Indian Journal of Biochemistry & Biophysics
Vol. 56, February 2019, pp. 81-85

Waterlogging tolerance in black gram [*Vigna mungo* (L.) Hepper] is associated with chlorophyll content and membrane integrity

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Received 28 November 2018; *revised 05 December 2018*

Black gram (*Vigna mungo* L.) is waterlogging sensitive legume crop. We studied the effect of waterlogging stress on membrane stability index (MSI), lipid peroxidation, superoxide dismutase (SOD) activity, chlorophyll content and chlorophyll fluorescence in four *Vigna* genotypes namely (Uttara, T-44, IC530491, IC519330). Stress was imposed for 10 days at vegetative stage (30 days after sowing). Thereafter, excess water was drained to allow recovery in stressed plants. Waterlogging treatment significantly increased lipid peroxidation and SOD activity in all the genotypes, which showed the oxidative injury posed by stress conditions. Chlorophyll content and fluorescence reduced under stress conditions. SOD activity, MSI and chlorophyll content was more in IC530491 and IC519330, T44 as compared to Uttara. Lipid peroxidation was high in Uttara. Though chlorophyll fluorescence reduced in all the genotypes under waterlogging, genotypic differences were non-significant. More efficient antioxidative scavenging to maintain membrane stability and chlorophyll content in black gram was found to be associated with tolerance to waterlogging.

Keywords: Antioxidants, Black gram, Chlorophyll, Membrane stability, Waterlogging

Water logging is a major limitation to crop production worldwide. It may result due to erratic rainfall, undulated land or poor drainage due to heavy soil texture. Oxygen diffusion is very much restricted (10000 times slower) in waterlogged conditions compared to air. In addition, respiration by plant roots and microorganisms exaggerate the oxygen deficiency in the rhizhosphere[1]. Short term waterlogging may result in hypoxia (oxygen deficiency) and if prolonged, it may lead to anoxia (absence of oxygen). Therefore, oxidative phosphorylation is hampered and due to low energy supply, plants adapt to anaerobic metabolism.

Due to unavailability of oxygen, the electron carriers in electron transport chain become reduced, affecting redox state of the cell[2]. Saturated electron transport carriers, altered redox potential of intracellular environment and energy deficit lead to oxidative stress and reactive oxygen species (ROS) are produced. ROS may damage the plant cell by causing lipid peroxidation, enzyme inactivation and oxidative damage to DNA[3]. Changes in ROS concentration, antioxidant enzyme activities, cell membrane permeability, lipid peroxidation, and hydrogen peroxide generations are well documented in legumes under waterlogged conditions[4,5]. Enzymatic as well as non-enzymatic sources generate ROS in plant cells under low O_2 concentrations[5-7]. An interplay between antioxidant activity and ROS production plays an important role in determining the response of plant to waterlogging[8]. The activities of antioxidant enzymes increased under waterlogging in pigeonpea[4], mungbean[5] and citrus[9].

India is the world's largest producer and consumer of pulses predominantly tropical and sub-tropical legumes such as chickpea, black gram, red gram, green gram and lentil. To ensure self- sufficiency, the requirement for pulses in the country is projected at 39 million tons by the year 2050 at an annual growth rate of 2.2%[10]. Black gram (*Vigna mungo* L.) is an important legume crop of rainfed agriculture. Crop is generally sown during *kharif* season. Being a legume, black gram is highly sensitive to waterlogged conditions. Present study was undertaken to study the effect of waterlogging stress on physiological and biochemical responses in selected black gram genotypes at early growth stage.

Materials and Methods

Plant material and waterlogging treatments

A pot experiment was conducted with four black gram lines namely Uttara, T-44, IC530491,

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IC519330) in *kharif* 2018. The experimental lines (Uttara, IC530491, IC519330) were selected from a set of 290 lines, which was screened in a preliminary screening in augmented block design during *kharif* 2016 and 2017 for waterlogging tolerance. During previous screening, Uttara did flowering but no pod formation occurred, while, IC530491 and IC519330 produced seeds. Since, we could not find any study on waterlogging tolerance in *Vigna mungo* L., we used T-44 (an already reported waterlogging tolerant *Vigna radiata* genotype) as check. A pot experiment was conducted in complete randomized design with three replications. Plants were grown in plastic pots (diameter 15 cm and height 15 cm) filled with 1 kg sand. At the time of sowing, five seeds were sown in each pot. After germination, they were thinned to one plant per pot. The plants were supplemented with half strength Hoagland solution on each alternate day[11]. Waterlogging stress was implemented 30 days after sowing by keeping the pots in water filled containers. Water level was maintained 2-3 cm above the soil surface. Control plants were watered as per the requirement.

