Indian J. Plant Physiol., Vol. 12, No. 4, (N.S.) pp. 344-352 (Oct.-Dec., 2007)

BIOCHEMICAL ALTERATIONS IN BUD AND ROOT BAND ZONE OF SUGARCANE SETTS DUE TO CHILLING TEMPERATURE

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Received on 31 Nov., 2007, Revised on 30 Dec., 2007

SUMMARY

The effect of chilling temperatures (0, 5 and 10°C) on the sugarcane bud sprouting were compared with 25°C (normal). At the chilling temperatures, in the buds and the root band zones of the single bud sugarcane sett, the reducing sugars, specific activities of acid invertase (AI), IAA (indole acetic acid) oxidase and ATPase were decreased whereas the sucrose and IAA contents were increased as compared with 25°C. However, there was no change in sucrose content at the chilling temperatures but it markedly decreased at 25°C. Sucrose immobilization caused by the suppression of the acid invertase, hence decreased content of reducing sugars and accumulation of IAA might affected the sprouting at chilling temperatures.

***Key words*: Acid invertase, ATPase, bud, chilling, IAA oxidase, sugarcane**

INTRODUCTION

Chilling temperatures are known to be one of the most important environmental stresses affecting the plant growth and the crop productivity (Xin and Browse 2000). In subtropical regions of India, sugarcane is also exposed to chilling temperatures after harvest of plant cane and subsequent stubble crop raising in the month of December (Shrivastava *et al*. 2006). In this region, the stubble buds are exposed to temperatures as low as 1-2°C, which either causes the mortality of the bud or leads to suppression of the bud sprouting (Mathur and Haider 1939). The mortality of buds caused by the chilling temperature generates significant gaps in the stubble crop while delayed bud germination, suppresses the plant growth and development and ultimately causes approximately 25-40% losses in overall crop yield (Chauhan and Motiwale 1989).

Sugarcane is known to be a cold sensitive plant. The magnitude of cold damage is dependent on the severity and duration of low temperature, cultivars resistant to post freezing deterioration and time lapse and temperature fluctuations between the freeze events and harvest (Tai and Lentini 1998). However field observations have shown that sensitivity of sugarcane to cold varies among varieties. Du *et al*. (1999) demonstrated that some subtropical hybrid species are more cold tolerant than tropical species.

Under chilling temperatures, the survival ability of the plants decreases due to cell damage caused by dehydration, biochemical and physiological changes involving changes in carbohydrate metabolism, lipid membrane composition, phenyl-propanoid content, respiration, photosynthesis and oxidative stresses (Thomashow 2001, Allen and Ort 2001). At chilling

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temperatures, the lipid and the protein of the membranes are damaged due to chilling injury and the reduced enzymatic activities, leading to decrease in the demand for ATP (Lynch and Thompson 1984). The effects of temperature on plasma membrane and plant tissues also provide information of ion transport through ATPase (Kami-Ike *et al*. 1986).

The buds attached to the root band zone of the sugarcane setts are dependent on the adjacent tissues for supply of nutrient and water to support sprouting (van Dillewijn 1952) and the early growth of the young shoots (Fig 1). On the root band zone are the root primordia, which develop into the sett roots and function till the young shoot produces its own roots. The sprouting of the bud is favored by the higher moisture content of the setts (Singh and Srivastava 1969). During the process of sprouting the biochemical changes take place in root band zone as well as in the buds. Kumar and Pande (1987) studied the changes in sucrose and reducing sugar contents in stubble buds. During germination, an increase in the acid invertase activity in the setts triggered the breakdown of the sucrose into hexoses (Jain *et al*. 2006).

