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Comparative Study on Nitrogen Metabolism in a Drought Tolerant and a Sensitive Cultivar of Groundnut (*Arachis hypogaea* L.) under Drought Stress

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The development of drought tolerant genotypes for peanuts has now become a priority due to the growing number of drought-prone areas. The effects of drought stress on nitrogen metabolism was studied in leaves of two groundnut cultivars with differential sensitivity to drought stress, K-134 (drought tolerant) and JL-24 (drought sensitive) subjected to different regimes of water stress conditions for a duration of 12 days. The total protein content in leaves of both cultivars declined with progressive accumulation of free amino acid levels. Concurrently, the protease activity in the tissues was also increased. Ammonia content was increased in both cultivars and comparatively higher ammonia levels were recorded for cv. JL-24. A gradual increase in the activities of key enzymes involved in nitrogen metabolism such as glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydrogenase (NADH-GDH and NADPH-GDH), aspartate aminotransferase (AAT) and alanine aminotransferase (ALAT) was observed in both cultivars subjected to water stress. The increase in enzyme activities was more pronounced in the drought tolerant than in the drought sensitive cultivar. Contrarily, the activities of nitrate reductase (NR) and nitrite reductase (NiR) were decreased in the stressed plants. The extent of decrease was more in cv. K134 than cv. JL-24. The results indicate that drought tolerance of cultivar K-134 may be attributed at least in part to the ability to shift the metabolic rate leading to a greater accumulation of amino acids coupled with lower levels of ammonia and largely by reassimilation as evidenced by relatively greater activities of GS and GOGAT in the tissue. The physiological importance of enzyme alterations under water stress was investigated in relation to plant metabolism.

Key words: Groundnut, nitrogen metabolism, ammonia, GS/GOGAT, amino acids

Groundnut (*Arachis hypogaea* L.) is a major species of the family Leguminosae grown globally under rain-fed conditions, especially in tropical and semi-arid climates, including India (Ajay *et al*., 2023). It is a rich source of oil (40–60%), protein (10–20%), carbohydrates, vitamins, minerals, antioxidants, and monounsaturated fatty acids, and a source of medicinally important compounds (Gundaraniya *et al*., 2020). It is cultivated on 20–25 million ha of land worldwide, yielding 35–40 million tons of pods per year. The Indian peanut industry ranks second after China, with 6–7 million tons of production and 5–6 million ha of cultivated land (Mishra *et al*., 2015). Almost 85 percent of the groundnut area remains rainfed, with approximately 80% falling under dryland with no irrigation facilities, resulting in considerable yield loss from pre-flowering to flowering, pegging, and pod formation (Lal *et al*., 2021). As a result, a greater focus is needed to accelerate research on drought stress tolerance mechanisms in order to create tolerant cultivars and implement effective mitigation techniques in the production of groundnuts.

