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Role of Proline and Glycinebetaine in Improving Chilling Stress Tolerance in Sugarcane Buds at Sprouting

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ABSTRACT

Sugarcane shows reduced crop stand under suboptimal conditions, the main reason amongst others is sensitivity of bud tissue to temperature fluctuations. The aim of these studies was to monitor the time course changes in some physiological and histological attributes and possible roles of proline and glycinebetaine (GB) in mitigating the effect of chilling stress in the sprouting nodal buds of sugarcane. Chilling stress reduced bud fresh and dry weights, led to the generation of H_2O_2, reduced the tissue levels of K^+ and Ca^{2+}; nonetheless, osmolytes synthesis was enhanced in a time course manner. As for the histological changes, chilling stress restricted and delayed the formation of new bud leaves and their expansion, which was mainly, because of reduction in the number and area of mesophyll cells and poor development of vascular bundles. The pretreatment of bud chips with proline and GB substantially reduced the production of H_2O_2, improved the synthesis of soluble sugars and protected the developing tissues from chilling stress effects. Correlation matrix indicated that pretreatment with proline and GB reduced H_2O_2 generation, improved the K^+ and Ca^{2+} nutrition, levels of free proline, GB and soluble sugars thus improving chilling resistance in sugarcane buds.

Key Words: Oxidative stress; Bud sprouting; Mesophyll cells; Pretreatment; Chilling

INTRODUCTION

Chilling and freezing are collectively are referred as cold stress. Chilling stress results from temperatures cool enough to produce injury without ice formation in plant tissues, whereas in freezing stress ice formation takes place in plant tissues. Chilling stress usually occurs at temperature between 2 and 10°C but few tropical species such as rice and sugarcane are exceptionally sensitive to chilling and show injury signs up to ~15°C (Thomashow, 1999). Chilling sensitive plants show transition of cell membrane from a flexible liquid-crystal to a solid gel phase physically, thus affecting the cellular function in many ways. The instantaneous effect is increased membrane permeability and ion leakage (Farooq *et al*., 2008a & b). As a result of aberrant metabolism, accumulation of toxic metabolites and reactive oxygen species (ROS) takes place in the injured cells (Farooq *et al.,* 2009c). On the basis of their ability to survive under cold stress, plants are categorized as tolerant and susceptible. Susceptible plants are confined to tropical and subtropical regions whereas tolerant ones mostly grow in temperate regions having the ability to survive at subzero temperatures. This survival is achieved with cold acclimation, wherein plants adjust their metabolism to cold (Thomashow, 1999).

Chilling injury is serious problem during germination and early seedling growth in a number of plant species including maize and rice (Bedi & Basra, 1993). There are many visible symptoms of chilling damage depending on the species, plant age and the duration of exposure. Young rice seedlings showed the signs of wilting, reduced leaf expansion and chlorosis upon exposure to chilling stress (Yoshida *et al*., 1996). In extreme cases, chilling stress results in accelerated senescence and eventually the plant death (Sharma *et al*., 2005).

The chilling tolerance is a complex phenomenon, which entails an array of physiological and biochemical processes at whole plant, organ, cell and subcellular levels. These processes are reduced water loss by stomatal resistance, enhanced water uptake with the development of prolific root systems and synthesis and accumulation of osmolytes (Farooq *et al.*, 2008a & c, 2009). Amongst endogenous plant hormones, roles of SA and ABA have been implicated in chilling stress tolerance (Farooq *et al*., 2008a). Of these, ABA has a more direct role in cellular desiccation caused by freezing stress and control of gene expression during cold acclimation (Anderson *et al*., 1994; Prasad *et al*., 1994; Aroca *et al*., 2003). AOS dousing using both enzymatic and non-enzymatic means, stabilization of cell membrane, biosynthesis of functional aquaporins and expression of stress proteins are vital mechanisms of cold tolerance (Bohnert & Sheveleva, 1998).

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Plants exposed to environmental stresses overproduce different types of compatible organic solutes (Sakamoto & Murata, 2002; Serraj & Sinclair, 2002). Generally, they protect the plants from stresses by cellular osmotic adjustment, detoxification of AOS, protection of membrane integrity and stabilisation of enzymes/proteins (Bohnert & Jensen, 1996; Verbruggen & Hermans, 2008). However, stress tolerance can be improved by exogenous use of osmoprotectants for proline and GB. For effective use of GB, proline and other compatible solutes as inducers of stress tolerance, the mechanisms of their action, optimum concentrations and pertinent stage of plant growth and developmental should be carefully assessed.

