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Assessing biochemical changes in exogenous application of osmoprotectants in amelioration of water stress in black gram (*Vigna mungo* L.)

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Abstract
The study was carried out for assessing the efficacy of exogenous applications of osmoprotectants - proline, glycine betaine, trehalose, mannitol in alleviating the adverse effects of water stress in blackgram. The seed material VBN (Bg) 4 was used for the study. Water stress was imposed for 7 days on 25 DAS followed by exogenous application of different osmolytes. On 35 DAS, the samples were analysed for biochemical attributes – proline, soluble proteins, catalase and peroxidase and physiological attributes – chlorophyll content, chlorophyll stability index and relative water content. Growth attributing parameters and yield attributing parameters were recorded at the time of maturity. Exogenous application of all osmoprotectants contributed in overcoming water stress and stress tolerance. However, specifically, the treatment with proline has greater impact on enhancing photosynthetic activity; Exogenous application of all osmoprotectants have resulted in increase in the activity of catalase and peroxidase and treatment with glycine betaine has enhanced catalase activity and treatment with mannitol has enhanced peroxidase activity. Based on yield attributes treatment with proline had an increased number of clusters/ plant and number of pods/plant and mannitol treatment yield maximum 100 seed weight.

Keywords: Water stress, osmoprotectants, blackgram, biochemical changes

1. Introduction
Drought is one of the major abiotic stresses which adversely affects crop growth and yield and thus a constraint for plant productivity worldwide (Hasanuzzaman *et al.* 2012a) [14]. It adversely affects a variety of vital physiological and biochemical processes in plants. The identification of adaptive mechanisms to drought is of considerable importance, especially for legumes, as they play significant ecological and economic roles. Plants have an inbuilt ability to adjust to environmental variables. They adapt to the water stress conditions with an array of biochemical and physiological interventions. In response to drought conditions, maintenance of cellular osmotic pressure - osmoregulation is the major mechanism. One effective mechanism to reduce damage from these stresses is the accumulation of high intracellular levels of osmoprotectant compounds which maintain plant growth and development by osmotic adjustment, and membrane stability and protecting protein and enzymes. They act as stress signals that elicit a series of protective responses in accumulation of anti-oxidants and other active metabolites that contribute to the adaptation to stress.
Metabolic adaptation via *de-novo* synthesis of osmoprotectants is the basic strategy. Not all plant species are capable of natural production or accumulation of these osmoprotectants in response to stress and extensive research on various approaches to introduce them into plants via metabolic engineering is in progress. Stress tolerance by genetic modification is difficult to achieve due to the involvement of complex traits in plants. Exogenous application of osmoprotective compounds/ growth promoters /antioxidant compounds by foliar spray promise to be a new, alternative way to genetic engineering to improve yield under environmental stress conditions (Demiral and Turkan, 2006) [9].
The abrupt climatic change, particularly the erratic rainfall is one of the major causes of reduced pulse production in India and black gram (*Vigna mungo* L. Hepper) being the third most important pulse crop in India, its productivity is adversely affected by various biotic and abiotic stresses. Hence, the objective of the study focusses in evaluating the efficacy of

different osmolytes – proline, glycine betaine, trehalose, mannitol on exogenous application in alleviating the adverse effects of water stress in blackgram and to assess the changes in the biochemical, physiological, growth and yield attributes on exogenous application of osmoprotectants.

2. Materials and methods

2.1. Seed material

Seeds of black gram (VBN 4) obtained from the Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Killikulam, Tamil Nadu, India was utilized for the study.

2.2. Pot experiment

The research experiment was conducted at Agricultural College and Research Institute, Killikulam, Tamil Nadu, India. The soil used for the experiment was red soil. The soil was dried and sieved with 5mm mesh and 10 kg soil was filled in 21 earthen pots. A basal fertilizer mixture of composition 25kg N, 50kg P_2O_5 and 25 kg K_2O per hectare was applied to each pot. Black gram seeds were sterilized with 70% (v/v) ethanol and rinsed thoroughly with distilled water. Three seeds were sown in each pot and watered daily to field capacity with normal ground water. After 15 days, seedlings were thinned to three plants per pot. Water stress and exogenous foliar spray of different osmoprotectants were applied as per the treatments given below:

T1- Control (unstressed with regular watering)
T2- Water stressed (withhold irrigation for a period of 2 weeks from 25 DAS)
T3 - T2+ Exogenous foliar spray of Proline (30mM) on 25 DAS
T4 - T2+ Exogenous foliar spray of Glycine betaine (100mM) on 25 DAS
T5 - T2 + Exogenous foliar spray of Mannitol (30 mM) on 25DAS
T6 - T2 + Exogenous foliar spray of Trehalose (30 mM) on 25 DAS
T7 - T2 + Foliar spraying of 2% KCl + 100 ppm Boron (TNAU recommendation)

Pods were harvested after maturity and used for determining yield and quality parameters. Fully expanded leaf samples from the apex were harvested on 35 DAS and were analyzed for various biochemical parameters. The experiment was performed as a CRD with three replications.