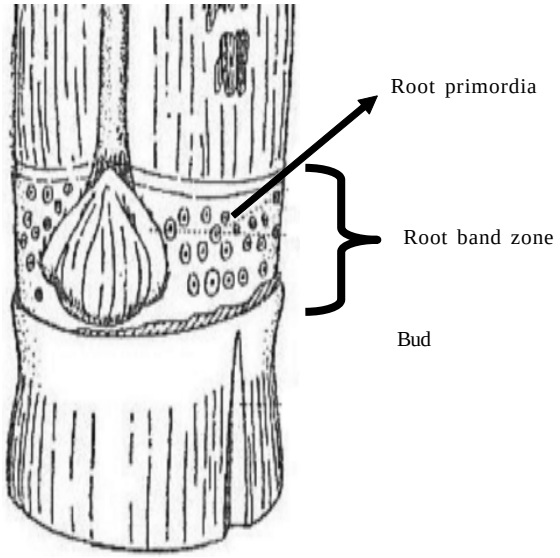

Fig. 1. A single bud sugarcane sett

It is also known that phenolic compounds play an active role under chilling stress conditions for the survival of the plant tissues (Solomon *et al*. 1990). IAA suppresses the germination of setts of sugarcane leading to dormancy (van Dillewijn 1952). During germination, the enzymatic oxidation of IAA is associated with superoxide dismutase (SOD) under low temperature conditions. The IAA oxidase system consisting of a flavo protein coupled with H_2O_2 production and release of superoxide molecules of oxygen has been implicated during low temperature stress conditions (Galston *et al*. 1950). Due to the low temperature stress, the lipid and the protein of the membranes are damaged, so the process of cold acclimation may include modifications of lipid and protein components of the plasma membrane (Lynch and Thompson 1984). The effects of temperature on plasma membrane and plant tissues also provide information of energy requirements of ion transport through ATPase (Kami-Ike *et al*. 1986).

Proteins, reducing sugar, sucrose, IAA, IAA oxidase, ATPase and acid invertase, therefore, have relevance in understanding biochemical changes during the process of sprouting at chilling temperatures in sugarcane setts. Since, these changes occur in both the root band zone and the buds, the present study was carried out under controlled conditions to quantify the chilling induced biochemical alterations in buds and root band zone to ascertain their roles in the sprouting of buds.

MATERIALS AND METHODS

Site, plant material and chemicals: The experiment was conducted at the Indian Institute of Sugarcane Research, Lucknow, India, located at 26° 56' N, 80° 52' E and 111 m above the sea level, which falls in the Agro-Ecoregion 4 (Northern plain and Central Highlands, Hot Semiarid Ecoregion with the Alluvial-derived (N8D2) soils of India (Sehgal *et al*. 1990). Single bud setts (approximately 5 cm in length) were cut from the middle portion of the stalks of the sugarcane variety CoSe 92423 (Saccharum spp hybrids) (after 10 months of growth), cultivated at the farm of the Institute. Care was taken while cutting to obtain the setts of nearly same girth (approx 2.3 cm). The cut setts were washed initially in running water to remove the extraneous particles, and then washed in the 5% Clorox solution for 10 min. The fine chemicals and the reagents used in this study were purchased from Sigma chemicals corporation, St, Louis, USA. All the other chemicals were of analytical grade.

Planting of the single bud setts: The washed sterile setts were layered in acid washed sand (particle size: 0.25-0.84 mm) in plastic trays (45 cm x 30cm x 15 cm) with equidistant holes plugged with glass wool. Forty setts were placed in each tray and covered with a thick layer (1.5 cm) of the sand. 100 ml of half strength Hoagland's nutrient solution was added in the trays after one day interval. Sterile water was applied to the sand culture so as to maintain 15-22% moisture in the sand medium and adequate moisture in the setts planted. Chloramphenicol and amphotericin B (30 and 100 µg/ml respectively) were added to avoid the microbial contamination. The setts were exposed to temperatures 0, 5 10 and 25^{0}C in incubators (Complab, India), with 14 h light period, light intensity of 5292 µw cm^{-2} and 73% relative humidity in three replications for 12 days. The buds and root band zone tissues were sampled for analysis after 3, 6, 9 and 12 days of growth. The moisture content in the sand was maintained from 16±1% by adding sterile water and ½ the strength Hoagland's nutrient solution (100 ml) in the sand on alternate days till the last day of the analysis.

