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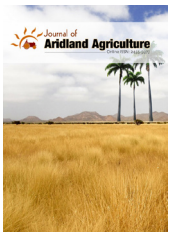

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The response of safflower genotypes to drought stress induced at the rosette stage

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ABSTRACT

Drought stress impact fuelled by climate change is becoming a global concern as it threatens food security in many arid and semi-arid lands (ASALs). However, planting drought-tolerant crops such as safflower (*Carthamus tinctorius* L.), in the ASALs can help ensure food security and sustainability. Greenhouse and field experiments were conducted during the 2021/2022 planting season to evaluate the response of safflower genotypes to drought stress. Factors under study were stress conditions and five safflower genotypes. The results revealed that drought stress reduced the chlorophyll content, leaf relative water content (LRWC), and plant height while the activities of ascorbate peroxidase (APX) and proline content increased. This trend was observed at different stress durations under both experiments with the effects being more noticeable when stress duration increases from 10 to 30 days. To rank genotypes based on their overall superiority, the genotype by trait (GT) biplot analysis was employed based on the mean values of all studied traits for five safflower genotypes stressed for 30 days. The results showed that genotype Kenya9819 was overall superior (drought tolerant) while Gila and Turkey ranked poorly (drought sensitive). This suggested that different safflower genotypes have different degrees of tolerance to drought stress depending on the duration of stress.

KEYWORDS: Safflower, Dought stress, GT biplots, Proline, Ascorbate peroxidase

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INTRODUCTION

Drought is one of the abiotic stress factors that seriously inhibit crop productivity and yield in arid and semi-arid lands (ASALs). The effects of drought stress are expected to worsen in the future as a result of progressive global warming with one of the negative results being the increasing margins of desertification in the ASALs (Singh & Laxmi, 2015). Fortunately, some plants have developed acclimation and adaptation mechanisms in response to drought stress. However, their response to drought stress depends on the species' intrinsic strategy along with the duration and severity of the stress period (Naderi *et al.*, 2014). Among oilseed crops, safflower is known to be the most drought-tolerant and can produce a good yield in ASALs (Weiss, 2000). Safflower genotypes vary in their response to drought stress and thus genotypes that exhibit excellent drought tolerance characteristics are better suited to be used by farmers because they can save the costs of implementing other drought management strategies (Mosupiemang *et al.*, 2022).

Photosynthetic pigments such as chlorophyll content have been reported to decrease with drought stress in many plant species including safflower (Mohammadi *et al.*, 2016; Farooq *et al.*, 2020). Plant height is one of the variables that indicates vegetative growth of plants. Plant height has been reported to be reduced by drought stress, especially if it occurs during the vegetative stage (Tayebi *et al.*, 2012; Kazemeini *et al.*, 2015; Joshan *et al.*, 2019). Similarly, safflower leaf relative water content was found to decrease with drought stress (Mohammadi *et al.*, 2016; Manvelian *et al.*, 2021). In response to drought stress, plants enhance the production of osmolytes such as proline for the protection of membranes, proteins, and enzymes against various stresses (Singh *et al.*, 2015). Studies on drought tolerance mechanisms of safflower have revealed an increase in proline levels with drought stress (Aeini *et al.*, 2018; Farooq *et al.*, 2020; Çulha *et al.*, 2021). Exposure of plants to unfavourable environmental conditions such as drought stress also increases the production of reactive oxygen species (ROS) which are highly reactive and toxic, causing damage to proteins, lipids, carbohydrates, and DNA which ultimately results in cell death (Gill & Tuteja, 2010). Ascorbate peroxidase has a high affinity to hydrogen peroxide (H_2O_2), and it is present in every cell compartment of plants, and its expression is highly regulated, making it important in the removal of H_2O_2 for signalling purposes (Mittler & Poulos, 2005). Studies on the activities of APX in safflower plants under drought stress are scanty. However,

the levels of APX were reported to increase with drought stress in safflower (Amini *et al.*, 2013; Çulha *et al.*, 2021).

