

Physiological and Biochemical Changes in Two Sunflower (*Helianthus annuus* L.) cultivars triggered by Drought stress

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Abstract

Drought stress is one of the primary environmental factors influencing sunflower (*Helianthus annuus* L.) productivity by disrobing physiological and metabolic homeostasis. By comparing the physiological and biochemical reactions of two sunflower cultivars, GangaKaveri and KBSH 42, under water deficiency regimes, the current study sought to assess the potential for drought resistance. The following important parameters were evaluated: relative water content (RWC), cell membrane integrity, total chlorophyll content, free proline content, lipid peroxidation, hydrogen peroxide (H_2O_2), and antioxidative superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) activities. In comparison to KBSH 42, the drought-tolerant cultivar GangaKaveri showed noticeably better RWC, membrane stability and chlorophyll content under drought stress. Additionally, GangaKaveri showed increased SOD, POX and CAT activities as well as higher proline accumulation, suggesting an effective antioxidative defence system. On the other hand, KBSH 42 showed increased amounts of H_2O_2 and lipid peroxidation indicating a poorer tolerance and more oxidative damage. These findings show that GangaKaveri's higher drought tolerance is linked to its capacity to preserve membrane integrity, preserve water balance and activate antioxidant defence pathways more successfully than the susceptible KBSH 42. These insights maybe useful for the improvement of abiotic stress tolerance through physiological marker traits and ROS regulation by means of antioxidants.

Introduction

Sunflower is a tropical and subtropical crop that is usually grown in semi-arid to arid climates with little to no irrigation and it is extremely vulnerable to negative environmental conditions like heat and drought stress (Robert et al., 2016). Drought occurrences are predicted to become more frequent and intense in the current period of climate change, which will have a significant impact on agricultural productivity (Debaeke et al., 2017). Drought significantly imposes water-deficit conditions, even crops like sunflower that are somewhat drought tolerant will experience large yield losses (Elliott et al., 2014). Drought first inhibits the growth of shoots and roots in plants (Celikkol Akcay et al., 2010). The next stage is stomatal closure, which lowers photosynthesis by limiting transpiration and CO_2 intake. The production of reactive oxygen species (ROS) is enhanced in most adverse situations, resulting in oxidative damage to biological components (Mohammadi et al., 2018). When ROS redox homeostasis is disrupted, excessive ROS accumulation can negatively impact plant genetic materials, including proteins, DNA, and RNA. This can result in chromosomal abnormalities, mutations, and even cell death (Apel and Hirt, 2004; Mittler et al., 2011; Hussain et al., 2019). In aerobic organisms, ROS, which are formed when molecular oxygen is excited or not completely reduced, include hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and singlet oxygen (1O_2). These are detrimental consequences of regular cellular metabolism (Mittler et al., 2011). Plants have developed a reactive oxygen species (ROS)-scavenging system that includes both nonenzymatic and enzymatic antioxidant mechanisms to lessen the detrimental effects of AOS on cellular components. Nonenzymatic antioxidant metabolites comprise β -carotenes, α -tocopherol, ascorbate (AsA), and reduced glutathione (GSH) (Halliwell, 1987); enzymatic

antioxidants consist of superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POX), catalase (CAT), and glutathione reductase (GR) (Bowler et al., 1992). In addition to benefiting from the protective function of antioxidant enzymes, plant cells can generate and accumulate organic osmolytes like proline and betaines to deal with osmotic stress.

Plants have developed a number of defenses against oxidative stress, such as the synthesis of antioxidants. Sunflower is extremely vulnerable to water scarcity, particularly during flowering and seed filling stages when leaf expansion and transpiration are poorly regulated under low soil moisture, despite having a moderate tolerance to drought due to its escape mechanism (Garcia-Lopez et al., 2014). Leaf withering and decreased production are caused by drought induced soil moisture depletion, especially in semi-arid regions with little rainfall (Aboudrare et al., 2006). Drought stress lowers achene yield, oil content and oil quality according to several research (Soleimanzadeh et al., 2010; Babaeian et al., 2011; Oraki and Aghaalkhana 2012 and Ibrahim et al., 2016). A 60% decrease in sunflower yields was noted when drought stress was imposed (Mazahery-Laghab et al. 2003).

Sunflower (*Helianthus annuus* L.) is a significant annual oilseed crop grown on around 24.77 million hectares worldwide and produces 44.31 million metric tons annually, or nearly 8% of the globally oilseed market (USDA, 2016). Due to its high nutritional content 40–50% oil and 17–20% protein, sunflower is a valuable crop for bridging the global gap between the production of animal feed and edible oil (Browsher et al., 2016). However, enhancing drought resistant necessitates better knowledge of the physiological, biochemical and molecular reactions to water stress as well as efficient use of germplasm. In the present study, the two sunflower cultivars with physiologically different levels of drought stress sensitivity, changes in growth indices, relative water content (RWC), lipid peroxidation, proline content, and activity of the antioxidant enzymes SOD, POX and CAT were investigated and contrasted.

Material and methods

A major environmental factor that restricts plant growth and productivity globally is drought stress. Although safflower (*Carthamus tinctorius* L.) a significant oilseed crop, is renowned for its capacity to thrive in semi-arid environments, its productivity is significantly impacted by water scarcity. Finding resilient genotypes requires an understanding of the physiological and biochemical processes that give drought tolerance. Two safflower cultivars, KBSH 42 and GangaKaveri were examined in the current study under varying soil moisture conditions to determine how they responded to drought stress.

Seeds from two sunflower varieties, KBSH 42 and GangaKaveri, were sourced from local farmers in nearby regions. The seeds were sterilized on the surface using 1% (v/v) sodium hypochloride solution for 5 minutes, rinsed thoroughly with distilled water, and soaked for 6 hours before planting. The seeds were then sown in pots containing a mix of 1 kg of field soil and 1 kg of coarse sand. The pots were positioned in the botanical garden and kept under natural garden conditions for a period of 21 days. During this time, the plants were watered with tap water to maintain field capacity until the drought treatments were initiated. Soil moisture level (SML) was measured gravimetrically before the imposition of stress.