Sugarcane is a tropical crop plant and requires relatively higher temperatures for growth. It is thus sensitive to chilling temperatures (~15°C) as seen from losses in its growth and productivity (Wahid *et al*., 2009). Various physiological and developmental processes of sugarcane have their own range of temperature for optimum growth and yield. Cold stress may also delay and suppress crop development in the spring, resulting in shorter growth season, poor crop stands and reducing yield (Moore, 1987; Chowdhary *et al*., 1998). Sugarcane leaves should be resistant to frost damage in order to prolong the growth and harvest season while resistance of lateral nodal buds is also very important in order to assure good germination of the bud chips (Wahid *et al*., 2009). The available literature shows that sugarcane buds have been rarely investigated for physiological and histological changes during sprouting and the effectiveness of some osmoprotectants in improving chilling tolerance. The objective of this study was to monitor the changes in sugarcane buds and effectiveness of proline and GB in improving chilling tolerance in sugarcane buds.

MATERIALS AND METHODS

Plant material: Stem nodes of sugarcane variety HSF-240 were obtained from Directorate of Sugarcane Research Institute (SRI), Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. Immature buds of similar age were selected from upper five nodes.

Pretreatments of bud chips sprouting: All determinations were made using single noded bud chips. The bud chips were pretreated with water and 10 mM solution each of proline and GB 25°C for 8 h, optimized using a range (5−40 mM) of their concentrations in a series of experiments. For control (25°C) and chilling stress (15°C), 25 bud chips were arranged in a double layer of moistened towel cloth in a plastic tray and kept in the growth chamber (FLI, Eyelatron, Rikkakai, Japan) and allowed to sprout. Design of the experiments was completely randomized with three replications.

Sampling and data recording: Since the histological changes and tissues differentiation begins quite early, the harvesting was done at 8, 16, 24, 32, 40 and 48 h after the buds were put to sprout. At each harvest time, the sprouting buds were excised from the bud chips using a sharp razor. Fresh weight of buds separated from the stalk was determined immediately. To determine dry weight, the excised buds were transferred to paper bags and put in an oven running at 70°C till constant weight.

For the analysis of free proline, GB, soluble sugars and hydrogen peroxide (H_2O_2) the freshly excised bud tissue was immediately frozen and stored at -40°C until analyzed. For the analysis of free proline according to Bates *et al.* (1973) method, 0.5 g of frozen fresh bud tissue was homogenized with 10 mL of aqueous sulphosalicylic acid (w/v), and homogenate was filtered. Two mL of filtrate was mixed with 2 mL each of acid ninhydrin and glacial acetic acid and incubated at 100°C in a water bath for 1 h. The reaction was terminated in an ice bath, immediately extracted with 4 mL of toluene after vortexing for 15-20 sec, chromophore containing the free proline aspirated, added to a test tube, warmed to room temperature and measure the absorbance at 520 nm on a spectrophotometer. The values of unknown samples were compared with standard curve constructed by running the proline standards (10 to 50 µg 2 mL^{-1}). The amount of free proline in the buds was calculated using the formula:

$$\mu\text{moles free proline } (g^{-1} \text{ fresh weight}) = \frac{[(\mu g \text{ proline } mL^{-1} \times mL \text{ toluene})}{115.5 \ \mu g \ \mu mole^{-1}]/[(g \text{ sample } 5^{-1}]}$$

For determination of GB using the method of Grieve and Grattan (1983), fresh extracts of buds were prepared by vigorously shaking in 2N H_2SO_4 and refrigerated. These extracts were mixed with equal volume of periodide prepared by dissolving excess of iodine in potassium iodide solution, vortexed and kept at 4°C for 16 h. The mixture was centrifuged at 10,000×*g* at 4°C for 15 min. and the supernatant discarded. The periodide crystals left in the bottom of the test tube were dissolved in 10 mL of 1, 2-dichloroethane, vortexed, left at room temperature for 15-20 min and absorbance of the colored solution taken at 365 nm.

To measure glucose equivalent soluble sugars, 0.1 g of chopped frozen fresh bud tissue was extracted in 5 mL of 0.2 M phosphate buffer (pH 7) for overnight. Next morning, to 0.1 mL of the aliquot 3 mL of freshly prepared anthrone reagent was added and carefully vortexed. Mixture was heated at 95°C for 15 min, cooled to room temperature under running tap water. The absorbance of the colored complex was taken at 625 nm. A standard series of glucose (20, 40, 60, 80 & 100 µg mL^{-1}) was prepared from AR grade glucose to calculate the exact amount of glucose equivalent soluble sugars in the sample (Yoshida *et al.*, 1976).