Different safflower genotypes have different degrees of tolerance to drought stress depending on the duration of stress. Moreover, studying different mechanisms that different safflower genotypes display under different environmental conditions may aid in the selection and breeding for drought tolerance. Therefore, this study sought to evaluate the effect of drought stress duration on the physiological and biochemical traits of safflower genotypes.

MATERIALS AND METHODS

Experimental Design and Setup

Field and greenhouse experiments were conducted at the Botswana University of Agriculture and Natural Resources (BUAN) Content Farm in Sebele during the winter of 2021/2022 planting season (May 2021 until September 2021). This site is located at the latitude of 24° 33' South and longitude of 25° 54' East in Sebele, Gaborone in the southern part of Botswana. The experiment was arranged in a 2x5 factorial design in the field and greenhouse. Factors under study were drought (stressed and non-stressed control plants) and five safflower genotypes (Turkey, Kenya9819, Sina, PI537636, and Gila) making a total of 10 experimental units replicated three times. For the greenhouse experiment, a total of 30 pots of 30 kg capacity were used. Firstly, soil was sieved and a total of 25 kg of soil was weighed into each pot. For a field experiment, plants were planted along the drip lines at an intra-row spacing of 25 cm. Each plot were 2 m x 2 m plots spaced 1m apart while the main plots (stress and non-stress) were spaced by 2 m apart. The experiments were conducted concurrently during winter which is the dry season.

Drought Stress Induction

Drought stress was imposed at the rosette stage (two weeks after emergence). In the non-stressed treatment, plants were irrigated throughout the crop cycle to satisfy plant water needs. Drought stress treatment was imposed by withholding irrigation completely. Two access tubes were installed in the middle of each sub-sub plot/pot at a depth of 5 cm and 20 cm (greenhouse experiment) and 20 cm and 40 cm (Field experiment) to measure soil moisture. The soil moisture was measured by inserting the moisture meter (Theta probe type ML2x, Delta-Devices, Cambridge England) into the access tubes and readings were taken at each assessment date. Data was collected every after 10, 20 and 30 days of stress imposition.

Determination of Leaf Relative Water Content (RWC)

The topmost fully expanded healthy leaves were used for measurements. Four leaf samples were collected from different plants in each plot or pot. Each leaf was labelled and weighed to determine the fresh weight. To obtain turgid weight, leaves were soaked in distilled water for 24 hours at room temperature. Then after gently wiping the water from the leaf surface with a paper towel, turgid weight was measured. Leaf dry weight was determined by oven drying the leaves for 48 hrs at 66 °C, then weighed to get dry weight. The LRWC was calculated as per the formula:

$$RWC\ (\%) = [(FW\text{-}DW)/(TW\text{-}DW)]\ X\ 100$$

Where FW is the initial fresh weight, TW is turgid fresh weight and DW is the dry weight.

Chlorophyll Content and Plant Height

Five fully expanded fresh leaves were measured, simultaneously using a chlorophyll meter (SPAD-502 plus, Konica Minolta). Plant height was measured from the ground level to the apex of the main stem at each assessment date.

Sampling for Proline and Ascorbate Peroxidase (APX)

At each assessment date, leaf samples for laboratory work were collected from intact leaves (leaves without injury) and they were quickly frozen in liquid nitrogen and stored at -80 °C until further analysis. Then, the stored leaf samples were ground to a fine powder in liquid nitrogen using a pre-cooled pestle and mortar and then used for quantification of APX and proline.

