# BIOCHEMICAL RESPONSES OF GROUNDNUT (*ARACHIS HYPOGEA* L.) TO DROUGHT STRESS: EVALUATION OF OSMOLYTE ACCUMULATION IN TWO CULTIVARS WITH CONTRASTING DROUGHT TOLERANCE

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**Abstract**
Groundnut (*Arachis hypogaea* L.) is an agriculturally valuable plant with widespread distribution in the world serving as a subsistence food crop as well as a source of various food products, and is frequently constrained by extreme environmental conditions such as drought. The study investigates the differential accumulation of osmolytes and leaf dry weight (LDW) along with relative water content (RWC) and lipid peroxidation in leaves of groundnut cultivars differing in their susceptibility to drought stress (cultivar K-134 and cultivar JL-24, drought tolerant and drought susceptible respectively) under water stress conditions, by keeping the soil moisture content as 100% (control), 75% (mild), 50% (moderate) and 25% (severe) of field capacity for a duration of 12 days. As the RWC was dropping progressively with the severity of treatment, the values of LDW were declined in all stress treatments and differed between the cultivars. The degree of osmolyte buildup in the two cultivars of groundnut was significantly altered as a result of water stress. The accumulation level of osmolytes such as proline, glycine betaine, soluble sugars, free amino acids and polyamines were increased significantly in both cultivars with increasing stress severity when compared with their controls. Nevertheless, cv. K-134 had a greater percentage increase in osmolyte accumulation than cv. JL-24, which was lower. In cultivar JL-24 compared to cultivar K-134, there was a higher amount of lipid peroxidation as indicated by MDA. The present study indicated that cv. K-134 is drought tolerant than cv. JL-24 based on dry mass and osmolyte accumulation. The physio-biochemical responses in relation to the drought tolerance of these cultivars was discussed.
**Keywords**: Groundnut, Osmolytes, Proline, MDA, Water stress, Drought tolerance.

## Introduction

Groundnut (*Arachis hypogaea L.*) is one of the most widely grown essential edible legume in the world and contains about 50% oil, 25–30% protein, 20% carbohydrates and 5% fiber.[1] The vulnerability of groundnut to drought varies depending on physiological characteristics, crop growth (reproductive) stages, and environmental conditions.[2] Drought stress imposed from flowering to the start of seed growth was shown to cause a severe reduction in yield of groundnut, as it decreases flower production, seed development.[3] and can cause plant death by inducing senescence. According to a recent estimate, world groundnut productivity incurred an annual loss of approximately 6 million tons due to drought alone among all abiotic stress factors.[4] Two-thirds of the global production occurs in rain-fed areas of the semiarid tropics which are characterized by unpredictable periods of water deficit.[5] The production of this crop is increasingly challenged by the growing population food demand and drought is a major abiotic constraint responsible for heavy groundnut production losses. In this context, proper selection of drought resistant cultivars and rootstock is one of the important strategies to reduce the impact of this stress and contribute to a more stable groundnut production for improving food security of small farmers.

