which proline and phenolic compounds are particularly prominent [23,24].

Proline, the predominant osmoregulatory solute in plants, accumulates markedly under water stress through either enhanced synthesis or reduced degradation [22]. This amino acid serves multiple protective functions, including free radical scavenging, stabilization of the cytoplasmic reductive environment, and preservation of membrane and enzyme integrity [25]. Additionally, proline acts as a reservoir for nitrogen and carbon, which the plant can mobilize during recovery after the stress subsides. Studies on cotton cultivars have demonstrated variable proline accumulation under water stress, with cultivar-specific differences reflecting variable degrees of drought tolerance [8,22]. Notably, wild species have been reported to accumulate proline more rapidly and at higher concentrations than their domesticated counterparts under equivalent stress conditions. This suggests that domesticated plant species selected for maximum yield under favorable conditions may have reduced their capacity for rapid osmotic adjustment [26].

Phenolic compounds are secondary metabolites that accumulate under water deficit and contribute to antioxidant defense, while aiding in the prevention of cellular water loss through covalent bonding with cell wall carbohydrates [22]. They are also involved in the biosynthesis of lignin and suberin, structural compounds that strengthen vascular tissues and form hydrophobic barriers against water loss, respectively [27,28]. Total phenolic content in cotton generally increases under moderate water stress, although the magnitude of the response varies with stress severity, duration, and cultivar [8,29]. Wild and ruderal plant species, including agricultural weeds, have been reported to constitutively maintain higher phenolic concentrations than domesticated crops, attributed to their evolutionary exposure to a broader range of biotic and abiotic stressors [30,31]. However, the extent to which the phenolic response of velvetleaf to drought differs from that of cotton under equivalent conditions has not been directly investigated, representing a key knowledge gap that the present study aims to address. In Fig. 1, we present the main response mechanisms of plants to water stress, where the key factors examined in this work are shown in bold and with a white font.

Despite extensive research on drought response in cotton, comparative studies examining how cotton and velvetleaf respond under equivalent conditions of limited water availability remain scarce. Understanding the differences in resilience between a domesticated crop and its primary weed competitor under water deficit provides valuable insights for integrated weed management and for breeding more drought-tolerant cotton cultivars. Therefore, the present study aims to assess the effects of water stress on these two co-occurring species and to examine their morphophysiological and biochemical responses. We hypothesized that velvetleaf, as a ruderal species, would exhibit greater adaptability to variable water availability. In contrast, we expected cotton plants to show reduced adaptability and plasticity under water stress as a consequence of domestication, and consequently, to experience greater impairment of their morphophysiological and biochemical characteristics.

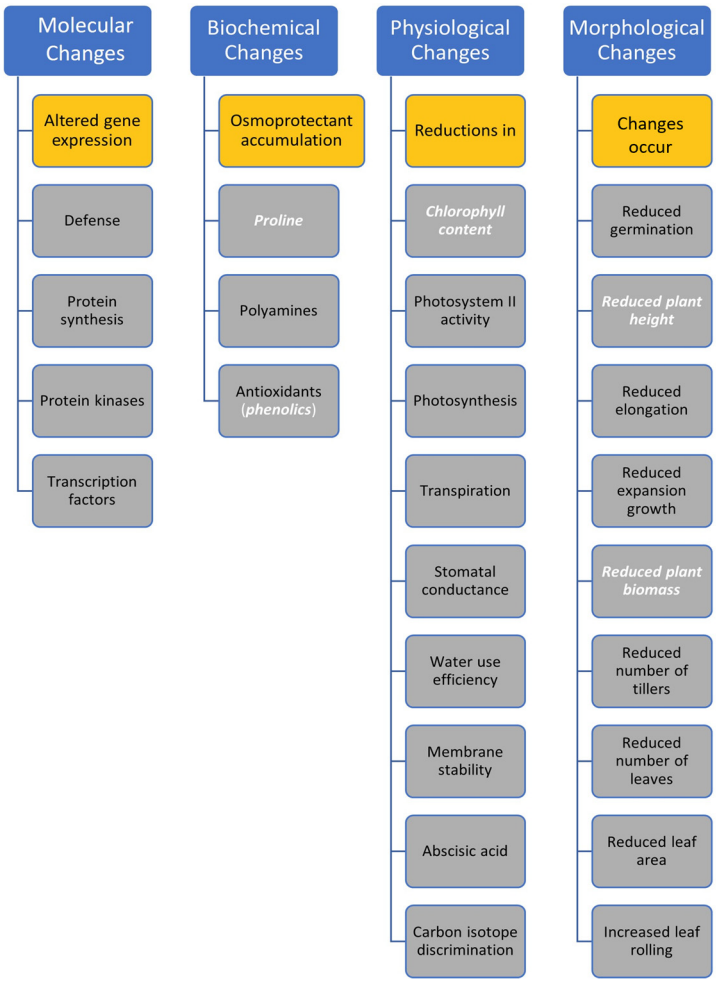

Figure 1: Conceptual diagram summarizing plant responses to water stress [32]. Factors examined in the current study are bold, italicized, and with a white font.

2 Materials and Methods

2.1 Plant Material and Growth Conditions

A greenhouse experiment was conducted at the University of Ioannina–Department of Agriculture, Arta, Greece, from May to October 2023, to assess the effects of water stress on the morphophysiological and biochemical characteristics of cotton and velvetleaf. After sowing, the plants were uniformly irrigated for eight weeks, followed by the stress imposition period (8 weeks). Subsequently, plants were again uniformly irrigated until harvest at 16 WAT (24 weeks after sowing). The experimental design comprised a randomized complete block design (RCBD) with four blocks (replications), with water stress as the treatment. The four irrigation regimes were 1000 mL (no stress, control) representing full capacity, 800 mL (mild stress), 600 mL (moderate stress), and 400 mL (severe stress). These irrigation regimes were selected to represent approximately 100%, 80%, 60%, and 40% of the estimated water requirements of both species at the growth stage studied. Similar water stress conditions have been imposed by other researchers [33,34].

A total of 192 plants in 5 L pots were used (96 cotton and 96 velvetleaf). Each block contained 48 pots (24 cotton and 24 velvetleaf), with six plants per treatment, per species, per block. Individual plants served as the experimental unit. Water stress was imposed from the pinhead square stage and maintained for

eight weeks. The experiment was conducted in a greenhouse (average day/night temperatures of 34/20°C and a light intensity of 1500 μmol m^{-2} s^{-1}), where the pots remained for the duration of the experiment. Before sowing, the pots were filled with equal amounts of growing substrate, and two seeds were sown in each pot to ensure germination. If both seeds germinated, the most vigorous plant was selected to continue the experiment. Before stress imposition, the plants were uniformly irrigated daily until they reached the pinhead square stage. Subsequently, the four irrigation regimes were initiated and applied every two days.