Drought stress was applied by adjusting the soil moisture levels in the pots to four targeted percentages relative to field capacity: 100% (control), 75%, 50% and 25% SML. To achieve the desired soil moisture levels, the weight of each pot was measured and the necessary volume of water either added or withheld to reach the target gravimetric moisture. The pots were kept at these moisture levels for 9 days. During the stress period, visible wilting symptoms were noted at the lower moisture levels. The experiment utilized a two-factor design, incorporating 2 cultivars (KBSH 42 and GangaKaveri) and varying soil moisture levels (100%, 75%, 50% and 25%). For each cultivar and moisture combination, three biological replicates (independently prepared pots) were used. Each biological replicate consisted of 15 seedlings per pot. For biochemical analysis, each biological replicate was measured in technical duplicate and the average was utilized for analysis. Sampling took place at the 9th day, drought period period when wilting symptoms had become noticeable. Fully expanded young leaves were collected between 9am to 11am, immediately frozen in liquid nitrogen for biochemical assays, or processed fresh for physiological evaluations.

Relative water content

The method of Baars and Weatherly (1968) was used to calculate the relative water content (RWC) of leaf discs. Leaf discs with 1 cm diameter were made from leaf samples taken from both stressed and control safflower plants. Fresh weight (FW) of leaf discs was noted for each of three replicates. The deflated weight (TW) was measured after the discs were floated in 10 ml of distilled water for 6 h to allow complete deflation. The discs were then dried in a hot air oven at 80°C for 24 h, and the dry weight (DW) was determined. The relative water content was calculated by the formula

$$\text{RWC (\%)} = [(\text{Fresh Weight} - \text{Dry Weight}) / (\text{Turgid Weight} - \text{Dry Weight})] \times 100$$

Cell membrane integrity

A cork borer was used to create 1 cm diameter leaf discs from both stressed and control safflower plants. After two hours of incubation in 10 ml of distilled water, the discs were filtered out of the solution, and the optical density (OD) was measured at 273nm to determine the initial OD. After 30 minutes of boiling in the same solution, the identical leaf discs were allowed to cool at room temperature, filtered and the final OD was measured at 273nm. The percentage of electrolyte leakage was calculated using the formula (Leopold et al 1981).

$$\text{Cell membrane integrity} = (\text{Initial OD} / \text{Final OD}) \times 100$$

Free Proline content

Fresh plant material was homogenized with 3% sulfosalicylic acid. Four layers of muslin cloth were used to filter the homogenate, and the filtrate was then collected. In a test tube, two millilitres of the filtrate were combined with two millilitres of glacial acetic acid and two millilitres of acid ninhydrin. To stop the process, the mixture was immediately moved to an ice bath after being incubated at 100°C for an hour in a boiling water bath. Then, using a test tube stirrer, 4ml of toluene was added to each tube and well

mixed for 15 seconds. Using a UV-Vis spectrophotometer, the absorbance of the toluene layer containing the chromophore was measured at 520nm in relation to toluene as a blank after it had been thoroughly separated from the aqueous phase. A standard curve made with real proline and expressed on a dry weight basis was used to calculate the proline content (Bates et al 1973).

Total chlorophyll content

The control and water stressed plants fresh leaves were collected, properly cleaned, and then blotted dry. Cold 80% acetone was used to homogenize the leaf material in a mortar and pestle that had been previously refrigerated. After centrifuging the homogenate for 30 minutes at 3000rpm, the supernatant was collected. All of the supernatant from the subsequent extractions were combined and made up to a specified volume with 80% acetone. In a UV-Vis spectrophotometer, the extracts absorbance was measured at 645 and 663nm with 80% acetone serving as a blank. The total chlorophyll (TCC) was collected using the formula (Arnon 1949)

$$\text{TCC} = 20.2 \times \text{OD (at 645nm)} + 8.02 \times \text{OD (at 663nm)}$$

Lipid peroxidation

1% trichloroacetic acid (TCA) was used to homogenize leaf tissues for assessment. The samples were spun at 5000rpm for 3 to 4 minutes. To 1 ml of the resulting supernatant was added 2.5 ml of incubation buffer and 0.5% thiobarbituric acid (TBA) in 20% TCA, which was taken in triplicate. After incubation for 30 minutes at 95°C, the mixture was allowed to cool to room temperature. After being adjusted for non-specific turbidity at 600 nm, the absorbance of the samples was measured at 532 nm (Yamamoto et al. 2001).

Hydrogen peroxide

0.1% trichloroacetic acid (TCA) was used to homogenize 0.1 g of leaf tissue to estimate hydrogen peroxide (H₂O₂) content. After that, the homogenate was centrifuged at 12000 g at 4°C for 15 min, 1 ml 1M potassium iodide (KI) and 0.5 ml 10mM potassium phosphate buffer (pH adjusted depending on the tissue type) were added to 0.5 ml of the resulting supernatant. After 10 min incubation at 4°C, the reaction mixture was allowed to cool to ambient temperature for 15 min. At 390 nm, the absorbance of each sample was determined. The H₂O₂ content was calculated using a standard calibration curve created using H₂O₂ solutions in 0.1% TCA. The results were expressed as µgg⁻¹ fresh weight (FW) according to Harinasut et al (2003).

Antioxidative enzyme extraction

Antioxidative enzymes were extracted by homogenizing lyophilized powdered plant tissue in 0.1mM EDTA containing ice-cold 100mM potassium phosphate buffer (pH 7.0). after passing through muslin cloth, the homogenate was centrifuged for 15minutes at 16000g. the resultant supernatant was utilized as the crude enzyme extract for measurement of superoxide dismutase (SOD), peroxidase (POX) and

catalase (CAT) activity. To ensure enzyme stability, extraction and assay processes were conducted at 4°C.