Abiotic stress factors and climate change are major contributors to crop losses globally. Drought has become a major abiotic stress factor that restrict plant growth, productivity, and survival while also posing a threat to global food production and security. Plants produce compatible solutes known as osmolytes to adapt themselves in such changing environment. Various low molecular weight substances or metabolites, such as sugars, polyamines, secondary metabolites, amino acids, and polyols, are referred to as osmolytes. Osmolytes contribute to homeostasis maintenance, provide the driving gradient for water uptake, maintain cell turgor by osmotic adjustment, and redox metabolism to remove excess level of reactive oxygen species (ROS) and reestablish the cellular redox balance as well as protect cellular machinery from osmotic stress and oxidative damage.[6] Plants respond to a variety of stresses by accumulating amino acids and the most conspicuous being proline. The role played by accumulated amino acids in plants varies from acting as osmolyte, regulation of ion transport, modulating stomatal opening, and detoxification of heavy metals.[7] Proline accumulation leads to stress tolerance by maintaining the osmotic balance (still controversial), cell turgidity and indirectly modulating metabolism of reactive oxygen species.[8,9] Furthermore, the crosstalk of proline with other osmoprotectants and signaling molecules, e.g. glycine betaine, abscisic acid, nitric oxide, hydrogen sulfide, soluble sugars, helps to strengthen protective mechanisms in stressful environments.[9] A quaternary ammonium compound identified as glycine betaine (GB), which predominates in higher plants under drought conditions, has been shown to protect photosynthetic machinery, stabilize the structure of Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase), and act as an oxygen radical scavenger.[8,10,11] The accumulation of sugars in plants in response to water stress is also quite well documented and is considered to play an important role in osmotic adjustment, [12,13,14] and also play an active role regulating growth, photosynthesis, carbon partitioning, and carbohydrate and lipid metabolism in response to various abiotic stressors.[15] Studies on the effects of sugars under various abiotic stressors represent an emerging field of research to be explored and sugars could play a pivotal role conferring tolerance against various abiotic stressors by modulating several physiological processes.[16] While many studies have indicated a positive relationship between accumulation of osmolytes and plant stress tolerance,[10,14,17,18] some have argued that the increase in their concentrations under stress is a product of, and not an adaptive response to stress.[8,19] Polyamines (PAs) are secondary metabolites that regulate physiological and metabolic progressions in plants to tolerate stress. Recent studies reported the beneficial roles of polyamines in plant development, including metabolic and physiological processes, unveiling their potential for inducing tolerance against adverse conditions.[20] Questions like whether osmolyte levels are accurate predictors of stress tolerance in breeding programmes or whether the maximum absolute concentrations of compatible solutes are always sufficient to account for significant mass-action effects have been the focus of many discussions and are still hotly contested today.[12] However, not all plants accumulate osmolytes compounds in sufficient amounts to avert adverse effects of drought stress.[21] Hence, the role of osmolytes in plant development and defense against drought stress needs to be critically examined. Plant must conform to extreme environmental conditions entailing adaptive changes in metabolism which is an interplay between various biochemical and physiological processes. Therefore, a comprehensive study has been initiated with an objective to look into water stress induced biochemical and physiological changes and a comparative analysis of tolerance potentials based on osmolyte accumulation in two cultivars of groundnut. The analysis of our findings allowed us to identify traits that might be useful in breeding programmes for developing groundnut genotypes resistant to water deficit stress or to find simple-to-use biological markers that could be used as indirect selection criteria for evaluating genotypes resistant to drought.

## Material and methods

### Experimental Design

Seeds of groundnut (*Arachis hypogaea* L.) cultivars namely (K-134 and JL-24) were procured from Andhra Pradesh Agricultural Research Station Kadiri, Anantapur district. Seeds were surface sterilized with 0.1 % (w/v) sodium hypo chlorite solution for 5 min, thoroughly rinsed with distilled water and then germinated in plastic pots containing 2 kg of soil and sand (2:1) mixture and allowed to grow for thirty days. The pots were maintained in the departmental botanical garden under natural photoperiod of 10-12 h and temperature 28 ± 4°C. Thirty-day-old plants were then divided into four-sets and arranged in randomized complete black design. One set of pots received water daily to field capacity and served as control (100 %). Water stress was induced by adding of water daily to 75, 50 and 25 % soil moisture levels respectively. Leaf samples were collected on day-12 after stress induction for analysis of various parameters.

### Leaf dry weight and Relative water content

For the determination of dry mass, the leaves were separately dried at 80°C in a hot air oven until a constant mass was formed. Fully expanded leaves were excised and fresh weight (FW) was immediately recorded from control and stressed plants. Then the leaves were immersed in distilled water and after 4h they were blotted dry and the turgid weight (TW) was taken. The leaves were kept at 80°C in a hot air oven for 48h and dry weights (DW) were recorded. The RWC was calculated using the following formula RWC (%) = [(FW – DW)/TW – DW)] X 100.[22]

