



Bioregulators protected the leaf anatomy and photosynthetic machinery under water deficit stress in chickpea (*Cicer arietinum* L.)

T.V. Vineeth, Pramod Kumar* and Jasvir Singh

Division of Plant Physiology,
Indian Agriculture Research Institute, New Delhi-110 012, India.

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ABSTRACT

Water deficit is undoubtedly one of the most important environmental stresses limiting the productivity of chickpea around the world. Exogenous application of bioregulators has been found to be a novel technology for imparting stress tolerance in crop plants. This study evaluated the changes brought about by two bioregulators viz., thiourea (TU) and thidiazuron (TDZ) under water deficit stress in chickpea leaf anatomy, chloroplast ultrastructure and photosynthesis. The experiment was conducted using Pusa 362 (*Desi* type) chickpea variety. Imposed water deficit treatment decreased relative water content (RWC), membrane stability index (MSI) and photosynthetic rate (P_N). However, bioregulators application maintained higher RWC, MSI and P_N under water deficit stress. Under imposed water stress, compact palisade layers of the mesophyll tissue were disrupted and cell size of the mesophyll cells displayed drastic reduction. Chloroplast, under water stress, displayed a number of grana with loose type of thylakoid, large increase in osmiophilic granules, reduction in the amount of starch granules and overall disruption of the thylakoid membrane. Foliar application of bioregulators maintained the integrity of mesophyll tissue and chloroplast structure thereby protected the chickpea plants from the detrimental effects of water deficit stress.

Key words: Bioregulators, Chickpea, Chloroplast, Leaf anatomy, Photosynthesis, Water deficit stress.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the fourth most important grain legume with a total global production of 13.1 million ton from 13.5 m ha area (FAOSTAT, 2013). India is the largest producer and consumer of chickpea contributing to 70% of world's total production (FAOSTAT, 2013, Rizvi *et al.* 2014). Approximately 90% of world's chickpea is grown in the arid and semi-arid zones under rainfed conditions (Kumar and Abbo, 2001). Thus, in these areas chickpea is constantly exposed to water deficit stress (Talebi *et al.*, 2013) due to deficient and erratic rainfall. Water deficit stress affects various morphological and physiological processes, resulting in poor growth, development and causes 40-50% reduction in yield of chickpea globally (Ahmad *et al.*, 2005).

Presently, there is a greater research concern in identifying the bioregulators which can be used to achieve the stress tolerance in crops under field conditions. Application of bioregulators provides an alternative approach to counter stress conditions by reverting inhibitory effects of water stress (Mehta *et al.*, 2006). Thidiazuron (TDZ) is a urea based cytokinin (CK), non-degradable by cytokinin oxidase enzyme and it has also been reported to stimulate endogenous cytokinin biosynthesis (Zhou *et al.*, 1994). TDZ, a phenyl urea derivative exhibiting cytokinin

activity in bioassays is also a highly stable compound (Mok *et al.*, 1982). In addition, certain thiol-molecule like thiourea is known to maintain the redox state ($-SH/-S-S-$ ratio) of the cell and its proper functioning under stress conditions (Srivastava *et al.*, 2011). In addition, thiourea is the most cost effective and eco-friendly in nature.

Water deficit stress in higher plants also leads to anatomical changes, which are better observed indicators as they can be directly applied to agriculture and handled easily (Shao *et al.*, 2008). Anatomical alterations occur in crop plants under water deficit stress in order to protect and adapt the crop under stress condition (Makbul *et al.*, 2011). Response of plant tissues to water deficit stress depends on the anatomic characteristics that regulate the transmission of the effect of water deficit stress to the cells (Olmos *et al.*, 2007). Understanding the anatomical and ultrastructural responses to water deficit stress is highly essential for a holistic perception of plant tolerance mechanisms to water-limited conditions.

Relative water content (RWC) is one of the important parameters to determine water status of the tissue and adaptive response (Barrs and Weatherley, 1962). Stress exposed plants immediately lower down RWC of their leaves (Grover *et al.*, 2004). Electrolyte leakage measurement is another commonly used criterion to assess the extent of

*Corresponding author's e-mail: pramodk63@yahoo.com.

oxidative stress and level of membrane stability, associated with leakage of solutes from the cells (Bandeoglu *et al.*, 2004). Moreover, photosynthesis is aptly regarded as the most important physiological process in plants which is particularly sensitive to effects of water deficiency (Ashraf and Harris, 2013). Soil water deficit (Schulze, 1986) and leaf water deficit (Hsiao, 1973) have been shown to induce stomatal closure, which is the primary cause of photosynthetic reduction (Chaves, 1991). Even a small decrease in the water potential of a plant causes its stomata to close, and decreases the intensity of photosynthetic assimilation of CO₂ (Rivero *et al.*, 2009). As the degree of hydration of the tissues decreases, the photosynthetic apparatus of plants undergoes functional changes, which further develop into disruption of its structure (Chernyad'ev, 2005).

