Physiological and biochemical effect of 24-epibrassinolide on cold tolerance in maize seedlings

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Abstract Germination and early seedling growth are important for establishment of maize because maize is chilling sensitive crop and low temperature during early period of growth can be detrimental to subsequent crop growth and productivity. Therefore, it is important to protect maize seedling from cold stress. A study was conducted on induced cold tolerance by 24-epibrassinolide (EBR) at the Indian Agricultural Research Institute, New Delhi, India. Maize seedlings were raised in green house condition (25/18 °C day-night temperatures). Ten days old seedlings were treated with EBR (0.0, 0.01, 0.1, 1.0 and 10 µM) and then divided into two sets, one set was kept in greenhouse (25/18 °C day-night temperatures) and another was transferred to net house (cold stress). Data on various morpho-physiological traits was recorded after 7, 14 and 21 days of treatment. Exogenous application of 1.0 µM EBR had significant effect on growth and morpho-physiological traits under both conditions. The maize seedlings treated with EBR were more tolerant to cold stress than the untreated one. Significant increase in plant height, dry matter accumulation, chlorophyll content, total soluble proteins and starch contents was observed under both conditions, however, the results were more pronounced under cold stress. 1.0 µM concentration being the most effective under both conditions. Maintenance of high tissue water content, reduced membrane injury index, increased total

chlorophyll, soluble sugar and protein content were taken as the possible indicators of EBR induced chilling tolerance.

Keywords 24-epibrassinolide · Cold tolerance · Maize · Glycine betaine · Total chlorophyll · Membrane injury index

Introduction

24-epibrassinolide (EBR) are polyhydroxylated steroids that are able to act at extremely low concentration and are pleiotropic with respect to their effect on plant development, morphology, and physiology (Rao et al. 2002). Studies have indicated that brassinosteroid (BRs) are essential for proper plant growth and development (Yokota 1997; Sasse 1997; Clouse and Sasse 1998). Hamada (1986) for the first time reported the ameliorative effects of BR-treatment of stressed plants and since then most of the work has been focused on chilling stress (Katsumi 1991; Wilen et al. 1995). The enhanced chilling resistance was attributed to BRs induced effects on membrane stability and osmoregulation (Wang and Zeng 1993) and highly effective in stimulating growth of young vegetative tissues (Sasse 1991). BRs could prevent loss of photosynthetic pigments, for example, by activation of enzymes that participate in chlorophyll biosynthesis (Hayat et al. 2007 and Ali et al. 2008a, b).

Maize is a thermophilic plant species and highly sensitive to low temperature at all stage of development (Staeblar 2001). The low temperature susceptibility of young maize plants is one of the most important limiting factors for expanding the maize production area (Janda et al. 2005). For maize plants even temperatures below 12–15 °C may induce chilling stress (Holla et al. 2003). Low temperature affects germination, seedling growth, early leaf development and overall maize crop growth and productivity

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(Zaidi et al. 2010). The present studies were conducted with an aim to improve tolerance of maize seedlings to low temperature.

Materials and methods

Growth conditions

Seedling of maize inbred line LM-17 (Erect plant with dark green narrow leaves) were raised in thermacole pots (10 cm length X 5 cm diameter) filled with 0.5 kg soil (soil + FYM + vermiculite in 2:1:1 ratio) in green house conditions at Indian Agricultural Research Institute, Pusa New Delhi, India, situated at 28.4° N latitude, 77.1° E longitude and 228.2 m altitude, during winter 2010-11 crop season in completely randomized design (CRD) with three replications. After germination, at first leaf stage, the pots were divided into two groups: one group was transferred in to the Green house (control) (Saver Biotech limited, New Delhi) other group of the seedlings were transferred in to the Net house (cold stress). After thinning single plant was retained in each pot. 10 mM stock solution of EBR was prepared by dissolving 0.048 g of EBR in 10 ml of absolute ethanol. Desired concentrations of EBR (0.1 µM, 1.0 µM and 10.0 µM) were prepared just before use and 10 ml solution was sprayed on each plant (10 days old) with a hand sprayer (Allevi et al. 1988). Prior to spray, 0.1 % teepol solution was mixed with EBR solution (which acts as surfactant and facilitates absorption of growth regulator by the leaf surface). For each parameter, three pots having single plant were sampled at a time. The plants were sampled after 7, 14 and 21 days of EBR treatments. The maximum and minimum temperature in net house ranged between 17.6 and 24.5 °C and 2.8–7.4, respectively.