### Amino acid, Proline and Glycine betaine content determination

The extraction and estimation of free amino acids was done according to method.[23] Free proline content was extracted from leaves of both cultivars in 3% aqueous sulphosalicylic acid and estimated using ninhydrin reagent.[24] Fresh leaves were homogenized using mortar and pestle in 3% aqueous sulfosalicylic acid and filtered through four layered muslin cloth. 2 $cm^3$ of the filtrate was then added to acidic ninhydrin and glacial acetic acid 2 $cm^3$ each and incubated in a boiling water bath for 1 h. The tubes were then transferred to an ice bath to terminate the reaction and 4 $cm^3$ of toluene was added and vortexed for 15s. Free proline content in the mixture was measured by reading the absorbance at 520 nm against toluene. Quaternary ammonium compounds (QACs) were extracted and measured as glycine betaine (GB) equivalents using KI-I2 reagent.[25]

**Sugars**

Carbohydrate fractions were extracted with 80% ethanol according to the method. [26] The reducing sugars were estimated by Nelson's [27] as modified by Scot.[28] The non-reducing sugars were estimated from the alcoholic extract. [29]

**Polyamine**

The extraction and estimation of total polyamine content was done according to the method.[30] 0.5 g of plant material was homogenized with 5 ml of 5% perchloric acid ($HClO_4$), centri- fuged at 12000 rpm for 20 min. Supernatant fraction was dansylated, and then benzene extract was separated on TLC plates coated with silica gel by using chloroform : triethylamine (25:2 v/v) as solvent system. Polyamines were identified with the help of authentic samples. The spots were eluted with ethyl acetate and quantified using a UV-visible spectrophotometer (Thermo- Spectronic, USA).

**Lipid peroxidation**

Lipid peroxidation was determined by measuring the amount of MDA produced by the thiobarbituric acid reaction as described.[31] One gram of tissue (FW) was homogenised in 5 ml of 0.1% (w/v) TCA. The homogenate was centrifuged at 10,000 g for 5 min and 4 ml of 20% TCA containing 0.5% (w/v) TBA was added to 1 ml of the supernatant. The mixture was heated at $95^0$C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10,000 g for 15 min and the absorbance was measured at 532 nm and 600 nm. After subtracting the non-specific absorbance (600 nm), the MDA concentration was determined by its molar extinction coefficient (155mM$^{-1}$ cm$^{-1}$).

**Statistical Analysis**

The data obtained in all parameters were subjected to analysis of variance (ANOVA) and the mean values were compared by Duncan's Multiple Range (DMR) test at 0.05% level as described.[32]

**Results and Discussion**

It has been shown that water deficit influences various physiological, biochemical, metabolic and molecular processes in various plants, including groundnut. [1,2,5,18] Drought stress caused a significant decline in the leaf dry mass accumulation in both cultivars at all stress treatments (Table 1), but with a greater degree of decline in cv. JL-24 than in cv.K-134. When severe drought stress occurred, LDW of cv. JL-24 was 0.540 g plant$^{-1}$ compared with 1.078 g plant$^{-1}$ in cv.K-134. Similar genotypic differences have been noticed in a variety of crop species. [13,33-35] Groundnut cultivars with vigorous early growth, a relatively large biomass accumulation and capacity for remobilizing stored assimilates to reproductive sinks may be better adapted to drought stress.[36] The altered carbon and nitrogen metabolism may be responsible for the reduced dry matter imposed by water stress[37] and also due to both senescence and death of leaves, which was considered as avoidance mechanism that allows minimizing water losses.[38] RWC is an important physiological trait that describes the water status of plants. It is the most important index for drought tolerance, as it is a measure of plant water status that reflects the metabolic activity of tissues.[39] Among the cultivars examined for RWC in the present study, cultivar K-134, registered high RWC and minimal reduction than cultivar JL-24 grown under all drought stress conditions, which is mainly because of the maintenance of osmotic regulation (Table 1). The significant variation in RWC observed across the cultivars in our study could be attributed to their genetic background and response to drought stress.[40] Although drought stress negatively affected dry weight accumulation in both cultivars, the remaining higher water content may have helped cv. K-134 maintain higher photosynthetic activity and consequently higher dry weight accumulation than cv. JL-24.