The growing substrate comprised soil and brown peat in a 1:1 ratio (v/v). The soil was collected from the experimental farm of the University of Ioannina in Arta. After the acquisition, the soil was analyzed to define its texture. It was classified as clay loam (CL), comprising 30.7% clay, 32.4% silt, and 36.8% sand.

Velvetleaf seeds were obtained from the Institute of Plant Breeding and Genetic Resources (IPGRB) in Thessaloniki, Greece. Cotton seeds (variety Olivia Stoneville, Stoneville, Mississippi, USA) were obtained from BASF Hellas S.A. To ensure the successful establishment and development of velvetleaf seeds under stress conditions, their germinability was examined according to the protocol developed by Begum et al. (2022) [35]. The Olivia Stoneville Cotton seeds were not subjected to quality testing, as their germination capacity and health status are certified by the supplier [36].

The germination test for velvetleaf seeds was conducted according to the protocol of the International Seed Testing Association (ISTA) [37]. Specifically, 10 sterilized plastic Petri dishes were lined with filter paper moistened with 5 mL of deionized water and Captan fungicide solution. Subsequently, 20 velvetleaf seeds were placed in each dish at equal intervals. The dishes were then transferred to a growth chamber that was set at a constant temperature of 25°C, a relative humidity of 90%, and a photoperiod of 14 h. The seeds remained in the chamber for 14 days, and germination was recorded every 2 days. Only seeds with a radicle ≥2 mm were considered germinated. The germination capacity was estimated to be greater than 90%.

Seed viability was estimated based on the tetrazolium test [38]. According to Lakon (1949) [38], tetrazolium (2,3,5-triphenyltetrazolium chloride) reacts with the hydrogen released during the enzymatic activity of dehydrogenase in living tissue. This reaction produces an insoluble red dye known as formazan. In non-living tissues, the color remains unchanged; hence, viable seeds can be distinguished from non-viable ones [39]. The methodology for assessing seed viability was identical to that for seed germination, with the exception that the seeds were immersed in a 2 mL solution comprising deionized water and tetrazolium. The seeds remained in the growth chamber for 2 days, and the percentage of viable seeds reached 98% (results not shown).

2.2 Parameters Measured

2.2.1 Plant Height and Dry Weight

Plant height was measured weekly and expressed as cm. At harvest (16 WAT), plants were cut at the substrate level and subsequently transferred to the laboratory to dry at room temperature. Total above and below-ground dry weights were measured using a precision scale and were expressed as g.

2.2.2 Chlorophyll Content Measurements

Chlorophyll content was measured weekly from the fourth uppermost fully expanded leaf of each plant, using a portable chlorophyll content meter (CL-01 Chlorophyll Meter, Hansatech Instruments, United Kingdom). Calculation of total chlorophyll content was performed using Eq. (1) [40].

$$y = 2.3636x + 4.2828 \quad (1)$$

where y = total chlorophyll content (a + b); x = CL-01 value.

2.2.3 Proline and Total Phenolic Content Measurements

Leaf proline and total phenolic content were estimated from the fourth and fifth uppermost fully expanded leaves of each plant, respectively. The leaves were sampled weekly, placed in air-tight plastic bags, and retained in the freezer (−20°C) until extraction and determination.

Proline extraction and determination were performed according to the methodology described by Bates et al. (1973) [41]. Initially, 0.1 g of fresh plant tissue was ground using a mortar and pestle. Subsequently, 2 mL of a 70% ethanol solution (70 mL ethanol and 30 mL deionized water) was added, and the samples were further homogenized. Then, another 2 mL of the 70% ethanol solution was added to ensure complete extraction. The resulting homogenate was transferred to Falcon tubes and retained in the refrigerator (4°C) for 24 h to achieve complete recovery of the proline fraction. Subsequently, 1 mL of the plant tissue extract was added to 2 mL of acid-ninhydrin solution and placed in new Falcon tubes. The mixture was vortexed for 15 s to ensure homogeneity. It was then incubated in a water bath at 95°C for 25 min to allow development of the proline-ninhydrin chromophore. Following incubation, the samples were placed in an ice bath to cool down. The samples were then centrifuged at 4000 rpm for 5 min to precipitate any remaining particulates. The resulting supernatant was transferred to a cuvette, and the absorbance was measured at λ = 520 nm using a UV-VIS spectrophotometer (Jasco-V630 UV-VIS). A solution of 2 mL of ninhydrin and 1 mL of 70% ethanol solution was used as a blank. The concentration of proline was estimated based on the calibration curve (R^2 = 0.9966), using proline solutions ranging from 0.025 to 0.8 mM. The data were reported in µmol of proline g^{-1} of fresh leaf weight (µmol g^{-1} FW), calculated from the µmol of proline in the leaf mass obtained during the extraction procedure.

The total phenolics extraction procedure was based on the method developed by Morsy & Abdel-Aziz (2014) [42]. According to their methodology, the leaves were placed in a drying oven at 40°C for 48 h. Dry plant tissue weighing 0.1 g was ground using a mortar and pestle. Then, 10 mL of 80% ethanol (80 mL ethanol and 20 mL deionized water) was added to the samples and homogenized. Finally, the samples were placed in Falcon tubes and retained in the refrigerator (4°C) for 24 h. For the determination of total phenolics, the Folin-Ciocalteu [43] method was employed. Specifically, 250 µL of the plant extract was transferred to new Falcon tubes and mixed with 0.5 mL of the Folin-Ciocalteu reagent. After 1 min, 5.25 mL of deionized water and 4 mL of Na_2CO_3 7.5% w/v were added and homogenized using a vortex mixer. After homogenization, the samples were incubated for 2 h in the dark at room temperature to allow color development. For the blank sample, the same procedure was followed, except that 250 µL of deionized water was used instead of plant extract. The absorbance was measured spectrophotometrically at 765 nm (Jasco-V630 UV-VIS) against the prepared blank. Gallic acid was used as a standard for the quantification of TPC, and the results were reported in mg GAE (Gallic Acid Equivalent) g^{-1} of leaf dry weight (mg GAE g^{-1} DW).

2.3 Experimental Design and Statistical Analysis

A randomized complete block design (RCBD) with four blocks (replications) was employed to investigate the effects of water stress on the parameters described above. Each block contained six plants per treatment, per species, which served as individual experimental units, yielding 24 observations per treatment, per species. Analysis of variance (ANOVA) was performed to detect statistically significant differences between treatments and plant species using JMP software, Pro 17 for Windows (SAS Institute Inc., Cary, NC, USA). Mean comparisons were performed using Fisher's least significant difference (LSD) test at α = 0.05. Before ANOVA, assumptions of normality and homogeneity of variance were tested

using the same statistical package mentioned above. Due to a statistically significant difference between species (i.e., cotton and velvetleaf), results on total chlorophyll, chlorophyll *a*, and chlorophyll *b* were analyzed separately.