Sprouting of buds and biochemical analysis: After every sampling, % sprouting of the buds was recorded. The buds, which showed excessive swelling, had broken scales and the initial shoot protrusion at least 2 mm in length (Prado *et al*. 2000), were considered to have sprouted. Freshly sampled buds were chopped and homogenized (10 % homogenate) in chilled pestle and mortar using chilled distilled water. This was filtered through four folds of muslin cloth and then centrifuged at 8000 x g for 20 min at 4^{o}C. The supernatant obtained after centrifugation was used for estimation of reducing sugars, sucrose and total soluble proteins, IAA and assay of acid invertase activity. The method for estimation of reducing sugars was adopted from the procedure reported by Nelson (1944) and Somogyi (1945). Sucrose contents were estimated by the resorcinol-thiourea method as described by Roe and Papadopoulos (1954). Total soluble proteins were estimated using (Lowry *et al*. 1951).

Extraction of indole acetic acid (IAA): Endogenous level of IAA was determined by the method of Nagar (1995). Freshly sampled buds were used for estimation of endogenous IAA levels. A sample (5 g fw) was separately homogenized in chilled 80% methanol three times. The homogenates were centrifuged at 10000 g at 5^{o}C for 30 minutes and the pH of the extract was maintained at 6.5 by checking it on a pH meter.

PVP column chromatography: The supernatant were concentrated in vacuo at 30^{o}C and then applied to polyvinypyrrolidine (PVP Column). The column (20cm X 1.5 cm internal diameter) was eluted with phosphate buffer (pH 8.0) and the resulting eluates were again adjusted to pH 8.0 with 1N HCl and partitioned against peroxide free diethyl ether (x3). The ether phases were discharged. The remaining aqueous fractions adjusted to pH 3.0 and partitioned against diethyl ether (x3). The ether phases were evacuated in vacuo and taken up in methanol (HPLC grade) for estimation of IAA.

Estimation of IAA by HPLC: The partially purified methanolic extracts were filtered through 0.54 µm Millipore filters and injected into 20 µl injector loop fitted over the Lichrosorb RP 18 (10 um)(250x4.6mm ID) protected by a guard column. Elution was carried out by a gradient of 30- 70 % methanol, (5 min) followed by 70- 100 % methanol (5 min) and finally with pure methanol for 15 min at the flow rate of 1ml per min. The column eluates were passed through an ultraviolet (UV) detector set at 254 nm and the IAA was estimated measuring the peak area and comparing it with standard curve of indole-3 -acetic acid (Sigma chemical company, St Louis, USA).

Enzyme assay: The acid invertase was assayed by the method of Hatch *et al*. (1965). IAA oxidase activity was determined by a modified version of the method described by Gordon and Weber (1951). The ATPase activity was assayed by the method as described by Fischer and Hodges (1969). The phosphorus estimation was done by the method as described by Fiske and Subbarow (1925).

Statistical analysis: Multivariate analysis of variance with Duncan's Multiple Range Test (DMRT) as post hoc analysis was used to compare the means (Snedecor and Cochran 1967).

