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Changes in sugars, osmolytes and antioxidants on exogenous application of Validamycin A, PPFM and KCl in black gram under drought stress

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Abstract
Black gram (*Vigna mungo* L.) is a popular pulse crop that is seriously affected by drought stress. A study was conducted to explore the changes associated with the carbohydrates, osmolytes and antioxidants and the associated drought tolerance in black gram with exogenous application of Validamycin A, Pink-pigmented facultative methylotrophic bacteria (PPFM) and Potassium chloride (KCl). The experiment had eight treatments: Control (C), Validamycin A (VM-A), PPFM, KCl, drought stress (DS), DS+VM-A, DS+PPFM, and DS+KCl. The highest trehalose contents were observed in DS+VM-A (173.69 µg/g) and DS+PPFM (175.12µg/g). DS+PPFM treatment had highest starch (37.02 mg/g) and sucrose (5.70 mg/g) contents. Fructose was higher in DS+PPFM and DS+KCl treatments. Treatments VM-A, PPFM and DS+PPFM showed a higher accumulation of fructans. The proline content ranged between 4.8 and 11.22 µM/g. Treatment DS+KCl exhibited higher carotenoids (8.51 µg/g) whereas DS+PPFM exhibited higher tocopherol content (26.02 µg/g). Though root biomass was affected by drought stress, exogenous application of Validamycin A significantly increased root biomass.

Keywords: Green gram, drought stress, trehalose, PPFM, Validamycin A, KCl

Introduction
Agriculture is severely affected by several environmental stresses of which drought is the most devastating factor that affects the growth and yield of various crops (Turner *et al*., 2001) [21]. For sustainable food production and food security, we are forced to produce more food in adverse environments. The reactions of plants to water stress vary significantly at different organizational levels based on the intensity and duration of stress (Sun *et al*., 2020) [20]. Drought stress results in the inhibition of cell enlargement, and decreased photosynthesis. It also disturbs the overall metabolism and finally reduces plant growth and yield. However, plants have evolved various acclimation mechanisms, together with osmotic adjustment and antioxidant defence systems to adapt to drought stress (Chen and Jiang, 2010) [7].
Blackgram (*Vigna mungo* L.) is one of the important pulse crops grown in India and Asian countries. Since black gram is mostly cultivated in rainfed conditions, they are more prone to drought stress due to unpredictable rainfall. In black gram, yield reduction under drought at the reproductive stage was reported as about 30 percent (Baroowa and Gogoi 2012) [3]. Exogenous applications of inorganic chemicals, antibiotics, and plant-growth-promoting bacteria (PGPB) are being studied to examine the metabolites in plant tolerance under stressful conditions (Fadiji *et al*. 2022) [8]. For instance, the application of PGPB increased drought tolerance in many plant species (cereals, beans, and oil seeds) by regulating the various enzymes, hormones, organic compounds, and metabolites concentrations (Forni *et al*., 2017; Fadiji *et al*. 2022) [9, 8]. It is previously established that foliar application of KCl imparts drought tolerance to black gram plants (Pushpam *et al*., 2020) [17]. Studies with Validamycin A have shown salt tolerance in rice (Hathout *et al*., 2014) [10]. In this study, we report the biochemical changes associated with foliar application of Validamycin A, PPFM and KCl to black gram plants under drought stress.

Materials and Methods
Pot experiment and root samples
A pot culture experiment was conducted in the net house protected with rainproof roofing. The soil mixture contained red soil, sand, and vermicompost in a ratio of 3:1:2. Six pots were packed with 10 kg of soil mixture. Black gram (VBN 6) seeds were sown in the pot and watered daily. The emerged seedlings were thinned to three seedlings per pot.