Proline Determination

Proline was determined as per Bates *et al.* (1973). Approximately 0.2 g of ground leaf samples were weighed and extracted by homogenizing in 3% (w/v) aqueous sulfosalicylic acid using a pestle and mortar. Then, the extracted leaf samples were transferred into 15 mL tubes and kept in ice until using as crude samples. The crude samples were centrifuged at 3,000 g for 20 min at 4 °C. Then 2 mL of supernatant was mixed with 2 mL of acid ninhydrin and 2 mL of glacial acetic acid in a test tube. Then samples were incubated in a boiling water bath for 60 min, followed by shocking in an ice-cold water bath for 15 mins then allowed to cool at room temperature. The reaction mixture was extracted with toluene and mixed vigorously for 15-20 seconds. Then 300 µL of chromophore (upper part) containing toluene was transferred into a 96-well microplate. Absorbance was read at 520 nm in a spectrophotometer (SPECTROstar Nano (BMG Labtech microplate reader, Ortenberg, Germany) set to endpoint mode and using toluene as a blank. Proline standards were made from pure proline and were put through the same process as of the sample. The proline concentration was determined from a standard curve and calculated on a fresh weight basis.

Determination of Ascorbate Peroxidase (APX) Activity

Ascorbate peroxidase (APX) was determined as per Nakano and Asada (1981) with some minor modifications. Approximately 0.2 g of ground leaves was weighed and extracted by homogenizing in 0.2 M potassium phosphate buffer (pH 7.8 with 0.1 mM EDTA) supplemented with 1 mM ascorbate. The samples were then centrifuged at 15,000×g for 20 min at 4 °C.

The supernatant was transferred to a new 2 mL Eppendorf tube, and the pellet was re-suspended in 0.8 mL of the potassium phosphate buffer, and the suspension was centrifuged for 15 min at 15,000×g at 4 °C. Then supernatant pipetted out and combined with the initial supernatant in 2 mL Eppendorf tubes then stored at -80 °C and used as a crude enzyme extract in the quantification of APX. The reaction mixture for APX analysis contained 50 mM potassium phosphate buffer (at pH 7.0), 0.5 mM ascorbate, 0.25 mM EDTA, 3% H_2O_2, and crude leaf extract was used to initiate the reaction. Then absorbance was recorded after every 30 sec for 3 minutes at 290 nm. The enzyme activity was calculated from the extinction coefficient (2.8 mM^{-1} cm^{-1}) for the reduced ascorbate. The APX activities were determined spectrophotometrically using SPECTROstar Nano (BMG Labtech microplate reader, Ortenberg, Germany) set to kinetic mode and expressed as micromoles of ascorbate per minute per gram of fresh weight.

Statistical Analysis

The GT biplot analyses for all studied traits of genotypes were executed on R-Software version 4.2.2 using the METAN package of Olivoto and Lúcio (2020).

RESULTS

Generally, drought stress increased the chlorophyll content by 4.2% and 13.5% on days 20 and 30, respectively relative to the control under the greenhouse experiment (Figure 1a). In contrast, drought stress reduced chlorophyll content by 6.7% at day 30 under the field experiment (Figure 1b). However, at day 10 there were no marked differences between the control and stress plants under the field and greenhouse experiments (Figure 1). Among the stressed plants, the highest chlorophyll content of 63.06 SPAD reading was observed at day 30 of stress induction while the lowest values (46.71 SPAD reading) were observed at day 10. Additionally, the leaves from stressed plants were wilting and dark green in colour while those of control plants were shiny with bright green colour.

During the early stages of stress induction (days 10), there were no marked differences in plant height among the stressed and control plants in both experiments (Figure 2). However, there was a substantial reduction (25.3%) in plant height as stress progressed to day 30 under greenhouse experiment (Figure 2a). At day 30, the control plants attained a height of 24.27 cm which was taller than the height (18.12 cm) of the stressed plants in the greenhouse (Figure 2a). The results further showed that the stressed plants stopped growing when they were stressed beyond 20 days, unlike the control which increased growth by 19.6% (Figure 2a). As drought period increased plant height was significantly ($P<0.05$) reduced linearly by 25.5% and 31.9% on days 20 and 30 respectively in the field experiment (Figure 2b). The results revealed that the stressed plants increased growth by 30.3% from day 20 to day 30 while the control increased growth by 42.5% during the rosette stage under field experiment (Figure 2b).