The most common osmolytes that play crucial role in osmoregulation are proline, glycine-betaine, polyamines, and sugars. These compounds stabilize the osmotic differences between surroundings of cell and the cytosol. The total free amino acid pool was highly elevated in leaves of both cultivars at all stress treatments (Table 1). The per cent increase in amino acid levels was dependent on intensity of stress. Nevertheless, the degree of accumulation was greater in the cultivar K-134 compared to JL-24 at all water stress regimes. A maximum increase (6.149 mg $g^{-1}$ FW) in the content of free amino acids was noticed in cultivar K-134 compared to JL-24 in severe stress treatment, thus making it the most drought-tolerant plant, while the comparatively less content (4.739 mg $g^{-1}$ FW) of these free amino acids noticed in cultivar JL-24 might lead to the loss of turgor and membrane damage and thus making this variety the most drought susceptible. Cultivar variations in the magnitude of accumulation of amino acids have been taken as an index for determining the drought tolerant potentials of groudnut.[18,33,40,41] Improved levels of free amino acids together with organic acids and quaternary ammonium compounds serve as compatible cytoplasmic solutes to maintain the osmotic balance under stress conditions.[7,42] The free proline content was significantly increased in the stressed plants of both cultivars at all stress regimes over controls (Table 1). There was a linear increase in proline accumulation with increasing severity of stress. However, a difference in the accumulation of free proline content was observed between the two cultivars, a more pronounced increase was observed in the cultivar K-134 compared to JL-24. Proline content was increased by about 2.5-fold and 3.7-fold in the leaves of cultivars JL-24 and K-134, respectively, on the 12$^{th}$ day at severe stress treatments. The accumulation of free proline in stressed plants has been found to be an adaptive mechanism for drought tolerance and a positive correlation between magnitude of free proline accumulation and drought tolerance has been considered as an index for determining drought tolerance potential of cultivars of peanut.[2,18,33,40,41] and other crops.[10,13,14,35,39] Proline is also known in plants as an osmotic and energy supplier, ROS scavenger, and stress reliever.[6,8,12]

The pool size of glycine betaine contents were increased with increasing stress severity and duration (Table 1). One of the important targets of metabolic engineering in plants is GB, a potent osmoprotectant that occurs among several flowering plants.[10] The amount of glycine betaine content increased by eleven-fold in cv. K-134 on day-12 at severe stress treatment, where as in cv. JL-24 we observed only eight-fold at same stress level when compared to respective controls. From the results it is also clear that at every stage of water stress the levels of glycine betaine were higher in the cv. K-134 compared to cv. JL-24. Previous study showed that accumulation of water stress-induced GB is correlated with drought resistance.[10,11,34,39] Similarly, in the present study it was observed that a variation in the magnitude of glycine betaine accumulation between the cultivars, being greater accumulation in the cultivar K-134, compared to JL-24, further supporting the drought tolerance of cultivar K-134. Several studies have reported GB as a key osmoprotectant in mediating several plant responses to drought stress, including growth, protein modifications, photosynthesis, gene expression, and oxidative defense.[8,10,11]

In addition to proline, soluble sugars are highly sensitive to environmental stress, as they act on the supply of carbohydrates from source organs to sink organs.[15] During water scarcity, higher availability of carbohydrates is also associated with drought stress tolerance and acclimation.[14]. Water stress increased the contents of reducing sugars and non-reducing sugars in leaves of both cultivars at all stress treatments (Table 2). The magnitude of increase in sugar levels was dependent on severity of water stress. However, the per cent increase was relatively more in the cultivar K-134, than in JL-24 at all stress levels and throughout the experimentation. Cultivar K-134 showed highest individual level up-regulation in the accumulation of reducing sugars and non-reducing sugars by 202.15%, and 249.93% respectively in severe stress treatment over their untreated controls. Cultivar JL-24 exhibited significant accumulation of reducing sugars (172.23%) and non-reducing sugars (190.48%) in severe stress treatment when compared to controls. Several investigators reported a rise in sugar levels in groundnut cultivars under water stress and further correlated the drought tolerance of groundnut cultivars with greater amounts of sugars, while drought sensitive cultivars accumulated less amounts of sugars.[18,40,41] Similar genotypic variations were also reported in other crops. [13,35] The shift in carbon partitioning from non-soluble carbohydrate (starch) to soluble carbohydrates could greatly contribute to osmotic adjustment capabilities by increasing glucose, fructose, sorbitol etc.[43]