Plants were supplied with NPK fertilizers and other cultivation practices as per the recommended practices of Tamil Nadu Agricultural University, Coimbatore. The experiment was laid out in a completely randomized block design with eight treatments: Control (C), Validamycin A (VM-A), PPFM, KCl, drought stress (DS), DS+VM-A, DS+PPFM, and DS+KCl and three replications. Drought (50% field capacity) was imposed in required treatments from the 25th day after sowing for 10 days. The foliar sprays of VM-A (30µM), PPFM (1%) and KCl (2%) were given two days after imposing drought stress as a single dose. The roots from each plant were harvested on the 10th day after imposing drought stress, and washed thoroughly with tap water followed by distilled water and stored at 20 °C until use.

Determination of starch and sugars

Leaf sample (0.5 g) was repeatedly extracted in hot 80% ethanol to remove soluble sugars. The residue containing starch was mixed with 5 mL of water and 6.5 mL of 52% perchloric acid and extracted at 0 °C for 20 min and centrifuged. The extraction was repeated once more and the supernatants were pooled and made up to 100 mL with distilled water. From this solution, starch was estimated by the anthrone method (Hodge and Hofreiter, 1962) [12].

Fresh leaf (250 mg) was homogenized in 5 ml of 80% hot ethanol, centrifuged at 15,000 g for 15 min, and the supernatant was dried at 80 °C. The residue was redissolved in 5 ml distilled water and used for analysis of various sugars.

Trehalose was estimated by the method of Müller *et al*. (1994) [15]. An aliquot of 100 µl of supernatant was mixed with 0.2 N H_2SO_4 and boiled at 100 °C for 10 min. To this solution, 150 µL of 0.6 N NaOH was added and boiled for 10 min, chilled, and added 2.0 mL of anthrone reagent and boiled for 10 min. The absorbance was measured at 630 nm with a spectrophotometer. The trehalose content was calculated using standard trehalose.

Sucrose was estimated by mixing 0.5 ml of sugar extract and 1 mL of invertase and placed in a water bath at 37 °C for 30 min. The released sugars were estimated by DNS method (Bernfeld, 1955) [6]. Glucose was determined in the supernatant by glucose oxidase peroxidase method according to the method described by Bergmeyer and Bernt (1974) [5].

Fructose was estimated by Seliwanoff's method (Sadasivam and Manickam, 2004). To 2 mL of sugar extract 1 mL of resorcinol reagent and 7 mL of dilute HCl were added and kept in boiling water bath for 10 min. The test tubes were removed from the water bath, cooled and absorbance was taken at a wavelength of 520 nm within 30 min. Fructose was used as standard.

Determination of osmolytes

Fructan was extracted by adding 250 µL HCl (0.2 M) to 250 µL of sugar extract, heated at 80 °C for 10 min. Samples were neutralized by adding 250 µL of 0.2 M NaOH and diluted by deionized H_2O. The released fructose was determined by Seliwanoff's method (Sadasivam and Manickam, 2004) [18].

Proline was estimated by acid ninhydrin method using proline as standard (Bates *et al*. 1973) [4]. Fresh leaves (0.5 g) 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 toluene layer was separated and the absorbance was measured at 520 nm against toluene as blank using spectrophotometer.

Determination of antioxidants

Carotenoid content was estimated according to Kirk and Allen (1965). About 500 mg of fresh sample was homogenized with 10 ml of 80 % acetone at 4 °C and was centrifuged at 2500 rpm for 10 minutes at 4 °C. The extraction was repeated until the residue became colourless. The supernatants were pooled and the absorbance was read at 480, 645 and 663 nm with a spectrophotometer against 80 % acetone as control. The following formula, Carotenoids (mg/g) = [A.480 + (0.114 × A.663) – (0.638 × A.645)] x V / 1000 x W] was used for the calculation of carotenoid content.

Determination of α-tocopherol was conducted as per the method of Baker *et al*., (1980) [2]. About 1.0 g of the sample was ground with a solution containing 20 ml of petroleum ether and ethanol (2 : 1.6, v/v) and centrifuged. To 1 ml of supernatant, 200 µl of 2 % 2, 2'- dipyridyl in ethanol was added and kept in dark for 5 minutes. Optical density was taken at 520 nm. A standard graph was made with α-tocopherol.