3 Results and Discussion

3.1 Effect of Water Stress on Plant Height and Dry Weight Accumulation

ANOVA revealed significant effects of species, water stress, and their interaction on plant height (p = 0.0041). Velvetleaf plants were significantly taller than cotton across all WAT, except during the first two WAT under the 400 and 600 mL H_2O pot^{-1} treatments, where both species exhibited similar heights. Under adequate irrigation (800 and 1000 mL H_2O pot^{-1}), velvetleaf exhibited rapid growth between WAT 5 and 8, increasing from ~1.5 to over 2 m, while severely stressed plants (400 mL) increased by only 3 cm between WAT 5 and 8. Cotton plants exhibited gradual height increases throughout the experiment, with the height increase between WAT 5 and 8 ranging from ~3 cm in the 400 mL treatment to ~8 cm in the control. Control velvetleaf plants were consistently taller than all other treatments, except at WAT 1 and 8, where the 800 mL treatment did not differ significantly from the control (Fig. 2).

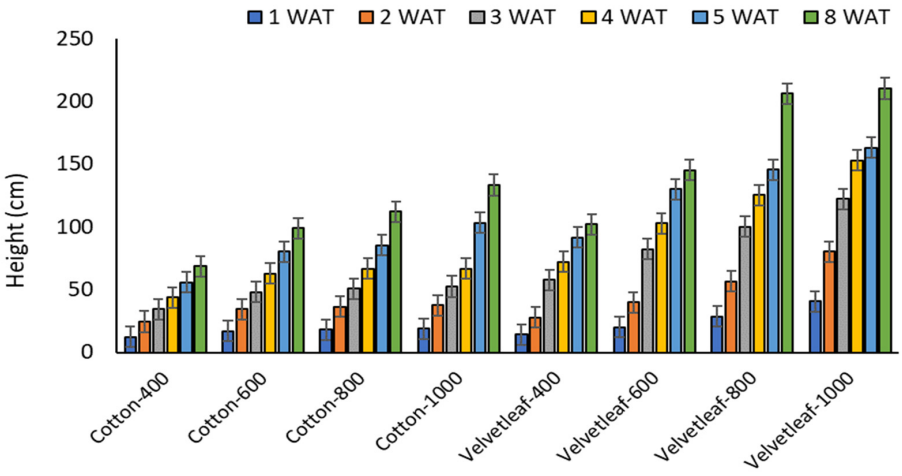

Figure 2: Effect of water stress × plant species × time on height of cotton and velvetleaf plants. Vertical bars depict LSD at α = 0.05 (where LSD bars overlap no significant differences occurred).

According to the literature, cotton and velvetleaf plants under water stress exhibit stunted growth and shorter stature due to reduced photosynthesis [44,45]. During water stress, plants prioritize root elongation while reducing the allocation of photosynthates to leaf production and stem elongation [46], thereby reducing transpirational surface area and improving water use efficiency.

Under optimal water conditions, velvetleaf exhibits a substantial height advantage that enables it to outcompete cultivated plants. Mausbach et al. (2022) [16] reported that velvetleaf reached heights of 108–123 cm and achieved 50% of its maximum height within just 6 weeks after transplanting, even under moderate water stress. Consistent with these findings, adequate water availability in our study led to rapid velvetleaf growth between 5 and 8 weeks after transplanting (WAT), whereas the 400 and 600 mL water treatments resulted in growth limitation comparable to that observed in cotton. This height advantage carries significant agronomic implications. Under well-watered or mildly stressed conditions, velvetleaf's rapid vertical growth allows it to overtop cotton during critical early developmental stages [16,18], thereby shading the crop, reducing light interception, and suppressing photosynthesis [44]. These competitive

effects can lead to substantial cotton yield losses [14,15]. Consequently, early and effective weed control prior to canopy closure is essential, particularly in irrigated cotton production systems where velvetleaf's growth advantage is most pronounced.

Water stress also significantly affected dry matter accumulation (p = 0.0002), with a significant species × water stress interaction. Cotton accumulated more dry weight than velvetleaf under the control treatment, while the opposite was observed at 800 and 600 mL H_2O. No significant differences between species were observed under severe stress (400 mL H_2O) (Fig. 3).

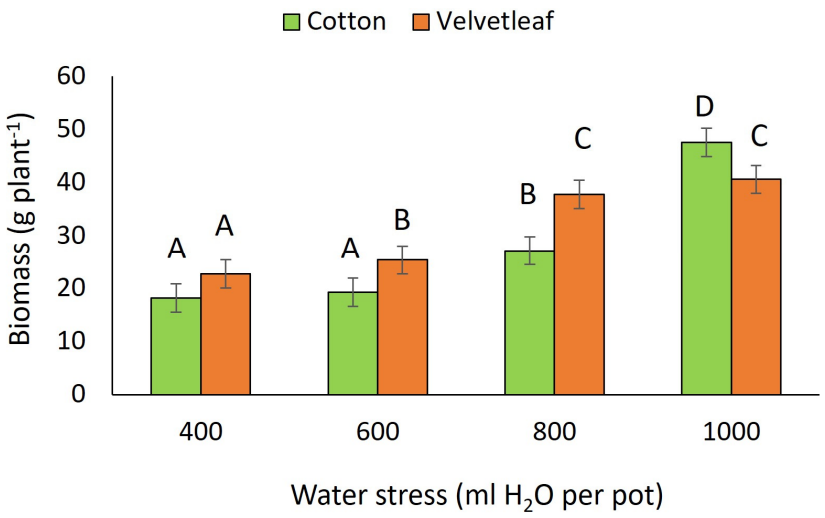

Figure 3: Effect of water stress × plant species on the biomass dry weight. Vertical bars depict LSD at α = 0.05. Columns with different letters are statistically different at a = 0.05.

The two species exhibited distinct patterns of biomass accumulation in response to water availability. Under high water availability, cotton accumulated more biomass, indicating that cotton has been selectively bred for maximum biomass accumulation under ideal water conditions [47,48], while velvetleaf is a ruderal weed adapted to persist across variable environmental conditions [49]. This divergence may be partly explained by differences in osmotic adjustment capacity, as domesticated crops generally exhibit a narrower range of osmotic adjustment compared to wild or ruderal species [50]. Consistent with this, velvetleaf maintained superior biomass production under moderate water stress (600–800 mL H_2O pot^{-1}), while cotton demonstrated significant reductions in dry matter accumulation even under moderate stress [45]. Mausbach et al. (2022) [16] showed that under continuous water stress at 50% of soil field capacity, velvetleaf can survive through reduced vegetative growth and shorter stature.