Physiological and biochemical traits

Chlorophyll and fluorescence were measured at the last day of stress on the first fully matured leaf from the top. Membrane stability index, lipid peroxidation and SOD activity was measured in root tissues. After recording physiological traits, the plants were uprooted carefully to avoid damage to the roots. Samples were stored in deep freezer (−20°C) and lipid peroxidation and SOD activity were measured as soon as possible.

Lipid peroxidation was determined by measuring MDA[12]. Root tissue (100 mg) was homogenized in 5 mL 5% (w/v) Trichloroacetic acid solution and centrifuged at 10000 × g for 20 min. 0.5 mL supernatant was added to 1 mL 0.5% (w/v) thiobarbituric acid in 20% TCA. The mixture was heated at 95°C for 20 min and immediately cooled in ice bath. The samples were again centrifuged at 10000 × g for 5 min. The absorbance was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated from the extinction coefficient as 155 mM^{-1} cm^{-1}.

Superoxide dismutase (EC1.15.1.1) assay was measured spectrophotometrically[13]. Root sample (100 mg) was homogenized in 10 mL extraction buffer (0.1 M phosphate buffer, pH 7.5 containing 0.5 mM EDTA). Enzyme extract was centrifuged at 10000 × g for 10 min at 4°C and supernatant was collected. Reaction mixture (3.0 mL) consisted of 0.1 mL 1.5 M sodium carbonate, 0.2 mL 200 mM methionine, 0.1 mL 2.25 mM NBT, 0.1 mL 3 mM EDTA, 1.5 mL 100 mM potassium phosphate buffer, 1.0 mL distilled water and 0.1 mL enzyme extract. The reaction was started by adding 0.1 mL riboflavin (60 µM) and placing the tubes below a light source of two 15W florescent lamps for 15 min. Absorbance was recorded at 560 nm in spectrophotometer. One unit of SOD activity was defined as 50% inhibition of the basic rate of the reaction. Total soluble protein was determined by Bradford method[14].

To measure MSI, samples (0.5 g) with 10 mL double distilled water in glass vial were incubated in a water bath at 40°C for 30 min. After cooling, electrical conductivity (C1) was recorded with a conductivity meter (Sanco, India). Samples were again incubated in a boiling (100°C) water bath for 10 min and conductivity (C2) was measured. Membrane stability index (MSI) was calculated as follows[15].

$$MSI = [1- (C1/C2)] \times 100$$

Chlorophyll content was recorded with a self-calibrating chlorophyll meter (Opti-science, USA) on the fully expanded leaves and expressed as SPAD units. Data was taken with three replications each from control and drought treatment.

Chlorophyll fluorescence (Fv/Fm) was measured on first fully expanded leaves using a handheld chlorophyll fluorometer (Opti-science Inc., Hudson, NH). Data was recorded after full dark adaptation for 30 min. Fluorescence is the ratio of variable fluorescence (difference between maximum and minimum fluorescence, Fv) and maximum fluorescence (Fm).

Data were subjected to analysis of variance for completely randomized design factorial. Differences at $P < 0.01$ were considered statistically significant[16].

Results and Discussion

It is well established that energy deficit induced under waterlogging leads to formation of reactive oxygen species (ROS) *viz*, free radicals (O_2, $OH^{\cdot}$), hydrogen peroxide (H_2O_2) and phenols. In response plants activates its ROS scavenging system involving enzymatic [peroxidase (POX), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR)] and non enzymatic antioxidants (ascorbate, glutathione)[17].

Waterlogging caused significant increase in MDA content after imposing stress in all the genotypes (Fig. 1A). MDA content was the maximum in Uttara (0.89 TBARS content) followed by T-44 (0.58 TBARS content). Percent increase ranged from 43.7-161.7 across the genotypes in comparison to control condition. MDA content was the lowest in genotype IC519330 (0.46 TBARS content) under stress.