Determination of Lipid peroxidation

Fresh samples (0.5 g) were homogenized with mortar and pestle in 10 ml of 0.1 % trichloroacetic acid (TCA) and centrifuged at 15000 g for 15 minutes. The obtained supernatant was used for the estimation of lipid peroxidation. Lipid peroxidation was measured as the amount of malondialdehyde (MDA) content (Heath and Packer, 1968). To 1 ml supernatant, added 4 ml of 0.5 % TBA in 20 % TCA and incubated at 95 °C for 30 minutes. Cooled in ice bath, centrifuged and the optical density of the supernatant was recorded at 532 nm and 600 nm. Using the difference in optical density and molar extinction coefficient (155 mM-1 cm-1), MDA content was arrived.

Results and Discussion

Different sugars were determined in root samples (Table 1). It was observed that trehalose and sucrose contents increased in treatments subjected to drought stress (Table 2). Highest trehalose contents were observed in DS+VM-A (173.69µg/g) and DS+PPFM (175.12µg/g). The high trehalose in DS+VM-A treatment might be due to inhibition of trehalase enzyme by VM-A as it is a competitive inhibitor. The highest sucrose (5.70 mg/g) was seen in DS+PPFM treatment. Increased photosynthesis with high starch was observed in DS+PPFM (37.02 mg/g) and KCl (35.81 mg/g). Treatments DS and DS+KCl recorded high glucose contents of 2.57 and 2.47 mg/g respectively. Fructose was higher in DS+PPFM and DS+KCl treatments. Sugars are the main source of energy during stress conditions and also provide the carbon skeleton for the synthesis of several secondary metabolites such as flavonoids, stilbenes, and lignins (Nabavi *et al*., 2020) [16]. In addition, sugars such as trehalose, sucrose, glucose and fructose participate in signaling which induce the expression of defense genes (Sami *et al.,* 2016) [19].

Table 1: Changes in root carbohydrates

Treatment	Trehalose (µg/g)	Sucrose (mg/g)	Starch (mg/g)	Glucose (mg/g)	Fructose (mg/g)
C	96.78^{c}	1.91^{d}	27.37^{b}	1.19^{d}	3.26^{d}
VM-A	140.63^{b}	2.37^{c}	18.19^{c}	1.06^{e}	3.57^{d}
PPFM	104.21^{c}	1.87^{d}	28.48^{b}	1.24^{d}	3.81^{d}
KCl	98.46^{c}	2.05^{cd}	35.81^{a}	1.20^{d}	2.64^{e}
DS	139.67^{b}	4.08^{b}	13.55^{d}	2.57^{a}	6.21^{b}
DS+VM-A	173.69^{a}	4.42^{b}	27.65^{b}	1.75^{c}	4.98^{c}
DS+PPFM	175.12^{a}	5.70^{a}	37.02^{a}	2.27^{b}	7.38^{a}
DS+KCl	147.33^{b}	4.34^{b}	19.95^{c}	2.47^{a}	7.79^{a}

All values are expressed as mean of three replications. Means followed by letters that are same are non-significant at $p \leq 0.05$ according to DMRT.