According to Patterson and Highsmith (1989) [49], drought early in the cultivation period reduces velvetleaf's competitive advantage over cotton as both species accumulate biomass at a comparable rate—a pattern consistent with our results under severe stress (400 mL H_2O). Therefore, in water-limited conditions where severe drought is frequent, the relative competitive pressure from velvetleaf may be lower than under well-irrigated conditions. However, as climate change projections for Mediterranean regions indicate an increasing frequency of moderate drought events [1], velvetleaf's superior performance under moderate stress is particularly concerning. Under these conditions, velvetleaf is likely to maintain a significant competitive advantage over cotton, potentially exacerbating yield losses and necessitating more intensive weed management interventions. Breeding programs aimed at improving cotton's osmotic adjustment

capacity and biomass maintenance under water deficit could therefore contribute not only to enhancing cotton's drought tolerance but also to improving its competitiveness against weed species such as velvetleaf.

3.2 Effect of Water Stress on Root Dry Weight

Water stress significantly affected root dry weight (p = 0.05), as did the interaction between water stress and species, whereas the main effect of species was not significant. Both species exhibited a significant reduction in root dry weight as water availability decreased. Adequately watered plants (800 and 1000 mL H_2O pot^{-1}), regardless of species, demonstrated the highest root dry weight (~10 and ~8.5 g $plant^{-1}$ for velvetleaf and ~8 and ~7.5 g $plant^{-1}$ for cotton, respectively) and did not present significant differences among them. Similarly, severely stressed plants (400 and 600 mL H_2O pot^{-1}) exhibited the lowest root dry weight (~4.5 and ~5 g $plant^{-1}$ for velvetleaf and ~3 and ~6.5 g $plant^{-1}$ for cotton, respectively) and no significant differences among them. Moreover, all examined velvetleaf plants, except those treated with 600 mL H_2O, showed greater root dry weight compared to the cotton plants (Fig. 4).

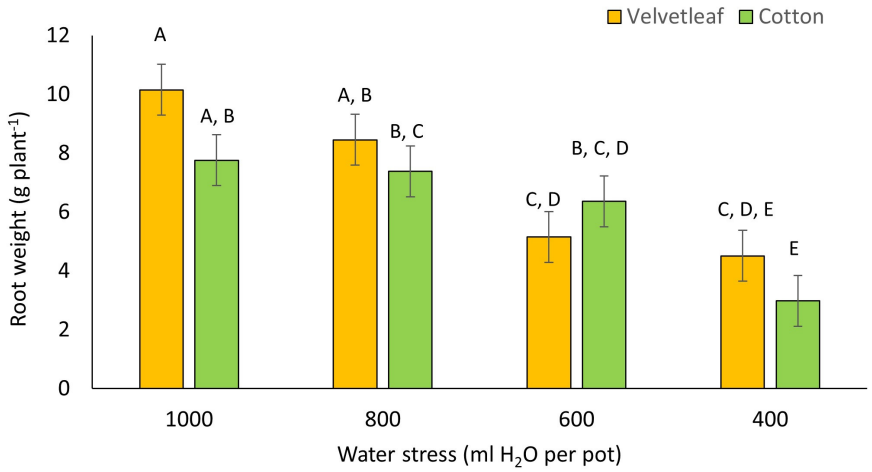

Figure 4: Effect of water stress on the root dry weight of cotton and velvetleaf. Vertical bars depict LSD at α = 0.05. Columns with different letters are statistically different at a = 0.05.

According to Aslam et al. (2023) [51], cotton plants that form longer roots during drought exhibit greater tolerance to water scarcity compared to genotypes producing shorter roots. While drought-sensitive genotypes may reduce root length as a resource-saving mechanism, drought-tolerant plants allocate resources to root elongation to access limited water sources more efficiently, exhibiting increased root length, root biomass, and higher relative water content [51].

Karkanis et al. (2011) [18] demonstrated that root growth in velvetleaf is not affected by water stress, while aboveground biomass, number of bolls, and leaf area are significantly affected. This pattern of maintaining root growth while reducing aboveground vegetative growth represents a fundamental survival strategy in ruderal species under limited water availability [18,46]. The greater root dry weight of velvetleaf compared to cotton across most irrigation treatments, coupled with velvetleaf's superior aboveground biomass under mild and moderate stress, suggests a higher overall biomass allocation capacity in velvetleaf relative to cotton under water deficit. Under severe stress, however, the two species exhibited comparable aboveground dry matter accumulation, indicating that velvetleaf's biomass advantage diminishes as water deficit intensifies. Ball et al. (1994) [52] further observed that water stress had a delayed effect on roots compared to leaf expansion. Specifically, the effect of stress on leaves was evident within 2 days, while only

after 6 days in the roots, with root dry weight remaining largely unaffected even at peak stress, suggesting that drought-tolerant cotton plants prioritize root growth under water deficit.

By preserving root architecture and volume, drought-tolerant plants sustain their capacity for water and nutrient uptake from deeper soil strata, partially compensating for reduced water availability in the upper soil layers. This is reflected in the higher root-to-shoot ratio typically observed in stressed compared to well-watered plants, as shoot growth is suppressed more rapidly and severely than root growth under water deficit [52].

Drought tolerance in cotton is a varietal trait, with certain varieties exhibiting high tolerance, others intermediate, and some being considerably more sensitive to water stress [53]. The drought-tolerant Olivia Stoneville cultivar used in the present study [36] demonstrated competitive root biomass growth under moderate stress, consistent with the drought tolerance mechanisms described above. These findings underscore the importance of cultivar selection in drought-prone cotton production systems, as drought-tolerant varieties with enhanced root biomass maintenance may aid in sustaining crop competitiveness against stress-resilient weeds such as velvetleaf.

3.3 Effect of Water Stress on Total Chlorophyll Content

Water stress, WAT, and their interaction all significantly affected total chlorophyll content in cotton (p = 0.0034, p = 0.0001, and p < 0.0001, respectively). Chlorophyll content in control plants increased progressively throughout the experiment, reaching a maximum of 45 mg g^{-1} FW at WAT 8. In contrast, total chlorophyll content in all stressed treatments declined. Initial and highest recorded values for the 400, 600, and 800 mL treatments were 28, 32, and 32 mg g^{-1} FW, respectively. Notably, even mild stress (800 mL H_2O) induced a marked decline in chlorophyll content, underscoring cotton's sensitivity to limited water availability (Fig. 5).