Waterlogging caused increase in activity of SOD in all studied black gram genotypes (Fig. 1B). Under waterlogged conditions, enzyme activities were higher in waterlogging tolerant genotypes (IC519330, T-44 and IC530491) compared to waterlogging

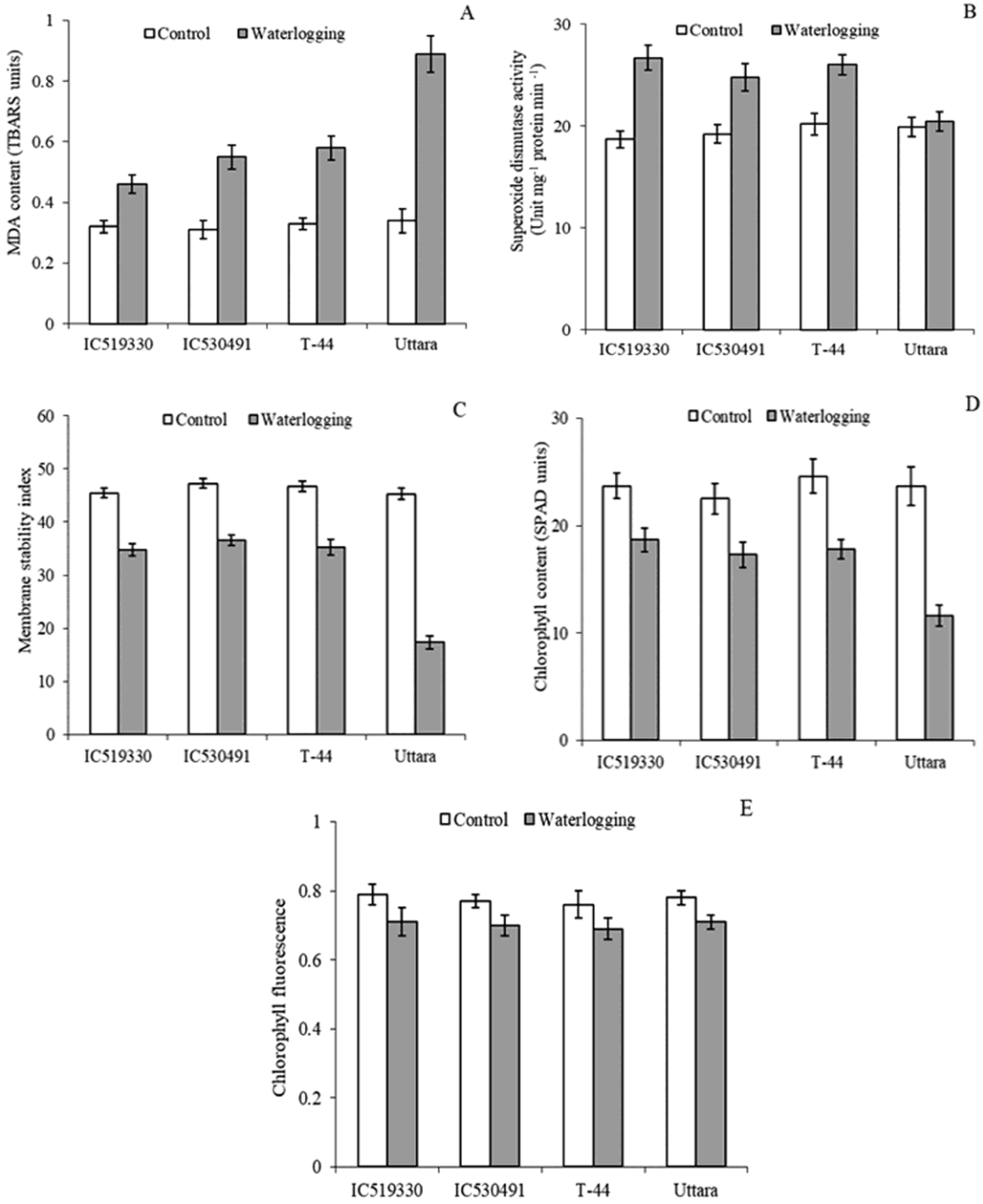

Fig. 1 — Effect of waterlogging stress on (A) Malondialdehyde contant; (B) Superoxide dismutase actiovity; (C) Membrane stability index;, (D) Chlorophyll content; and (E) Chlorophyll fluorescence in different black gram genotypes

susceptible genotype. Stress increased SOD activity from 2.6 to 42.7% compared to control in studied genotypes. SOD activity was 26.71 units mg^{-1} protein min^{-1} in IC519330 and 26.3 units mg^{-1} protein min^{-1} in T-44 but was least in Uttara (20.45 units mg^{-1} protein min^{-1}) at final stage of observation.