2.3 Biochemical Analysis

Chlorophyll was extracted from fresh leaf samples (0.2 g) with 80% acetone and the total volume of supernatant was made up to 25 ml (Arnon, 1949) [4]. The absorbance was read at 645 (A_{645}) and 663 nm (A_{663}) in a spectrophotometer and chlorophyll content was determined using the equation; Total Chlorophyll (mg L^{-1}) = (20.2 × A_{645}) + (8.02 × A_{663}).

Protein content was estimated based on Lowry's method using bovine serum albumin as standard (Lowry *et al.*, 1951) [18]. Fresh leaf sample (0.2 g) was homogenized with 0.1 M phosphate buffer (pH 7.0) and centrifuged. About 0.1 ml of supernatant was pipetted out and made up to 2.5 ml with distilled water. To this 5 ml of alkaline copper reagent was added and was incubated at 37ºC for 10 minutes. Folin-ciocalteau reagent (0.5 ml) was then added and again incubated at 37ºC for 20 minutes and the absorbance was measured at 620nm.

Proline was estimated by acid ninhydrin method using proline as standard (Bates *et al.* 1973) [5]. About 0.5 g fresh leaves were extracted with 10 ml 3% sulphosalicyclic acid and filtered. To 2 ml of the filtrate, 2 ml acid ninhydrin reagent (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml orthophosphoric acid) followed by 2 ml glacial acetic acid were added. The solution was kept in boiling water bath for 1 hour and cooled in ice bath. Toluene (4 ml) was added and mixed vigorously and the coloured upper layer was separated and the absorbance was measured at 520 nm using toluene as blank.

For the determination of chlorophyll stability index, leaf samples (0.25 g) were immersed in normal water (25ºC) and hot water (65ºC) for 30 minutes and the total chlorophyll contents were first estimated (Arnon, 1949) [4]. Chlorophyll stability index (CSI) was then calculated using the formula CSI= Total Chlorophyll of heated leaf sample/Total Chlorophyll of unheated sample (Sairam *et al.* 1997) [23]. Leaf RWC was estimated from the turgid mass (TM) of 0.5 g of fresh leaf samples (FM) after being kept in water for 4 h, followed by drying in hot air oven till constant mass (dry mass, DM) was achieved (Weatherley 1950) [25]. It was calculated as: RWC = [(FM – DM)/(TM – DM)] × 100.

About 1 g fresh leaf sample was homogenized with 10 ml of 0.01 M phosphate buffer (pH 7.0) and centrifuged at 4ºC. The supernatant was used for catalase assay A mixture of 2.6 ml of 50 mM phosphate buffer (pH 7.0) and 0.4 ml of 15 mM H_2O_2, was taken and to this 40 µl enzyme extract was added. The decrease in optical density was observed at 240 nm. CAT activity was expressed as U mg^{-1} protein. One CAT unit is defined as the µmoles of H_2O_2 oxidized per minute. (Barber, J.M, 1980) [19].

About 1g of fresh leaf sample was homogenized in 3 ml of ice cold 0.1M phosphate buffer. The homogenate was centrifuged at 5^0 C for 15 minutes and the supernatant was used as enzyme source and the peroxidase activity was measured by adding 0.1 ml of o-dianisidine and 0.2 ml of 0.2M H_2O_2. Increase in absorbance was measured at 436 nm for every 30 sec for 3 minutes and enzyme activity was expressed in terms of increased absorbance per unit time per g tissue fresh weight (Malik and Singh, 1980) [19].

3. Results and discussion

3.1. Effect of exogenous application of osmoprotectants on biochemical attributes in water stress induced blackgram

From the analysis (Table1), it was observed that water stress (T2) resulted in reduction of total chlorophyll content than control (T1). However exogenous application of all osmoprotectants except Trehalose (T6) application have resulted in enhanced chlorophyll synthesis and prevented chlorophyll degradation due to osmotic stress. Among all the treatments, exogenous application of proline (T3) has pronounced impact on increased chlorophyll content (1.117 mg/g) than water stressed treatment (0.739 mg/g). It is because proline plays a beneficial role in plants exposed to various stress condition by stabilizing the mitochondrial electron transport complex II, membranes and proteins and enzymes such as RUBISCO (Allen *et al*, 1999).