**Table 1.** Effect of water stress on leaf dry weight (gm plant$^{-1}$), relative water content (%), free amino acids (mg/gm$^{-1}$ fresh wt), proline contents (μg g$^{-1}$ fresh wt) and glycine betaine (μ mol g$^{-1}$ dry wt.) in the leaves of control and water stressed groundnut cultivars.

| **Parameters** | **JL-24** | | | | **K-134** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Control** | **Mild** | **Moderate** | **Severe** | **Control** | **Mild** | **Moderate** | **Severe** |
| Leaf dry weight (gm plant$^{-1}$) | 1.078a (100) ±0.036 | 0.9432b (87.50) ±0.058 | 0.7755c (71.94) ±0.064 | 0.5400d (50.09) ±0.048 | 0.6872a (100) ±0.029 | 0.6214b (90.42) ±0.047 | 0.5326c (77.50) ±0.042 | 0.4217d (67.37) ±0.040 |
| RWC in leaves | 91.58a ±2.08 | 83.52b ±3.21 | 62.34c ±3.56 | 37.68d ±2.54 | 90.12a ±1.09 | 81.62b ±1.72 | 65.01c ±2.94 | 48.53d ±3.88 |
| Free Amino acids | 1.828a (100) ±0.350 | 2.251b (123.16) ±0.380 | 3.323c (181.78) ±0.281 | 4.739d (259.23) ±0.301 | 1.695a (100) ±0.281 | 2.365b (139.52) ±0.268 | 4.293c (253.26) ±0.314 | 6.149d (362.79) ±0.368 |
| Free Proline | 30.37a (100) ±5.42 | 40.91b (134.72) ±7.65 | 55.38c (182.37) ±6.40 | 77.04d (253.67) ±5.98 | 36.75a (100) ±5.61 | 54.78b (149.07) ±6.82 | 86.53c (235.47) ±7.43 | 138.40d (376.82) ±6.91 |
| Glycine betaine | 1.42a (100) ±1.06 | 3.16b (221.96 ±1.10 | 6.67c (470.8) ±0.98 | 11.42d (804.58) ±1.12 | 1.66a (100) ±0.99 | 4.52b (272.08) ±1.12 | 10.11c (609.35) ±1.01 | 18.27d (1101.04) ±1.28 |

Means from 5 experiments ± SD. The mean values in a row followed by a different letter for each plant species are significantly different ($P \leq 0.05$) according to Duncan's multiple range (DMR) test.

Sugars and their derivatives may have accumulated in response to stress and can function as osmolytes to maintain cell turgor and provide a hydration shell around proteins, thereby providing the first line of defense against further water loss and may assist to maintain a water balance in drought tolerant plants. [44] Sugar also acts as a signaling molecule and helps to modulate the plant's growth, development, and response to multiple stresses.[45] Since tolerance must depend on the energy status of cells in which appropriate responses are induced, many tissues of stressed plants are likely to have an increased demand for rapidly metabolizable carbohydrate. This must be satisfied because a likely decrease in carbon fixation and increased diversion of carbon from growth or storage to osmolyte synthesis.

