International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
www.chemijournal.com
IJCS 2020; 8(4): 1360-1364

Received: 18-05-2020
Accepted: 22-06-2020

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Water stress mitigation of blackgram (*Vigna mungo* L.) with exogenous application of mannitol

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DOI: https://doi.org/10.22271/chemi.2020.v8.i4l.9790

Abstract
The study was undertaken for standardizing the effective concentration of the osmolyte mannitol through exogenous application for mitigating 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 concentrations of osmolyte mannitol. On 35 DAS, the samples were analysed for biochemical attributes – proline, soluble proteins, free aminoacids, catalase and peroxidase Growth attributing parameters and yield attributing parameters were recorded at the time of maturity. After optimization based on biochemical analysis, 30 mM mannitol was found to be the effective dose for amelioration of water stress in blackgram. Further, impact of foliar application of 30 mM mannitol during three different stages (Vegetative, Flowering, Vegetative and Flowering) of application was evaluated and the appropriate stage for application was standardized based on analysis.

Keywords: Water stress, mannitol, Blackgram, biochemical changes

1. Introduction
Pulses are very crucial in human diet as it is a potent source for protein. Legumes ranked second after cereal in terms of food production, which accounted for 27% of the world's primary crop production and contributed 33% of protein needs. With rise in atmospheric temperature, the biotic and abiotic stresses are predicted to become more severe and adversely affect the stability and productivity in pulse crops. Moisture stress during reproductive stage is often the most critical phase that reduced the yield by 43.4% by reducing cell division, cell elongation, leaf area, leaf area index, photosynthetic rate, increased the membrane damage and disturbed the activity of various enzymes. (Subbaramamma., 2017) [25]. It adversely affects a variety of vital physiological and biochemical processes in plants. All the plants have an inbuilt ability to adjust to environmental variables. The identification of adaptive mechanisms to drought is of considerable importance, especially for legumes, as they play significant ecological and economic roles. Metabolic adaptation via *de-novo* synthesis of osmoprotectants is the basic strategy. Over decades, several technologies to mitigate drought at farm level are evolved and among which exogenous application of osmoprotectants, growth promoters and antioxidant compounds through seed soaking, rooting medium or as a foliar spray has gained a significant ground during the last decade because it is a shotgun approach to improve stress tolerance in different crops. (Demiral and Turkan, 2006). Among the osmoprotectants, mannitol plays an important role in osmotic adjustment and is normally synthesized in large amount in many plant species (Mitoi *et al*. 2009) [21]. Its proportion is about 50% of the total translocated photoassimilates (Loester *et al.* 1992) [18]. Mannitol also function as scavengers of membrane-damaging hydroxyl radicals generated during stress. It acts as an antioxidant to scavenge hydroxyl radicals (OH•). Polyols can perform this function because their water-like OH groups mimic the structure of water and maintain an artificial sphere of "hydration" around the macromolecule.
Currently, the economically viable approaches to support crop production under drought are still limited. (Daryanto, S, 2015) [6]. Experimental evidence suggests beneficial effects of exogenous K application under soil moisture deficit. However, potassium alone as a means of alleviating drought stress is inefficient. Currently, the economically viable approaches to support crop production under drought are still limited Under such condition, exogenous application of compatible organic solutes like mannitol is a novel approach to minimize the

yield losses of agricultural crops caused by drought stress. Accumulation of compatible solutes allow turgor maintenance and stabilization of proteins and membranes against destabilizing effects of abiotic stresses including salinity, drought and extremes temperature, all of which cause cellular water depletion. Among the compatible solutes assessed for their hydroxyl radical scavenging activity, sorbitol, mannitol, myo-inositol and proline were effective hydroxyl radical scavengers. (Smirnoff and Cumbes, 1989) [24]. Moreover, there is little information available in the literature on the role of mannitol in stress tolerance in plants of agronomic importance. Hence, the present investigation is an attempt to evaluate the effectiveness of mannitol in mitigation of water stress.