Membrane stability index decreased significantly as a consequence of waterlogging stress (Fig. 1C). In Uttara, cell membrane injury was drastic and stability reduced more than two folds after imposing the stress. Study showed that extent of injury to cell membrane was less in T-44 (35.24), IC519330 (36.56) and IC530491 (34.76) compared to susceptible genotype (17.32).

In the present study, lipid peroxidation and cell membrane injury increased immediately in black gram genotypes after imposing stress. It reflects reduced membrane stability and damage to biomolecules caused by ROS generated during waterlogging. Previous study suggested that free radical-induced peroxidation of membrane lipids indicates stress-induced damage at the cellular level[18]. Waterlogging caused significant increase in MDA content in stress susceptible genotypes. Present results were consistent with prior studies conducted in pigeonpea[4,19], mungbean[20], Malus[21], and cotton[22]. High membrane stability in IC519330 and IC530491 accompanied with less lipid peroxidation shows efficient ROS detoxification in these genotypes. Membrane stability was reduced due to enhanced electrolytic leakage. Maintenance of cell membrane stability in tolerant genotypes revealed efficient scavenging of ROS in these genotypes compared to others.

SOD is the enzyme, which dismutates superoxide radical to H_2O_2 and is usually considered the first line of defense against oxidative stress. Increased SOD activity was associated with increased protection from damage associated with oxidative stress[23]. In this study, SOD activity increased on account of waterlogging stress and was higher in T-44, IC519330 and IC530491. Increase in SOD activity was also demonstrated in citrus[9], pigeonpea[4] and maize[24] during waterlogging. Induction of SOD expression was recorded during waterlogging in pigeonpea[5]. In this study also, high SOD activity increased the ROS scavenging efficiency in waterlogging tolerant genotypes.

As duration of waterlogging stress increases, leaf yellowing starts followed by wilting of the plant. Hence, chlorophyll content reduced significantly in all the genotypes in response to stress (Fig. 1D). Percent reduction varied 21.0-51.0 among different genotypes. Chlorophyll content was 18.7 SPAD units in IC519330, followed by 17.8 SPAD units in T-44. Chlorophyll content reduced by 51% in Uttara (11.6 SPAD units) under stress.

Chlorophyll fluorescence shows the quantum efficiency of photosystem II. Fluorescence reduced under waterlogging, but reduction was non-significant with respect to genotype as well as treatment (Fig. 1E). Fluorescence values ranged 0.70 to 0.79 across different genotypes under normal and waterlogged conditions, which reflect normal functioning of PSII.

Chlorophyll is the most important pigment required for photosynthesis. Its loss under flooding is well documented and is visible by increased yellowing of leaves[25,26]. Chlorophyll content reduced in all the genotypes, but tolerant one sustained the stress by relatively maintaining the chlorophyll content. Similar results were reported in mungbean[26] and pigeon pea[27]. Chlorophyll fluorescence, which shows the maximum photosynthetic efficiency of photosystem II has been used in phenotyping studies against different abiotic stress. Damage to light-harvesting complex was reported in tomato[28] and mung bean under waterlogging[29]. In the present study, we could not observe significant differences in fluorescence under control and stress conditions. During waterlogging, photosynthetic parameters are affected adversely and stomatal as well as non stomatal components posed limitation to photosynthesis in pigeonpea[27]. In present study, reduction in chlorophyll was found to be associated with waterlogging susceptibility, while fluorescence remained unaffected during the stress.

Conclusion

Present study showed that membrane stability as evident by SOD activity and MDA content was directly related to waterlogging induced oxidative injury. Furthermore, more chlorophyll content sustained photosynthetic capacity in black gram genotypes under waterlogging stress.

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