A positive correlation between proline accumulation and plant stress is supported by a large body of data. Besides acting as an excellent osmolyte, proline plays three major roles during stress, as a metal chelator, an antioxidative defense molecule and a signaling molecule. Proline contributes to stabilizing sub-cellular structures, membranes and proteins, scavenging free radicals and buffering cellular redox potential under stress conditions and act as protein compatible hydrotrope, alleviating cytoplasmic acidosis and maintaining

appropriate $NADP^+$/NADPH ratios compatible with metabolism. Similar result was reported by Ben Ahmed C., 2010 [6] on exogenous proline application that mitigated the reduction in photosynthetic activity and leaf water relations under salt stress in *Olea europaea* L. cv Chemlali and the mitigating effect of proline was found to be concentration dependent. Gadallah., 1999 [11] also reported that exogenous proline application to *Vicia faba* significantly increased leaf water potential during salinity stress.

The efficacy of pure proline and *Lolium perenne* L. (LP) leaf extract against the phytotoxicity generated by nickel and salinity stress have been investigated in pea plants (*Pisum sativum* L.) by M.A.Shahid *et al*, 2014 [24]. The exogenously applied proline and LP leaf extracts significantly overcame the nickel and salinity-induced toxic effects on growth, RWC, and various photosynthetic attributes and pure proline are efficacious in improving growth and polyamine metabolism in pea under nickel and salinity stress.

Table 1: Impact of exogenous application of osmoprotectants on biochemical attributes in water stress induced blackgram

Treatments	Total chlorophyll (mg/g)	Protein (mg/g)	Proline (µmol/g)	Catalase (U/mg protein)	Peroxidase activity (Δ^A/min/g)
T1 : Control	0.890^{ab}	7.48^{a}	10.82^{a}	77^{a}	92^{a}
T2 : Water stress	0.739^{a}	8.37^{a}	32.47^{d}	154^{cd}	138^{b}
T3 : T2 + Proline	1.117^{b}	6.99^{a}	22.94^{bc}	134^{bc}	154^{bcd}
T4 : T2 + Glycine betaine	0.895^{ab}	7.04^{a}	24.68^{c}	163^{d}	177de
T5 : T2 + Mannitol	0.962^{ab}	7.71^{a}	16.45^{ab}	138^{bc}	187^{e}
T6 : T2 + Trehalose	0.692^{ab}	7.33^{a}	36.36^{d}	124^{b}	153^{bc}
T7 : T2 + 2% KCl	0.876^{ab}	7.89^{a}	18.18^{bc}	124^{b}	163^{cd}
SEd	0.203	2.14	3.33	10	15
CD (0.05)	NS	NS	7.26**	21**	31**

**Significant at 1% level

In the present study, 30mM proline was effective against water stress imposed. Ali *et al*., 2008 [1] also demonstrated that the exogenous application of proline counteracted the adverse effects of water stress on nutrient uptake because it promoted the uptake of K^+, Ca^{2+}, N and P in maize cultivars and application of 30 m*M* proline concentration has been more effective as compared to the other levels in up-regulating ion transport.

In the present study, proline accumulation was increased in T2 (32.47 µmol/g) as a adaptive mechanism to water stress, however exogenous application of all osmoprotectants has greater impact on proline accumulation and in trehalose treatment the accumulation was found to be the highest (36.36 µmol/g). Similarly, during water stress, the soluble protein content has been increased in T2 (8.37 mg/g) indicating that water stress has induced enhanced synthesis of new enzymes and stress related proteins. However in exogenous application of other treatments, the increase in protein content is less than T2 indicating that stress imposition has not affected the normal metabolic reactions and exogenous osmoprotectants were effective in stress management.