Chickpea is planted over a wide range of conditions but information related to bioregulators mediated anatomical and ultrastructural changes in the plant tissues under water deficit stress is totally lacking. Therefore, an attempt was made to interlink bioregulators modulated leaf anatomical and chloroplast ultrastructure alterations with photosynthetic response under water deficit stress.

MATERIALS AND METHODS

Plant material and growth conditions: The present study was conducted in the Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi (latitude of 28°N, longitude of 77°E and about 250 m above mean sea level). Pusa 362 (*Desi* type) variety of chickpea was selected for the study whose seeds were obtained from Division of Genetics, IARI, New Delhi. Sowing was done in 12 inch diameter earthen pots filled with clay loam soil and farmyard manure in 4:1 ratio during winter season. Experiment was conducted in factorial completely randomized design (CRD) with 4 replications. All recommended agronomic practices were followed to raise the healthy crop plants. Recommended basal dose of fertilizers in the form of urea, single super phosphate and murate of potash were applied.

Water stress treatment: Present pot experiment was divided into two sets. In the first set of potted plants (half of the total number of pots), water stress was imposed at the reproductive stage by withholding water until the symptoms of wilting were visible. This stage was obtained by withholding water continuously for 8 days when soil moisture diminished to around 5% and leaf relative water content around 60% (Fig. 1a). While the remaining second set of potted plants (half of the total number of pots) was used for providing well watered condition, to maintain optimal soil moisture for healthy plant growth.

Bioregulators treatment: Before imposition of water stress treatment, plants of both sets were treated exogenously with foliar application of optimum dose of thiourea (1,000 mg L⁻¹)

and thidiazuron (10 mg L⁻¹) (Vineeth *et al.*, 2015) and water spray was used as control.

Measurement of soil moisture: The soil moisture content was estimated periodically during the imposed water stress treatment. For the estimation of soil moisture 25 g fresh mass soil sample was taken from the root zone of the potted plants. Then the sample was kept in the oven at 105°C, and dried till constant mass was achieved. The soil moisture content was calculated according to Faulkner *et al.* (1989) and expressed in percentage [%]. Fully saturated soil at 100% capacity (FC) had 30% moisture. Soil moisture content = [(fresh mass – dry mass)/dry mass] × 100

Determination of relative water content (RWC): Leaf RWC was determined from fresh mass (FM), dry mass (DM), and turgid mass (TM) using the formula $RWC [\%] = [(FM - DM)/(TM - DM)] \times 100$. Leaf tissue (1 g) was immersed in deionized water and kept in the dark for 12 h at 4°C. After this, leaves were removed from deionized water, gently blotted dry and weighed for TM. Samples were then dried in an oven set to 80°C for at least 72 h and weighed for DM (Barrs and Weatherley, 1962).

Membrane stability index: Membrane stability index (MSI) was estimated according to the method described by Premachandra *et al.* (1990). For estimation of membrane stability index 100 mg leaf material, in two sets, was taken in test tubes containing 10 ml of double distilled water. One set was heated at 40°C for 30 min in a water bath, and the electrical conductivity of the solution was recorded on a conductivity bridge (C₁). Second set was boiled at 100°C on a boiling water bath for 10 min, and its conductivity was measured on a conductivity bridge (C₂). Membrane stability index (MSI) was calculated as:

$$MSI = [1 - (C_1/C_2)] \times 100$$

Determination of photosynthesis: Rate of photosynthesis was measured using portable Infrared Gas Analyzer (IRGA), LI-6400XT model (Li-COR, USA). The rate of photosynthesis was measured by operating the IRGA in the closed mode. The top most fully expanded leaf was enclosed in the assimilation chamber. Photosynthesis was measured while the CO₂ concentration changed over a definite time interval. The system automatically calculated photosynthesis on the basis of pre-loaded flow and leaf area. All the parameters were determined during the course of the experiment between 10 to 11.30 am by providing artificial light source of saturated intensity 1,000 μmol m⁻² s⁻¹. Three readings were taken per pot on each sampling day. Rate of photosynthesis was expressed as [μmol (CO₂) m⁻² s⁻¹].