Drought stress did not cause reductions in LRWC during the early stages of stress (days 10 and 20) but noticeable reductions were observed when stress was beyond 20 days and the reduction in LRWC increased linearly up to 30 days under the greenhouse (Figure 3a). Drought stress reduced LRWC by 43.6% at day 30 compared to control plants under the greenhouse (Figure 3a). The control plants had higher LRWC by 3.41-6.14% depending on number of days water stress was imposed in the field

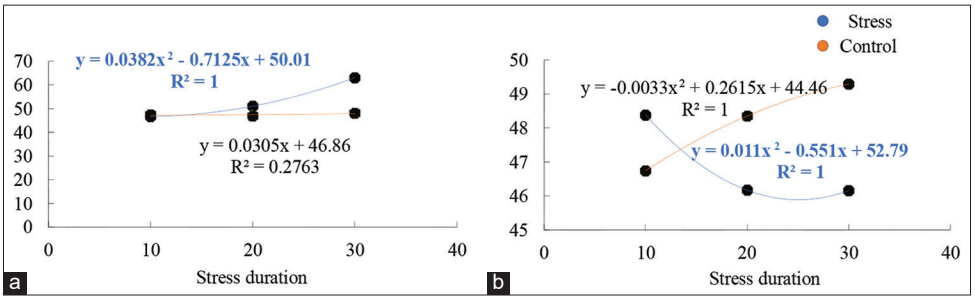

Figure 1: The effect of stress duration on the chlorophyll content of safflower grown under a) greenhouse and b) field experiment.

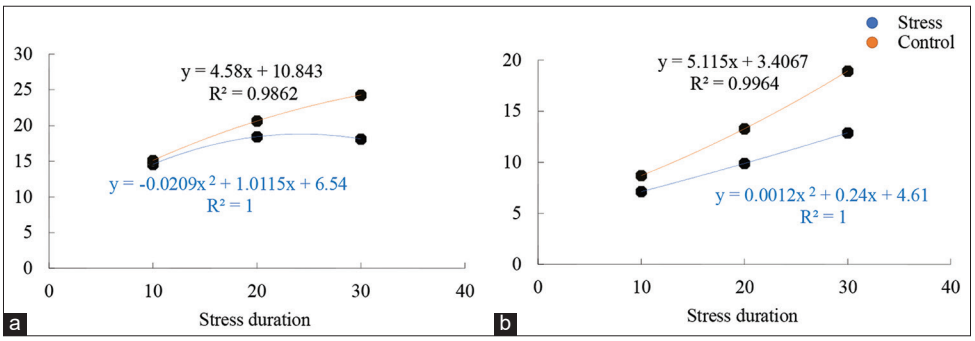

Figure 2: The effect of stress duration on the plant height of safflower grown under a) greenhouse and b) field experiment.

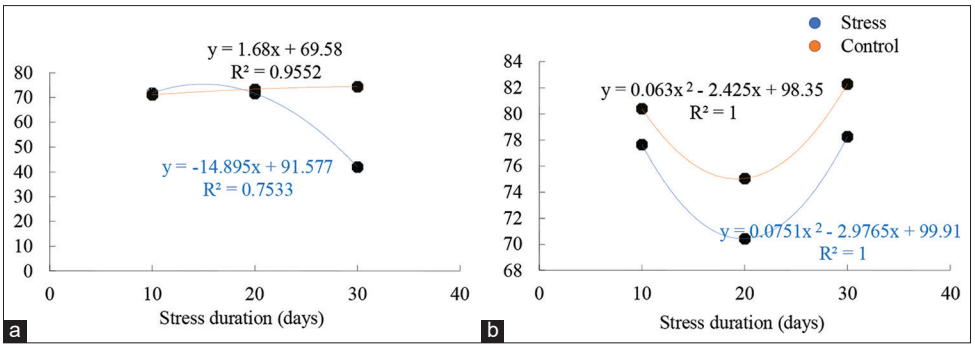

Figure 3: The effect of stress duration on the LRWC of safflower grown under a) greenhouse and b) field experiment.

experiment (Figure 3b). The highest reduction in LRWC (6.14%) was observed on stressed plants on day 20 in the field experiment (Figure 3b).