The modulation of polyamine metabolism has been revealed to correlate with induced tolerance to a wide range of environmental stress factors such as salt, drought, flooding, heavy metal and UV-light stress.[46] Polyamine content increased significantly in both cultivars at all water stress levels (Table 2). The magnitude of increase was dependent on severity and duration of water stress. However, the per cent increase was relatively more in the cultivar K-134, than in JL-24 at all stress regimes. A number of studies have demonstrated that polyamines function in stress tolerance largely by modulating the homeostasis of reactive oxygen species (ROS) due to their direct, or indirect, roles in regulating antioxidant systems or suppressing ROS production.[20] Therefore, from the results pertaining to the accumulation of proline, glycine betaine, sugars and polyamines, it can be said that a cultivar-specific differences in the accumulation of osmolytes under the different stress levels was observed, with cultivar K-134 showing the greater capability to accumulate those organic osmolytes under stress. MDA is a product of membrane-lipid peroxidation and an index of oxidative injury of plants. Lipid peroxidation in the leaves of the control and stressed samples were measured in both cultivars (Table 2). The MDA content was gradually increased with increase in stress intensity from mild to severe stress in both cultivars. Lower levels were observed in cv. K-134 than in cv. JL-24 plants during drought stress, indicating that less oxidative injury was occurred in cv. K-134 under drought stress. The general increase in membrane lipid peroxidation is proportional to the severity of drought stress and may result from the spontaneous interactions of ROS with organic molecules found in the membranes.[47] In the present study, osmolyte accumulation was negatively correlated with the accumulation of MDA in groundnut cultivars and a smaller per cent injury was observed in cv. K-134 compared to cv. JL-24.

**Table 2.** Effect of water stress on reducing sugars (mg $gm^{-1}$ dry wt), non-reducing sugars (mg $gm^{-1}$ dry wt), polyamine (μ $gm^{-1}$ fresh wt) and malondialdehyde (μ mol $gm^{-1}$ fresh wt.) in the leaves of control and water stressed groundnut cultivars.

| **Parameters** | **JL-24** | | | | **K-134** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Control** | **Mild** | **Moderate** | **Severe** | **Control** | **Mild** | **Moderate** | **Severe** |
| Reducing Sugars | 10.30a (100) ±0.80 | 12.23b (118.72) ±0.82 | 14.68c (142.52) ±0.90 | 17.74d (172.23) ±1.01 | 9.92a (100) ± 0.92 | 12.89b (129.97) ±0.87 | 16.87c (170.07) ±0.64 | 20.25d (202.15) ±1.10 |
| Non Reducing Sugars | 29.96a (100) ±0.80 | 37.06b (123.71) ±0.82 | 46.22c (154.26) ±1.24 | 57.07d (190.48) ±1.01 | 26.02a (100) ±1.31 | 35.58b (136.73) ±0.87 | 47.50c (182.56) ±0.68 | 65.03d (249.93) ±1.28 |
| Polyamines | 13.82a (100) ±0.370 | 15.24b (110.27) ±0.272 | 17.42c (126.05) ±0.324 | 21.61d (156.36) ±0.401 | 13.02a (100) ±0.362 | 16.36b (125.65) ±0.269 | 20.25c (155.52) ±0.223 | 25.94d (199.23) ± 0.378 |
| MDA | 10.04a (100) ±0.84 | 14.98b (149.17) ±0.86 | 20.84c (207.53) ±1.02 | 27.29d (271.86) ±1.32 | 9.01a (100) ±0.91 | 12.43b (137.53) ±1.10 | 16.08c (178.46) ±1.21 | 19.83d (220.15) ±1.34 |

Means from 5 experiments ± SD. The mean values in a row followed by a different letter for each plant species are significantly different ($P \leq 0.05$) according to Duncan's multiple range (DMR) test.

The accumulation of osmolytes like amino acids, GB, proline, sugars, and polyamines seems to have positive correlation in preventing oxidative damages triggered by drought through their capacity to scavenge reactive oxygen species, protecting important cellular macromolecules from oxidative deterioration.[48] Hence, the role of these compatible osmolytes under drought stress strongly supports their involvement in redox-regulation for better preparedness to combat drought-induced secondary oxidative stress in the cultivars studies.[14]

## Conclusions

In the present study, a comparative evaluation of two groundnut cultivars for their ability to produce osmolytes, accumulate bio mass and maintain turgor revealed a clear distinction. Cultivar K-134 showed an elevated drought tolerance with higher levels of relative water content, leaf dry weight, osmolyte accumulation and lower levels malondialdehyde (MDA) under drought stress as compared with cultivar JL-24. The results will provide useful information for genetic improvement of peanut under drought tolerance.

## Conflicts of Interest:

The author declares no conflict of interest.

## Authors' contributions:

KVM: Performed the research, collected data, analyzed the data, writing-original draft preparation CS: Designed and supervised the study, writing- reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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