Morphological observations

Plant height (that is the distance from the ground to ligules of the youngest fully developed leaf) was recorded at 7, 14 and 21 days. Seedling fresh biomass was determined by uprooting the whole plant from the pot. An uprooted plant was cleaned with double distilled water and after cleaning excess water on plant was soaked with tissue paper. Whole plants were dried in labelled paper envelopes in an oven at 80 °C until constant weight is achieved and their dry weights were recorded and expressed as g plant⁻¹.

Physiological observations

Relative water content (RWC %) of leaf was calculated according to Barr and Weatherley (1962) by using the

equation:

$$RWC = (FW - DW)/(TW - DW) \times 100$$

Where, FW is the fresh weight of the leaves, TW is leaf weight at full turgor measured after floating the leaves for 24 h in water in the light at room temperature and DW is the weight estimated after drying the leaves for 4 h at 80 °C or until a constant weight is achieved.

Membrane Injury Index was measured by the method of Sullivan (1972), modified by Ibrahim and Quick (2001). Leaf membrane injury index is determined by recording the electrical conductivity of leaf lichgates in double distilled water at 40 °C and 100 °C. Leaf sample (100 mg) were cut into discs of uniform size and taken in test tubes containing 10 ml of double distilled water in two sets. One set was kept at 40 °C for 30 min and their respective electric conductivities C₁ and C₂ were measured by digital conductivity meter. Membrane injury index was recorded at different determine growth stages. Membrane injury index is determined by given formula:

$$MII = \left(\frac{C_1}{C_2} \times 100 \right)$$

Where,

MII Membrane injury index
C₁ EC before autoclaving
C₂ EC after autoclaving

Biochemical observations

Total chlorophyll content was estimated by using the method of Wellburn (1994). The amount of chlorophyll content was expressed as mg g⁻¹ fresh weight of leaf and calculated by using following formula:

Total chlorophyll content

$$= \{20.2(\text{O.D at } 645) + 8.02(\text{O.D at } 663) \times V\} / \{1000 \times W\}$$

The protein content was determined by the method of Lowry et al. (1951) and values were expressed in mg g⁻¹ fresh weight of leaf. Glucose content was measured by the method described by Milller (1959); using dinitrosalicylic reagent. Sucrose was estimated by using dinitrosalicylic reagent for the determination of reducing sugar as described by Milller (1959) and expressed in mg g⁻¹ fresh weight of leaf. Hodge and Hfreiter (1962) procedure was followed for starch estimation and expressed in mg g⁻¹ fresh weight of leaf.

For determination of glycine betaine, procedure of Grieve and Grattan (1983) was followed and expressed in µmol g⁻¹ fresh weight of leaf. The extract of finely ground leaf material (500 mg) was mechanically shaken with 20 ml

Table 1 Effect of 24-epibrasinolide on plant height, fresh biomass and dry biomass of maize seedlings under controlled and cold stress conditions