There were no marked differences in proline content observed between control and stressed plants 10 days after water stress induction under the greenhouse experiment and field experiments (Figure 4a & b). At 30 days after water stress induction, stressed safflower plants had 5x more proline content (51 µmoles/g FW) than control plants (10.16 µmoles/g FW) in the greenhouse (Figure 4a). The increase in proline content in both stressed and control plants with increase in water stress duration was quadratic (Figure 4a). Generally, water stress increased the proline content by 100% and 402% relative to control plants at 20 and 30 days of water stress, respectively in the greenhouse experiment (Figure 4a). Proline content of stressed plants was higher by 31% than control plants after 20 days of water stress in the field experiment (Figure 4b).

In the greenhouse and field experiment, an increase in water stress duration significantly ($P \leq 0.05$) increased APX content of stressed plants compared to control plants (Figure 5a & b). The APX content increased by 227, 137, and 173% after 10, 20, and 30 days of stress imposition, respectively compared to control plants in the greenhouse experiment (Figure 5a). Under the field experiment, the increase in APX was quadratic (Figure 5b).

The Associations Among Traits and the Trait Profiles of the Genotypes

The genotype by trait (GT) biplots presented in Figures 6 and 7 are traits means of five safflower genotypes drought stressed for 30 days under greenhouse and field conditions. Notably, only stressed genotypes were used. The Pearson correlation between any two traits is estimated by the cosine of the angle between the vectors of the traits. In this regard, vectors of two traits with acute angle represent positive correlation, those of obtuse angle represent negative correlation, while those of right angle indicate the absence of correlation (Yan & Frégeau-Reid, 2018). They further stated that the angle between a genotype and a trait designates the approximate level of the genotype for the trait. Therefore, an acute angle designates that the genotype is above average for the trait; an obtuse angle designates that the genotype is below average for the trait while a right angle designates that the genotype is average for the trait. Figure 6a displays that proline and APX correlated positively with each other and they both correlated negatively with chlorophyll content. Therefore, genotype Kenya9819 which had above-average plant height, proline and APX, had below-average chlorophyll content and LRWC. Additionally, genotype PI537636 which accumulated above-average LRWC, proline, and APX had below-average chlorophyll content and plant height (Figure 6a). Under field experiment the chlorophyll content was negatively correlated with plant height and APX (Figure 6b). Therefore, genotype Gila which had high chlorophyll and proline content accumulated below-average APX and plant height. Analogously, genotypes Sina and PI537636 which had above-average APX and plant height accumulated below-average proline and chlorophyll content (Figure 6b). Genotype Kenya9819 accumulated above-average proline, LRWC, and APX but it had average plant height and chlorophyll content (Figure 6b).

Superiority Rank of the Genotypes based on their Trait Profiles

An ideal genotype is denoted by a small circle with an arrow pointing to it and has a superior trait profile overall. A superior genotype is located closest to the ideal genotype. Figure 7 ranks genotypes based on their overall superiority and it shows that under the greenhouse, genotype Kenya9819 was considered as the best genotype followed by genotype PI537636 as they were very close to the ideal genotype (Figure 7a). The genotypes are ranked as follows; Kenya9819 > PI537636 > Sina > Turkey > Gila (Figure 7a). Additionally, under field conditions, genotype Kenya9819 was the best while Turkey and Gila ranked poorly (Figure 7b). Generally, genotypes ranked as follows Kenya9819 > Sina > PI537636 > Gila >Turkey under field conditions (Figure 7b). Notably, genotype Kenya9819 outperformed other genotypes under greenhouse and field conditions. On the contrary, overall, genotype Gila and Turkey performed poorly when compared with other genotypes under greenhouse and field experiments.