2. Materials and Methods

2.1. Plant material and treatments

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 used for the study. The pot experiment was conducted during February to April, 2016 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. Pot culture experiment was conducted to standardize the effective concentration of exogenous application of the osmolyte, mannitol in mitigation of water stress in blackgram. Water stress and exogenous foliar spray of different concentrations of mannitol were applied as per the treatments given below.

T1- Control (unstressed with regular watering)
T2- Water stressed (for a period of 2weeks from 25 DAS)
T3 - T2+ Exogenous foliar spray of Mannitol (10mM) on 30 DAS
T4 - T2+ Exogenous foliar spray of Mannitol (20mM) on 30 DAS
T5 - T2 + Exogenous foliar spray of Mannitol (30mM) on 30 DAS
T6 - T2 + Exogenous foliar spray of Mannitol (40mM) on 30 DAS
T7 - T2 + Foliar spraying of 2% KCl + 100 ppm Boron

Imposed water stress on all treatments except control by withholding irrigation for two weeks from 25 DAS. Leaf samples were collected after 10 days (35 DAS). The changes in the biochemical, photosynthetic, antioxidant and yield attributes on exogenous application of different concentrations of mannitol were determined.

2.2. Effect of exogenous application of mannitol (30 mM) during different growth stages in blackgram

To standardize the growth stage for foliar application of mannitol, a pot culture experiment was conducted. The experiment was conducted with VBN4 blackgram with five treatments and four replications as mentioned below:

T1- Control (Unstressed with regular watering)
T2- Water stress imposed by with holding water for 2 weeks from 25 DAS
T3- T2 + Exogenous foliar spray of mannitol (30mM) during vegetative stage
T4 - T2 + Exogenous foliar spray of mannitol (30mM) during flowering stage
T5 – T2 + Exogenous foliar spray of mannitol (30mM) during vegetative and flowering stage

The treatments were imposed and the observations were recorded.

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). 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). 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.

The method described by Bates et al. (1973) was employed to determine free proline in fresh leaves (third leaf from top). Fresh leaf material (0.5 g) was triturated in 10 ml of 3% aqueous sulfosalicylic acid. An aliquot (2 ml) of the filtrate was reacted with 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid. After heating the mixture for 1 h at 100°C in a water bath, the mixture was extracted with 4 ml toluene. The chromophore containing toluene was aspirated, cooled down to room temperature, and the optical density was read at 520 nm using toluene as blank.

For estimation of total free aminoacids, extracted one g of finely ground plant material in 10 mL of 80% ethanol at 55˚C for 5 min. Centrifuged at 10,000 rpm for 5 min. Re-extracted the residue in 5 mL alcohol. To 0.5 mL of plant extract, added 4 mL of ninhydrin - citrate - glycerol reagent and mixed well. Heated the tubes in a boiling water bath for 10 min. Cooled the tubes to room temperature and measured the absorbance at 570 nm within 1 h against the reagent blank and concentration of the total free amino acids in the sample was found out and expressed as percentage equivalent of leucine. (Lee and Takahashi, 1966) [13].

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) [4].

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) [20].

3. Results and Discussion

3.1. Impact of exogenous application of different concentration of mannitol on biochemical constituents in water stressed Blackgram

Under drought stress, plants respond and adapt to and survive by the induction of various morphological, biochemical and physiological responses. From the analysis, it was found out that water stress (T2) has resulted in reduction of chlorophyll a and total chlorophyll contents than control (T1). Shortage of water, produces changes in the ratio of chlorophyll 'a' and 'b' and carotenoids (Anjum *et al.*, 2003b; Farooq *et al.*, 2009) [1, 9]. However, exogenous application of different concentrations of mannitol has resulted in enhanced chlorophyll a, chlorophyll b and total chlorophyll which is evident in Table1. Similar increase in chlorophyll contents of the salt-stressed maize plants on mannitol application was recorded by Kaya. C. *et al.* 2013 [15] and he reported that mannitol was more effective in improving salinity tolerance of maize plants in terms of growth and physiological attributes than thiourea.

