

## RESEARCH ARTICLE

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## Proline, glycine betaine, total phenolics and pigment contents in response to osmotic stress in maize seedlings

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### ABSTRACT

In order to evaluate the fresh weight, RWC, pigment content, total phenolics, proline and glycine betaine responses of maize inbred lines to osmotic stress, a factorial experiment was carried out under laboratory conditions with two maize inbred lines (B73 and MO17) and two osmotic stress levels induced by PEG (control and -0.6 MPa). Fresh weight significantly decreased under drought stress. On the basis of percent inhibition in fresh weight at the osmotic stress MO17 was ranked as tolerant (inhibition 45.30%), and B73 drought sensitive (inhibition more than 50%). Leaf relative water content (RWC) was significantly decreased in both inbred lines under osmotic stress. The pigment concentrations were substantially declined in both maize inbreds under osmotic stressed conditions. However, this reduction was less in B73 than MO17. Osmotic stress declined the levels of total phenolics in both maize inbreds. On the other hand, the osmotic stress markedly enhanced the levels of proline and glycine betaine in both maize inbreds, but this was more pronounced in MO17. The present results showed that osmotic stress retards the growth and some biochemical attributes of maize inbreds. In conclusion, the level of proline and glycine betaine in maize could improve drought tolerance.

**Key words:** drought stress, glycine betaine, maize, pigment content, proline

## Introduction

Maize (*Zea mays* L.) is the world's third most important cereal crop, and moisture is a crucial factor for successful production of maize (Konopka *et al.*, 2009). Drought is one of the most important abiotic stresses that severely reduce crop productivity. Changes in plant productivity due to changes in gas exchange, especially photosynthetic rate, have received much attention worldwide. The ability of crop plants to acclimate to different environments is directly or indirectly linked with their ability to acclimate at the level of photosynthesis (Chandra, 2003).

Drought stress damage the thylakoid membrane, disturb its functions, and ultimately decrease photosynthesis and crop yield (Huseynova *et al.*, 2007). The reduction of the photosynthetic activity under drought stress can be ascribed to both stomatal and non-stomatal factors. From a

physiological perspective, leaf chlorophyll concentration is a parameter of significant interest in its own right. Studies by Randall *et al.* (1977) revealed that the majority of chlorophyll lost in response to water deficit occurs in the mesophyll cells with a less amount being lost from the bundle sheath cells. The strategy of exogenous application of various organic osmolytes, osmoprotectants, and growth regulators such as proline (Ali *et al.*, 2007), glycine betaine (Anjum *et al.*, 2011), and secondary metabolites (Dolatabadian *et al.*, 2009) to plants was regarded vital technique to alleviate the deleterious effects of drought stress in plants. Of various secondary metabolites, terpenes and phenolics are more important to abiotic stress tolerance than the others due to their structural properties (Ruiz & Romero, 2001).

Relative leaf water content (RWC) is an integrative index of plant water status, which is used to evaluate the tolerance to water stress. Reduction in RWC under drought stress leads

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to stomatal closure, which further resulting in decreased CO<sub>2</sub> assimilation (Gindaba *et al.*, 2004). Proline and glycine betaine (GB) amino acid have been found to act as osmoprotectants and improve the growth and development of plants exposed to a variety of abiotic stresses including drought, temperature and salinity. Previous studies have demonstrated that biochemical, physiological and morphological changes occur in plants in response to water deficit (Basu *et al.*, 2010; Chaum *et al.*, 2010). In response to water deficit/drought conditions, maintenance of cellular osmotic pressure/osmoregulation is the major mechanism (Thapa *et al.*, 2011). Earlier studies have reported that molecules like glycine betaine, proline, mannitol, sorbitol, and trehalose act as osmoprotectants under water stress and help to maintain plant growth and development (Hadiarto & Tran, 2011). But osmolyte accumulation mainly depends upon water status, crop growth stage and cultivar (Shao *et al.*, 2006). It has been argued that exogenous proline and glycine betaine application could be a promising way to directly maintain and enhance the growth and yield in monocot crops such as rice (Farooq *et al.*, 2008), wheat (Ma *et al.*, 2006) and maize (Anjum *et al.*, 2011). Previous studies have reported that foliar spray of glycine betaine significantly improves growth performances of fine grain aromatic rice seedlings subjected to drought stress (Farooq *et al.*, 2010). However, the aim of this study was to investigate the effect of osmotic stress on proline, glycine betaine, pigment contents, total phenolics, RWC and fresh weight of maize plant.