DISCUSSION

Although safflower is known to be drought tolerant, there is a need to select genotypes that better adapt to drought-stress conditions. Bahadori *et al.* (2025) highlighted that drought stress causes a decline in yield and oil content of safflower

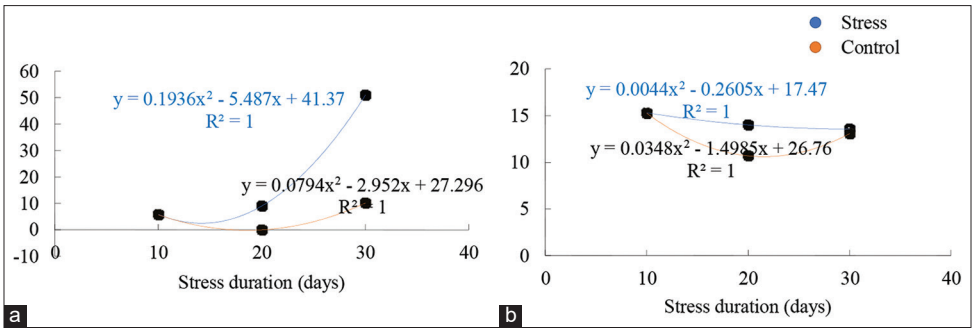

Figure 4: The effect of stress duration on the proline content of safflower grown under a) greenhouse and b) field experiment.

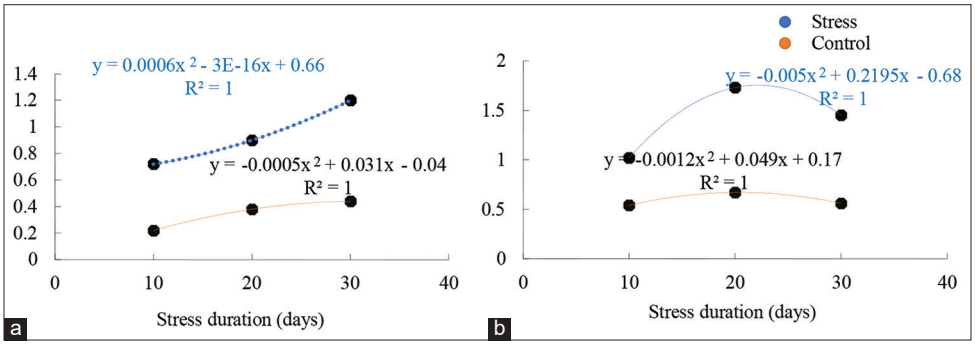

Figure 5: The effect of stress duration on the APX level of safflower grown under a) greenhouse and b) field experiment.

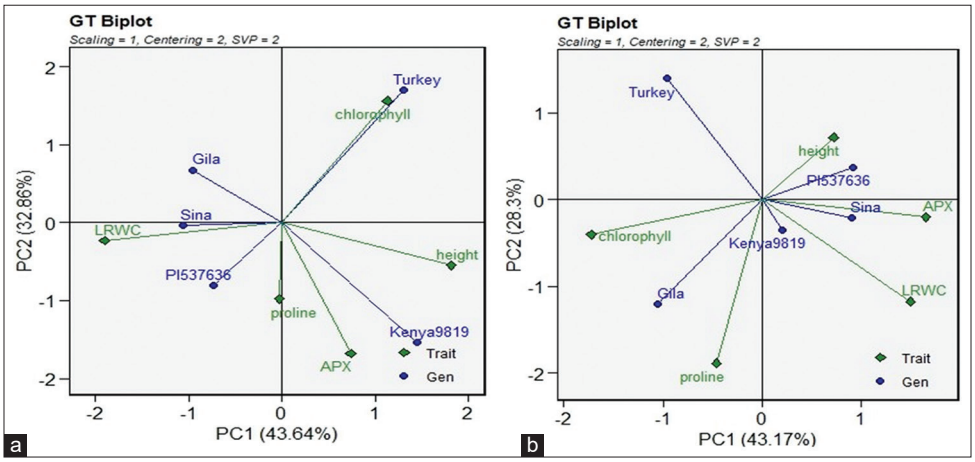

Figure 6: GT biplot showing associations among drought traits under a) greenhouse and b) field experiment.