Treatments	Control			Cold stress		
	7 DAT	14 DAT	21 DAT	7 DAT	14 DAT	21 DAT
Plant height (cm)						
0.0 μ M EBR	14.7 $\pm$ 0.18	16.6 $\pm$ 0.07	18.3 $\pm$ 0.27	12.0 $\pm$ 0.40	11.8 $\pm$ 0.24	11.9 $\pm$ 0.38
0.1 μ M EBR	16.2 $\pm$ 0.29	17.4 $\pm$ 0.23	20.7 $\pm$ 0.35	12.1 $\pm$ 0.44	12.6 $\pm$ 0.62	12.4 $\pm$ 0.09
1.0 μ M EBR	16.3 $\pm$ 0.33	17.7 $\pm$ 0.50	21.2 $\pm$ 0.41	12.3 $\pm$ 0.19	13.0 $\pm$ 0.18	13.7 $\pm$ 0.29
10.0 μ M EBR	16.1 $\pm$ 0.37	16.9 $\pm$ 0.45	19.5 $\pm$ 0.32	12.0 $\pm$ 0.31	12.0 $\pm$ 0.12	12.4 $\pm$ 0.41
Fresh biomass (g)						
0.0 μ M EBR	4.33 $\pm$ 0.077	6.83 $\pm$ 0.353	11.78 $\pm$ 0.210	2.29 $\pm$ 0.172	5.86 $\pm$ 0.277	8.96 $\pm$ 0.423
0.1 μ M EBR	6.01 $\pm$ 0.310	8.89 $\pm$ 0.346	12.23 $\pm$ 0.632	3.70 $\pm$ 0.175	6.44 $\pm$ 0.241	9.39 $\pm$ 0.705
1.0 μ M EBR	7.07 $\pm$ 0.276	9.58 $\pm$ 0.171	14.78 $\pm$ 0.466	4.41 $\pm$ 0.165	6.49 $\pm$ 0.069	12.22 $\pm$ 0.456
10.0 μ M EBR	5.36 $\pm$ 0.064	8.09 $\pm$ 0.097	14.22 $\pm$ 0.171	3.89 $\pm$ 0.042	6.45 $\pm$ 0.484	10.49 $\pm$ 0.112
Dry biomass (g)						
0.0 μ M EBR	0.443 $\pm$ 0.008	0.728 $\pm$ 0.012	1.105 $\pm$ 0.020	0.452 $\pm$ 0.008	0.673 $\pm$ 0.098	1.139 $\pm$ 0.086
0.1 μ M EBR	0.568 $\pm$ 0.009	0.730 $\pm$ 0.011	1.264 $\pm$ 0.065	0.483 $\pm$ 0.006	0.728 $\pm$ 0.105	1.153 $\pm$ 0.021
1.0 μ M EBR	0.688 $\pm$ 0.006	0.990 $\pm$ 0.008	1.421 $\pm$ 0.055	0.618 $\pm$ 0.089	0.774 $\pm$ 0.027	1.157 $\pm$ 0.055
10.0 μ M EBR	0.600 $\pm$ 0.009	0.762 $\pm$ 0.012	1.341 $\pm$ 0.016	0.591 $\pm$ 0.021	0.687 $\pm$ 0.038	1.116 $\pm$ 0.012

of distilled water for 48 h at 25 °C. The samples were then filtered and the filtrate was stored in freezer until analysis. Thawed extracts were diluted to 1:1 with 2 N sulphuric acid. 0.5 ml aliquot was cooled in ice water for 1 h. 0.2 ml cold potassium iodide-iodine reagent was added and the mixture was gently mixed with vortex mixture. The samples were stored at 0–4 °C for 16 h. The samples were transferred to centrifuge tubes and then centrifuged at 10,000 $\times$ g for 15 min at 0 °C. The supernatant was carefully aspirated with 1 ml micropipette. The periodite crystals were then dissolved in 9 ml of 1, 2-dichloroethane and were mixed properly using vortex mixture and kept at room temperature for 2 h. The absorbance was measured at 365 nm with UV–vis spectrophotometer.

Statistical analysis Data were analyzed by means of analysis of variance (ANOVA) by using Statistical Analysis System (SAS 9.2) software, significant level $P=0.01$ was used.

Result and discussion

Chilling stress reduced the plant height, fresh biomass and dry biomass of maize seedling (Table 1, Fig. 1). Foliar application of EBR improved all these attributes under both optimal and stress conditions, maximum plant height, fresh biomass and dry biomass were recorded from plant treatment with 1.0 μ M concentration of EBR in both conditions and was found significant only at 7 DAT & 21 DAT in control condition. These results indicate that growth of the maize seedlings increase by the application of EBRs

(Bhardwaj et al. 2007 and Arora et al. 2008). EBR increase, the plant fresh and dry biomass of sugar beet (Schilling et al. 1991). Chilling stress reduced the relative water content (RWC) and increased the membrane injury index (Table 2).