## Materials and Methods

### *Plant material and experimental conditions*

The experiment was conducted in factorial form, using a completely randomized design with four replications. Two inbred lines (MO17 and B73) were evaluated under laboratory conditions. Seeds of maize were surface sterilized with 0.01% HgCl<sub>2</sub> solution for three minutes, followed by washing several times by distilled water. Ten seeds of each inbred lines then were placed on the moist Whatman germination papers in Petri dishes and were germinated using distilled water for 3 days under control conditions (light/dark regime of 16/8 h at 25/20°C, relative humidity of 60-70%, Light intensity during the daytime was 350 μmol m<sup>-2</sup> s<sup>-1</sup>). After 3 days, osmotic stress was imposed by application of PEG-6000 (polyethylene glycol) for 5 days. Using the Michel Kaufmann equation, 171 g of PEG-6000 was dissolved in 200 ml of distilled water and total volume was raised up to

one liter to produce solutions of -0.6 MPa osmotic potential (Michel & Kaufmann, 1973)

### *Relative water contents (RWC)*

To determine RWC, fresh leaves (1.5 g) were weighed, and then these leaves were placed in water for 20 h to regain full turgor then weighed the turgid weight. These leaves were dried in oven for 72 h at 70°C to measure dry weight. RWC was then assessed as:  $RWC = [(fresh\ weight - dry\ weight) / (turgid\ weight - dry\ weight)] \times 100$ .

### *Pigment content*

Chlorophyll-*a*, chlorophyll-*b*, carotenoids and anthocyanins were determined in leaves. About 200 mg weight of fresh leaf was placed in a mortar half full with liquid nitrogen and it was ground to powder. Then, pigments were extracted from the powdered sample by adding 2.0 ml of the extraction solvent 85% acetone and 15% Tris stock buffer (1% w/v Tris final concentration; adjusted to pH 8 with HCl) previously cooled in ice. The extract was centrifuged at 12,000 g for 3 min. A defined quantity of supernatant (1 ml) was removed and diluted to 3.0 ml. Its absorbance was measured at 537, 663, 647 and 470 nm in a 1-cm path length cell (Yaryura *et al.*, 2009).

### *Total phenolics*

Fresh leaf tissue (50 mg) was homogenized with 80% acetone and centrifuged at 10,000 g for 10 min. One-hundred microlitres of the supernatant were diluted with 2 mL of water and 1 mL of Folin-Ciocalteu's phenol reagent and shaken vigorously. Then 5 mL of 20% sodium carbonate solution was added and the volume was made up to 10 mL with distilled water. The contents were mixed thoroughly and the absorbance was read at 750 nm (Noreen & Ashraf, 2009). The results were expressed as mg/g of fresh leaf.

### *Proline*

Proline content was quantified by following the method of Bates *et al.* (1973). Fresh leaf samples (500 mg) were homogenized in 3% (w/v) sulphosalicylic acid, and centrifuged at 4000 g for 10 min at 4°C. The supernatant was added with acid ninhydrin and glacial acetic acid in a test tube. The mixture was heated for 30 min at 98°C in a water bath and then allowed to cool at room temperature. The mixture was extracted with toluene and absorbance was read at 520 nm.