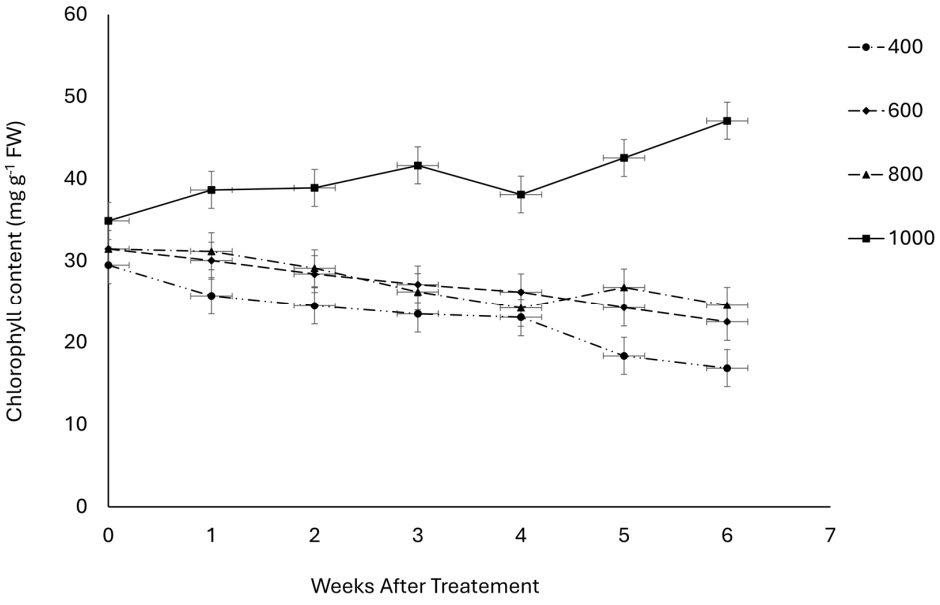

Figure 5: Effect of the interaction between water stress × sampling occasion (weeks after treatment) on total chlorophyll content in cotton. Vertical and horizontal bars represent the LSD values at a = 0.05 (where LSD bars overlap, no significant differences occurred).

Our results showed that even mild stress can have a detrimental impact on chlorophyll content in cotton plants, consistent with Hasan et al. (2018) [19], who reported significant reductions in chlorophyll under water stress. Water deficit limits chlorophyll accumulation by inhibiting its biosynthesis and accelerating its degradation [45,54], while enhanced ROS generation further damages chloroplasts and promotes pigment breakdown [55,56]. Stomatal closure under water stress reduces photosynthetic rate and chlorophyll production [57]. Genotype also plays a crucial role, with some genotypes exhibiting greater resilience through enhanced root elongation [51,58]. Stressed plants reached their maximum chlorophyll content prior to stress imposition, whereas control plants continued to accumulate chlorophyll throughout the experiment. This suggests that water availability is a primary determinant of chlorophyll dynamics in cotton and that even modest reductions in irrigation are sufficient to shift the plant from a growth-oriented to a stress-response mode.

In contrast, the main effect of water stress on the total chlorophyll content of the velvetleaf plants was not significant ($p = 0.069$), while WAT and their interaction were significant ($p < 0.0001$). Chlorophyll content in control plants increased progressively until WAT 3, reaching a maximum of 37 mg g^{-1} FW, before gradually declining until the end of the experiment. In all stressed treatments, chlorophyll content steadily declined throughout the experiment, with the highest recorded values appearing before treatment imposition. The maximum chlorophyll content in the 800, 600, and 400 mL treatments was 27.5, 26, and 26 mg g^{-1} FW, respectively. Notably, chlorophyll measurements in the 400 mL treatment could not be performed after WAT 4 due to leaf abscission (Fig. 6).

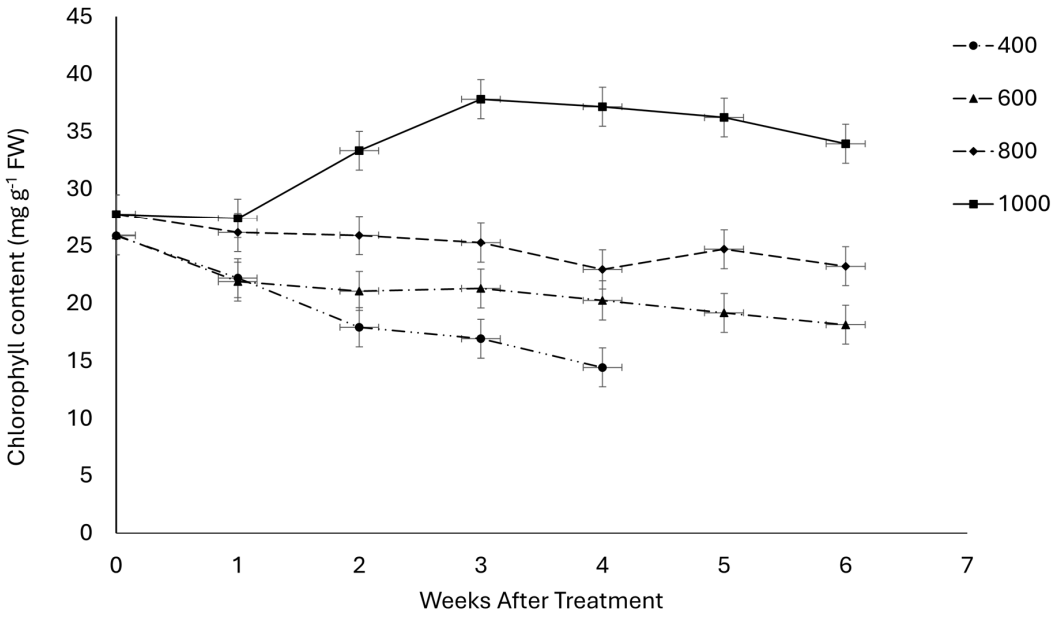

Figure 6: Effect of the interaction between water stress × sampling occasion (weeks after treatment) on total chlorophyll content in velvetleaf. Vertical and horizontal bars represent the LSD values at $a = 0.05$ (where LSD bars overlap no significant differences occurred).

The non-significant effect of water stress on velvetleaf chlorophyll content, in contrast to the significant effect observed in cotton, indicates that velvetleaf maintains relatively stable chlorophyll concentrations across a wide range of water deficit levels. This stability likely reflects velvetleaf's broader biochemical resilience under abiotic stress, consistent with its higher accumulation of antioxidant compounds, including

phenolics and proline [59,60], which protect chloroplast membranes from ROS-mediated oxidative damage [55,56]. By maintaining photosynthetic pigment concentrations under moderate stress, velvetleaf preserves carbon assimilation capacity at a level that cotton cannot sustain, potentially conferring a significant photosynthetic competitive advantage under moderately water-limited field conditions. Leaf abscission in severely stressed velvetleaf plants represents an adaptive strategy to minimize transpirational water losses and redirect resources toward reproductive development [61]. This is consistent with Karkanis et al. (2011) [18], who reported that velvetleaf prioritizes early inflorescence and seed maturation even when vegetative growth is severely curtailed. From an ecological perspective, this strategy is characteristic of ruderal weed species [50].