Nitrogen (N) metabolism is a fundamental process in determining the growth and productivity of plants and it has special relevance to legumes, because these plants are symbiotically associated with *Rhizobium* bacteria in the root nodules. N is mainly absorbed by plant roots in the form of, nitrate (NO_3^-) and transported to leaves for N assimilation. NO_3^- is reduced to, nitrite (NO_2^-) by nitrate reductase (NR; EC 1.6.6.1) in the cytoplasm. Subsequently, nitrite (NO_2^-) is transported to the chloroplast and then transformed into ammonium (NH_4^+) by nitrite reductase (NiR; EC 1.7.2.1) with reduction-ferredoxin as an electron donor (Rajasekhar and Oelmüller, 2010). Afterward, the ammonium (NH_4^+), derived from NO_3^- reduction, photorespiration and/or other metabolic processes is assimilated into glutamine by the glutamine synthase (GS: EC 6.3.1.2)/glutamine oxoglutarate aminotransferase or glutamate synthase (GOGAT: EC 1.4.1.13) (GS/GOGAT) cycle or the alternative glutamate dehydrogenase (NADH-GDH: EC 1.4.1.2 and NADPH-GDH: EC 1.4.1.4) pathway with 2-oxoglutarate (2-OG) and reducing equivalents provided by photosynthesis (Chardon *et al*., 2012). The subsequent process involves using glutamate as an amino group donor to create additional amino acids, which are then utilized to create a variety of biological molecules such as chlorophyll, proteins, and nucleic acids. The reactions are catalyzed by aminotransferases such as aspartate aminotransferase (AAT: EC 2.6.1.1) and alanine aminotransferase (ALAT: EC 2.6.1.2) (Slattery *et al*., 2017). As far as higher plants are concerned, the GS/GOGAT pathway constitutes in usual conditions the main pathway of ammonium assimilation, particularly the pathway that proceeds from nitrate reduction (Lea *et al.*, 1990). Nonetheless, whenever internal ammonium concentration increases for some reason, a second pathway, controlled by another enzyme, the GDH seems to contribute to reduce this internal concentration, however, the function of alternative GDH pathway remains obscure in plants. Nitrogen and its metabolism in and outside of plants plays an essential role as a signal in closely regulating the response associated with the resistance/tolerance/ adaption of plants to environmental challenges, including drought stress (Fang *et al*., 2016). Several studies have reported that drought stress affects N absorption and consequently inhibits enzymes through alteration in the transcriptional levels of transporters implicated in N-metabolism (e.g., nitrate (NO_3^-) transporters (NRTs), ammonium (NH_4^+) transporters (AMTs), NR, NiR, GS, GOGAT, GDH (Pawar *et al*., 2015; Ye *et al*., 2022). NO_3^- and NH_4^+ concentrations have distinct effects on plant performance under drought stress; the application of NH_4^+ mitigates the impact of drought on plant growth, while NO_3^- has the opposite effect (Saud *et al.,* 2017). Compatible solutes or osmolytes accumulate in organisms in response to osmotic stress. Many of these compatible solutes are N-containing compounds, such as amino acids and amides or betaines are important in osmotic adjustment, including maintaining cell turgor and subcellular structures, quenching free radicals, and buffering cellular redox potential (Madhusudhan and Sudhakar, 2023a), hence the nitrogen metabolism is of central importance under stressful conditions. Interestingly, enzyme responses often (up or down regulation) differ

among species, cultivars, analysed tissue, duration and intensity of stress in several crop plants (Ramanjulu and Sudhakar, 1997; Du *et al*., 2020; Xia *et al*., 2020). The precise enzymes involved and the parameters influencing plant N-metabolism are still frequently unknown, despite the fact that several biochemical investigations have advanced our understanding of this process. Therefore, studying the effect of drought on enzyme activities involved in nitrogen metabolism could provide valuable information on the physiological significance of its contribution in plants to cope with stress. In the present study, we report on a comparative analysis in regard to nitrogen metabolism in two groundnut cultivars differing in drought tolerance. Further, the information obtained will enable the characterization of these cultivars under water shortage and will contribute to breeding programs aiming at drought adaptation in groundnut.

MATERIALS AND METHODS

Plant material and water stress treatments

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 % of field capacity and characterized as mild (T_1), moderate (T_2) and severe (T_3) stress treatments, respectively. Leaf samples were collected on day-12 after stress induction for analysis of various parameters.

Determination of proteins, free amino acids, ammonia content and nitrogen content.

The total protein content was estimated following precipitation with TCA (10%) using BSA as standard (Lowry *et al.,* 1951). The extraction and estimation of free amino acids was done according to Moore and Stein (1948). Ammonia content was estimated using 5% homogenated tissue prepared in cold distilled water (Bergmeyer, 1965). Total nitrogen was estimated according method of Markham (1942)

Nitrogen Metabolism Enzyme extraction and assays

Protease activity

The activity was assayed following the method described by Snell and Snell (1971) and modified by Biswas and Choudhuri (1978). 1 g of frozen leaf material was suspended with 8.0 ml of 0.1 M sodium phosphate buffer (pH 6.5) centrifuged (10,000 rpm at 4 ºC for 25 min) in a high-speed refrigerated centrifuge (Sigma 3K18, Germany). The supernatant was passed through sephadex G.25 column, active fractions were collected and used as enzyme source (Kar and Mishra, 1976). The reaction mixture to measure the protease activity consisted of 1.0 ml enzyme extract, 0.1 ml of M magnesium sulphate and 1.0 ml bovine serum albumin (BSA, 50 µg ml−1). The reaction was stopped by adding 1.0 ml of 50% TCA after incubation for 1 h at 37 ºC and residual protein was measured by Folin–Phenol reagent (Lowry *et al.,* 1951) as mentioned earlier. A zero time control was taken as a blank. Enzyme activity was expressed as mg BSA hydrolysed mg^{-1} protein h^{-1}.