Superoxide dismutase (SOD) activity

According to the Giannopolitis and Ries's (1977) methodology, the activity of total superoxide dismutase (SOD; EC 1.15.1.1) was measured by its capacity to prevent the photochemical reduction of nitroblue tetrazolium (NBT). 50mM phosphate buffer (pH 7.8), 0.1µM EDTA, 13mM methionine, 75µM NBT, 2µM riboflavin, and the enzyme extract made up the reaction mixture (1.5ml). After adding riboflavin last, the tubes were gently shaken and exposed to two 20W fluorescent lights. After allowing the reaction to continue for fifteen minutes, the light source was turned off, and a black cloth was placed over the tubes. A spectrophotometer was used to measure the reaction mixtures absorbance at 560nm. The quantity of enzyme needed to provide a 50% inhibition of the NBT photoreduction rate was referred to as one unit of SOD activity.

Peroxidase (POX) activity

According to Urbanek et al (1991), total peroxidase (POX, EC 1.11.1.7) activity was determined by incubating 2.0 ml of a reaction mixture containing 100 mM phosphate buffer (pH 7.0), 0.1µM EDTA, 5 mM guaiacol, 15 mM H₂O₂ and enzyme extract. Enzyme was added to initiate the reaction, and the increase in absorbance at 470nm was noted for one minute. The enzyme activity was calculated based on the formation of tetraguaiacol using its molar extinction coefficient (26.6 mm⁻¹ cm⁻¹).

Catalase (CAT) activity

Using 1.5ml reaction mixture comprising 100mM phosphate buffer (pH 7.0), 0.1µM EDTA, 20mM H₂O₂ and the enzyme extract, total catalase (CAT, EC 1.11.1.6) activity was measured, with minor modifications, in accordance with Beers and Sizer (1952). The enzyme was added to start the reaction, and the breakdown of H₂O₂ was observed at 240nm, and its molar extinction coefficient (36mM⁻¹cm⁻¹) was used to quantify the enzyme (Beers and Sizer 1952).

Results

The fresh and dry weight of leaf tissues in the two types of sunflower under stress were significantly lower than the respective controls. However, the amount of reduction varied between types. While the sensitive variety KBSH 42 demonstrated a large decline in leaf biomass, the tolerant variety GangaKaveri displayed relatively higher dry weight (Fig 1A). A more noticeable decline in dry weight was observed in the sensitive type, suggesting severe dehydration and tissue mass loss under stress. Improved water status and metabolic stability under stress is suggested by the sustained greater dry matter formation for the tolerant variety.

Relative water content

The relative water content (RWC) of the leaves of the two sunflower cultivars varied notably under different stress condition. RWC gradually decreased as stress intensity increased, suggesting that leaf tissues were becoming dehydrated and had less water available. Nonetheless, there were significant variations in the levels of decrease among the cultivars. Both Gangakaveri and KBSH 42 maintained high-water content under control, indicating typical physiological activity and ideal hydration state. The RWC gradually decreased in both the cultivar's as stress increased, however Gangakaveri's reduction was substantially less and it maintained a higher RWC throughout, even under extreme stress. Gangakaveri's capacity to maintain a greater RWC under stress is indicative of its improved water absorption, effective osmotic adjustment, and decreased transpirational water loss (Fig 1B). The steep drop in RWC in KBSH 42 is indicative of its restricted ability to preserve cellular turgor and store water. Gangakaveri's shows more efficient water retention mechanism under stress, and this experiment clearly shows that maintaining a higher relative water content is a major physiological sign of drought resistance.

Cell membrane integrity

According to the cell membrane intensity assay, the two cultivars responses to stress, significantly different. Both the varieties show an increase in electrolyte leakage as the stress level elevated, suggesting that the cell membranes were gradually being damaged. However, there were notable differences in the degree of leakage among the cultivars. Both the cultivars exhibited minimal leakage under control treatment, indicating that the membranes were sound and undamaged. Electrolyte leakage progressively increased as stress increased from 75% soil moisture level to 25% soil moisture level, indicating the extent of membrane damage. Gangakaveri's reduced leakage suggests that it has a greater capacity to protect cell membrane integrity by halting lipid peroxidation and preserving ion balance even in the face of stress (Fig 1C). The increased leakage in KBSH 42 leads to more membrane damage and a weakened resistant to oxidative stress. Therefore, this experiment unequivocally shows that membrane stability and stress tolerance are strongly related, and that Gangakaveri functions better under stress by preserving the structural and functional integrity of its membranes.

Total chlorophyll content

With increasing stress intensity, both sunflower cultivar's total chlorophyll content gradually decreased, suggesting that photosynthetic pigments were degraded as a result of stress. Nevertheless, there were notable differences in the rate of decrease between the two genotypes. Both Gangakaveri and KBSH 42 showed high level of chlorophyll content under control conditions, although the chlorophyll content gradually dropped in both the cultivars as the stress severity increased. Gangakaveri variety maintained a significantly higher amount of total chlorophyll than KBSH 42 (Fig 2A). Gangakaveri's superior protection of the photosynthetic apparatus is demonstrated by its capacity to sustain higher level of chlorophyll under stress, which may be the result of decreased oxidative damage and increased antioxidant activity. On the other hand, stress-induced damage to chloroplast membranes and increased pigment degradation are the causes of the sharp drop in chlorophyll content in KBSH 42. Thus, this

experiment shows that Gangakaveri has a more effective mechanism to preserve photosynthetic pigments under adverse condition, and that chlorophyll stability under stress is a crucial indicator of tolerance.

Free proline content

Both sunflower varieties free proline content in their leaves significantly increased as the level of stress increased, which is consistent with the plant's normal reaction to stress. However, there was a significant difference in the proline accumulation level in two different cultivars. Both Gangakaveri and KBSH 42 showed comparatively low proline levels under control, suggesting typical metabolic activity. Proline content gradually increased in both varieties as the stress severity increased. The variety Gangakaveri showed a significantly higher accumulation than the KBSH 42. Gangakaveri's proline concentration was around 1.5-2 times higher than KBSH 42 at the maximum stress level (Fig 2B). This experiment clearly shows that proline accumulation and stress tolerance are positively associated, and Gangakaveri's higher proline concentration suggests it is more capable to adapt and endure harsh environments than the KBSH 42 variety.