The differential chlorophyll response between the two species also has practical implications. Cotton chlorophyll content could serve as a reliable early indicator of water stress, enabling timely irrigation management decisions. Furthermore, velvetleaf's greater chlorophyll stability underscores the importance of proactive weed management before water availability becomes scarce.

3.4 Effect of Water Stress on the Accumulation of Proline and Total Phenolics

Proline accumulation in cotton was not affected by water stress (p = 0.083), whereas WAT and the water stress × WAT interaction exerted significant effects (p < 0.0001 and p = 0.041, respectively). The highest proline accumulation was recorded in severely stressed plants (~120 µmol g^{-1} leaf DW), followed by the 600 mL treatment (~20 µmol g^{-1} leaf DW), while both the 800 mL and control treatments accumulated approximately 17 µmol g^{-1} leaf DW (Fig. 7).

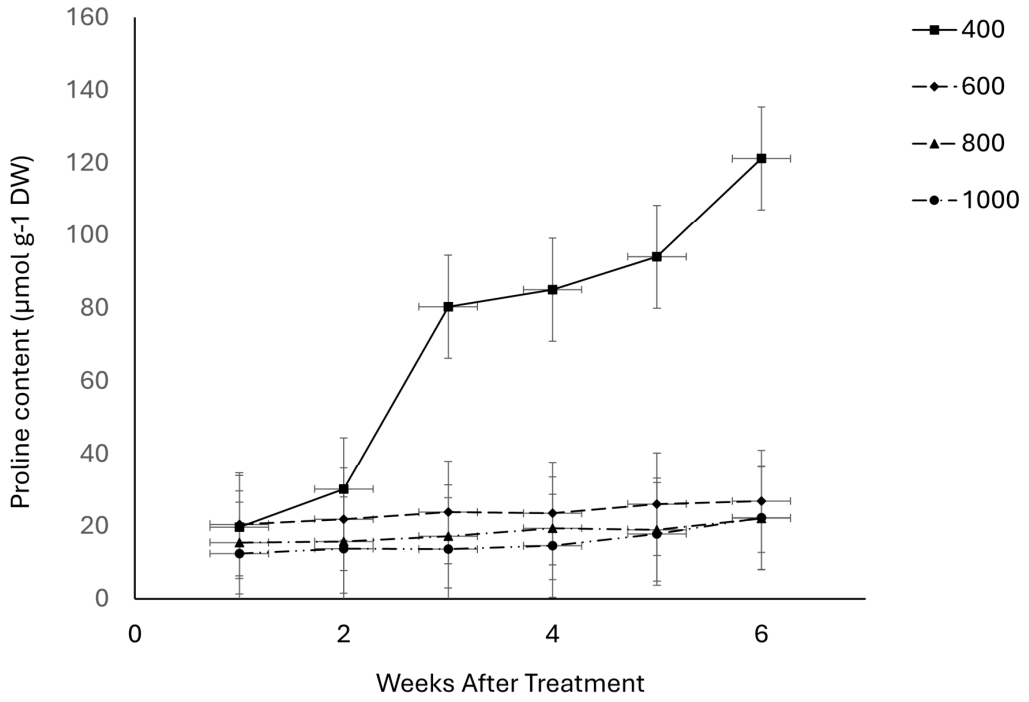

Figure 7: Effect of the interaction between water stress × sampling occasion (weeks after treatment) on proline content in cotton. Vertical and horizontal bars represent the LSD values at a = 0.05 (where LSD bars overlap no significant differences occurred).

Proline is an amino acid that accumulates in plants under osmotic stress conditions, including drought and salinity. It helps plants regulate osmotic pressure, maintain cellular turgor, and stabilize membrane integrity under drought [62]. Proline also has enhanced osmoprotectant abilities and is able to mitigate ROS-induced damage [63,64]. Furthermore, exogenous proline application in cotton under water stress has been shown to enhance chlorophyll content, dry matter accumulation, and leaf water content [65]. Proline accumulation is also reported in the roots of cotton plants under drought [66].

In our study, proline accumulation was significantly elevated only in the severely stressed cotton plants (~120 μmol g^{-1} leaf DW). The three remaining treatments maintained relatively stable and comparable levels (~17–20 μmol g^{-1} leaf DW) throughout the experiment. This threshold-type response suggests that cotton's osmotic adjustment capacity is activated primarily under severe water deficit. Stress levels sufficient to reduce growth and chlorophyll content were therefore insufficient to trigger osmotic adjustment mechanisms. This pattern may partly explain cotton's greater vulnerability to moderate drought compared to velvetleaf [50].

In velvetleaf, the main effect of water stress on proline accumulation was not significant ($p = 0.091$), while WAT and the water stress × WAT interaction were significant ($p < 0.0001$ and $p = 0.05$, respectively). Severely stressed plants exhibited the highest proline accumulation (~200 μmol g^{-1} leaf DW), increasing significantly from WAT 2 until leaf abscission at WAT 4. Moderately stressed plants accumulated ~75 μmol g^{-1} leaf DW, while the 800 and 1000 mL treatments remained low throughout the experiment (~40 and ~30 μmol g^{-1} leaf DW, respectively) (Fig. 8).