Table 1: Impact of exogenous application of different concentration of mannitol on biochemical constituents in water stressed Blackgram

Treatments	Chloro phyll a mg/g	Chloro phyll bmg/g	Total chloro phyllmg/g	Proline µmol/g FW)	Protein (mg/g)	Amino acid (mg/g)	Catalase µ mol/min/mg Protein	Peroxidase activity ΔA min^{-1} g^{-1} FW
Control (T1)	0.565^{b}	0.183^{bc}	0.748^{b}	11.20^{e}	7.58^{ab}	0.54^{e}	73^{d}	87^{c}
Water stress (T2)	0.395^{e}	0.242^{ab}	0.637^{c}	31.24^{a}	6.01^{b}	0.71^{d}	166^{a}	131^{b}
T3: T2 + 10mM mannitol	0.447^{d}	0.279^{a}	0.726^{b}	28.18^{ab}	7.71^{ab}	0.87^{c}	164^{ab}	157^{ab}
T4: T2 + 20mM mannitol	0.509^{c}	0.248^{ab}	0.757^{b}	25.13^{b}	7.84^{ab}	1.03^{b}	140^{bc}	166^{a}
T5: T2 + 30mM mannitol	0.648^{a}	0.233^{ab}	0.881^{a}	17.32^{cd}	8.24^{a}	1.10^{ab}	140^{bc}	179^{a}
T6: T2 + 40mM mannitol	0.643^{a}	0.221^{abc}	0.864^{a}	14.94^{d}	7.97^{ab}	1.18^{a}	125^{c}	188^{a}
T7: T2 + 2% KCl	0.563^{b}	0.139^{c}	0.702^{bc}	19.35^{c}	8.10^{ab}	0.74^{d}	133^{c}	169^{a}
SEd	0.013	0.033	0.030	1.43	0.87	0.05	10	15
CD (0.05)	0.028	0.070	0.065	3.07	1.86	0.11	21	31

Proline content has increased remarkably in water stressed sample compared to control. Though the mannitol treated samples showed an elevated proline level than control, it was noticed that increasing the concentration of mannitol decreased proline content. With the accumulation of solutes, which mostly consists of organic substrates (sugars - sucrose, sugar alcohols mannitol, sorbitol and aminoacid, proline, quartenary amine-glycine betaine, organic acids, calcium, potassium, chloride ions), the osmotic potential of the cell is lowered, which attracts water into the cell and helps in maintenance of turgor. Osmotic adjustment allows the cell to decrease osmotic potential and, as a consequence, increases the gradient for water influx and maintenance of turgor. Improved tissue water status may be achieved through osmotic adjustment and changes in cell wall elasticity. This is essential for maintaining physiological activity for extended periods of drought (Kramer and Boyer, 1995) [17].

Protein content significantly reduced in water stressed sample (T2) compared to control (T1). Though protein content increased in other treatments with foliar application of mannitol, the changes were statistically non-significant and were on par with each other. Generally the accumulation of compatible solutes in osmotic adjustment to protect the plants from stress contributed towards osmotic adjustment, detoxification of reactive oxygen species, stabilization of membranes, and native structures of enzymes and proteins and that had resulted in increased protein content. The study showed that amino acid contents of all water stressed treatments were higher than that of control without water stress. Amino acid content gradually increased with increasing mannitol concentration. This is because both osmotic and drought stress influenced different aspects of nitrogen metabolism, resulting in a decline in the activities of aspartate aminotransferase, alanine amino-transferase and an increase in protease activity accompanied by increased free proline and alterations in other amino acid content. (Pandey *et al.*, 2004) [26].

All water stressed samples showed increased catalase and peroxidase activity. These enzymatic antioxidants scavenge reactive oxygen species mainly hydrogen peroxide and other organic and inorganic peroxides. Highest catalase activity was observed in water stressed treatment (T2) without any foliar application of osmolytes. Peroxidase activity was highest in treatment T6 (Water stressed + 40mM mannitol). It was observed that peroxidase activity gradually increased with increased application of mannitol; whereas catalase activity gradually decreased. In a similar study, among the osmoprotectants assessed for water stress, treatment with mannitol has demonstrated enhanced peroxidase activity (187 ΔA/min/g) and moreover exogenous application of mannitol has greater impact on maintaining RWC (92.3%). (Kavithapushpam and Mini, 2020) [14]. Increase in catalase and peroxidase activity in all the treatments might be due to upregulation of antioxidant defense and glyoxalase system.