Fig. 1 Effect of different concentrations of 24-Epibrassinolide on plant height of 21 days old maize seedlings under control and cold stress conditions. (A: 0.0 μ M EBR, B: 0.1 μ M EBR, C: 1.0 μ M EBR, D: 10.0 μ M EBR)

Table 2 Effect of 24-epibrasinolide on relative water content, membrane injury index and canopy temperature of maize seedlings under controlled and cold stress conditions

Treatments	Control			Cold stress		
	7 DAT	14 DAT	21 DAT	7 DAT	14 DAT	21 DAT
Relative Water Content (RWC %)						
0.0 μ M EBR	66.9 $\pm$ 1.12	65.4 $\pm$ 1.83	65.7 $\pm$ 1.61	31.6 $\pm$ 0.69	36.3 $\pm$ 1.10	32.5 $\pm$ 1.04
0.1 μ M EBR	79.4 $\pm$ 1.33	66.5 $\pm$ 1.89	74.3 $\pm$ 1.85	40.3 $\pm$ 0.78	46.5 $\pm$ 1.35	41.3 $\pm$ 1.14
1.0 μ M EBR	80.8 $\pm$ 1.36	69.5 $\pm$ 2.10	76.1 $\pm$ 1.91	42.7 $\pm$ 0.84	47.2 $\pm$ 1.36	43.7 $\pm$ 0.58
10.0 μ M EBR	69.9 $\pm$ 1.18	65.4 $\pm$ 1.96	67.6 $\pm$ 1.74	39.5 $\pm$ 0.75	45.5 $\pm$ 1.33	40.6 $\pm$ 0.44
Membrane Injury Index (MII)						
0.0 μ M EBR	20.4 $\pm$ 0.30	15.6 $\pm$ 0.12	21.0 $\pm$ 0.31	39.6 $\pm$ 1.38	42.9 $\pm$ 1.53	43.5 $\pm$ 0.53
0.1 μ M EBR	15.4 $\pm$ 0.29	12.9 $\pm$ 0.44	13.5 $\pm$ 0.46	36.8 $\pm$ 0.44	37.6 $\pm$ 1.35	30.0 $\pm$ 1.73
1.0 μ M EBR	12.2 $\pm$ 0.19	12.1 $\pm$ 0.19	12.6 $\pm$ 0.19	28.1 $\pm$ 1.07	32.8 $\pm$ 0.83	27.5 $\pm$ 1.43
10.0 μ M EBR	15.7 $\pm$ 0.15	15.2 $\pm$ 0.25	15.8 $\pm$ 0.29	37.8 $\pm$ 2.48	39.8 $\pm$ 0.46	41.7 $\pm$ 1.76
Canopy temperature (CT °C)						
0.0 μ M EBR	21.8 $\pm$ 0.35	22.9 $\pm$ 0.09	19.4 $\pm$ 0.29	20.7 $\pm$ 0.29	19.3 $\pm$ 0.21	19.2 $\pm$ 0.44
0.1 μ M EBR	21.6 $\pm$ 0.29	22.6 $\pm$ 0.18	19.2 $\pm$ 0.49	20.0 $\pm$ 0.32	18.9 $\pm$ 0.25	19.2 $\pm$ 0.24
1.0 μ M EBR	21.5 $\pm$ 0.29	22.0 $\pm$ 0.42	18.9 $\pm$ 0.47	19.9 $\pm$ 0.52	18.2 $\pm$ 0.09	18.9 $\pm$ 0.47
10.0 μ M EBR	21.5 $\pm$ 0.35	22.4 $\pm$ 0.33	19.2 $\pm$ 0.24	20.6 $\pm$ 0.53	18.5 $\pm$ 0.15	19.2 $\pm$ 0.49

However, all the EBR treatments significantly improved the RWC under both conditions. Maize plants exposed to chilling stress often show water stress symptoms because of chilling induced water stress (Melkonian et al. 2004). Chilling induced water stress begins with decreased root hydraulic conductance followed by large decrease in leaf water status (Aroca et al. 2001). If root and shoot are exposed to low temperature, stomata cannot be closed in spite of root leaf water status. This failure of stomatal control in addition to decreased root hydraulic conductance can further aggravate chilling induced water stress (Lee et al. 1993). All EBR treatments decreased the membrane injury index in maize leaves at both control and stress condition except at 7DAT in stress condition. However, highest RWC and minimum membrane injury index was recorded with foliar application of 1.0 μ M EBR under both conditions (Table 2). Enhanced