Nitrate reductase (NR) and nitrite reductase (NiR)

The frozen leaf tissue powder was suspended in two volumes of buffer containing 50 mM HEPES-KOH (pH 7.6), 20 mM $MgCl_2$, 10 µM FAD and 1 mM DTT. After thawing the suspension was cleared by centrifugation (15,000 rpm at 4 ºC for 10 min) and the supernatant was passed through sephadex G-25 column, active fractions were collected and kept on ice until use. For measuring NR, 200 µl of the supernatant was injected into 800 µl of the reaction medium, which consists of the extraction buffer, containing 5 mM KNO_3 and 0.2 mM NADH. Nitrate reduction was allowed to proceed for 3 min and was terminated by addition of 0.5 M zinc acetate (125 µl). After removal of unreacted NADH with 10 µM phenazine methosulphate (20 min at 25 ºC), nitrate was measured at 546 nm spectrophotometrically (Shimadzu-1601, Japan) (Hagemann and Reed, 1980). The NiR

enzyme activity was estimated according to Losada and Panique (1971).

Glutamate dehydrogenase (NADH and NADPH - GDH), glutamate synthase (GOGAT) and glutamine synthetase (GS) activities

Both NADH and NADPH-specific GDH activities were determined by the method of Sadler and Scott (1974). For NADH- dependent GDH, 1 g of frozen leaf material was suspended with 0.03 M Tris–HCl buffer (pH 8.2) containing 10 mM mercaptoethanol. The extract was centrifuged (3000 rpm at 4 ºC for 15 min) in a refrigerated centrifuge and the supernatant was passed through a sephadex G-25 column, active fractions were collected and used as enzyme source. The reaction mixture (3.0 ml) consisted of sodium ketoglutarate (10 µmol), ammonium chloride (10 µmol), calcium chloride (10 µmol), NADH (2 µmol), Tris–HCl buffer (100 µmol, pH 8.2) and enzyme 0.1 ml. The reaction was followed by measurement of the decrease in absorbance at 340 nm in UV–vis spectrophotometer (Shimadzu-1601, Japan). The enzyme activity was expressed as nano mole of NADH oxidised per mg protein. For NADPH-dependent GDH, the extraction buffer consisted of M Tris–HCl (pH 8.2) containing 1% triton X-100. The homogenate was centrifuged and passed through sephadex G-25 as mentioned earlier. The reaction mixture consists of (3.0 ml) sodium α-ketoglutarate (4 µmol), calcium chloride (10 µmol), ammonium chloride (30 µmol), NADPH (2 µmol) and potassium phosphate buffer (20 µmol, pH 7.4). The reaction was followed by measurement of the decrease in absorbance at 340 nm in UV–vis spectrophotometer (Shimadzu-1601, Japan). GOGAT activity was determined according to the method of Rachina and Nicholas (1985). GS enzyme activity was determined using the hydroxylamine dependent synthetase assay described as per the method of O'Neal and Joy (1973). Following a 30 min incubation period at 30 ºC in 20 µmol $MgSO_4$, 80 µmol α-ketoglutarate, 6 µmol hydroxylamine, 8 µmol ATP and enzyme extract, the enzymatic reaction was terminated by the addition of 1.5 ml of a solution containing 0.37 mol/l $FeCl_3$, 0.2 mol/l TCA and 0.67 mol/l HCl and the amount of glutamyl hydroxamate determined at 540 nm.

Aminotransferases (AAT and ALAT)

Aspartate aminotransferase (AAT) and alanine aminotransferase (ALAT) were extracted and estimated according to Reitman and Frankel (1957) and Hedley and Stoddart (1971) respectively.

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 by Duncan (1955).

RESULTS

Nitrogen (N) metabolism is a fundamental process in determining the growth and productivity of plants. The total protein content, free amino acid pool and protease activity in the leaves of groundnut cultivars were determined and the data were showed in Table 1. The leaf total protein content in both the cultivars decreased significantly under water deficit and they differed in the degree of their response to the severity of stress. Total protein content was 81.28, 71.09, 59.74, and 48.96 mg g^{-1} fresh wt. in cv. K-134 and 85.36, 72.82, 59.51, 45.21 mg g^{-1} fresh wt. in cv. JL-24 under control, mild, moderate and severe stress treatments respectively. In general, the leaf protein content in both the cultivars declined and the magnitude of decrease was found to be dependent on stress severity. The free amino acid levels of the normal leaves the cultivars in K-134 and JL-24 is 1.695 and 1.828 mg g^{-1} fresh wt. respectively and it was significantly increased in the stressed plants of both cultivars at all stress regimes over controls. There was a linear increase in amino acid accumulation with increasing severity of stress. However, a difference in the accumulation of amino acid pool was observed between the two cultivars, a more pronounced increase was observed in the cultivar K-134 compared to JL-24. Toral free amino acids was increased by about 3.6-fold and 2.6-fold in the leaves of cultivars K-134 and JL-24 respectively, at severe stress treatments. A significant elevation in protease activity was registered at all stress regimes in both cultivars and the increase was found to be dependent on severity of stress. However, the percent in increase was relatively less in cv. JL-24 compared cv. K-134. The percent increase was 145,