Roots also exhibited an accumulation of osmolytes under water-stressed conditions (Table 2). Treatments VM-A, PPFM and DS+PPFM showed higher accumulation of fructans in roots and the values are 3.36, 3.63 and 3.32 mg/g respectively. Recent research has provided important evidence of fructans in the adaptation of plants to abiotic and biotic stresses (Jeandet *et al.,* 2022) [13]. High proline accumulation was seen in DS+PPFM (11.22 µM/g) followed by DS+KCl (10.39 µM/g). The proline content ranged between 4.8 and 11.22 µM/g. Proline is an important amino acid that act as an osmolyte and also stabilizes protein and membrane structures and detoxify reactive oxygen species (ROS) under dessication stress (Ashraf and Foolad, 2007) [1]. Treatment DS+KCl exhibited higher carotenoids (8.51 µg/g) whereas DS+PPFM exhibited higher tocopherol content (26.02 µg/g). Tocopherol content ranged between 8.46 and 26.02 µg/g in different treatments in root tissue. Both carotenoids and tocopherol are involved in scavenging ROS. The highest lipid peroxidation was seen in DS (3.66 nM/ml) followed by DS+VM-A (2.80 nM/ml) and the least in PPFM (1.63 nM/ml). The root structure revealed that drought stress significantly affected root growth (Figure 1). Treatment with validamycin A under drought stress (DS+VM-A) showed better root growth compared to that of DS.

Table 2: Changes in osmolytes, antioxidants and membrane stability in root

Treatment	Fructan (mg/g)	Proline (µM/g)	Carotenoids (µg/g)	Tocopherol (µg/g)	LPO (nM/ml)
C	2.53^{b}	4.46^{f}	5.50^{bc}	8.46^{e}	1.94^{e}
VM-A	3.36^{a}	5.90^{d}	5.03^{c}	19.96^{bc}	2.20^{cd}
PPFM	3.63^{a}	5.25^{e}	5.61^{bc}	18.19^{cd}	1.63^{f}
KCl	2.62^{b}	4.81^{ef}	5.88^{bc}	18.23^{cd}	2.05^{de}
DS	1.40^{c}	7.82^{c}	5.64^{bc}	15.08^{d}	3.66^{a}
DS+VM-A	2.06^{bc}	7.36^{c}	5.90^{bc}	18.81^{bc}	2.80^{a}
DS+PPFM	3.32^{a}	11.22^{a}	6.14^{b}	26.02^{a}	2.21^{cd}
DS+KCl	2.28^{b}	10.39^{b}	8.51^{a}	22.13^{b}	2.44^{c}

All values are expressed as mean of three replications. Means followed by letters that are same are non-significant at $p \leq 0.05$ according to DMRT

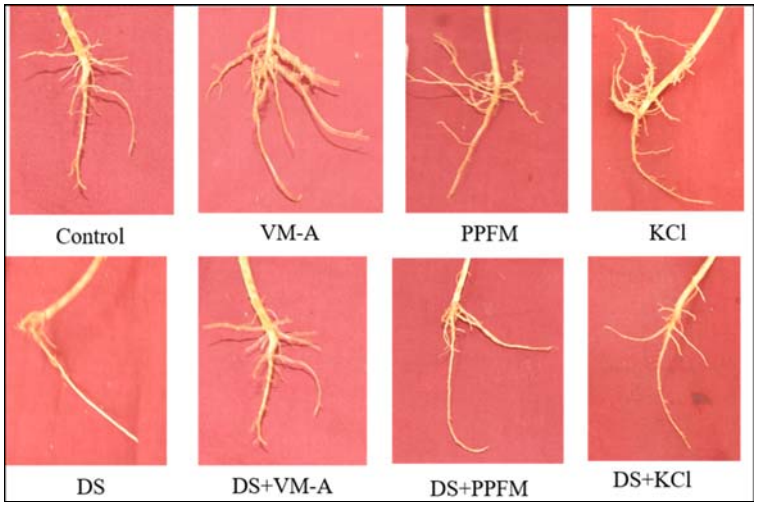

Fig 1: Changes in root growth in the different treatments

Conclusion

The study showed that foliar spray of Validamycin A, PPFM and KCl showed a protective effect against drought stress by altering the sugar, osmolyte, and antioxidant contents. Trehalose content was increased by both Validamycin A and PPFM. Osmolytes proline and fructans accumulated in high amounts in PPFM treatment under drought stress which enhanced the drought tolerance in these treatments. Foliar sprays also enhanced root growth.

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