RESULTS AND DISCUSSION

Sprouting: At the chilling temperatures, no sprouting in buds occurred except that at 10°C, they were found to be swollen after 6, 9 and 12 days of growth (Fig 2). However, at 25 °C after 3, 6, 9 and 12 days, the sprouting was 75, 80, 90 and 100 % respectively (Fig 3). The initial moisture content, reducing sugars, IAA

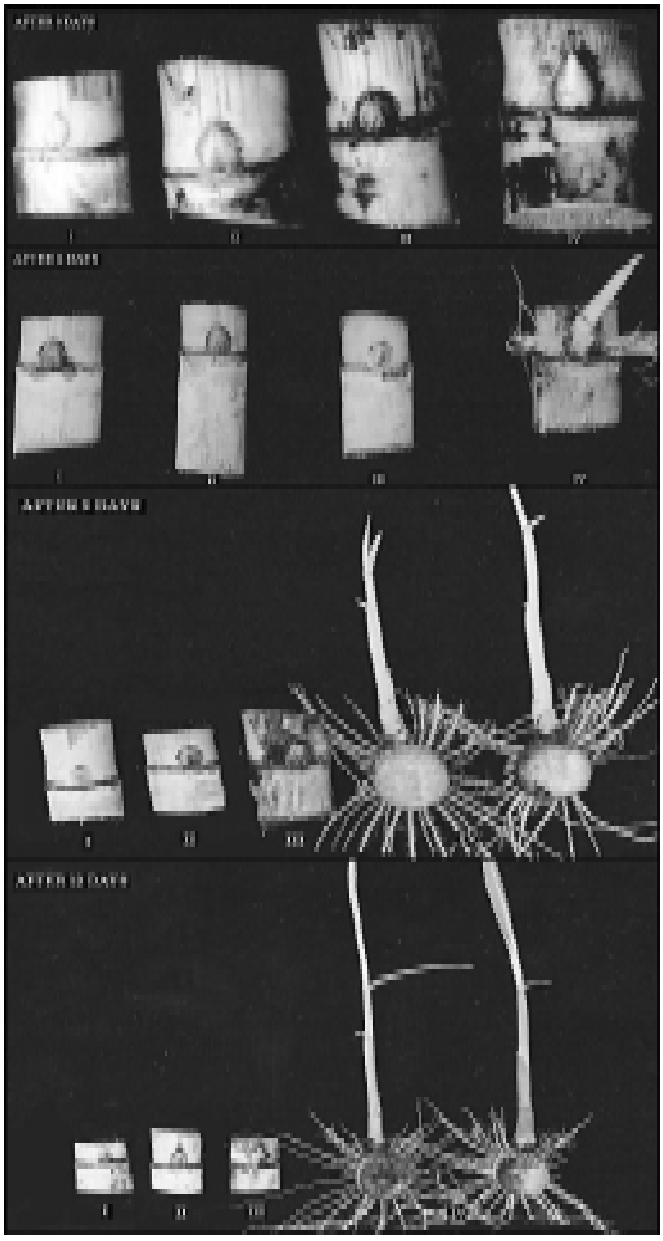

Fig. 2. Sugarcane bud sprouting at 25°C and chilling temperatures (I: 0°C, II: 5°C, III: 10°C, IV: 25°C; a: after 3 d, b: after 6d, c: after 9 d and d: after 12 d)