suggesting that this crop is vulnerable to water deficit conditions. Greenhouse and field experiments revealed that the chlorophyll content, plant height and LRWC of safflower were not significantly affected by drought stress during the early days of stress induction (at day 10). This indicated that the available moisture was not limiting for plant growth during that period. However, as drought stress duration increased to 20 and 30 days, stressed plants under the greenhouse experiment accumulated a substantially high chlorophyll content relative to the control. A higher chlorophyll content under stress could be a result of mechanisms such as the activation of the antioxidant system (Monteoliva *et al.*, 2021). Similarly, Canavar *et al.* (2014) revealed that during the vegetative stage, higher chlorophyll content was observed in drought-stressed safflower than in non-stressed safflower. Zhang *et al.* (2020), Monteoliva *et al.* (2021) and Yang *et al.* (2021) contended that not all plants under drought reduce their chlorophyll content and that their ability to maintain chlorophyll content may indicate higher drought tolerance but this varies with the plant genotype, stress duration, and intensity. It is also worth noting that these leaves were rolling and were dark green in colour showing signs of stress while those of the control looked shiny and bright green in colour. On the contrary, on day 30 of the field experiment, a significant reduction in chlorophyll content was observed in stressed plants relative to control plants. This highlighted that the effect of stress was significant when exposed for a long duration under low moisture conditions. Similarly, a decline in chlorophyll content of safflower plants exposed to drought

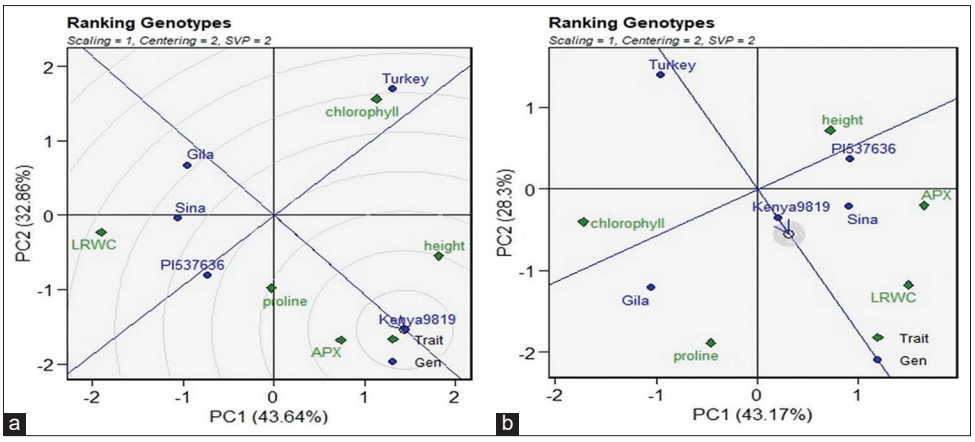

Figure 7: GT biplot ranking genotypes based on overall superiority. Safflower genotypes stressed for 30 days in the a) greenhouse and b) field experiment.

stress during the vegetative stage was reported in the literature (Amini *et al.*, 2013; Bortolheiro & Silva, 2017). Drought stress reduced plant height by 14.5% on day 30 under the greenhouse experiment while it reduced plant height by 14.6% and 19% on day 20 and 30 respectively under the field experiment. This showed that drought stress inhibited the apical growth of stressed plants relative to control plants with the effect being more severe under long exposure to stress conditions. Plant growth retardation under drought stress is a result of lower cell enlargement and higher leaf senescence rate (Hussain *et al.*, 2019). Moreover, towards the end of the rosette stage, plants critically require more water to facilitate stem elongation and extensive branching. Therefore, water limitation at this period can seriously hamper plant growth and yield. This explains why there was a significant reduction in plant height at day 30 as plants were elongating at a period when available soil moisture was limiting. Kazemeini *et al.* (2015) found that the effect of drought stress on plant height at the vegetative stage was more compared to reproductive stages of safflower. This emphasized the importance of sufficient soil moisture at the early stages of plant growth. Gültaş (2025) indicated that to increase yield in safflower, sustainable water resources should be ensured through supplementary irrigation in all development periods in dry years when rainfall is low.