192 and 249% in cv. K-134 and 134, 173 and 220% in cv. JL-24 under mild, moderate and severe stress respectively as compared to their control plants. The activities of foliar NR and NiR were significantly declined during the drought period in both cultivars, except the NiR activity in cv. K-134 at mild stress treatment (Table 1). Compared to cv. K-134, drought stress induced an higher inhibition for the two indices in cv. JL-24. Further, the NiR activity was not as much affected as that of NR in both cultivars during stress. Mild, moderate and severe stress treatments reduced NR and NiR by about 87-71-54% and 92-81-65% in cv. K-314 respectively and by about 82–63-42% and 88-77-57% in cv. JL-24 respectively.

The total nitrogen content and ammonia content were estimated in control and stressed groundnut cultivars and the results are presented in table 2. The total nitrogen content was decreased in both cultivars with an increase in drought level treatments. Severe drought stress resulted in decrease in the percentage of concentration by 70.96 and 58.72% in cv. K-134 and cv. JL-24, respectively. A significant accumulation of ammonia was noticed in both cultivars subjected to water stress. The magnitude of the increase was found to be stress intensity dependent. However, the percent increase was relatively less in cv. K-134 at all stress regimes. In mild, moderate and severe stress treatment, ammonia content in leaves was increased by 126.38%, 159.62 %, and 204.93 %, respectively, for cv. K-134, 150.65` %, 215.73 %, and 306.94 %, respectively, for cv. JL-24. GS activity significantly increased in leaves of both the cultivars at all stress intensities (Table 2). However, the percent increase in glutamine synthetase activity was relatively higher in the cultivar K-134 compared to JL-24 at all stress treatments. The percent increase in GS activity was about 3-fold and 2-fold in cultivar K-134 and cultivar JL-24 respectively at the end of experimentation. An elevated level of GOGAT activity was noticed as a response to drought stress (Table 2). The magnitude of increase was dependent on severity of stress. However, the percent increase in GOGAT activities were relatively greater in the cv. K134 compared to cv. JL-24 at all stress treatments. The percent increase was about 149, 212, and 333% in cv. K-134 and 131, 167 and 220 % in cv. JL-24 under mild, moderate and severe stress treatments respectively.

Table 1: Total protein (mg g^{-1} FW), free amino acids (mg/gm^{-1} FW.) and activities of protease (mg BSA hydrolyzed mg^{-1} protein h^{-1}), nitrate reductase (µ moles nitrate formed mg^{-1} DW h^{-1}) and nitrite reductase (mg nitrite reduced mg^{-1} DW h^{-1}) in the leaves of control and water stressed groundnut cultivars.

Parameter	K-134				JL-24			
	T1	T2	T3	T4	T1	T2	T3	T4
Total Protein	81.28a (100) ±1.96	71.09b (87.46) ±1.08	59.74c (73.50) ±1.31	48.96d (60.24) ±1.23	85.36a (100) ±1.68	72.82b (85.31) ±1.31	59.51c (69.72) ±1.70	45.21d (52.96) ±1.52
Amino acids	1.695a (100) ± 0.28	2.365b (139.52) ±0.26	4.293c (253.26) ±0.31	6.149d (362.79) ±0.36	1.828a (100) ±0.35	2.251b (123.16) ±0.38	3.323c (181.78) ±0.28	4.739d (259.23) ±0.30
Protease	3.08a (100) ±0.18	4.48b (145.39) ±0.30	5.92c (192.13) ±0.25	7.68d (249.51) ±0.28	3.21a (100) ±0.35	4.31b (134.27) ±0.21	5.55c (173.06) ±0.27	7.07d (220.35) ±0.38
NR	3.25a (100) ±0.19	2.84b (87.52) ±0.17	2.32c (71.39) ±0.19	1.78d (54.83) ±0.18	2.98a (100) ±0.16	2.46b (82.50) ±0.21	1.89c (63.62) ±0.29	1.27d (42.57) ±0.16
NiR	1.58a (100) ±0.12	1.46a (92.48) ±0.15	1.29b (81.52) ±0.23	1.03c (65.21) ±0.16	1.72a (100) ±0.16	1.52b (88.48) ±0.18	1.33c (77.41) ±0.14	0.98d (57.21) ±0.21

Figures in parenthesis represent the percent of control.
Mean values (n=5) in a row followed by different letter for each parameter and cultivar are significantly different (P≤0.05) according to Duncan's multiple range (DMR) test.