Hydrogen peroxide (H_2O_2) levels were determined as reported by Velikova *et al.* (2000). Bud tissue (0.1 g) was homogenized on an ice bath with 1 mL 0.1% (w/v) trichloroacetic acid (TCA) using a pre-chilled mortar and pestle. The homogenate was centrifuged at 12,000×*g* for 15 min and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M

potassium iodide. The supernatant was vortexed and absorbance read at 390 nm on spectrophotometer (Hitachi U-2001, Japan) using water as blank. The content of H_2O_2 was computed by comparing with a standard curve constructed from a standard series containing 20, 40, 60, 80 and 100 µM H_2O_2.

To estimate K and Ca with the method of Tendon (1993), oven dried buds were grinded to fine powder with a grinding mill. Out of this, 0.5 g of the material was digested in a mixture of concentrated HNO_3 and $HClO_4$ (3:1 ratio) on a heating block by gradually increasing the temperature to 250°C. After about 1 h, when the samples became clear, the volume was made up to 50 mL with distilled water. Analysis of K^+ was carried out using flame photometer (Sherwood Model 410, Cambridge). The unknown sample values were compared with the standard curve prepared from standard series (10, 20, 30, 40 & 50 mg L^{-1}). The quantity of Ca^{2+} from the above extracts was estimated with atomic absorption spectrophotometer (Perkin Elmer, Model AAnalyst 3000, Norwalk, Connecticut) as instructed by the manufacturer. The unknown sample values were determined by comparing with standard curve prepared from standard series (10, 20, 30, 40 & 50 mg L^{-1}).

Histological studies: Tissue processing for microtomy of the buds was done with the methods of Ruzin (1999). For fixation and dehydration, sugarcane buds were immediately fixed in formaldehyde, acetic acid, alcohol (ethanol) and water (FAA; 10:5:1:4) for 48 h and then transferred to 70% ethanol for storage. While processing for section cutting, the tissues were dehydrated in graded alcoholic series i.e., 50, 70, 90 and 95% followed by absolute ethanol and then xylene (each step for 25 to 30 min) at room temperature. Infiltration and embedding of the dehydrated tissues in paraffin wax contained in pastic molds was done as described by Ruzin (1999). The trimmed paraffine blocks containing bud tissues were adjusted on the microtome (Shandon, Germany) for cutting 5-7 µm thick sections. The cut sections were deparaffinized with xylene and rehydrated after affixing the ribbon on the adhesive coated glass slides, and stained with toluidine blue stain. The photographs of the stained tissues were taken on a camera equipped microscope (DG3 LaboMed, USA) and stained sections measured for various cells/tissues using standard procedure.

Statistical analysis: Analyses of variance (ANOVA) of all parameters were made using COSTAT computer package (CoHort software, 2003, Monterey, California) and DMR test was applied to determine the differences among various factors and their interactions separately at individual growth stages (Steel *et al*., 1996). Correlations were drawn between different attributes at each growth stage.

RESULTS

Biomass of sprouting buds: Results on bud fresh and dry weight revealed significant ($P<0.01$) differences of harvest times and various treatments, although there was no interaction ($P>0.05$) of time of harvest and treatments. Although there was a time course increase in the fresh weight of buds in all treatments, it was the lowest in chilled followed by control buds. Pretreatment of the bud chips with proline and GB pretreatment significantly increased the bud fresh weight both under control and chilling conditions. Of the two osmoprotectants, pretreatment with proline was more effective (~5−7%) than GB under chilling stress. Bud dry weight increased linearly in all the treatments. Under control, at initial time points the bud dry weight was comparable in all the treatments except untreated bud, which manifested reduced dry weight. However, under cold stress, untreated buds showed lowest dry weight, which improved with pretreatment of bud with proline and GB; the latter being more effective (Table I).