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***Glycine betaine***

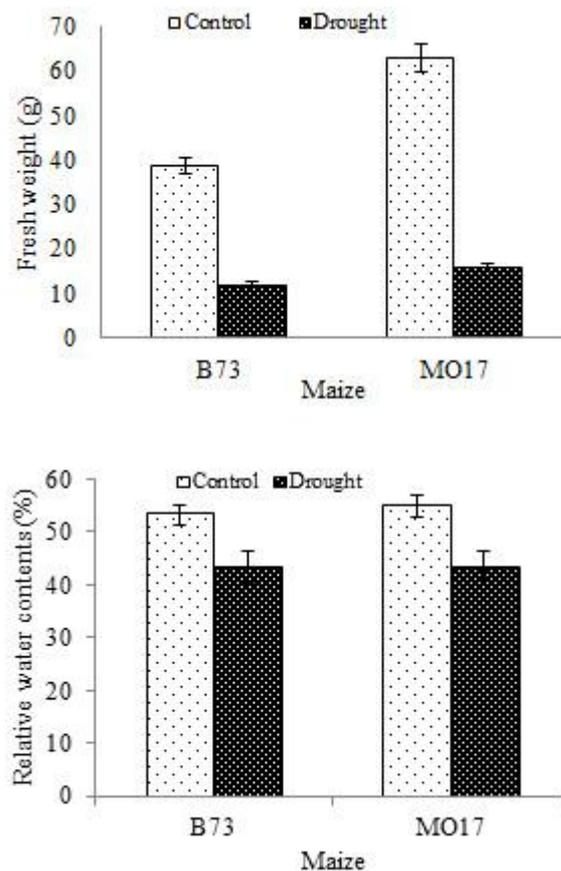
For glycine betaine, fully expanded upper most leaves were taken from the plants grown under normal and water stressed conditions, and analysis was carried out according to the method of Grieve & Grattan (1983). Leaf extract was prepared in 20 mL test tubes by chopping 0.5 g leaves in 5 mL of toluene-water mixture (0.05% toluene). All the tubes were mechanically shaken for 24 h at 25°C. After filtration 0.5 mL of extract was mixed with 1 mL of 2 N HCl solution then and 0.1 mL of potassium tri-iodide solution (containing 7.5 g iodine and 10 g potassium iodide in 100 mL of 1 N HCl) was added and shaken in an ice cold water bath for 90 min and then 2 mL of ice-cooled water was added after gentle shaking 10 mL of 1, 2 dichloroethane (chilled at -10°C) was pour in it. By passing continuous stream of air for 1-2 minutes two layers were separated, upper aqueous layer was discarded and optical density of organic layer was recorded at 365 nm.

***Statistical analysis***

Data was analyzed by using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). The assumptions of variance analysis were tested by ensuring that the residuals were random and homogenous, with a normal distribution. For treatment showing a main effect, means ( $\pm$  SE) compared by Duncan test.  $P \leq 0.05$  was considered as significant differences between treatments.

**Results and Discussion*****Fresh weight and leaf relative water content (RWC)***

The fresh weight of maize was decreased under osmotic stress in both maize inbred lines, however, this impairment was lower in B73 as compared to MO17, while maximum fresh weight was recorded in plants raised under well-watered conditions (Figure 1). The difference among the inbreds with respect to fresh weight was prominent at the osmotic stress, percent fresh weight inhibition at condition was used to rank the maize inbreds for drought tolerance. Thus, MO17 (45.3% inhibition) demonstrated drought-tolerance due to less reduction in the fresh weight than B73 (56.7% inhibition) under osmotic conditions (Table 2). Over the experimental period, the progressive drought stress caused subsequent reduction in RWC of maize plants as compared to well water control (Figure 1).



**Figure 1.** Fresh weight and relative water content pattern of two maize inbred lines under water stress conditions.

Maize yield losses in the tropics range from an average of 17% up to 80% depending on the severity and timing of the drought (Araus *et al.*, 2008). Robins & Domingo (1953) working with maize in pots reported that even 2- or 7-day long water stress in the tasselling stage leads to grain yield reduction up to 22% and 50%, respectively. NeSmith & Ritchie (1992) evaluated short- and long-term responses of corn to a pre-anthesis water deficit and described yield losses of 15–25% as long-term consequences of water stress. One of the mechanisms associated with drought tolerance is the ability of a plant to retain cellular water under water deficit stress (Erice *et al.*, 2010). RWC is a physiological measurement of plant cellular water status influenced by both leaf water potential and osmotic adjustment (Barrs & Weatherley, 1962). Reduction in RWC results in loss of turgidity, which leads to stomatal closure and reduced

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photosynthetic rates (Kramer & Boyer, 1995). Leaf RWC is a reliable indicator of leaf water deficit status at the time of sampling. It is often used to examine the response of a plant to the progress of drought stress (Erice *et al.*, 2010). Tolerant inbred lines (Tx205, C2A554-4, and B76) were able to maintain relatively high leaf relative water content when subjected to drought stress, while sensitive lines (B73 and C273A) showed a rapid reduction in leaf relative water content at very early stage of drought (Chen *et al.*, 2012), supporting our results in the present study.