Table 2: Impact of exogenous application of mannitol on yield attributes in water stressed Blackgram

Treatments	Number of Pods /plant	Yield/ plant	100 seed weight (g)
T1: Control	36.33^{a}	10.39^{a}	4.89^{ab}
T2: Water stress	23.67^{c}	5.19^{d}	3.74^{c}
T3: T2 + 10mM mannitol	26.67^{b}	7.00^{c}	4.49^{b}
T4: T2 + 20mM mannitol	30.67^{ab}	8.30^{abc}	4.58^{ab}
T5: T2 + 30mM mannitol	33.67^{a}	8.90^{ab}	4.63^{a}
T6: T2 + 40mM mannitol	28.00^{ab}	7.53^{abc}	4.64^{a}
T7: T2 + 2% KCl	29.00^{ab}	7.95^{bc}	4.59^{ab}
SEd	2.00	0.14	0.15
CD (0.05)	4.28	0.30	0.32

The study showed that the number of pods per plant was maximum in T1 (control), but was on par with 20mM and 30 mM concentration of mannitol indicating that those foliar treatments were effective in mitigating water stress. The yield was highest for T1 (control) followed by T5 (foliar application of 30mM mannitol). Statistical analysis of seed weight data indicated that water stressed sample (T2) showed significant decrease compared to other treatments indicating the positive impact of foliar application of osmolytes in increasing seed weight. 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 and mannitol treatment yielded maximum 100 seed weight (4.23 g) in a similar study reported by Kavithapushpam and Mini, 2020 [14]. The study revealed that foliar application of 30 mM Mannitol is effective in mitigating water stress in black gram.

Table 3: Impact of exogenous application of mannitol on biochemical constituents in water stressed blackgram at different growth stages

Treatments	Chloro phyll a mg/g	Chloro phyll b mg/g	Total chloro phyll mg/g	Proline µmol/g FW)	Protein (mg/g)	Catalase µ mol/ min/mg Protein	Peroxidase activity $\Delta A\ min^{-1}\ g^{-1}$ FW
T1: Control	0.547	0.256	0.803	12.4	7.06	77.54	78
T2: Water stress	0.404	0.173	0.577	33.45	6.47	159.83	143
T3: T2 + 30mM mannitol at vegetative stage	0.455	0.316	0.772	28.53	8.33	151.98	153
T4: T2 + 30mM mannitol at flowering stage	0.497	0.318	0.815	31.88	8.82	181.17	171
T5: T2 + 30mM mannitol at both vegetative and flowering stage	0.515	0.307	0.821	32.07	8.33	160.06	179
SEd	0.019	0.0273	0.028	1.133	0.814	6.25	9.256
CD (0.05)	0.04	0.0583	0.060	2.415	1.735	13.31	19.729

Impact of exogenous application of mannitol on biochemical constituents in water stressed blackgram at different growth stages was analysed and **i**t was found that highest chlorophyll was observed in control followed by T5 (Water stress imposed 30mM mannitol during both vegetative and flowering stage) but both control and T5 were on par with each other. Significantly higher chlorophyll b was observed in T3, T4 and T5 that is in those treatments which involved exogenous foliar spray of mannitol (30mM). Except T2, in all other treatments there was a significant increase in total chlorophyll indicating that foliar application of 30 mM mannitol during vegetative, flowering and vegetative and flowering stages improved total chlorophyll content. From the data, it was observed that Proline content was found to be high in treatments T2, T4 and T5. The control (T1) with water unstressed treatment showed the least proline content. Similarly, the treatments T3, T4 and T5 had higher protein content indicating that the exogenous application of mannitol resulted in increase of stress related proteins and enzymes. It was observed that Catalase activity was the highest during exogenous application of mannitol in flowering stage whereas highest peroxidase activity was observed during T4 (exogenous application of 30 mM mannitol in flowering stage) and T5 (exogenous application of 30 mM mannitol in flowering stage and Flowering and Vegetative stage). Mannitol has positive impact on antioxidant defense mechanism and hence increases in enzymatic antioxidants *viz*., catalase, various peroxidases and peroxiredoxins, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase which might have resulted in scavenging of superoxide radicals and H_2O_2.