Lipid peroxidation

Lipid peroxidation level measured in terms of malondialdehyde (MDA) content. Stress progression suggesting increased oxidative damage to the lipids in the membrane. However, there were significant difference in the two different cultivars. Gangakaveri and KBSH 42 both are revealed low MDA levels under control, indicating less oxidative stress. The MDA content gradually increased in both varieties as the stress intensity increased but the increase in KBSH 42 was significantly greater than in Gangakaveri variety, at the extreme stress level (25% soil moisture level). Reduced lipid peroxidation and improved defense of cellular membrane oxidative damage are reflected in Gangakaveri variety (Fig 2C). On the contrary increased oxidative stress damage and membrane lipid breakdown are reflected in KBSH 42. Therefore, Gangakaveri protects membranes better under stress condition than KBSH 42.

Hydrogen peroxide

As the level of stress increased, the hydrogen peroxide content increases significantly in both the sunflower cultivars, which suggests that increases reactive oxygen species production. But there was clear difference in the level of accumulation in the two different cultivars. Both the cultivars showed low H_2O_2 levels under control, indicating the cellular redox balance was normal, and it increased gradually as stress increased, but in the KBSH 42 cultivar accumulation was significantly larger than Gangakaveri cultivar (Fig 3A). Comparatively Gangakaveri cultivars shows lower amount of H_2O_2 highlighted the presence of more effective antioxidant defense mechanism that can scavenge ROS and prevent oxidative damage. On the other hand, increased H_2O_2 levels in KBSH 42 indicate excessive production of ROS and inadequate detoxifying ability. It confirms Gangakaveri cultivar has enhanced stress tolerance and stronger antioxidative defense system compared to the KBSH 42.

Superoxide dismutase (SOD) activity

Superoxide dismutase activity is increased with increasing stress intensity, in both the sunflower cultivars, indicating its critical function in scavenging reactive oxygen species (ROS) produced under stress. However, there were noticeable differences in the level enzyme activity of the different cultivars. Under control conditions, Gangakaveri and KBSH 42 cultivars shows baseline level of SOD activity, but it increases gradually under stress condition. Comparatively Gangakaveri cultivar exhibited a significantly greater induction than KBSH 42 (Fig 3B). Gangakaveri's increased SOD activity indicates that it has a higher ability to reduce oxidative damage by converting the extremely reactive superoxide radicals (O_2^-) into less harmful hydrogen peroxide (H_2O_2). This experiment shows that increased SOD activity is closely related to the stress tolerance, Gangakaveri cultivar has a more effective antioxidant defence system when stressed.

Peroxidase (POX)

Under a control condition the POX activity is relatively slow in both the cultivars, which is consistent with normal metabolic conditions. POX activity progressively enhanced in both varieties as the stress intensity increased. However, the tolerant variety Gangakaveri showed noticeably higher enzyme activity than the KBSH 42 (Fig 3C). The higher peroxidase activity in Gangakaveri cultivar suggests a more efficient enzymatic defense mechanism for breaking down excess H_2O_2 preserving redox balance and averting lipid peroxidation and membrane damage. However, in the KBSH 42 cultivar reduced POX activity suggests inefficient ROS detoxification, which increases oxidative stress and cellular damage. Thus, this experiment makes it abundantly evident that increased peroxidase activity is essential for stress tolerance, and that the tolerant Gangakaveri cultivar has a more robust defensive mechanism against oxidative stress than the sensitive KBSH 42 cultivar.

Catalase (CAT) activity

The two sunflower cultivars Gangakaveri and KBSH 42 showed comparatively modest basal levels of CAT activity under control which is consistent with typical physiological conditions. CAT activity gradually increased in both the cultivars as the stress intensity increased from 75% soil moisture level to 25% soil moisture level, nevertheless, the tolerant variety Gangakaveri showed considerably greater induction than KBSH 42 cultivar (Fig 3D). Gangakaveri's enhanced capacity to scavenge hydrogen peroxide by dissolving it into water and oxygen and preventing oxidative damage to cellular components are demonstrated by its higher CAT activity. The reduced CAT activity seen in KBSH 42, on the other side, indicates that H_2O_2 is not adequately detoxified, which leads to excessive buildup and increased oxidative stress.

Discussion

In the current investigation, the sensitive sunflower variety (KBSH 42) retained considerably lower leaf fresh and dry weights under stress than the tolerant sunflower variety (GangaKaveri) (Fig. 1A). Reduced

photosynthetic activity, restricted cell growth, decreased cell turgor are the primary causes of the fresh weight loss during stress, which eventually restricts biomass accumulation (Ashraf et al., 2024). The sensitive variety showed a larger decrease in dry weight, which may have been caused by increased membrane damage and poorer carbon assimilation, which inhibited growth (Ameen et al., 2024).