**Pigment content**

Pigment content of leaves from both stressed and control maize plants are presented in Table 1. Although drought stress decreased chlorophyll-*a*, chlorophyll-*b*, total chlorophyll, anthocyanins and carotenoids concentration in both maize inbreds, the reduction was smaller in MO17 than in B73. MO17 was able to better withstand the water stressed conditions by maintaining higher concentration of chlorophyll than B73. Furthermore, anthocyanins and carotenoids in the leaves of MO17 2.48% and 26.37% inhibition, while in the B73 37.87% and 37.57% inhibition,

respectively under drought stress (Table 1). As a general rule, anthocyanins are considered light attenuators and antioxidants. In this context, it is believed that under stress situations, their main function is the quenching of the reactive oxygen species generated by stress (Neill & Gould, 2003). The efficacy of light captured to drive photosynthesis is strongly related to the chlorophyll concentration in the leaf. The change in chlorophyll contents was used to evaluate the influence of environmental stress on plant growth and yield. Many studies indicated that high chlorophyll concentrations are associated with improved yield under water-limited conditions (Verma *et al.*, 2004). In our study, the observed reduction of chlorophyll in water stressed plants might be due to a reduction in the lamellar content of the light harvesting chlorophyll protein (Randall *et al.*, 1977). The decreased chlorophyll-*a*, chlorophyll-*b* and total chlorophyll contents under drought stress are consistent with Anjum *et al.* (2011) who reported the reduced chlorophyll-*a*, chlorophyll-*b* and total chlorophyll contents under progressive drought stress in maize. The decrease in chlorophyll-*a* may be caused by the inhibition of biosynthesis of precursors of chlorophyll-*a* under moisture stress as reported by Makhmudov (1983).

**Table 1.** Pigment content (mean  $\pm$  SE) and percent inhibition in maize leaves under control and drought stress.

Pigment content ( $\mu\text{mol g}^{-1}$ fresh weight)	Maize inbred lines					
	B73			MO17		
	Control	Drought	Percent inhibition	Control	Drought	Percent inhibition
Chlorophyll-a	0.021 $\pm$ 0.0014	0.0088 $\pm$ 0.002	58.10	0.022 $\pm$ 0.0006	0.0131 $\pm$ 0.0002	40.45
Chlorophyll-b	0.0025 $\pm$ 0.0002	0.0014 $\pm$ 0.0002	44.00	0.004 $\pm$ 0.0007	0.0018 $\pm$ 0.0001	59.10
Total chlorophyll	0.024 $\pm$ 0.002	0.0102 $\pm$ 0.002	58.37	0.025 $\pm$ 0.001	0.015 $\pm$ 0.0001	40.94
Anthocyanin	0.120 $\pm$ 0.016	0.0735 $\pm$ 0.009	37.87	0.122 $\pm$ 0.006	0.120 $\pm$ 0.012	2.48
Carotenoid	0.009 $\pm$ 0.0004	0.006 $\pm$ 0.0009	37.50	0.009 $\pm$ 0.0009	0.007 $\pm$ 0.0008	26.37

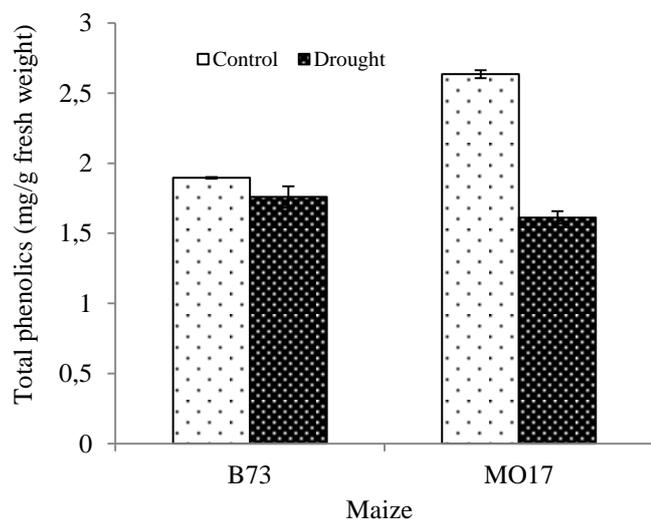
**Table 2.** Drought percent increase/decrease in fresh weight, RWC, proline, glycine betaine and total phenolics two maize.