membrane injury index was considered to be a symptom of stress induced membrane injury and deterioration (Feng et al. 2003). EBR increased the resistance against chilling stress (1–5 °C) and the tolerance was associated with increased ATP, proline levels and SOD activity, thus indicating brassinosteroid involvement in membrane stability and osmoregulation. Total chlorophyll was negatively affected by the exposure of plants to long-term cold stress (Table 3). Under cold stress, chlorophyll content reduced in maize leaves (Lee et al. 2002). The degradation of photosynthetic pigments is other symptom frequently associated with cold induced stress (Haldimann 1998). The total chlorophyll content was significantly increased by the foliar application of EBR except at 7 DAT in stress condition. The total chlorophyll content or its fraction increased in the leaves of *Cucumis sativus* by EBR, applied directly to their foliage

Table 3 Effect of 24-epibrasinolide on total chlorophyll and protein content in leaf of maize seedlings under controlled and cold stress conditions

Treatments	Control			Cold stress		
	7 DAT	14 DAT	21 DAT	7 DAT	14 DAT	21 DAT
Total chlorophyll (mg/g Fresh weight)						
0.0 μ M EBR	1.77 $\pm$ 0.013	1.77 $\pm$ 0.024	1.84 $\pm$ 0.021	1.34 $\pm$ 0.012	1.34 $\pm$ 0.008	1.34 $\pm$ 0.006
0.1 μ M EBR	2.06 $\pm$ 0.006	2.02 $\pm$ 0.018	2.60 $\pm$ 0.017	1.37 $\pm$ 0.019	1.36 $\pm$ 0.015	1.37 $\pm$ 0.016
1.0 μ M EBR	2.54 $\pm$ 0.021	2.49 $\pm$ 0.020	3.48 $\pm$ 0.019	2.14 $\pm$ 0.006	2.04 $\pm$ 0.020	2.12 $\pm$ 0.020
10.0 μ M EBR	1.92 $\pm$ 0.073	2.19 $\pm$ 0.016	1.93 $\pm$ 0.014	1.84 $\pm$ 0.030	1.83 $\pm$ 0.032	1.86 $\pm$ 0.017
Protein content (mg/g Fresh weight)						
0.0 μ M EBR	3.63 $\pm$ 0.033	3.63 $\pm$ 0.033	3.92 $\pm$ 0.036	3.17 $\pm$ 0.033	3.17 $\pm$ 0.016	3.20 $\pm$ 0.034
0.1 μ M EBR	4.07 $\pm$ 0.330	4.07 $\pm$ 0.036	4.20 $\pm$ 0.034	3.70 $\pm$ 0.058	3.63 $\pm$ 0.013	3.70 $\pm$ 0.062
1.0 μ M EBR	4.73 $\pm$ 0.033	4.67 $\pm$ 0.033	4.80 $\pm$ 0.034	4.50 $\pm$ 0.068	4.57 $\pm$ 0.067	3.90 $\pm$ 0.054
10.0 μ M EBR	4.23 $\pm$ 0.067	4.20 $\pm$ 0.058	4.20 $\pm$ 0.058	4.17 $\pm$ 0.043	4.20 $\pm$ 0.058	3.89 $\pm$ 0.067

(Yu et al. 2004). However, overall 1.0 μM concentration of EBR was found best treatment with respect to increase in total chlorophyll content (Fariduddin et al. 2009). Decrease in chlorophyll a and b content in plants when plants subjected to cold treatment has been reported by Wise and Naylor (1987). The observed decline of chlorophyll content induced by low temperature confirms our results. Seedlings transferred from control to cold stress condition resulted in increase in leaf total soluble protein content during low temperature period (Table 3). It has been found in the present investigation that the different treatments of EBR significantly increased protein content as compared to control (Table 3). Cold stress induced an accumulation of soluble proteins in *Brassica juncea* (Hayat et al. 2000, 2001). Synthesis of specific proteins is an important mechanism involved in increasing freezing tolerance during cold acclimation (Antikainen et al. 1996). Low temperature stress increased glucose, starch and sucrose contents (Table 4). At both temperatures, all EBR treatment improved soluble sugars compared with control. However, maximum glucose, sucrose and starch content were recorded from plant treatment with 1.0 μM concentration of EBR under both conditions. Soluble sugars have been found to play an important role during cold acclimation process (Yuan et al. 2009). Sucrose, glucose and fructose content increased quickly