In the present study, exogenous application of all osmoprotectants have contributed to increase in the activity of catalase and peroxidase as they scavenge reactive oxygen species. However, treatment with glycine betaine has enhanced catalase activity (163 U/mg protein) and treatment with mannitol has enhanced peroxidase activity (187 Δ^A/min/g). Similar effects have been observed on exogenous applications of Pro and GB under salt stress. This is because, besides osmoprotection, Pro and GB also showed their roles in elimination of oxidative stress by triggering the antioxidant defense and also glyoxalase system. Hasanuzzaman, M *et al.*, 2014 [13] opined that exogenous proline and glycine betaine mediated upregulation of antioxidant defense and glyoxalase systems provides protection against salt-induced oxidative stress in two rice (*Oryza sativa L.*) varieties. Plants have well-developed enzymatic and nonenzymatic antioxidant defense system ready to encounter the deleterious effects of ROS. The enzymatic system includes enzymes of the ascorbate-glutathione cycle: ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase as well as superoxide dismutase, catalase, glutathione peroxidase, and glutathione *S*-transferase. The nonenzymatic antioxidants include ascorbic acid, glutathione, phenolic compounds, alkaloids, nonprotein amino acids and tocopherols. They act together in scavenging or detoxifying ROS and subsequent protection of plant cells from oxidative damage. Similarly, exogenous Pro also enhanced the activity of APX in PK, and POX, CAT and APX in KDML105 during both stress and recovery period (Hasanuzzaman, M *et al.*, 2014) [13]. Increase in catalase and peroxidase activity in all the treatments might be due to upregulation of antioxidant defense and glyoxalase system.

Park *et al.*, 2006 [22] reported that majority of endogenous GB was found in the cytosol and only 0.6-22.0% of the total leaf GB was localized in chloroplasts. Immediately after GB application, levels of H_2O_2, catalase activity and expression of the catalase gene were all higher in GB-treated than in control plants. GB-treated plants maintained lower levels of H_2O_2, but had higher catalase activity than the controls. This is a clear evidence that GB in addition to protecting macromolecules and membranes directly, GB-enhanced tolerance involve the induction of H_2O_2 mediated antioxidant mechanisms resulting in enhanced catalase expression and catalase activity. Findings of the study is well substantiated by the findings of previous studies wherean GB can reduce the impact of stress on growth and senescence, enhance the photosynthetic efficiency of PS-II and increase the protein content of rice seedlings. (Demiral., T, and Türkan, I. 2006) [9].

3.2. Effect of exogenous application of osmoprotectants on physiological attributes of water stress induced blackgram

In the present study, it was observed that chlorophyll stability index (CSI), a measure of integrity of membrane or heat stability of the pigments under stress conditions was comparatively very low in T2 (30.05%). (Table 2)

Table 2: Impact of exogenous application of osmoprotectants on physiological attributes of water stress induced blackgram

Treatments	Chlorophyll stability Index (%)	Relative Water Content (%)
T1 : Control	42.62^{ab}	87.5^{ab}
T2 : Water stress	30.05^{a}	84.6^{a}
T3 : T2 + Proline	46.67^{bc}	83.3^{a}
T4 : T2 + Glycine betaine	60.05^{cd}	83.3^{a}
T5 : T2 + Mannitol	48.56^{bc}	92.3^{b}
T6 : T2 + Trehalose	53.26b^{cd}	90.0^{ab}
T7 : T2 + 2% KCl	62.07^{d}	85.7^{ab}
SEd	7.50	2.92
CD (0.05)	16.35*	NS

* Significant at 5% level

It was found that in all other treatments, CSI was greater than that of control (42.62%) indicating that exogenous application of all osmoprotectants has profound effect on stabilizing the chlorophyll content and CSI under water stress. A higher CSI helps plants to withstand stress through better availability of chlorophyll by maintaining more dry matter production, and higher productivity. Treatment with 2% Kcl and Glycine betaine reported the maximum CSI. Similarly, in the present study, RWC was also reduced in T2 (84.6%) when compared to control (87.5%) whereas the exogenous application of mannitol (T5) has greater impact on maintaining RWC (92.3%). More beneficial effects of foliar-applied mannitol might be due to the reason that mannitol is a primary product of photosynthesis and hence a source of energy in a number of plant species. Although mannitol plays an important role in osmotic adjustment, it acts as an antioxidant to scavenge hydroxyl radicals The enhancement in biomass production due to foliar application of mannitol and thiourea have been earlier reported wherein they might have acted as a source of C and N, respectively and energy for plant growth. (Kaya. C. *et al*, 2013) [16].