Leaf anatomy: Chickpea leaf sections were cut in suitable planes using a very sharp razor. Then the sections were stained with principal stain safranin and thereafter washed with water till no more stain dissolved in water. Then the sections were passed through a graded series of alcohol for

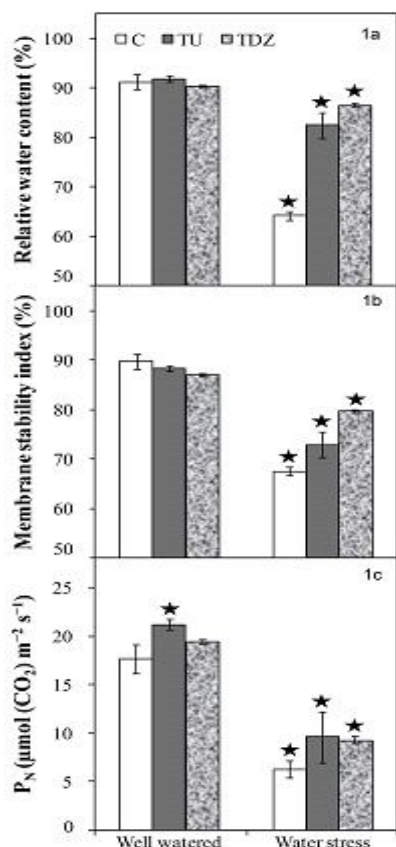


Fig 1: 1a,b,c: Effect of bioregulators (TU and TDZ) under well watered and water deficit stress conditions on [A] relative water content (RWC) [B] membrane stability index (MSI) and [C] photosynthetic rate (P_n) in chickpea. Data represent the mean of four replicates $\pm$ standard deviation (error bars). The bars with star symbol are significantly different at $p \leq 0.05$ level of probability. [C: control, TU: thiourea, TDZ: thidiazuron].

dehydration. To begin with sections were transferred to a watch glass comprising 50% alcohol, then without disturbing the sections, used alcohol was removed by a glass dropper and the 50% alcohol was replaced with 70% alcohol. At that stage counter stain was employed using fast green prepared in 90% ethanol and was further destained with 90% ethanol. Now the sections were placed in 90% and then 100% ethanol to complete the dehydration process. Now as a part of the clearing process, sections were gradually passed through xylol series of 25, 50 and 75% and finally transferred to pure xylol. Then the sections were mounted on to the centre of the slide using Canada balsam mounting medium and subsequently viewed through a compound microscope as described elsewhere (Iftikhar *et al.*, 2009).

Chloroplast ultrastructure: Fresh leaves of chickpea collected were cut properly (2 mm), washed and then fixed in 2% para-formaldehyde, 1% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 6.9) for 2 h at room temperature (Gunawardena *et al.*, 2001). Leaf segments were washed in the buffer, and post-fixed in 1% aqueous osmium tetroxide

for 1 h, before being stained in 0.5% uranyl acetate at 4°C overnight. Tissue sections were dehydrated in a graded ethanol series (30 min each) and then embedded through ethanol: spur resin mixtures (3:1, 1:1, 1:3) for 1 h each. Finally, they were embedded in 100% Spur resin and polymerized at 60°C for 9 hours in flat silicon rubber moulds. Ultra thin sections (60- 80 nm) were cut on a Reichert Jung Ultra-cut E microtome (Leica, Milton Keynes, UK), collected on Formavar-coated grids (200-mesh copper hexagonal mesh: Agar scientific, Stansted, UK), stained in lead citrate and examined with a transmission electron microscope (Phillips CM- 10) operated at 60-80 kV.

Statistical analysis: The experiment was conducted as completely randomized design. Statistical analysis of the data was done using standard statistical procedure (Panse and Sukhatme, 1967). The critical difference was calculated at 5% probability level.