One of the most accurate assessments of a plant's water status under stress is its relative water content (RWC), which represents the equilibrium between transpiration rate and water supply to the leaf tissue (weatherly 1950). According to the current study, RWC significantly decreased under drought stress as compared to the control, demonstrating the detrimental effects of water deprivation on cellular hydration. In contrast to the sensitive cultivar KBSH 42, the tolerant cultivar Gangakaveri retained a comparatively higher RWC (Fig. 2A), indicating a stronger ability to retain water under stress. According to the Yamasaki and Dillenburg (1999), the tolerant cultivar's ability to maintain a higher RWC can be attributed to effective osmotic adjustment through the accumulation of suitable solutes such as proline, carbohydrates and other osmoprotectants that support metabolic processes and cell turgor. Similar results were reported by Anjum et al 2011, and Farooq et al 2012, who showed that higher RWC was maintained by drought tolerant cultivars of different crops than by sensitive ones, indicating improved water retention and stress tolerance. According to Sairam and Saxena (2000), the sensitive cultivar's decreased RWC may be the result of oxidative stress induced membrane damage and decreased leaf water potential. The tolerant cultivar's capacity to sustain a higher RWC even in the face of extreme stress suggests improved water conservation techniques, which are probably bolstered by effective osmotic management and antioxidative defence. Together, these systems improve resistance to drought and maintain physiological functions under stress. One of the most crucial physiological markers of a plant's overall health and photosynthetic effectiveness under stress is its chlorophyll concentration. In the present study, plants under stress showed a significantly lower total chlorophyll content than the control. Chlorophyll pigments may decrease under stress due to oxidative degradation of chlorophyll molecules, inhibition of enzymes involved in the biosynthesis of chlorophyll or increased activity of the enzyme chlorophyllase, which catalyses the breakdown of chlorophyll (Sudakar et al., 2001; Anjum et al., 2011). One of the well-known adaptive response to stress is the loss of chlorophyll, which restricts light absorption to avoid photo-damage (Ashraf and Harris, 2013). The tolerant cultivar showed a higher chlorophyll concentration than the sensitive cultivars in the current study (Fig. 2A), indicating that it may sustain stronger photosynthetic machinery and membrane stability under stress. The activation of antioxidative defence mechanisms, which shield chloroplast structures from reactive oxygen species (ROS), has been linked to the maintenance of greater pigment content under stress (Foyer and Noctor., 2005). Several crops under abiotic stress have shown a similar pattern. For example, Hussain et al., 2019 found that when sunflower plants were exposed to salinity and drought their chlorophyll concentration decreased, while resistant genotypes retained more pigments. Similarly, resistant wheat cultivars retained a greater chlorophyll stability index (CSI) under stress than susceptible ones, as shown by Sairam et al., 2002. Thus, one of the most important markers of tolerance and photosynthetic resilience is the capacity to maintain chlorophyll under stress. The tolerant variety's higher chlorophyll content indicates increased stability of pigment-protein complexes and decreased oxidative damage, which

enhance photosynthetic efficiency and improve growth performance under stress. Proline accumulation is a well-documented physiological response in plants subjected to various abiotic stresses, serving as a reliable biochemical marker of stress tolerance (Szabados and Savoure, 2010). In this study, proline levels increased significantly under stress conditions compared to the control, with the stress-tolerant cultivar demonstrating the highest accumulation (Fig. 2B). This finding suggests that proline plays a crucial role in stress adaptation, functioning as both an osmoprotectant and an antioxidant molecule. The tolerant cultivar's greater proline synthesis indicates an improved capacity to preserve osmotic balance and shield cellular structures from damage imposed by dehydration. Proline is a scavenger of reactive oxygen species, a stabilizer of proteins, membranes, and subcellular structures, and an osmolyte for osmotic adjustment (Ashraf and Foolad, 2007). By controlling NADP⁺/NADPH ratios, increased proline synthesis under stress may also aid in maintaining redox equilibrium (Kavi Kishor et al., 2005). Similar results were found by Bates et al., (1973), Sairam and Tyagi (2004), Hayat et al., (2012) and Naveed et al., (2025) who found that proline accumulation was higher in cultivars of several crops that were resistant to salt and drought than in sensitive ones. Higher accumulation in tolerant cultivar reflects their improved ability to respond to changes in osmotic pressure, which allows them to preserve metabolic processes, preserve enzyme activity, and maintain cell turgor when under stress condition. In the present investigation, lipid peroxidation was found to be significantly increased under stress conditions, as indicated by the amount of malondialdehyde (MDA) compared to controls (Fig. 2C). Increased oxidative breakdown of polyunsaturated fatty acids in membrane lipids, resulting in loss of membrane fluidity and integrity, is shown by elevated levels of MDA (Dhindsa et al., 1981). Overproduction of ROS, such as superoxide radicals (O₂^{•-}), hydrogen peroxide (H₂O₂) and hydroxyl radicals (•OH) that target membrane lipids, proteins and pigments, causes increased lipid peroxidation under stress (Gil and Tuteja 2010). In contrast to the sensitive variety, MDA concentrations were lower in the tolerant variety in this study, indicating a more effective antioxidant defence system that limits ROS generation and avoids the critical membrane activity of enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX), which together scavenge ROS detoxify, the tolerant genotype displays better oxidative stress management (Appel and Hurt 2004). Anjum et al (2011) in maize and Sairam et al (2002) in wheat showed a similar result, with the tolerant genotype displaying much less lipid peroxidation under stress due to its increased antioxidant activity. Similarly, Hameed et al (2014) found that drought tolerant sunflower genotypes accumulated less MDA than sensitive ones. Therefore, reduced lipid peroxidation is a hallmark of stress tolerance in plants, as confirmed by the results of the present study, which are consistent with previous reports. Overall, the ability of the tolerant variety to maintain low MDA concentrations reflects its superior capacity for ROS scavenging and membrane protection under stress, whereas the sensitive variety. In plants, hydrogen peroxide H₂O₂ is a crucial reactive oxygen species (ROS) that serves as a signalling molecule and an indicator of oxidative stress. According to the current study, plants under stress had a much higher H₂O₂ content than the control, which suggests that oxidative damage occurred as a result of an imbalance between the production of ROS and detoxifying processes (Fig. 3A). Excessive generation of ROS, including superoxide radicals and H₂O₂ under stressful situations causes oxidative damage (Gill and Tuteja 2010). The tolerant cultivar has

a more effective antioxidative defence system that can scavenge ROS, as seen by the increased accumulation of H_2O_2 in the sensitive cultivar relative to the tolerant one. On the other hand, high H_2O_2 build up in susceptible plants can cause cell membrane damage and increased lipid peroxidation, which can lead to cellular dysfunction (Apel and Hirt 2004). Controlled H_2O_2 levels are essential for signalling pathways that activate defensive mechanisms and control genes that respond to stress (Neill et al., 2002). However, oxidative stress occurs when H_2O_2 accumulates above the threshold levels due to the inability of antioxidant enzymes to maintain redox balance. In order to maintain redox equilibrium, effective enzymatic detoxification systems that convert H_2O_2 to water and oxygen are activated, as evidenced by the tolerant cultivar's decreased H_2O_2 concentration in the current study. Similarly, stress tolerant cultivar maintained decreased H_2O_2 formation due to improved antioxidative activity as shown by Sairam et al., 2002 in wheat and Hameed et al 2014 in sunflower. In this study the tolerant cultivar shows lower H_2O_2 level indicates its greater ability to prevent ROS accumulation and shield cellular components from stress.