Table 2: Total nitrogen content (mg g-1 DW), Ammonia (μg^{-1} DW) and activities GS (units mg^{-1} enzyme protein h^{-1}; 1 OD = 1 unit), and GOGAT (μ moles NADH oxidized mg^{-1} protein min^{-1}) in the leaves of control and water stressed groundnut cultivars.

Parameter	K-134				JL-24			
	T1	T2	T3	T4	T1	T2	T3	T4
Total Nitrogen	15.84a (100) ±0.34	14.66a (92.57) ±0.60	13.04b (82.30) ±0.56	11.24c (70.96) ±0.45	16.32a (100) ±0.49	14.51b (88.93) ±0.68	12.25c (75.07) ±0.66	9.583d (58.72) ±0.59
Ammonia	92.08a (100) ±8.41	116.37b (126.38) ±9.20	146.98c (159.62) ±9.58	188.71d (204.93) ±8.30	99.83a (100) ±6.32	150.40b (150.65) ±8.98	215.36c (215.73) ±7.42	306.42d (306.94) ±9.34
GS	17.98a (100) ±2.23	23.65b (131.52) ±1.78	34.53c (192.08) ±2.34	52.81d (293.67) ±2.50	19.84a (100) ±0.90	23.62b (119.05) ±1.73	29.45c (148.45) ±2.14	37.76d (190.36) ±2.02
GOGAT	182.42a (100) ±18.42	272.02b (149.12) ±24.62	387.53c (212.44) ±25.29	607.90d (333.24) ±27.94	178.30a (100) ±17.18	234.14b (131.32) ±19.37	297.99c (167.13) ±21.29	392.40d (220.08) ±28.12

Figures in parenthesis represent the percent of control.
Mean values (n=5) in a row followed by different letters for each parameter and cultivar are significantly different (P≤0.05) according to Duncan's multiple range (DMR) test.

Table 3: Activities of NADH-GDH (n mols NADH oxidized mg^{-1} protein min^{-1}), NADPH-GDH (n mols NADH oxidized mg^{-1} protein min^{-1}), AAT and ALAT (μg ketoacids mg^{-1} protein h^{-1}) in the leaves of control and water stressed groundnut cultivars.

Parameter	K-134				JL-24			
	T1	T2	T3	T4	T1	T2	T3	T4
NADH-GDH	2.19a (100) ±0.28	3.39b (154.63) ±0.32	5.74c (262.36) ±0.37	9.34d (426.38) ±0.28	2.04a (100) ±0.27	2.99b (146.58) ±0.31	4.79c (234.80) ±0.26	7.71d (377.80) ±0.34
NADPH-GDH	2.26a (100) ±0.41	3.82b (169.24) ±0.31	6.52c (288.74) ±0.58	10.28d (455.22) ±0.87	2.11a (100) ±0.32	3.22b (152.65) ±0.98	5.26c (249.15) ±0.72	8.32d (394.42) ±1.04
AAT	402.18a (100) ±31.24	447.53b (111.28) ±32.72	521.24c (129.60) ±35.36	616.48d (153.28) ±39.50	391.12a (100) ±29.10	423.23ab (108.21) ±32.77	480.62c (122.88) ±25.36	553.36d (141.48) ±41.08
ALAT	229.91a (100) ±13.52	266.97b (116.12) ±16.62	336.22c (146.24) ±18.32	396.41d (172.42) ±20.92	218.30a (100) ±14.32	243.43b (111.51) ±18.36	283.00c (129.64) ±19.37	331.92d (152.05) ±21.58

Figures in parenthesis represent the percent of control.
Mean values (n=5) in a row followed by different letter for each parameter and cultivar are significantly different (P≤0.05) according to Duncan's multiple range (DMR) test.