Inbreds	Fresh weight	RWC	Proline	Glycine betaine	Total phenolics
	Drought	Drought	Drought	Drought	Drought
	decrease/increase (%)	decrease/increase (%)	decrease/increase (%)	decrease/increase (%)	decrease/increase (%)
B73	-56.76	-21.33	9.04	5.88	-7.21
MO17	-45.30	-24.89	26.93	10.64	-38.79

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**Total phenolics**

Addition of varying levels of water deficit to the growth condition caused a consistent decrease in the accumulation of phenolics in both maize hybrids. Enhanced synthesis of total phenolics has been directly correlated with salt and heat tolerance of sugarcane (Wahid & Ghazanfar, 2006). In the present study, drought stress decreased total phenolics of both maize inbreds. Although maize inbreds differed in growth performance at varying drought conditions (Figure 2), the relationship between drought tolerance and accumulation of total phenolics was not found, MO17 was maximal in accumulating total phenolics at the both conditions.



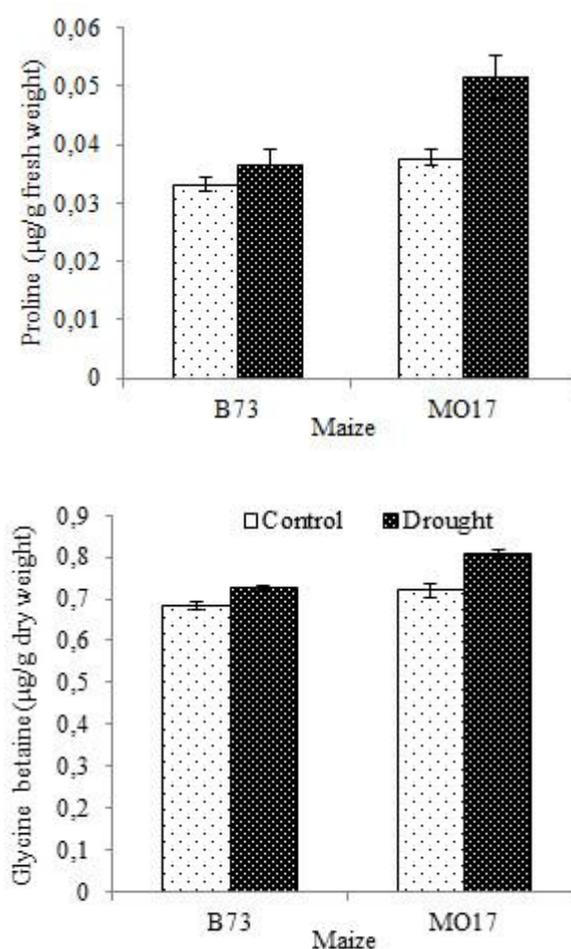
**Figure 2.** Total phenolics of two maize inbred lines under water stress conditions.

**Proline and glycine betaine**

Under progressive drought stress the proline and glycine betaine concentration in the leaves of maize inbreds enhanced at beginning of drought stress. Drought stress increased proline and glycine betaine content in leaves of both hybrids. However, constitutive level of proline and glycine betaine was higher in MO17 (Figure 3). Proline and glycine betaine are thought to play adaptive roles in inducing osmotic adjustment and protecting subcellular structures in stressed plants (Ashraf & Foolad, 2007). In response to water stress, proline accumulation generally occurs in the cytosol where it plays significant role in cytoplasmic osmotic adjustment (Anjum *et al.*, 2011) and glycine betaine is abundant mainly in chloroplast where it plays a vital role in adjustment and

protection of thylakoid membrane, thereby maintaining photosynthetic efficiency (Genard *et al.*, 1991).

Increased proline and glycine betaine accumulation under osmotic stress in MO17 more than B73 is in accordance with previous data (Manivannan *et al.*, 2007), which is suggested to be associated with drought tolerance (Moussa & Abdel-Aziz, 2008). By decreasing water potentials, proline accumulation involved in osmoregulation appeared to allow additional water to be taken up from the environment, thus counteracting the influence of drought stress on the plant tissues (Kumar *et al.*, 2003).



**Figure 3.** Proline and glycine betaine of two maize inbred lines under water stress conditions.

The glycine betaine content increased under drought stress in barley (Nakamura *et al.*, 2001) and in higher plants (Jun *et al.*, 2000). High levels of proline enabled the plant to maintain low water potentials. By lowering water potentials, the accumulation of compatible osmolytes, involved in

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osmoregulation allows additional water to be taken up from the environment, thus buffering the immediate effect of water shortages within the organism (Kumar *et al.*, 2003). Cell membrane integrity undergoes diverse changes such as increase in penetrability and decrease in sustainability under drought stress (Blokhina *et al.*, 2003).

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