The accumulation of osmolates like proline is essential for osmotic protection and osmoregulation to prevent the swelling and dehydration of cells resulting from conditions of water deprivation (Chen *et al.*, 2025). The results revealed that there was no significant difference in proline content between control and stressed plants at early stages of stress induction (Day 10) under greenhouse and field experiment but significant differences were observed at days 20 and 30. This was probably because adequate moisture was still available in the soil at day 10, hence water limitation was not experienced. Additionally, LRWC was similar among stressed and control plants during that time which could explain why this osmolyte was similar among the stressed and control plants. In fact, Khan *et al.* (2025) highlighted that RWC is a vital regulator of metabolic activities in plant tissues and a measure of a plant's degree of hydration. High proline accumulation may indicate the condition of the plant regarding drought stress. Likewise, Chavoushi *et al.* (2019) found that safflower plants stressed during the vegetative stage had fivefold higher proline content in the leaves and roots of drought-stressed plants than that of non-stressed ones. This is because accumulation of proline content in plants plays a role in maintaining the intracellular water content by reducing intracellular osmotic potential (Yang *et al.*, 2021). The results of this study showed that the activities of APX increased significantly under drought stress irrespective of stress duration under greenhouse and field experiments. This observation implied that stressed plants produced APX earlier to scavenge ROS even before visual symptoms of stress were observed. Chen *et al.* (2025) found that under drought stress conditions, drought resistant safflower cultivar BH had an increased activities APX while the YN cultivar had lower values. Similarly, Slabbert and Krüger (2014), found that increased activity of APX in stressed Amaranthus plants occurred earlier (7 days) than the activities of SOD and GR which occurred 10-12 days after. Moreover, Zhang *et al.* (2020) reported that the highest level of APX activity in different roof greening plant species were recorded under moderate drought stress, suggesting that APX activities were first activated under moderate drought stress to scavenge ROS.

Although the studied traits were all useful in discriminating genotypes for drought tolerance, no single trait was considered the best as they were all equally important. In particular, the GT biplots revealed that plants of genotype Gila under water stress had high proline content under field experiments but it had lower levels of the other traits when compared with other genotypes which showed that it was not competitive. Water stressed plants of the genotype Kenya9819 had above-average proline, LRWC, and APX which could have contributed to its less reduction in height when compared with other genotypes (Figure 6a). To evaluate the overall superiority of the genotypes for all the studied drought stress traits, the GT methodology was used. This approach is based on the hypothesis that an ideal genotype should have superior levels for multiple desired traits (Yan & Frégeau-Reid, 2018). Generally, the GT biplots showed that genotype Kenya9819 was superior under both the greenhouse and field experiment. Hence, genotype Kenya9819 was considered as the most drought tolerant when compared with other genotypes. On the other hand, genotype Gila and Turkey was the least drought tolerant because it ranked poorly

under greenhouse and field experiments. The GT biplots also revealed that genotypes PI537636 and Sina ranked fairly (moderately drought tolerant).

CONCLUSION

The results of this study showed the differential response of safflower genotypes to drought stress as demonstrated by their chlorophyll content, plant height, LRWC, proline content, and APX activities as stress progressed. Therefore, to increase the productivity of safflower especially in arid and semi-arid areas supplemental irrigation should be provided and the use of tolerant genotypes should be incorporated into the cropping system to curb the effects of drought stress. The genotype, Kenya9819 was an overall superior/competitive genotype under drought stress conditions and hence, considered drought stress tolerant. On the other hand, genotype Gila and Turkey ranked poorly in most of the traits and hence, they are considered susceptible to drought stress. Generally, drought stress tolerance is very complex and it involves several mechanisms either working synergistically or independently. Moving forward, the selection of drought-tolerant safflower genotypes should be conducted at longer drought stress durations where stress severity is high and multiple traits should be used to make more informed choices.

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