Physiological changes in sprouting buds: Data for the accumulation of free proline, GB and soluble sugars indicated significant ($P<0.01$) difference in the harvest times and treatments. However there was significant ($P<0.01$) interaction of harvest times and treatments for free proline and soluble sugars but not ($P>0.05$) for GB. Under control condition, free proline was the lowest in untreated and GB treated buds, but was increased in proline pretreated buds. Chilling stress further enhanced the free proline level in the proline treated buds, as well as in chilling stressed untreated and and GB treated buds at all time points (Fig. 1). The accumulation of GB was the lowest in control and GB untreated buds. It accumulated highly in a time dependent manner in the GB treated buds followed by cold and GB treated and only chilling stressed buds, respectively. Proline pretreated buds accumulated some GB when under chilling stress (Fig. 1). For soluble sugars, bud pretreatment had no effect on the soluble sugar accumulation at all times under control condition. Under cold stress, however, treated and untreated buds indicated a time course accumulation of soluble sugars, which was the highest in proline treated buds followed by GB treated and untreated buds (Fig. 1).

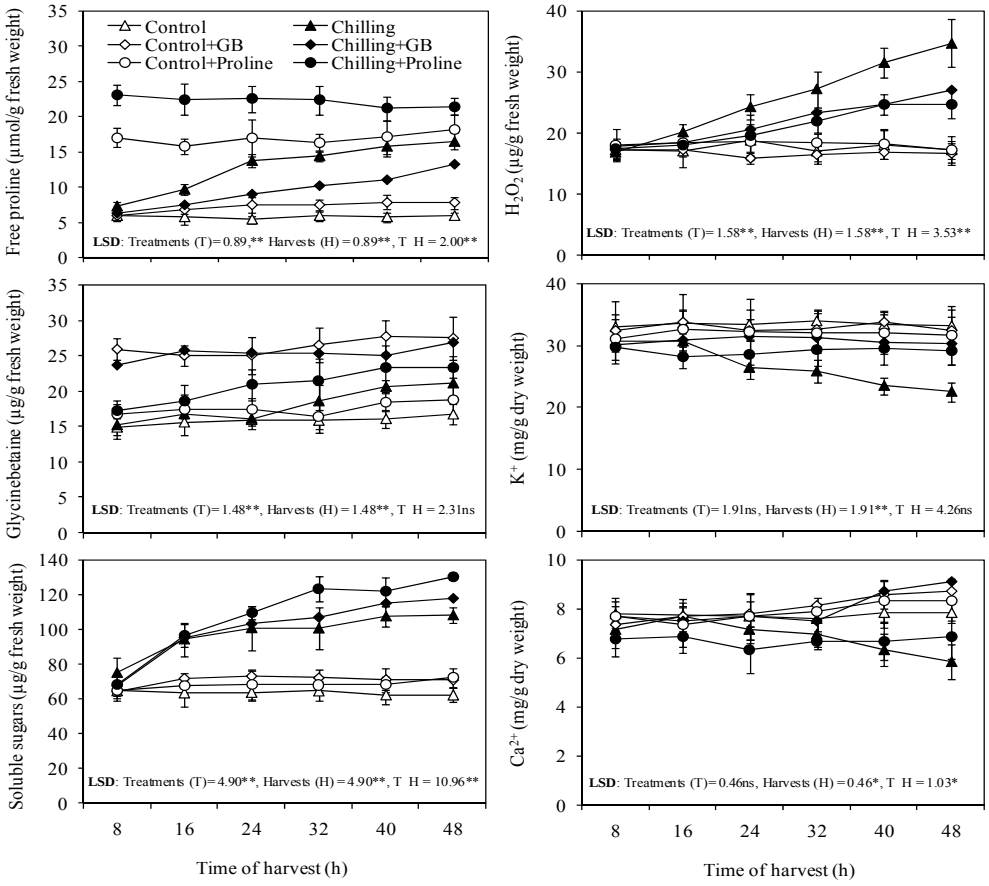
As regards H_2O_2 concentration, results revealed significant ($P<0.01$) difference in the harvest times and treatments with a significant ($P<0.01$) interaction of these factors. Under control condition, the H_2O_2 contents were similar in the untreated or treated buds at all time periods irrespective of bud pretreatment. However under chilling stress, the untreated buds indicated a time related accumulation of H_2O_2, while pretreatment with proline and GB was effective in substantially restricting this accumulation (Fig. 1). For K^+ accumulation, treatments, not the harvest times, significantly ($P<0.01$) while there was no interaction of both these factors. Treated buds (with proline & GB) under control indicated no difference in K content. However, pretreatment with GB and proline allowed the cold stressed sugarcane buds to display K concentrations equivalent to control samples (Fig. 1). For bud Ca^{2+} contents, data revealed no significant ($P>0.05$) difference in the harvest times but a significant ($P<0.01$) one in the treatments with a significant ($P<0.05$) interaction of both