GDH (NADH and NADPH) activities were also significantly elevated with the aggravation of the drought intensity (Table 3). Further, the increased degrees of cv. K-134 in among different stress treatments were higher than cv. JL-24. Furthermore, the NADPH-GDH activity was relatively more pronounced than NADH-GDH. By the end of the experiment, the elevated activities of NADH-GDH and NADPH-GDH in cultivar K-134 is 426.38 and 455.22% whereas in cultivar JL-24 it is 377.80 and 394.42%. Elevated activities of AAT and ALAT were observed during salt stress in both the cultivars (Table 3). The increased activities of AAT and ALAT were comparatively greater in cv. 134 than in cv. JL-24 at all stress treatments. The percent increase in the activities of AAT and ALAT reductase in leaves of severe water-stressed plants was 153.28 and 172.42% in cv. K-134, and 141.48 and 152.05 in cv. JL-24, respectively.

DISCUSSION

The regulation of nitrogen metabolism is very important for plant stress tolerance as most plant physiological processes are associated with it (Lawlor, 2002). Protein degradation and its regulatory processes in plants are crucial for maintaining cellular homeostasis under water deficits; otherwise, damaged proteins would accumulate and disrupt cell structure and metabolism (Moloi *et al*., 2023). Increased protease-mediated proteolysis and rapid protein breakdown are responses to drought in plants (Du *et al*., 2020). In this study, the total protein content in both cultivars decreased over control in all stress treatments. Further, the decline in protein content is associated with an increase in the net pool of free amino acids. The increased levels of proteolytic activity observed here and elsewhere in the leaf tissue in response to drought, are consistent with the idea that the decrease in protein is a result of enhanced proteolytic degradation leading to an increase in net pool of free amino acids (Xie *et al*., 2019; Du *et al*., 2020; Lian *et al*., 2023) However, no clear correlation was observed between enhanced proteolytic activity and drought tolerance. Similar to previous reports in drought tolerant and sensitive varieties of wheat (Simova-Stoilova *et al*., 2009), the tolerant cv. K-134 had proteolytic response similar to that of sensitive cv. JL-24. Plant proteases are related to the increment in reactive oxygen species (ROS) production detected in the plant biochemical response to drought (Moloi *et al*., 2023). Plant proteases mitigate this process by degrading damaged, denatured, and aggregated proteins, remobilizing amino acids, and generating molecules involved in signal transductions (D'Ippólito *et al*., 2021). Dubey (1994) suggested that the high protease activity in water stressed plants appears to be of adaptive significance because it leads to the accumulation of amino acids, which together with organic acids and quaternary ammonium compounds serve as compatible cytoplasmic solutes to maintain the osmotic balance under stress conditions. Amino acids, as an important osmotic substance, play a vital role in maintaining the cell turgor pressure and in the reduction of tissue dehydration. The increase in protease activity in the stressed plants of cultivar K-134 and JL-24 could contribute to amino acid accumulation at the expense of total proteins, that might contribute to the tolerance of the plants to water stress through an increase in the osmotic potential, which is similar to published results in mung bean (Lian *et al*., 2023). Further, the accumulation amino acids in peanut was correlated with their drought tolerance in groundnut (Padmavathi and Rao, 2013; Madhusudhan and Sudhakar, 2023a) and other plants (Du *et al*., 2020). Similarly, in the present study it was observed that variation in the magnitude of amino acid accumulation between the cultivars, being greater accumulation in the cultivar K-134 in all the treatments, thus making it as drought tolerant cultivar. It is suggested that amino acids, sugars, and polyamines act as important compatible solutes to maintain osmotic balance, to protect cellular macromolecules, and scavenge free radicals under water stress conditions in groundnut and other plants (Lian *et al*., 2023). It was reported that increase in the free amino acids creates a reserve of nitrogen leading to dynamic adjustment of nitrogen metabolism, principally for the synthesis of drought specific enzymes (Navari-Izzo *et al*. 1990).