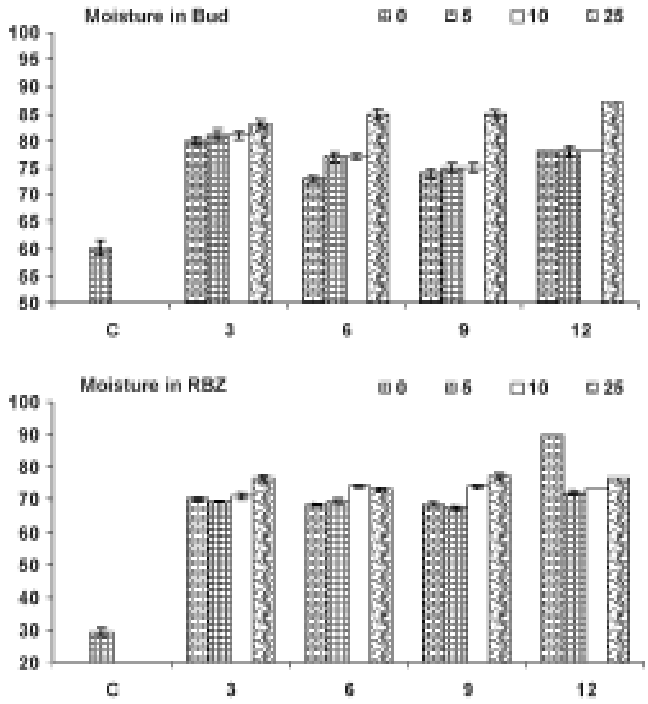

Fig. 3. Moisture contents in buds and root band zone at different temperatures and days of growth

and the enzymes were higher in the buds as compared to the adjacent root band zone (RBZ) except sucrose contents (Table 1).

Table 1. Moisture, sugars and enzyme activities in sugarcane buds and root band zone before subjecting to different temperatures.

Parameter units	Bud	Root band zone
Moisture (%)	60.2-±5.16	29.2 ± 5.71
Reducing sugars (mg/g fw)	8.92 ± 1.19	3.14 ± 0.64
Sucrose (mg/g fw)	35.75 ± 2.68	83.33 ± 8.54
Protein (mg/g fw)	17.72 ± 2.17	7.97 ± 0.89
IAA (µg/g fw)	0.83 ± 0.01	0.43 ± 0.01
Acid invertase (mmol/ min/mg protein)	0.116 ± 0.01	0.02 ± 0.002
IAA oxidase (µg IAA oxidized/mg protein)	9.0 ± 0.90	10 ± 1.10
ATPase (µg Pi liberated /mg protein/10min)	3.67 ± 0.08	0.015 ± 0.001

Each value is mean of three replications ± SE

Effect of different temperatures on moisture content: Variation in moisture content in the buds and the RBZ of the planted setts with time are given in Fig 3. In the buds, moisture contents increased from an initial value of 60.2% to 76-82% in all the temperature regimes (0°C to 10°C) but at 25°C, moisture % was much higher as compared to the chilling temperatures. In the RBZ, at the various temperatures, the moisture contents also increased with time from an initial level of 29.2% to 70-80%. Initially the RBZ had half the moisture of the buds but subsequently it absorbed moisture and no appreciable differences could be observed in the moisture contents with time at the different temperatures.

Reducing sugars, sucrose and acid invertase activity: In the buds, the initial level of reducing sugars were 8.92 mg/g fw and at 0, 5 and 10°C, it decreased with time and approached around 3 mg/g fw at 12th day after the planting. But the differences were not significant at 25°C. However, it decreased after 3 days of growth (DAG) and then gradually minimized and approached a significantly higher value of 9.16 mg/g (P= 0.05) as compared to the values at 0, 5 and 10°C at 12 days. In the RBZ, after the 3rd day, reducing sugars increased irrespective of the temperature and decreased afterwards with time, the decrease being minimal at 10 and 25 °C (Table 2).

The levels of sucrose in the buds decreased significantly (p=0.05) at 25 °C after 3rd day. At 0°C, there was no difference in the sucrose content after 3, 6 and 9 days. However after 12 DAG, the sucrose content decreased. At 25°C, sucrose content increased after 6 and 9 DAG, but declined after 12 DAG (Table 2). In the RBZ, sucrose content gradually decreased with time and at the 12th day, no significant difference was recorded (Table 2).