Table I: Time course changes in bud fresh and dry weight during sprouting and the effectiveness of proline and glycinebetaine pretreatment under chilling stress

Parameters	Treatments	Harvests (h)					
		8	16	24	32	40	48
Fresh weight	Control	1.39±0.22	1.440±0.13	1.54±0.15	1.68±0.16	1.80±0.13	1.98±0.12
	Chilling stress	1.48±0.11	1.521±0.17	1.57±0.07	1.60±0.13	1.66±0.11	1.74±0.08
	Control + GB	1.60±0.23	1.684±0.27	1.66±0.33	1.78±0.22	1.98±0.34	2.17±0.27
	Chilling stress + GB	1.43±0.13	1.508±0.12	1.57±0.14	1.71±0.15	1.84±0.09	1.88±0.18
	Control + Proline	1.41±0.08	1.516±0.15	1.63±0.15	1.76±0.22	1.94±0.20	2.28±0.27
	Chilling stress + Proline	1.49±0.17	1.540±0.20	1.61±0.17	1.67±0.20	1.72±0.11	1.85±0.29
Dry weight	Control	0.30±0.04	0.36±0.03	0.41±0.04	0.44±0.06	0.46±0.07	0.50±0.06
	Chilling stress	0.34±0.03	0.37±0.04	0.38±0.03	0.40±0.03	0.41±0.05	0.44±0.04
	Control + GB	0.36±0.03	0.40±0.05	0.43±0.04	0.46±0.03	0.54±0.05	0.61±0.05
	Chilling stress + GB	0.37±0.04	0.40±0.02	0.44±0.04	0.45±0.05	0.49±0.04	0.54±0.04
	Control + Proline	0.36±0.03	0.42±0.05	0.44±0.04	0.48±0.06	0.53±0.05	0.57±0.04
	Chilling stress + Proline	0.35±0.03	0.39±0.03	0.41±0.05	0.48±0.04	0.50±0.05	0.52±0.04

LSD values for fresh weight: Harvests (H) 0.137**, treatments (T) 0.137 & H × T 0.306ns,
LSD values for fresh weight: H 0.033**, T 0.033 & H × T 0.073ns

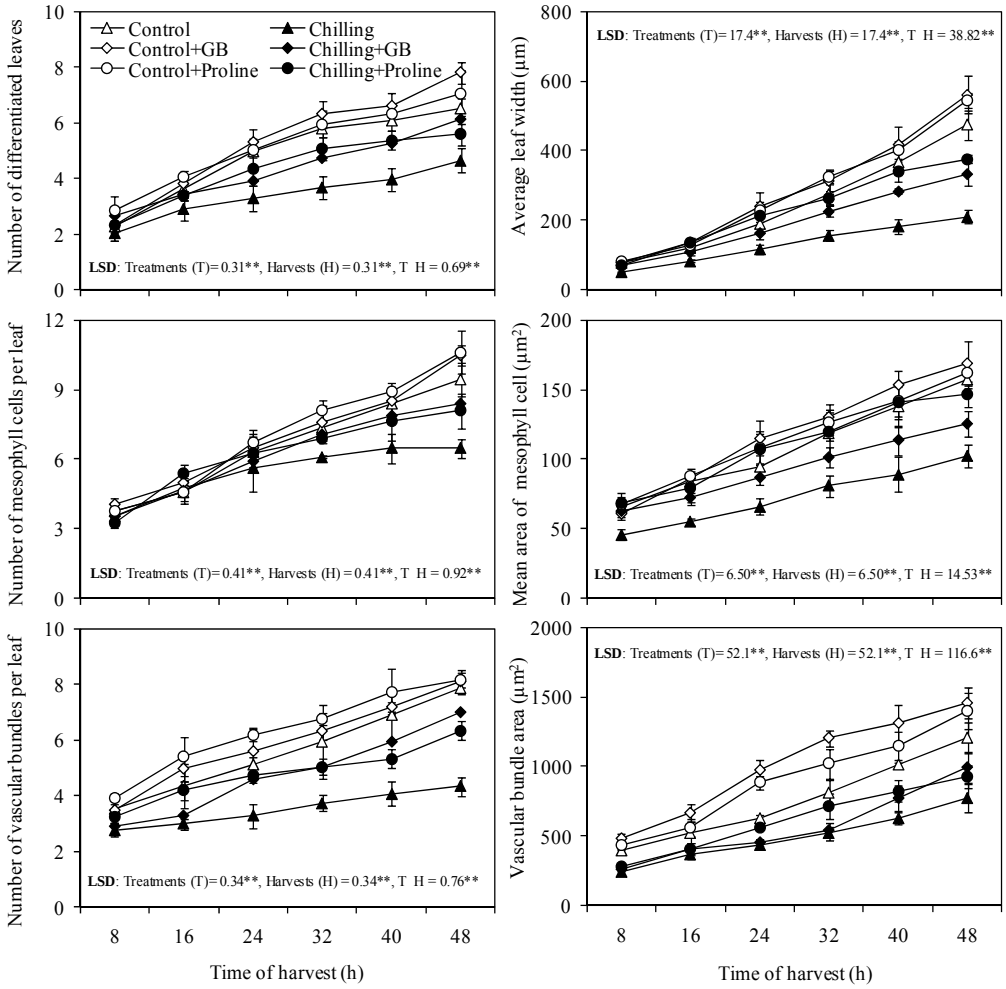
Fig. 1: Time course changes in bud fresh and dry weight during sprouting and the effectiveness of proline and glycinebetaine pretreatment under chilling stress