The primary defence against reactive oxygen species (ROS) in plants is provided by superoxide dismutase (SOD), it prevents oxidative damage to biological components by catalysing the dismutation of the extremely hazardous superoxide radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Apel and Hirt 2004). In the current study, plants under stress shows considerably higher SOD activity than the control, suggesting greater ROS production and subsequent activation of antioxidant defence systems (Fig. 3B). In contrast to the sensitive variety, the tolerant one showed increased SOD activity, indicating that it is better able to scavenge superoxide radicals preserve redox equilibrium under stress, according to Gill and Tuteja 2010, elevated SOD activity is a typical adaptive response that guards against oxidative damage. Stress induced increases in SOD activity have also been documented in a number of crop species (Sairam et al 2002). For example, wheat genotypes that were resistant to drought had higher SOD activity than those that were susceptible. In a similar vein, Khali et al., 2020 found that salt tolerant sunflower types had higher SOD activity, which was associated with reduced lipid peroxidation and better membrane integrity. These results confirm the exiting data that increased SOD activity helps reduce oxidative stress. However, if stress intensity increases, a significant accumulation of ROS can overwhelm the antioxidant system and cause oxidative damage, even in resistant genotypes (Foyer and Noctor 2005). To preserve cellular integrity and safeguard photosynthetic machinery under stress, the tolerant variety of the current study exhibits higher SOD activity which is indicative of its effective ROS detoxification mechanism. This mechanism probably functions in tandem with other enzymes like catalase (CAT) and peroxidase (POX). The increased SOD, POX and CAT activities under drought stress was reported in safflower (Thippeswamy et al., 2021) Peroxidases uses a variety of electron donors, including ascorbate and phenolic substances, to detoxify hydrogen peroxide (H_2O_2). It protects against oxidative damage brought on by various abiotic stressors and aids in the maintenance of cellular redox homeostasis (Gill and Tuteja 2010). In the present study peroxidase activity significantly increased under stress as compared to the control, suggesting that the plants antioxidant defence mechanism was activated in response to increases to increased ROS formation.

Compared to the sensitive variety, the tolerant one showed noticeably increased POX activity, indicating that it was better able to scavenge H_2O_2 and prevent oxidative damage (Fig. 3C). Increased POX activity contributes to the integrity of cell membrane (Apel and Hirt 2004). Hameed et al., 2014 found that drought tolerant sunflower cultivars had higher POX activity, which was linked to improved membrane integrity and less MDA content accumulation. According to Passadi et al., 2005 peroxidase et al 2005 peroxidase also contributes to the strengthening of cell walls by lignin production, which increases mechanical stiffness and prevents oxidative damage. In the tolerant cultivar the POX activity is increased, it indicates that an effective ROS scavenging mechanism is an essential part of the antioxidative defence system. The maintenance of cellular homeostasis in challenging circumstances and enhanced resistance to oxidative stress are two benefits of this enzymatic overexpression. Catalase (CAT) is essential for detoxifying hydrogen peroxide (H_2O_2). It prevents oxidative damage to plant cells by catalysing the quick breakdown of H_2O_2 into oxygen and water (Gill and Tuteja 2010). In the current investigation, CAT activity dramatically increased under stress when compared to the control, suggesting that the antioxidative defence mechanism is more effectively activated in response to elevated H_2O_2 levels (Fig. 3D). In contrast to the sensitive variety, the tolerant one showed increased CAT activity, indicating that it is better able to scavenge H_2O_2 and preserve redox equilibrium under stress. Similar findings have been documented for a number of crops under environmental stress. Drought tolerant wheat genotypes retained higher CAT activity than sensitive ones, as shown by Sairam et al 2002, which helped to improve oxidative stress management. Similar findings were made by Ahmad et al 2019 and hammed et al 2014 who found that tolerant sunflower cultivars exhibited increased CAT activity in response to salinity with reduced lipid peroxidation and membrane integrity. However, decreased CAT activity in the sensitive cultivar might be the result of enzyme breakdown caused by an excess of ROS accumulation (Foyer and Noctor 2005).

Conclusion

The analysis of several physiological and biochemical parameters revealed that cultivar Gangakaveri seemed to be less physically impacted, the reactions of both sunflower cultivars to drought stress similar traits, particularly with regard to MDA, H_2O_2 , and proline levels. Proline and POX, two of the characteristics examined in both cultivars, were crucial in protecting the tissues under extreme stress, which led to a negligible rise in MDA. Although the tissues displayed an increase in free proline concentration and SOD, POX and CAT activities, the antioxidant system was unable to stop membrane damage and excessive ROS production in the KBSH 42 cultivar compared to the tissues of cultivar Gangakaveri. Thus, methods for increasing the activity of the SOD, POX and CAT enzymes in sunflower tissues may offer a useful defense against drought stress in this significant species of oilseed crop. Future research will use various sunflower cultivars to examine how drought stress affects the composition of fatty acids.

Declarations

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Author's contributions

VNH and TM contributed to the design of the study, analysis of the data and drafted the manuscript. VNH and RV executed the experiments. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

Not applicable

Consent for publication

All the authors listed in this manuscript have read and approved the final version for publication.