RESULTS AND DISCUSSION

In the present study, imposed water deficit stress adversely affected the chickpea leaf anatomy. Under water deficit stress, compact palisade layers of the mesophyll tissue were disrupted. However, the thickness of the epidermal cells of leaf was found relatively higher than that of under well watered condition. Moreover, size of the mesophyll cells showed considerable reduction under water stress condition (Fig. 2). Tissues exposed to environments with low water availability have earlier been reported to have reduced cell size and increased cell wall thickness (Pitman *et al.*, 1983, Guerfel *et al.*, 2009). Guerfel *et al.* (2009) and Ennajeh *et*

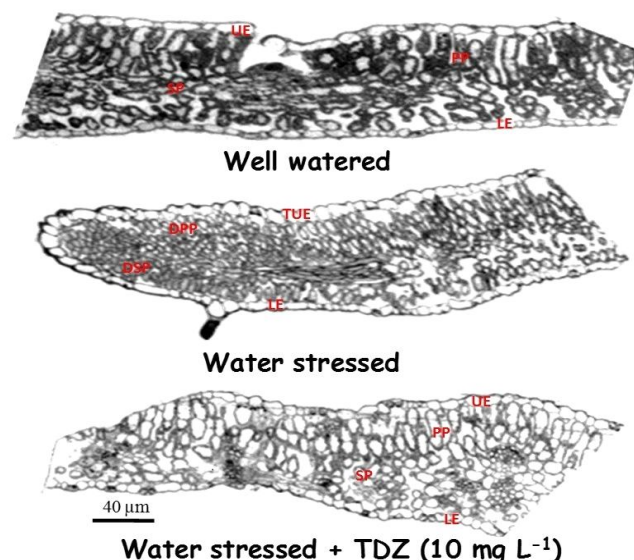


Fig 2: Leaf anatomical changes in desi chickpea under well watered, imposed water stress conditions and imposed water stress + TDZ (10 ppm) treatment. UE: upper epidermis, PP: palisade parenchyma, SP: spongy parenchyma, LE: lower epidermis, TUE: thickened upper epidermis, DPP: disrupted palisade parenchyma, DSP: disrupted spongy parenchyma.

al. (2010) also reported that olive leaves reveal an increase in palisade parenchyma thickness under low water conditions. A limitation of cell growth takes place because division and cell expansion are affected by osmotic and water stress in the mesophyll cells (Carcamo *et al.*, 2012). Foliar application of cytokinin (TDZ) and thiourea maintained the compactness and integrity of palisade layers of mesophyll tissue (Fig. 2) which indicated that bioregulators application improved the tolerance by maintaining the integrity of photosynthetic tissue.

Under imposed water stress condition, destruction of thylakoid, appearance of lipid droplets, disappearance of starch inclusions, changes in the lamellar structure, number of grana with loose type of thylakoid, large increase in osmiophilic granules and reduced amount of starch in the bundle sheath chloroplast were noted (Fig. 3). Similar symptoms were also reported earlier under water stress in *Sorghum bicolor* (Giles *et al.*, 1976), willow (Vapaavuori *et al.*, 1984) and *Cynodon dactylon* (Utrillas and Alegre, 1997). However, water stress with foliar application of cytokinin (TDZ) and thiourea maintained the integrity of the chloroplast structure. Grana were recorded with compact type of thylakoid. Water stressed with foliar application of thiourea maintained the chloroplast structure similar to the control. Moreover, TDZ treatment had large and higher number of starch grains and low number of osmiophilic granules in chloroplast (Fig. 3). Similar reports on chloroplast structure have also been made earlier with the application

of BA under water stress in bean and sugarbeet (Haisel *et al.*, 2006).

RWC reduced under water deficit stress (Fig. 1a). Similar results on reduction in RWC under water stress have been made earlier (Grover *et al.*, 2004). Foliar application of bioregulators (TU and TDZ) during water deficit stress maintained higher RWC values over the water deficit stress control. Maximum RWC was estimated with the application of thidiazuron (Fig. 1a). Similar results on RWC [%] were also reported with the application of thiourea in wheat (Anjum *et al.*, 2011). MSI response of chickpea under water stress also showed the similar pattern as to RWC. Membrane stability was drastically reduced under imposed water deficit stress (Fig. 1b). Foliar application of bioregulators (TU and TDZ) during water deficit stress maintained higher MSI values over the water deficit stress control. Maximum MSI was maintained with the application of thidiazuron (Fig. 1b). It might be because cytokinins are well recognized for enhancing the antioxidant activities, which scavenge the ROS production and ultimately reduce the membrane damage and improve the cell membrane thermostability (CMT) (Sayd *et al.*, 2010). Furthermore, TDZ has also been reported to stimulate endogenous cytokinin biosynthesis (Guo *et al.*, 2011) and thus probably improved the membrane stability. Similarly, thiourea is a direct scavenger of reactive oxygen species and a potential antioxidant thereby improved cell membrane stability (Hammed and Sheikh, 2007 and Srivastava *et al.*, 2011).