these factors. Pretreatment with GB and proline resulted in a small ($P>0.05$) increase in Ca^{2+} over time in control buds, which reduced with time in buds subjected to cold stress. Cold stressed buds responded better to GB than proline pretreatment; GB treatment showing Ca^{2+} contents equal to or more than unstressed and untreated buds (Fig. 1).

Developmental changes in sprouting buds: For number and width of differentiated leaves, data indicated significant ($P<0.01$) differences in the harvest times with a significant ($P<0.01$) interaction of both these factors for the number of differentiated leaves. There was no great difference in the treatments at 8 and 16 h time periods for the differentiation

Fig. 2: Time course changes in some histological characteristics of buds during sprouting and possible effectiveness of pretreatment with proline and glycinebetaine under chilling stress



of leaves, but these differences were well marked later on. Despite treatment with GB or proline, cold stressed buds were not able to differentiate as many leaves as the unstressed/untreated controls (Fig. 2 & 3). Although the width of differentiating leaves increased with time in all the treatments, chilling stress greatly reduced this attribute at all harvests. Pretreatment with both GB and proline increased leaf width under chilling condition, although there was no difference in the effectiveness of GB and proline. Nonetheless, proline was more effective (though non-significant) than GB (Fig. 2 & 3).

For number of mesophyll cells and mesophyll cell area, results revealed significant ($P<0.01$) differences in the harvest times, treatments and their significant ($P<0.01$) interaction. Up to 24 h, there were no clear differences in treatments for the number of mesophyll cells between lower and upper epidermis, while these differences were well evident at later time points. The GB and proline overlapped for their effectiveness in improving this number under control condition at all harvest. However under chilling stress this number increased almost similarly both under proline and GB pretreatment and was markedly higher than control buds (Fig. 2 & 3). Area of individual mesophyll cells did not differ between both the treated and untreated buds with time under control, while under chilling stress this area was the lowest in the untreated buds while GB and proline were effective in improving it at all time points. The improvement produced by proline was nearly at par with the control buds (Fig. 2 & 3).

Number and area of vascular bundle per leaf revealed significant ($P<0.01$) differences at all the harvest times and treatments with a significant ($P<0.01$) interaction of both these factors. Under control, the number of vascular bundles per leaf was lower in un-treated buds but higher in proline followed by GB treated buds at all harvests. Under cold stress, however this number was the lowest in un-treated control buds. However GB showed a great improvement followed by proline (Fig. 2 & 3). Area of vascular bundles although increased in all treatments in a time course manner, this attribute increased highly in GB

Table II: Correlation of dry weight and some physiological attributes with the development of buds as affected by various treatments at 8 and 48 h after exposure to cold stress

X-variable	Y- variable	8 h	48 h
Dry weight	Ca^{2+}	-0.158ns	0.825*
	Number of differentiated leaves	0.486ns	0.933**
	Width of differentiating leaves	-0.030ns	0.854*
	Number of mesophyll cell	-0.004ns	0.884*
	Number of vascular bundle per leaf	-0.179ns	0.846*
	Area of vascular bundles	-0.062ns	0.862*
H_2O_2	Number of differentiating leaves	0.991ns	-0.903*
	Width of differentiating leaves	0.696ns	-0.977**
	Number of mesophyll cell	0.100ns	-0.956**
	Area of individual mesophyll cells	0.738ns	-0.976**
	Number of vascular bundle per leaf	0.490ns	-0.956**
	Area of vascular bundles	0.297ns	-0.921*
K^+	Number of differentiated leaves	-0.184ns	0.858*
	Width of differentiating leaves	0.457ns	0.873*
	Number of mesophyll cell	0.796ns	0.881*
	Area of individual mesophyll cells	0.033ns	0.889*
	Number of vascular bundle per leaf	0.531ns	0.974**
Ca^{2+}	Number of vascular bundle per leaf	0.367ns	0.828*