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Figures

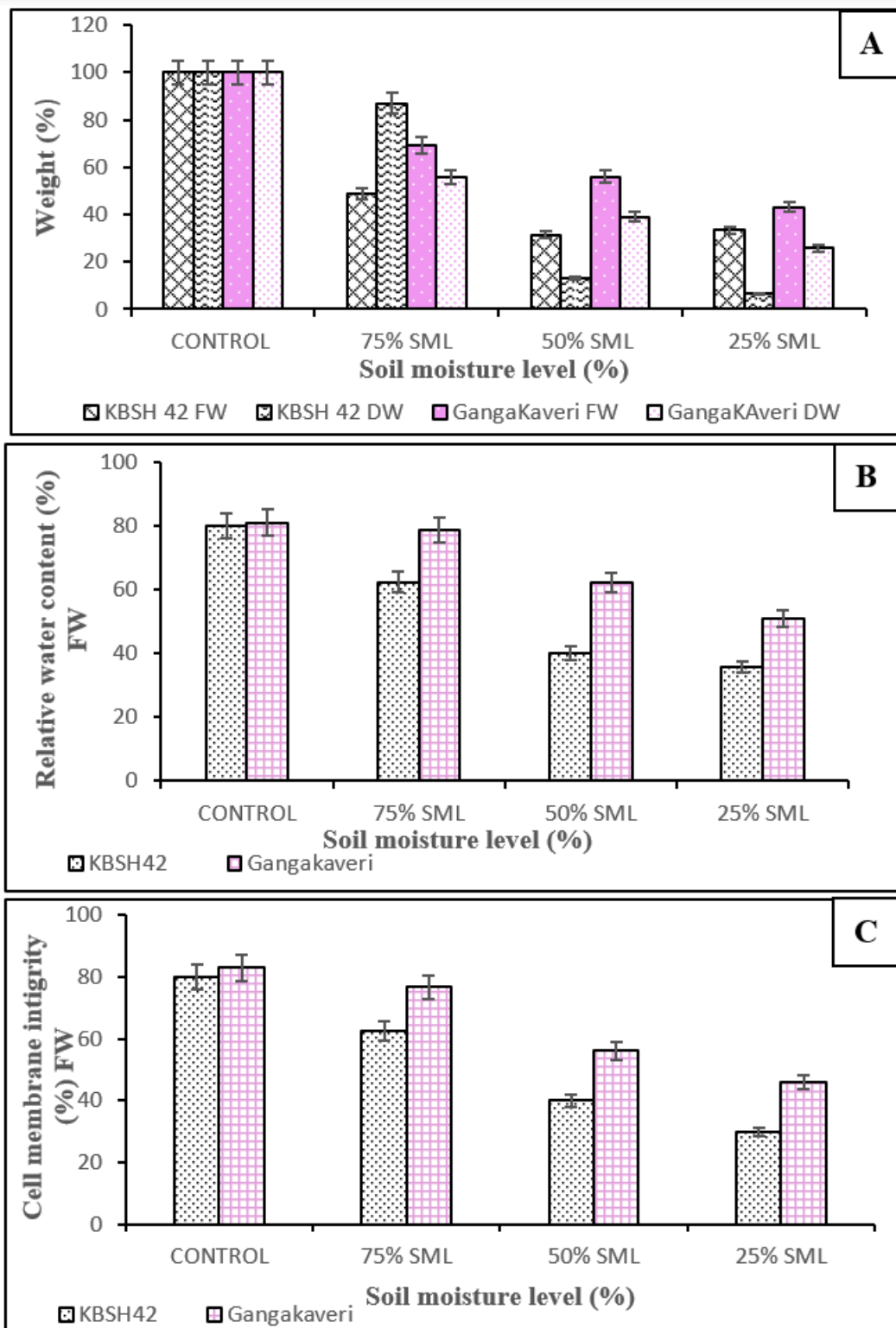


Figure 1

A-Fresh weight and Dry weight,

B-Relative water content,

C-Cell membrane integrity

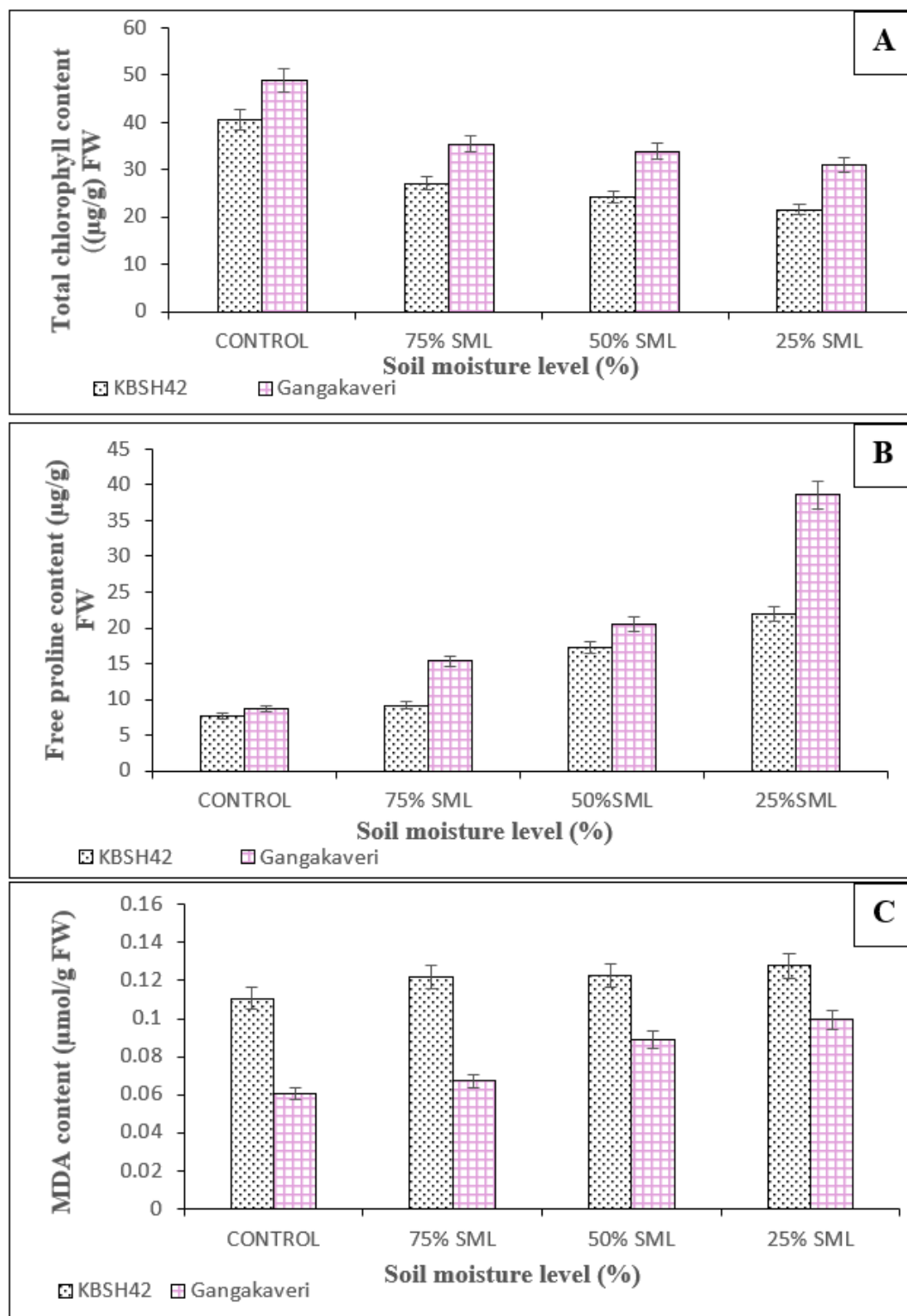