3.3. Effect of exogenous application of osmoprotectants on growth and yield attributes of water stress induced blackgram

Based on growth attributing parameters, in the present study (Table 3), when compared to other osmoprotectants, treatment with Glycine betaine recorded increase in plant height (51.6 cm), root length (14.43 cm) and total dry matter production (23.57 g). Similar increase in yield parameters and growth performance have been reported by Miri and Armin, 2013 [20] on effect of exogenous glycine betaine in corn under drought condition and found out that external glycine betaine with 150ppm concentration while spraying before flowering had great positive effects and its usage is affected by time of application, concentration and more stress severity. Foliar application of glycine betaine reduced effect of stress conditions on the plant so that extent of chlorophyll (a and b), plant height, yield and 1000 seed weight increased significantly. Spraying glycine betaine thus caused improving plant performance in stress conditions. Similar results were obtained by Farooq *et al*., 2008 in rice, Ma *et al.*, 2006 in wheat and Anjum *et al*., 2011 [3] in maize on exogenous GlyBet application. Foliar spray of GlyBet significantly improved growth performances of fine grain aromatic rice seedlings subjected to drought stress (Farooq *et al.*, 2008, 2010) [10] and Farooq *et al*., (2009) found that foliar application of glycine betaine at the rate of 100 mg L^{-1} at 5-leaf stage improved drought tolerance in rice crop both under well-watered and stressed conditions and this could be a promising way to directly maintain and enhance the growth and yield in monocot crops. In the present study, 100 mM of Glycine betaine had a positive impact on growth attributes in blackgram.

Ali *et al*., 2007 reported that Glycine betaine (GB) and proline, compatible osmolytes could be used as foliar spray to improve water stress and sustain the productivity of arable crops. based on their findings. Cuin and Shabala (2007) [8] also reported that glycine betaine (GB), proline, and trehalose have a mitigating effect on K^+ efflux in *Arabidopsis* under stressed conditions and low concentrations of these organic osmolytes have a role in osmotic adjustment due to the accumulation of inorganic ions, as exogenous application of low concentrations of glycine betaine or proline significantly reduces the extent of the stress induced K^+ efflux from barley roots.

Table 3: Impact of exogenous application of osmoprotectants on growth attributes in water stress induced blackgram

Treatments	Plant height (cm)	Root length (cm)	Total dry matter production/plant (g)
T1 : Control	51.8^{b}	15.13^{a}	19.47^{c}
T2 : Water stress	41.6^{a}	19.37^{b}	10.07^{a}
T3 : T2 + Proline	44.6^{ab}	14.30^{a}	15.17^{b}
T4 : T2 + Glycine betaine	51.6^{b}	14.43^{a}	23.57^{d}
T5 : T2 + Mannitol	45.7^{ab}	14.37^{a}	22.53^{d}
T6 : T2 + Trehalose	44.9^{ab}	14.23^{a}	21.73^{cd}
T7 : T2 + 2% KCl	50.8^{b}	13.77^{a}	22.07^{cd}
SEd	2.93	2.75	2.22
CD (0.05)	6.38*	1.26**	1.02**

In the present study, based on yield attributes treatment with proline under water stress reported increased number of clusters/ plant (12.33) and number of pods/plant (36.33) and mannitol treatment yielded maximum 100 seed weight (4.23 g). (Table 4). The findings of Kaya. C. *et al*, 2013 [16] demonstrated similar improvement of salinity tolerance in maize plants in terms of growth and physiological attributes on treatment with mannitol. Cha-um, *et al.*, 2013 [7] reported that even at the harvest stage, plant height and yield traits such as panicle length and weight, fertility percentage and one-hundred grain weight of GlyBet pre-treated plants were better than those in the control plants when exposed to water

stress. However, in the present study the effect of Gly bet treatment on yield parameters was not pronounced whereas positive impact of proline and mannitol on yield attributes was observed.

Table 4: Impact of exogenous application of osmoprotectants on yield attributes in water stress induced blackgram

Treatments	No of clusters /plant	Number of Pods /plant	100 seed weight (g)
T1 : Control	10.67^{bcd}	30.67^{bc}	4.03^{a}
T2 : Water stress	8.33^{a}	22.33^{a}	3.87^{a}
T3 : T2 + Proline	12.33^{d}	36.33^{d}	4.13^{a}
T4 : T2 + Glycine betaine	9.33^{ab}	28.67^{b}	4.07^{a}
T5 : T2 + Mannitol	11.33^{bcd}	32.67^{bcd}	4.23^{a}
T6 : T2 + Trehalose	9.67^{abc}	29.33^{b}	3.97^{a}
T7 : T2 + 2% KCl	11.67^{cd}	35.33^{cd}	3.90^{a}
SEd	0.77	2.20	0.53
CD (0.05)	1.67	4.8	NS

4. Conclusion

Overall, the present work emphasizes that exogenous application of osmoprotectants – Proline, Mannitol, Glycine betaine and Trehalose have pronounced impact on amelioration of water stress and could be utilized for improvement of stress tolerance in blackgram.

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