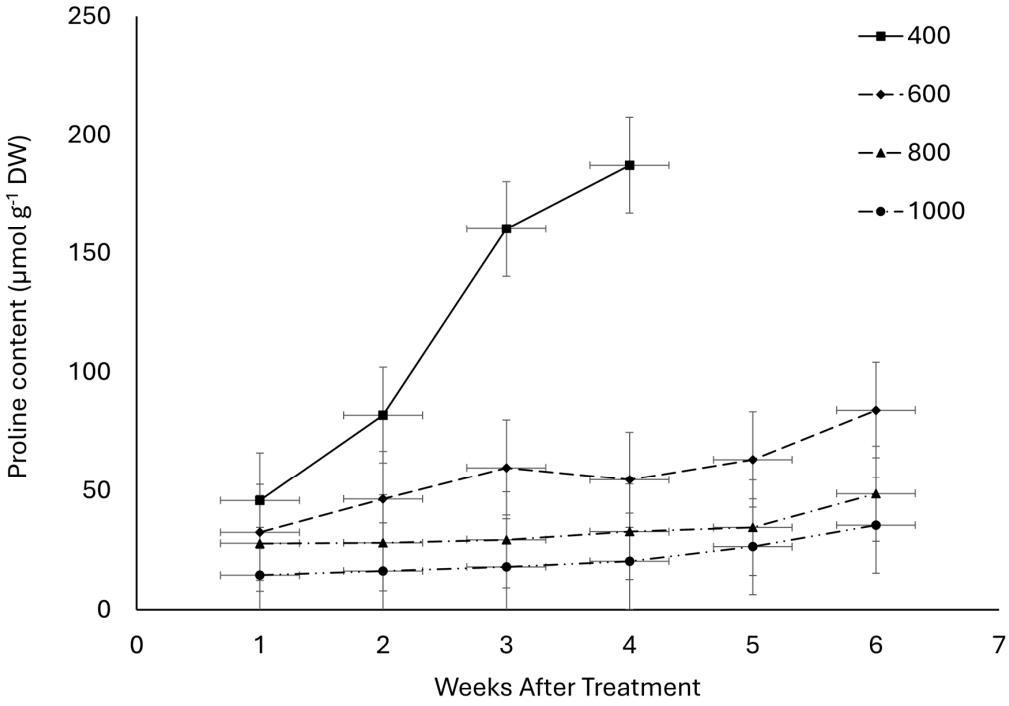

Figure 8: Effect of the interaction between water stress × sampling occasion (weeks after treatment) on proline content in velvetleaf. Vertical and horizontal bars represent the LSD values at $a = 0.05$ (where LSD bars overlap no significant differences occurred).

The markedly higher proline accumulation in severely stressed velvetleaf plants (~200 μmol g^{-1} leaf DW) compared to cotton (~120 μmol g^{-1} leaf DW) highlights the ability of weeds to better cope with

limited water availability than cultivated plants [60]. Leaf abscission in velvetleaf further emphasizes the prioritization of survival through propagation over vegetative growth under severe stress [18]. Similar results were reported by Mejri et al. (2016) [67], who observed that wild barley (*Hordeum maritimum*) exhibited higher proline accumulation during water stress compared to cultivated barley (*Hordeum vulgare* L.). Muzammil et al. (2018) [26] identified the allele P5cs1 as the driver of rapid and higher proline accumulation in wild barley, while Parida et al. (2008) [68] similarly reported enhanced activities of the biosynthetic enzymes P5CS and P5CR in drought-tolerant cotton varieties under water stress. Hence, wild species appear to possess specific alleles that aid them in responding rapidly to water stress by accumulating secondary and primary metabolites.

Greater proline accumulation in velvetleaf confers superior osmotic adjustment capacity, enabling its cells to maintain turgor pressure and membrane integrity under more severe water stress than cotton [62,63]. This broader osmotic adjustment range is consistent with velvetleaf's classification as a stress-adapted ruderal species that has evolved under conditions of unpredictable water availability [50,60]. From a practical perspective, proline concentration could serve as a reliable biochemical indicator of water stress severity in both species, potentially aiding irrigation management decisions and enabling early stress detection before visible morphological symptoms appear.

Total phenolic content in cotton was not significantly affected by water stress, whereas WAT and the water stress × WAT interaction were significant ($p < 0.0001$ and $p = 0.0056$, respectively). Phenolic accumulation peaked at WAT 3 in all treatments, with maximum values of approximately 21, 19, 14, and 9 mg GAE g^{-1} DW for the 400, 600, 800, and 1000 mL treatments, respectively, before converging to similarly low values by WAT 6 (Fig. 9).

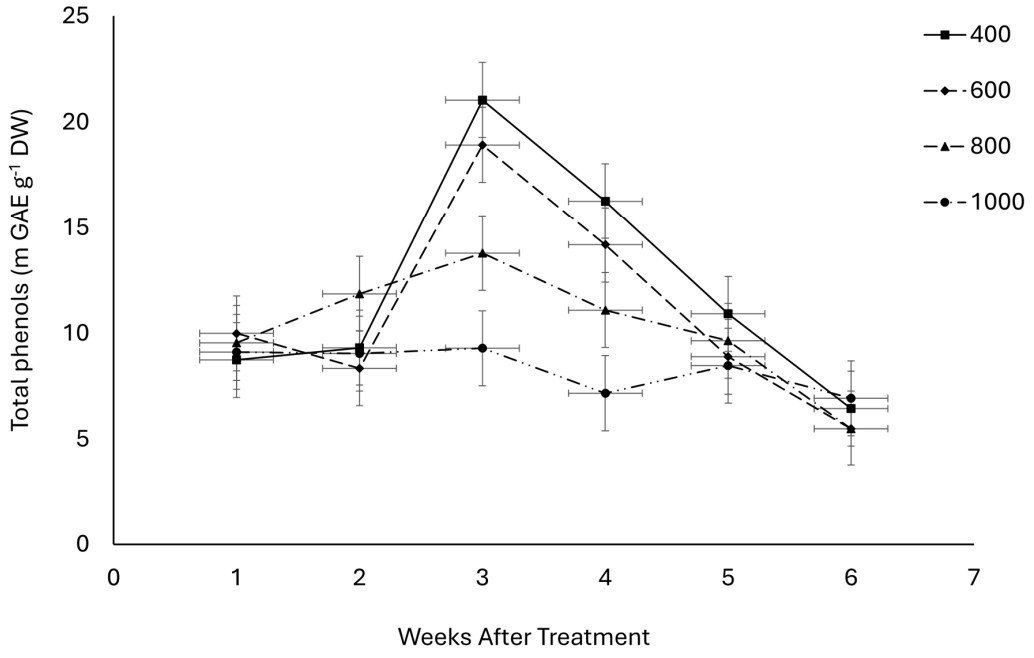

Figure 9: Effect of the interaction between water stress and weeks after treatment on total phenolic content in cotton. Vertical and horizontal bars represent the LSD values at $a = 0.05$ (where LSD bars overlap no significant differences occurred).

Plants produce phenolics to mitigate ROS accumulation and prevent water loss. These compounds are synthesized from excess carbohydrates, acting as antioxidants through radical scavenging and maintaining carbon homeostasis [22]. Phenolics are also involved in the production of lignin, which strengthens vascular tissues and prevents water diffusion into surrounding tissues [69]. Phenolic compounds also contribute to the biosynthesis of suberin, a hydrophobic barrier that limits water loss from plant tissues [28]. Turgorins, which partly comprise phenolic derivatives, are synthesized in certain plant species and induce a sudden loss in turgor in the leaves, causing rapid folding in response to injury, touch, and other external stimuli [27]. The initial peak in phenolic accumulation at WAT 3, especially in the severely and moderately stressed plants, suggests that phenylpropanoid biosynthesis was activated in response to ROS accumulation. The subsequent decline likely reflects the progressive depletion of photosynthates available for phenolic biosynthesis as water stress persisted and carbon fixation became limited [22,69].