Table 2. Reducing sugars, sucrose content and acid invertase activity in sugarcane buds and root band zone subjected to different temperatures after 3rd, 6th, 9th and 12th DAG

Temp	Buds				Root band zone			
	Days							
	3	6	9	12	3	6	9	12
	Reducing sugars (mg/g fw)							
0	5.46^{abc}	4^{bc}	3.2^{c}	2.22^{c}	3.68^{abc}	1.62^{c}	1.18^{cde}	1.76^{cde}
5	5.7^{abc}	5^{abc}	3.78^{bc}	2.72^{c}	3.52^{a}	1.42^{cde}	1.08^{de}	1.34^{cde}
10	5.24^{abc}	2.5^{c}	2.88^{c}	3.2^{c}	4.44^{a}	1.08^{de}	0.62^{e}	0.86^{de}
25	6.26^{abc}	6.54^{abc}	7.72^{a}	9.16^{a}	4^{ab}	1.06^{de}	0.88^{de}	0.84^{de}
	Sucrose (mg/g fw)							
0	38.48^{a}	30.3^{ab}	22.42^{b}	25.4^{b}	11.15^{a}	83.23^{bc}	82.72^{b}	35.75^{e}
5	14.24^{c}	26.98^{b}	19.89^{b}	12.12^{cde}	122.72^{a}	86.66^{b}	69.99^{bcd}	48.78^{de}
10	9.99^{cde}	12.4^{cd}	10.6^{cde}	11.21^{cde}	126.36^{a}	86.36^{b}	65.75^{bcd}	53.33^{de}
25	5.15^{de}	8.48^{cde}	12.72^{cd}	3.63^{e}	126.66^{a}	89.39^{b}	47.27^{b}	34.24^{e}
	Acid invertase (mmol/min/mg proteins)							
0	0.94^{d}	0.66^{d}	1.12^{d}	4.04^{cd}	1.15^{de}	3.2^{bc}	3.8^{cd}	5.81^{c}
5	2.6^{cd}	1.15^{d}	5.56^{cd}	3.65^{d}	0.78^{de}	1.93^{de}	3.23^{cde}	4.79^{cd}
10	2.56^{cd}	1.48^{d}	3.37^{cd}	4.09^{cd}	0.26^{e}	1.9^{c}	1.15^{de}	1.8^{de}
25	4.38^{cd}	11.44^{b}	9.00^{bc}	18.75^{a}	0.73^{de}	9.63^{b}	11.5^{b}	16.48^{a}

Values in the same column bearing different letters significantly differ at p=0.05 based on Duncan's Multiple Range Test (DMRT)

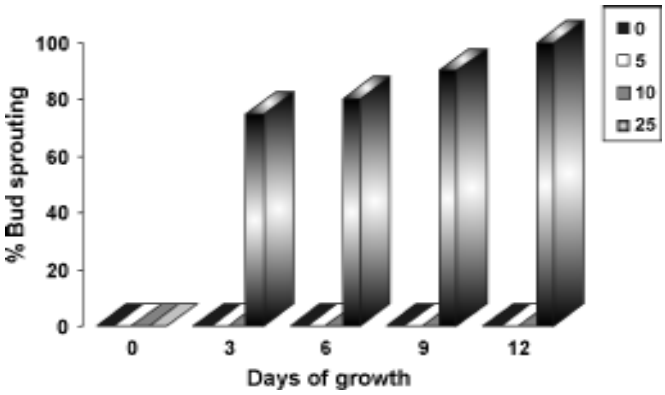

Fig. 4. Bud sprouting at different temperatures and days of growth

In the buds, specific activity of acid invertase was initially 0.73 mmole/min/mg proteins which increased with time irrespective of the temperatures, but the increase was more at 25°C as compared to 0, 5 and 10°C. As compared to the initial level on the 12th day, it increased to four and a half times at chilling temperature (0.5 and 10°C) and more than 25 times at 25°C. In the RBZ, the initial activity of acid invertase was 0.32 mmole/ min/ mg proteins and it increased gradually with time up to the 12th day. At 0 and 5°C, after 12 DAG, the acid invertase activity increased 15-18 folds where as at 10°C and 25°C, this increase was only 5-6 folds (Table 2).