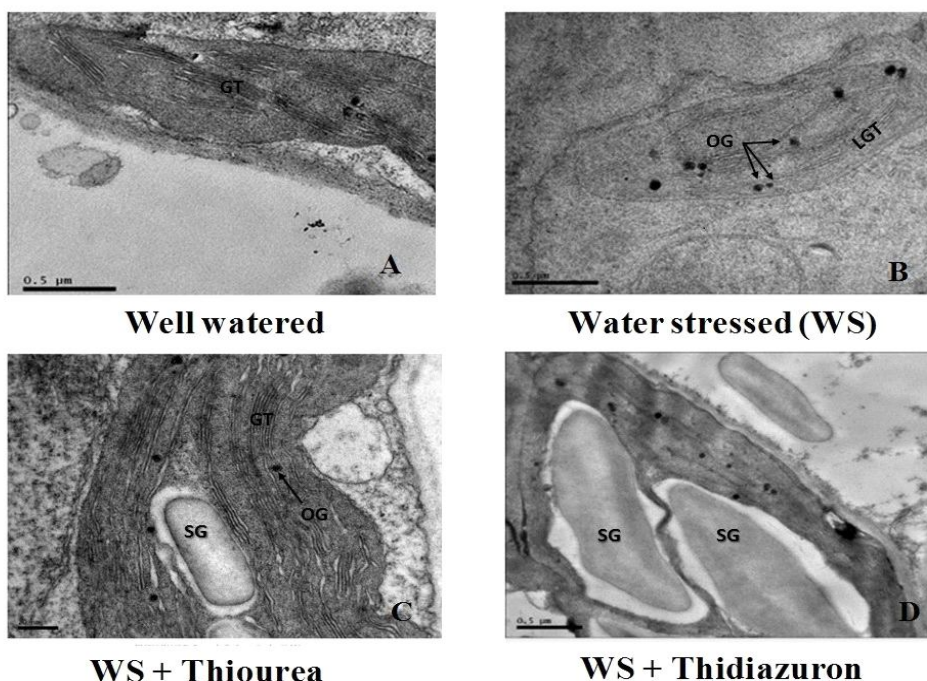


Fig 3: Effect of bioregulators on chloroplast structure under well watered (A), water stress (B), water stress + thiourea (1000 ppm) (C) and water stress + TDZ (10 ppm) (D) conditions. GT: grana thylakoid, OG: osmiophilic granule, LGT: loose grana thylakoid, SG: starch granule.

Photosynthetic rate drastically decreased under water deficit stress (Fig. 1c). Drought stress has been reported to decrease the rate of photosynthesis (Lawlor, 2002). The reduction in photosynthetic activity under stress takes place due to decline in CO₂ availability caused by the restriction of CO₂ diffusion (Flexas *et al.*, 2007) and inhibition of ribulose-1,5-bisphosphate (RuBP) synthesis (Lawlor, 2002, Rivero, *et al.* 2009). Mild water stress reduces photosynthesis due to stomatal limitations while under severe drought stress reduction in CO₂ fixation is caused by the breakdown of photosynthetic machinery (Tambussi *et al.*, 2002).

Under well watered condition, thiourea and TDZ enhanced photosynthetic rate and also maintained higher rate of photosynthesis under water stress condition (Fig. 1c). Thiourea has been reported to increase photosynthetic efficiency (Sahu *et al.*, 1993, Mathur *et al.*, 2006). Thiourea appears to have more diverse biological activities because of its –SH group (Sahu *et al.*, 1993) which favoured larger green photosynthetic surface and hence large accumulation

of photosynthates (Shanu *et al.*, 2013). Cytokinins increase the stability of photosynthetic apparatus during water stress (Chernyad'ev, 2005). Phenylurea cytokinins have been reported to improve photosynthetic activity in maize (Lazova and Yonova, 2010). TDZ treatment was also found to induce photosynthetic CO₂ assimilation in soybean plants (Vakilionová *et al.*, 1991).

CONCLUSION

From the present study, it may be concluded that bioregulators application maintained the integrity of leaf mesophyll tissue and chloroplast structure, improved RWC, membrane stability and photosynthetic rate and as a result protected the photosynthetic machinery under water deficit stress condition.

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