Table 4: Impact of exogenous application of mannitol on yield attributes in water stressed blackgram at different growth stages

Treatments	Number of Pods /plant	Yield/ plant	100 seed weight (g)
T1: Control	40	11.27	5.00
T2: Water stress	25	5.54	3.65
T3: T2 + 30mM mannitol at vegetative stage	30	8.22	4.59
T4: T2 + 30mM mannitol at flowering stage	35	9.38	4.77
T5: T2 + 30mM mannitol at both vegetative and flowering stage	36	9.34	4.69
SEd	1.94	0.389	0.18
CD (0.05)	4.14	0.83	0.38

In the present study, the results of yield attributes (Table 4) showed that treatments T4 and T5 (30mM mannitol at flowering stage and 30mM mannitol at vegetative and flowering stage) had on par values for number of pods per plant, yield and 100 seed weight. These treatments showed higher values for yield attributes compared to treatment T3 (30mM mannitol at vegetative stage). Since both T4 and T5 showed on par values for yield attributes, and considering the economically low cost treatment for mitigating water stress, exogenous application of mannitol (30mM) during flowering stage (T4) could be selected as the best treatment. Nam *et al*., 2001 reported that in pigeonpea, coincidence of drought stress with flowering stage reduced the seed yield by 40–55% and in this study, 50.84% reduction (T2 - water stressed) in yield was observed and however application of mannitol during flowering stage (T4), the yield reduction was minimum (16.77%).

Moisture stress during flowering stage is detrimental for yield of the pulse crops. In legumes, moisture stress has drastic effects on nitrogen fixation besides plant growth. Also the number of rhizobia in soil declines drastically as soil dries and foliar nitrogen nutrition mitigate this effect and increase drought tolerance. Drought during flowering stage has often resulted in bareness due to a reduction in the flux of assimilate to the developing seeds below the threshold level necessary to sustain optimal growth. Similarly, reduction in the assimilate partitioning and activity of starch synthesizing enzymes (i.e., sucrose synthase, adenosine diphosphate glucose pyrophosphorylase, starch synthase and starch branching enzyme) occur during the grain-filling period

(Farooq *et al*., 2009) [9]. Drought usually reduces the yield in grain legumes by one or the combination of following mechanisms during late vegetative phase: (i) shortening of the duration of reproductive development, (ii) reducing branching and consequently the number of pods (Frederick *et al*., 1991; Frederick *et al*., 2001) [10, 11]; (iii) reducing seed weight and the number of seeds per pod (Dogan *et al*., 2007) [7] which was reflected in the present study too. Drought that occurred during the early reproductive stage (i.e., flowering) were more devastating as compared to those that occurred during the late generative stage i.e., pod filling to maturity. Therefore, drought that happens during the later vegetative periods (e.g., trifoliate formation) was relatively more tolerable to the plants even though they might have experienced retarded cell division, elongation and differentiation (Farooq *et al*., 2009) [9]. The plants are still able to maintain their growth functions under stress because early drought may lead to immediate survival or acclimation where the plants modify their metabolic and structural capabilities mediated by altered gene expression (Chaves *et al*., 2002) [5].

4. Conclusion

To conclude, mannitol accumulate in response to environmental stresses and can act as osmolytes, compatible solutes and function as scavengers of membrane-damaging hydroxyl radicals generated during stress. The effective dose and stage for exogenous application of mannitol was optimized in this study and demonstrated that foliar application of 30 mM mannitol once during flowering stage can mitigate water stress in an economical way.

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