ATPase: At the chilling temperatures up to 12th day, there was only 1.5 times increase in the ATPase activity where as at 25°C at the end of this period, nearly 7 times higher activity was observed in the buds (Table 3). Initially in the RBZ, the activity of this enzyme was at par to that of the buds, but with time, the activity increased and the increase was around 45 times (as compared to the initial activities at the chilling temperatures and it was 141 times at 25°C after the 12 DAG.

Table 3. IAA content, activities of IAA oxidase and ATPase in sugarcane buds and root band zone at different temperatures after 3rd, 6th, 9th and 12th DAG

Temp (°C)	Buds				Root band zone			
	Days							
	3	6	9	12	3	6	9	12
	IAA (µg/g fw)							
0	0.546^{c}	1.686^{a}	1.023^{b}	1.230^{c}	0.359^{cde}	1.153^{a}	0.633^{bc}	0.429^{cde}
5	0.703^{bc}	0.753^{bc}	0.889^{bc}	0.733^{bc}	0.853^{a}	0.085^{e}	0.339^{cde}	0.403^{cde}
10	0.766^{bc}	0.973^{bc}	1.093^{bc}	0.836^{bc}	0.273^{cde}	0.106^{e}	0.403^{cde}	0.516^{bcd}
25	0.633^{bc}	0.623^{bc}	0.679^{bc}	0.836^{bc}	0.559^{bcd}	0.233^{de}	0.419^{cde}	0.409^{cde}
	IAA Oxidase (µg/mg protein)							
0	8.69^{abc}	8.70^{edef}	11.52^{ab}	12.05^{a}	41.79^{ab}	60.89^{abcd}	28.63^{efg}	6.01^{g}
5	10.58^{bcdef}	4.35^{bcde}	6.71^{def}	3.05^{ef}	43.29^{abc}	37.11^{bcde}	24.59^{efg}	13.86^{g}
10	11.56^{bcde}	9.66^{bcde}	7.17^{bcde}	15.84^{ab}	15.86^{efg}	69.89^{bcde}	14.29^{fg}	18.36^{g}
25	14.39^{ef}	17.01^{f}	20.4^{f}	22.07^{abc}	19.91^{def}	28.49^{a}	49.45^{abc}	7.25^{ab}
	ATPase (µg Pi liberated/mg protein/10 min)							
0	0.16^{bcd}	0.18^{bcd}	0.37^{bcd}	0.28^{bcd}	0.21^{e}	1.40^{e}	1.16^{b}	3.51^{de}
5	0.15^{bcd}	0.17^{abcd}	0.39^{abcd}	0.38^{abc}	0.62^{e}	1.73^{de}	1.17^{b}	11.89^{c}
10	0.15^{cd}	0.13^{cd}	0.281^{cd}	0.29^{bcd}	0.48^{de}	3.21^{de}	1.55^{b}	7.64^{c}
25	0.24^{bcd}	1.35^{bc}	0.75^{abc}	1.32^{ab}	0.82^{de}	10.54^{de}	6.76^{b}	23.98^{a}

Values in the same column bearing different letters significantly differ at p=0.05 based on Duncan's Multiple Range Test (DMRT)

IAA and IAA oxidase: IAA contents in the buds were initially 8.3 µg/g fw. At the chilling temperatures, it increased to 10.31 µg/g fw after 12 DAG, which was 11 % higher as compared to the initial value. At 25°C, there was no change in the IAA content with time. In the RBZ at the chilling temperatures and 25°C with time, there was no change in the IAA contents. IAA oxidase activity in the buds increased two times after 12 DAG, at the chilling temperature and it increased 4 times at 25°C. In the RBZ, the IAA oxidase activity increased to 9 times after 12 days at the chilling temperatures while it increased to 57 times at 25°C.