The effects of water stress on leaf N status are erratic, with increase, decrease, or constant in different species (Jangam and Raghuram, 2015). Decrease in total nitrogen content during water stress has been reported in Pakchoi (Xiong *et al*., 2018), soya bean (Du *et al*., 2020); Indian mustard (Sahay *et al*., 2021) and *Cynodon dactylon* (Zhang *et al*., 2023) and it may be attributed to the decreased nitrate reduction. In the present study similar results were obtained. NR, a key point of metabolic regulation and rate limiting enzyme in nitrogen assimilation, is known to modulate rapidly and equally sensitive to environmental stress conditions (Kaiser and Huber, 1994). The inhibition in NR activity under water stress conditions was reported in groundnut (Sharma *et al*.,1990; Jharna *et al*., 2001) and in several plant species (Meng *et al*., 2016; Xie *et al*., 2019; Sahay *et al*., 2021 Lian *et al*., 2023). Further, under conditions of restriction in nitrate flux induced by salt stress or water deficit, NR activity could be lowered initially on account of enzyme degradation/inactivation

and the reduction in gene expression and NR protein synthesis (Kaiser *et al.,* 2002; Carillo *et al.,* 2005). In agreement with the earlier reports in the present study, NR enzyme activity decreased with increasing in the intensity of water stress in both the cultivars. Further, lesser magnitude of inhibition in NR activity was recorded for the drought tolerant cultivar K-134. Jharna *et al*. (2001) observed that groundnut genotypes retaining the ability to preserve the activity of nitrate reductase activity in water stress condition could withstand and recover from the stress better. Similar to NR, NiR activity was also decreased under water stress. Compared to NR activity, NiR activity was relatively less affected in this study, which is also in agreement with the earlier report (Rao and Gnanam, 1990). However, higher NiR activity compared to NR activity seems to be related with more stable nature of NiR proteins in plants (Bray, 1997) as showed in the present study. In the present investigation, though both cultivars registered a decline in NR and NiR activities during water stress conditions, the magnitude of inhibition in NR and NiR activity was relatively less in the cultivar K-134, that appears to be a drought tolerant cultivar than JL-24, which may be due to cultivars' ability to deal with stress effectively and thereby avoid the accumulation of ammonia in excessive amounts mostly by reassimilation (Ramanjulu and Sudhakar, 1997; Pawar *et al*., 2015; Du *et al*., 2020).

The elevated ammonia levels in both cultivars during water stress in this study, presents an additional evidence of altered nitrogen metabolism during stress. Givan (1979) suggested that, either rapid nitrate reduction or enhanced proteolysis might be the cause for the increased levels of endogenously generated ammonia under stressful conditions. The involvement of rapid nitrate reductase activity as the basis for accumulated ammonia can be completely ruled out in this study, as evidenced by a decline in NR activity. The other possibility, i.e. increased protein hydrolysis, however, is strongly supported by the elevated levels of protease activity in the tissue. In addition to proteolysis, the accumulation of ammonia in the present study, was paralleled by a sharp rise in GDH activity (NADH-deamination and NADPH-amination), probably to compensate the inhibition in NiR activity and to provide the required NH_4^+ needed for amino acids synthesis. This is evident from an increased level of total free amino acids in response to water stress. Further, Robinson *et al.,* (1992) and Xie *et al*., (2019) also suggested that the GDH activity is altered in response to shifts in carbon rather than nitrogen metabolism, which they postulated that the modulation in GDH is related to the carbon supply, as the elevated activity of GDH is correlated with reduced carbon availability. The decreased photosynthetic CO_2 assimilation in both groundnut cultivars in our previous studies further supported the above hypothesis (Madhusudhan and Sudhakar 2023c). There are two ways of metabolic detoxification of ammonia in tissues of higher plants, in which plants able to assimilate disposal of ammonia at higher concentrations: (1) detoxification of excessive ammonia by simple acceleration rate of nitrogen assimilation via usual pathway or (2) supplementing the normal pathway by additional ammonia utilizing reactions, initiated only at times when there was excessive levels of ammonia in tissues (Givan, 1979). Both these mechanisms seem to be operative during certain environmental conditions with the "additional ammonia utilizing reactions" which are particularly important during accumulation of nitrogen containing compounds (Rabe, 1994).

It was suggested that GDH activity could be a measure of utilizing accumulated ammonia under stress when GS/GOGAT system is less efficient, particularly at severe stress condition. Similar to previous studies (Ramanjulu and Sudhakar, 1997; Xie *et al*., 2019; Sahay *et al*., 2021), drought led to marked increase in the activities of foliar NAD-GDH and NADH-GDH in both groundnut cultivars, but were differently affected by water shortage with the increment more pronounced in cultivar K-134. These results suggest that accelerated NH_4^+ assimilation in groundnut may be an adaptive mechanism to produce more glutamate and eliminate the accumulation of excess foliar NH_4^+. In plants cells, excessive levels of NH_4^+ are destructive, and the major NH_4^+ assimilation pathway is the GS/GOGAT cycle in higher plants. GS in conjunction with