Significant at ** P<0.01, * P<0.05 and ns non-significant

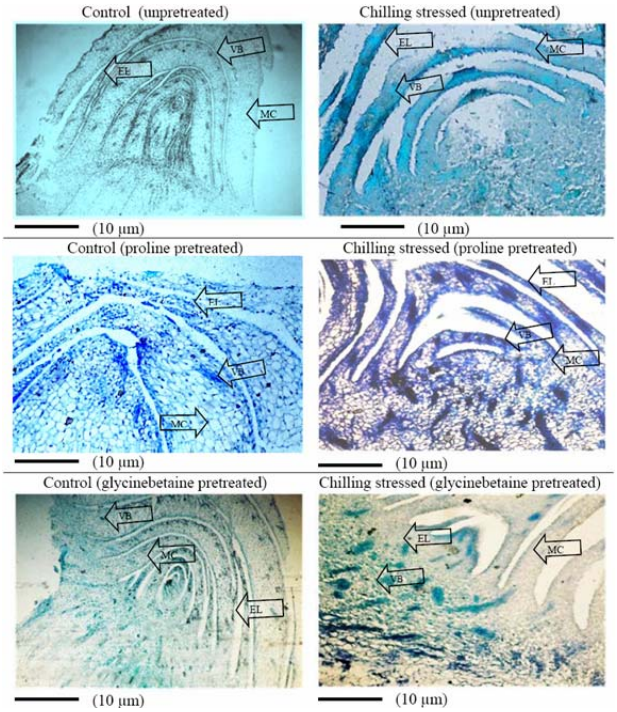
treated buds followed by proline and untreated buds. However under cold stress, pretreatment with proline was more effective than GB in increasing the vascular bundler area up to 32 h time point beyond, which GB was more effective (Fig. 2 & 3).

Correlations: The validity of above changes was substantiated by establishing the relationships of dry weight and various physiological and developmental attributes of bud and those of changes in some physiological attributes with the developmental attributes of buds at 8 h and 48 h time points (Table II). At 8 h, no relationship of various attributes could be seen of soluble sugars with the width of differentiating leaves (data not shown) except a negative relationship. However at 48 h, dry weight was positively correlated with Ca^{2+}, number and width of differentiating leaves, area of individual mesophyll cells and number and area of vascular bundles. H_2O_2 was negatively related to all the developmental parameters of buds, K^+ was positively paralleled with all the developmental parameters of buds except area of vascular bundles and positive relationship of Ca^{2+} with n umber of vascular bundles per leaf (Table II).

DISCUSSION

Sugarcane is a C_4 plant, originated from tropical climates and grows at relatively higher temperature (~30°C) than temperate region C_3 plants. Thus it shows more sensitivity to low temperature stress as compared to high temperature (Wahid *et al*., 2009). In Pakistan, sugarcane bud chips are sown usually in the months of February and March, when the night temperature for sugarcane growth is often lower (12-16°C) than that required for optimum growth (25-30°C), thus low temperature being a stress factor for sugarcane sprouting. Some studies show that stress

Fig. 3: Diagrammatic presentation of the changes in the development of various cells and tissues in the transverse sections of buds under control (left panel) and cold stress (right panel) conditions after 36 h, the buds were treated with water (control) and 10 mM solution each of proline and glycinebetaine (treated). MC, mesophyll cells; VB vescular bundles; EL, elongating bud leaves, the photographs were taken at 36 h harvest time



tolerance in sugarcane can be improved by presowing treatment of buds with osmoprotectants (Grover *et al*., 1999; Verbruggen & Hermans, 2008; Farooq *et al*., 2009c). In this research, the effect of 15°C on the sugarcane sprouting buds and effectiveness of GB and proline in reducing the low temperature stress was studied. Results revealed no great differences in the fresh weight of buds showing that cold stress did not much influence the fresh weight of buds (Table I). This is likely in view of the fact that, contrary to heat stress, chilling stress does not act like a dehydrative force, although there are changes in tissue water status (Farooq *et al*., 2009c). Likewise, there was no clear difference amongst the treatments for changes in the dry weight of buds, although pretreatment with GB and proline was effective in enhancing this attribute at later time points (Table I). These data suggested that chilling stress does not have osmotic strain on the buds rather it affects fundamental physiological phenomena independent of water stress.