Water stress also did not significantly affect total phenolic content in velvetleaf, while WAT and their interaction were significant, i.e., $p < 0.0001$ and $p < 0.05$, respectively. Similar to cotton, phenolic accumulation increased in all treatments up to WAT 3, then declined, stabilizing at WAT 4 and remaining constant until the end of the experiment. The highest phenolic content was exhibited by the moderately stressed plants (600 mL) at WAT 3, reaching ~47 mg GAE g^{-1} DW, while plants treated with 400, 800, and 1000 mL H_2O pot^{-1} exhibited ~50, 40, and 33 mg GAE g^{-1} DW, respectively. The 400 mL treatment showed no detectable phenolic content after WAT 4 due to leaf abscission (Fig. 10).

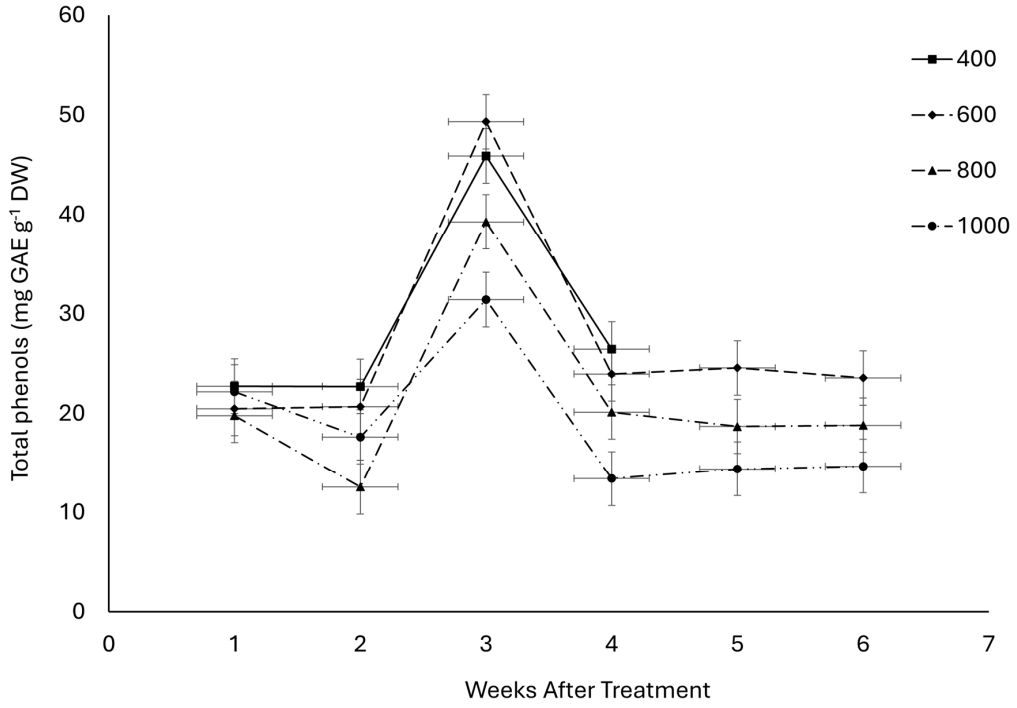

Figure 10: Effect of the interaction between water stress and weeks after treatment on total phenolic content in velvetleaf. Vertical and horizontal bars represent the LSD values at $a = 0.05$ (where LSD bars overlap, no significant differences occurred).

The consistently higher phenolic content in velvetleaf compared to cotton across all irrigation treatments, regardless of stress level, suggests that velvetleaf maintains a constitutively elevated phenolic defense system, rather than relying solely on stress-inducible phenolic production. Wild and ruderal

species that have evolved under broad biotic and abiotic pressure tend to maintain constitutively elevated secondary metabolites [30,31,59]. In contrast, domesticated crops such as cotton rely predominantly on inducible phenolic responses, reflecting their selection primarily for yield under managed, low-stress conditions [31,50]. This higher baseline phenolic content implies greater intrinsic protection against oxidative stress, enabling velvetleaf to sustain photosynthetic and metabolic function under drought or other abiotic stress conditions.

Disciglio et al. (2017) [59] reported higher concentrations of total polyphenols and antioxidants in the wild counterparts of *Cichorium intybus* and *Borago officinalis* compared to their cultivated relatives, with all 11 wild species examined showing significantly higher antioxidant accumulation. Similarly, Salhi et al. (2025) [30] noted that domesticated *Origanum compactum* exhibited nearly half the polyphenol content of its wild counterpart. Likewise, in our study, velvetleaf accumulated approximately double the phenolic content of cotton across all irrigation treatments.

The synchronous peak in phenolic concentration at WAT 3 in both species indicates a temporal response to ROS accumulation under water stress [45]. The subsequent decline after WAT 3 likely reflects two concurrent processes. Firstly, the reduced photosynthetic carbon availability under prolonged water deficit, which limits carbon supply for the phenylpropanoid pathway, and secondly, the oxidative consumption of phenolics in ROS scavenging at a rate exceeding their biosynthesis [22,69].

Collectively, the proline and phenolic analyses demonstrate that velvetleaf possesses both a broader osmotic adjustment range and a higher constitutive antioxidant defense capacity than cotton. These biochemical advantages likely underpin velvetleaf's superior resilience under moderate water stress. As climate change projections indicate an increasing frequency of moderate drought events in Mediterranean cotton-growing regions [1], velvetleaf's biochemical resilience is likely to become an increasingly important factor in weed management. Therefore, developing cotton cultivars with enhanced constitutive antioxidant capacity and broader osmotic adjustment range could represent a key breeding strategy for reducing competition from drought-tolerant weed species, such as velvetleaf. In addition, optimized irrigation scheduling that minimizes the duration of moderate water stress should be considered as a complementary agronomic approach [1,50].

4 Conclusions

Water stress reduced plant height, biomass, and chlorophyll in both cotton and velvetleaf, while triggering proline and phenolic accumulation. Velvetleaf showed stronger drought resilience than cotton—maintaining better growth and accumulating ~200 μmol/g proline and ~47–50 mg/g phenolics versus lower levels in cotton. Key implications of this research are mostly related to crop-weed competition, where moderate water stress may boost velvetleaf performance over cotton, especially as the Mediterranean climate especially changes in precipitation pattern and consequent drought periods intensify. In addition, proline and phenolic compounds could be used as biochemical markers for monitoring water stress in cotton well in advance before macroscopic symptoms become visible. Finally, the genetic pathway of velvetleaf's water resilience pathway for proline synthesis and the phenylpropanoid pathway for phenolic biosynthesis offers a broader research avenue for developing drought-tolerant cotton cultivars.

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Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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