GOGAT catalyzes the assimilation of ammonia in to glutamate to glutamine, which then serves as the nitrogen donor for the biosynthesis of all nitrogenous organic compounds in the plants. Several studies have shown that water stress induces an increase in the activity of GS and GOGAT (Ramanjulu and Sudhakar, 1997; Lian *et al*., 2023; Zhang *et al*., 2023). In parallel, in this investigation, higher GS and GOGAST activity was recorded under water stress conditions. Further, GS activity under stress conditions may also be important for the increased pool of glutamate as a precursor of proline and biosynthesis of other compatible solutes used by the plant for osmotic regulation (Venkamp *et al.,* 1989). In contrast, the decreased activity of GS and GOGAT under water stress was reported (Pawar *et al*., 2015; Xie *et al*., 2019; Du *et al*., 2020; Sahay *et al*., 2021). Further, Miranda *et al.*, (1994), noticed that in maize plants water and salt stresses did not affect GS activity, whereas the NADH-GDH activity was reduced, suggesting that the change in GS activity was not involved in the plant responses to water and salt stress. In the present study, water stress altered the NH_4^+ assimilation pathway, favored the enhancement of GS/GOGAT cycle and suppressed the amination and deamination of GDH pathway, which may contribute to maintaining the NH_4^+ conversion to glutamine and glutamate, and eliminate excess NH_4^+. Ramanjulu and Sudhakar (1997) in mulberry reported an increase in GS and GOGAT activities in tolerant cultivars than the susceptible cultivars over the controls during water stress. Furthermore, Veeranagamallaiah *et al.,* (2007) confirmed the increased GS activity with anti GS antibodies in westernblot analysis and observed more cross reaction in salt tolerant cultivar over salt susceptible cultivar in parallel to increase to GS activity. Relatively, greater activities of GS and GOGAT were recorded in the cv. K-134, supporting a better reassimilation and thus contributing for relatively low accumulation of ammonia in this cultivar. Transamination is a key step in the biosynthesis of various amino acids from glutamate, with the availability of carbon skeletons from the Krebs cycle (Hodges , 2000). In our studies, both the aminotransferases studied, AAT and ALAT, showed increased activities in groundnut during the water stress treatments. However, marked differences between the cultivars in the elevation of AAT and ALAT was observed, having higher elevation in cv. K-134 than cv. JL-24. Such increases in aminotransferases activities under drought conditions might help in the synthesis of increased amounts of amino acids that act as compatible cytoplasmic solutes and protect cell organelles and biomolecules, thus reducing the adverse effects of drought (Ramanjulu and Sudhakar, 1997; Xie *et al.*, 2019). Increase in the AAT and ALAT mediated the utilization of excessive ammonia by converting ketoacids into amino acids under stress conditions (Sheoran *et al.,* 1981). Relatively greater activities of AAT and ALAT in the cultivar K-134, supported a greater accumulation of amino acids, compared to cultivar JL-24. The transaminases play an adaptive role under environmental stress conditions and forms an important link between carbohydrate and protein metabolism under stressful conditions (Surabhi *et al*., 2008).

In the current study, the elevated activities of GS, GOGAT, AAT, and ALAT reduce the accumulated ammonia content and increase the amino acid pool. The combined direct and indirect evidence from the literature of accumulation of ammonia and nitrogen containing compounds (amino acids, amides and poly amines) during various environmental stress conditions, support the hypothesis that of sequestering toxic levels of free cellular ammonia (Rabe, 1994). Further, increased amino acid pool under stress conditions in the present study, suggest that in addition to serving an osmoregulatory function may also serve as mechanism for preventing the buildup of toxic levels of ammonia. This phenomenon could be viewed as a biochemical adaptive feature of plants and possibly play a protective role under water stress conditions. In conclusion, drought stress has an impact on the nitrogen metabolism of groundnut, which differs between cultivars. It seems the drought tolerance of cv. K-134 may be attributed at least in part to the ability to shift the metabolic rate: greater accumulation of amino acids coupled with lesser accumulation of ammonia, largely by reassimilation as evidenced by relatively greater

activities of GS, GOGAT, AAT and ALAT in the tissue.

ABBREVIATIONS:

AAT: aspartate aminotransferase, **ALAT**: alanine aminotransferase, **GDH**: glutamate dehydrogenase, **GS**: glutamine synthetase, **GOGAT**: glutamate synthase/glutamine oxoglutarate aminotransferase), **NiR**: nitrite reductase, **NR**: nitrate reductase

CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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