Figure 2

A-Total chlorophyll content,

B-Free proline content,

C-MDA content

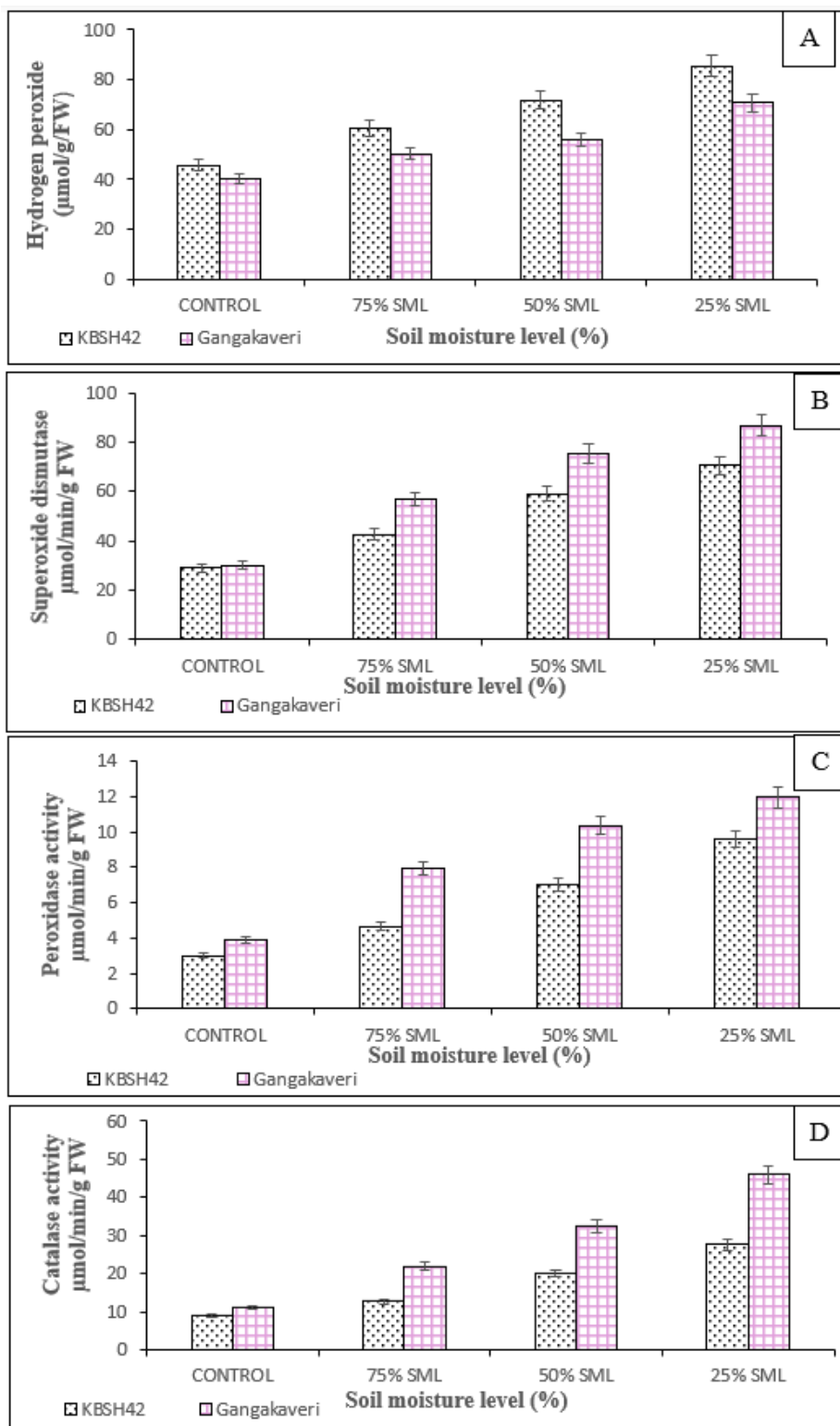


Figure 3

A-Hydrogen peroxide,

B- Superoxide dismutase activity,

C- Peroxidase activity,

D – Catalase activity.