Like many other environmental stresses, production of AOS is an important facet of low temperature stress (Hasegawa *et al*., 2000; Xing & Rajashekar, 2001; Guo *et al*., 2006). In this experiment, chilling stress caused a linear

increase in the level of H_2O_2 (Fig. 1), an important and relatively long-lived ROS (Hasegawa *et al*., 2000). Both GB and proline were effective in reducing the level of H_2O_2, which indicated their specific role in up-regulating the antioxidative system as suggested earlier by many workers (Sakamoto & Murata, 2002; Serraj & Sinclair, 2002). Determination of changes in the levels of GB and free proline indicated their higher and persistent levels upon bud pretreatment, which showed that these compounds were not utilized, rather prevailing low temperature condition further increased their accumulation (Fig. 1) and appeared to protect the cellular structures from the cold damage (Farooq *et al*., 2009). The role of proline and GB appears to be alleviation of oxidative damage, since pretreatment with proline and GB led to reduced H_2O_2 contents over time.

An important facet of cold stress is slowing down the physiological phenomena such as reduced activities of membrane transporters, leading to reduced acquisition and translocation of mineral nutrients (Taiz & Zeiger, 2006). Here changes in the accumulation of K^+ and Ca^{2+} were monitored. It was important to note that chilling stress declined the level of K^+ in untreated buds but pretreatment with both proline and GB improved it (Fig. 1). Likewise Ca^{2+} indicated a declining trend under chilling stress, while GB pretreatment improved it the most and greater than the control, while proline was not as effective (Fig. 1). Both these ions have protective role and they stabilize the membranes against low temperature injury (Zhu, 2003; Farooq *et al*., 2008b, 2009). These findings suggested that the GB had a more profound role than proline in assisting the sugarcane buds to withstand cold stress.

Immature bud in sugarcane is a mass of vegetative cells, which differentiate into the leaves when sprout (Alexander, 1973). Sprouting of buds linked to the expansion of differentiating leaves and formation of vascular connections determines the vigor of the seedling. No study is available on the development of various tissues of sugarcane bud from immature to mature state under normal or stressful conditions. Changes were monitored in the GB and proline treated or untreated sugarcane buds under cold stress, which indicated that there was a progressive development of various tissues including the number and expansion of differentiating leaves, number and area of mesophyll cells and number and area of vascular bundles. There was a great effect of cold stress on these attributes while treatment with proline and GB had well pronounced effect in improving the development of these structures (Fig. 2 & 3). Main effect of chilling stress was reduced by expansion of mesophyll cells and number of vascular bundles, whilst both osmoprotectants were effective in improving these parameters.

Finding meaningful relationships of various parameters highlight the type of association and their potential in changing one another (Steel *et al*., 1996). The relationships were developed in pretreatment of buds and effect of cold stress at 8 h and 48 h time points. These relationships were non-significant at 8 h for all attributes except a negative relationship of soluble sugars with width of differentiating leaves. However at 48 h presence of positive correlations in dry weight and Ca^{2+} contents, number and width of differentiating leaves number of mesophyll cells and number and area of vascular bundles suggested that bud pretreatment triggered maintenance of requisite levels of Ca^{2+} and led to a greater development of buds as compared to untreated buds under clod stress (Table II). Presence of a negative correlation of H_2O_2 with developing bud tissues indicated the alleviation of oxidative damage by pretreatment with proline and GB. However, positive relationship of K^+ with all the bud structures revealed the specific role of K^+ in protection of bud tissues from low temperature stress by repair of physiological and biochemical phenomena, since it acts as a cofactor in many enzyme activities (Leigh *et al*., 1981; Anderson *et al*, 1994). No correlations of free proline, GB or sugars with dry weight or differentiation of the bud tissues revealed their indirect roles in the low temperature tolerance of sugarcane.

In conclusion, chilling stress produced oxidative damage, whilst pretreatment of bud chips with GB and proline partly (25-30%) counteracted chilling stress effect by enhancing the tissue levels of K^+ and Ca^{2+} and reducing the H_2O_2 production. Thus pretreatment of sugarcane buds at the used levels can be employed to accomplish requisite plant density in the field in relatively cool season.

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