At 25°C, a consistent supply of reducing sugars was maintained possibly by the hydrolysis of sucrose mediated by the higher levels of acid invertase activity. Stimulation of acid invertase activity in germinating sets of sugarcane was reported by Naik *et al*. (2001). The higher levels of reducing sugars in the bud helped in the initiation of the metabolic processes for germination / sprouting of the bud. A positive correlation has been reported between germination of sugarcane setts and reducing sugars (Singh and Kanwar 1986). Soluble sugars have been reported to regulate the osmotica and metabolic activities in germinating cereal grains (Yu *et al*. 1996). With growth, the accumulated sucrose appeared to have undergone breakdown by the acid invertase activity in bud as well as root band zone. Enhancement of reducing sugars and decrease in sucrose during sprouting of sugarcane sett has been reported by Ali (1997). Since the acid invertase activity was higher in the root band zone, it triggered the sucrose breakdown but there was a marked decline in the reducing sugars in the root band zone with the days of growth. This reduction in the reducing sugars in the root band zone was in sharp contrast with increase in reducing sugars in the buds indicating the parallel mobilization of reducing sugars from root band zone to the buds essential for initiating the bud sprouting.

Lower acid invertase activity at the chilling temperatures led to accumulation of higher sucrose at chilling temperatures. Williams and Leopold (1989) have reported that higher sucrose contents are known to facilitate vitrification, a phenomenon in which intracellular water hardens like glass with no ice crystal formation during freezing or chilling stress and thus avoiding the damage caused by crystallization, as water is withdrawn. Accumulation of sugars in different parts of the plants has been reported to enhance in response to a variety of environmental stresses (Wang *et al*. 1996, Prado *et al*. 2000). The lesser availability of reducing sugars at the low temperatures thus becomes a limiting factor in sprouting of the buds. Reducing sugars play an important role in osmotic regulation of cells during germination and are involved in the expression of some genes involved in the germination of seeds (Gorham *et al*. 1981, Yu *et al*. 1996). At low temperatures, the sharp decline in the acid invertase activity renders the availability of reducing sugar very low, suppressing the bud sprouting.

The increase IAA oxidase activity may inactivate or destroy IAA and thus retard the germination/growth (Omran 1980). A marked increase in the IAA oxidase activity during chilling improved the endogenous auxin levels in the buds. In view of known regulation of cell growth by IAA and inverse correlation of IAA oxidase activity with growth showed that enzymatic destruction of IAA is important in regulating the amount of growth substance in plants. Hence, the increase in IAA oxidase may cause the destruction of IAA, therefore affecting the growth of buds. Bolduc *et al*. (1970) reported an increase in IAA oxidase activity during cold treatment of winter wheat seedlings. These results suggest that the increase in the IAA oxidase activity by cold treatment kept the endogenous auxin at low level. IAA oxidase may be an inducible enzyme (Bohnsack and Albert 1977, Galston and Dalberg 1954) and thus chilling is one of the stimuli that can cause its induction. Our results appear to be in agreement with the reported work (Galston *et al*. 1950, Goldacre 1951 and Biggs *et al*.1955).

At chilling temperatures, reduced ATPase activity in buds and enhanced activity in RBZ indicated that during this process, the generated energy was transported from RBZ to buds. Similarly at 25°C the flow of energy was more towards bud from RBZ helping in sprouting of buds. Kasamo (1988) have reported the decrease in ATPase activity at chilling temperatures while increase at the ambient temperatures in rice.

The study indicated that the chilling temperatures suppressed the sugarcane bud sprouting which was associated with the alterations involving relatively higher

levels of IAA and decreased acid invertase, ATPase and IAA oxidase activity (which otherwise is increased under favorable conditions of temperatures, in this case 25°C) in the buds and the adjacent root band zone. These alterations at chilling temperatures appear to be responsible for suppressing the sprouting under the chilling conditions. Further investigations are needed to improve our understanding on accumulation of reactive oxygen species and translocation of sugars from the root band zone to the bud during the early development of the shoot under low temperatures.

ACKNOWLEDGEMENTS

Authors thank the Director, Indian Institute Sugarcane Research, Lucknow for encouragement and providing the facilities.

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