following exposure to lower temperature in cloudberry (Kaurin et al. 1981) and in spinach (Guy et al. 1992). The present study with maize plant has shown that glucose and sucrose contents also increased on exposure to low temperature. Soluble sugars exert their positive effects to protect plant cells from damage caused by cold stress through several ways, including serving as osmo-protectants, nutrient as well as interacting with the lipid bi-layer. Lukatkin (2003) observed a gradual increase in leakage of ions from the cells upon prolongation of chilling exposure, with the maximum attained by the end of 24-h chilling treatment. Farooq et al. (2008) found that reduced membrane permeability contributed towards chilling tolerance in maize hybrids. Starch serves generally as the dominant storage carbohydrate in highest plants. In maize seedling, starch accumulated during cold acclimation and decreased during deacclimation. Guy et al. (1992) showed that chloroplastic starch in the leaves of spinach increased under cold acclimation but the increase was not related to freezing tolerance. At optimal and low temperatures, EBR treatments improved the content of glycine betaine as compared to control. However, the maximum glycine betaine content was recorded from the treatment with 1.0 μM concentration of EBR under both conditions. In maize, chilling tolerance has been associated with buildup of glycine betaine (Chen et al. 2000).

Table 4 Effect of 24-epibrasinolide on glucose, starch, sucrose and glycine betaine contents in leaf of maize seedlings under controlled and cold stress conditions

Treatments	Control			Cold stress		
	7 DAT	14 DAT	21 DAT	7 DAT	14 DAT	21 DAT
Glucose content (mg/g Fresh weight)						
0.0 μM EBR	3.17 $\pm$ 0.041	3.33 $\pm$ 0.121	3.20 $\pm$ 0.088	3.33 $\pm$ 0.019	3.37 $\pm$ 0.029	3.30 $\pm$ 0.042
0.1 μM EBR	3.70 $\pm$ 0.058	3.77 $\pm$ 0.088	3.90 $\pm$ 0.062	3.37 $\pm$ 0.049	3.47 $\pm$ 0.042	3.50 $\pm$ 0.082
1.0 μM EBR	4.17 $\pm$ 0.032	4.37 $\pm$ 0.102	4.40 $\pm$ 0.017	3.77 $\pm$ 0.102	3.87 $\pm$ 0.120	3.80 $\pm$ 0.077
10.0 μM EBR	3.67 $\pm$ 0.071	3.93 $\pm$ 0.091	4.00 $\pm$ 0.067	3.37 $\pm$ 0.083	3.53 $\pm$ 0.097	3.60 $\pm$ 0.63
Starch content (mg/g Fresh weight)						
0.0 μM EBR	43.7 $\pm$ 1.96	45.0 $\pm$ 2.19	43.0 $\pm$ 0.89	46.7 $\pm$ 1.72	46.0 $\pm$ 1.92	44.0 $\pm$ 2.92
0.1 μM EBR	46.0 $\pm$ 2.51	44.7 $\pm$ 3.02	49.0 $\pm$ 3.30	48.3 $\pm$ 3.02	47.0 $\pm$ 1.06	46.0 $\pm$ 2.13
1.0 μM EBR	62.7 $\pm$ 2.31	64.0 $\pm$ 2.97	66.0 $\pm$ 2.17	65.7 $\pm$ 2.97	68.0 $\pm$ 1.78	64.0 $\pm$ 1.88
10.0 μM EBR	57.0 $\pm$ 1.72	58.3 $\pm$ 1.93	57.0 $\pm$ 2.79	57.3 $\pm$ 1.73	60.0 $\pm$ 2.41	60.0 $\pm$ 1.62
Sucrose content (mg/g Fresh weight)						
0.0 μM EBR	11.43 $\pm$ 0.13	11.33 $\pm$ 0.37	11.20 $\pm$ 0.09	11.17 $\pm$ 0.07	11.57 $\pm$ 0.04	11.60 $\pm$ 0.79
0.1 μM EBR	12.00 $\pm$ 0.29	12.13 $\pm$ 0.17	12.40 $\pm$ 0.07	12.27 $\pm$ 0.06	12.37 $\pm$ 0.07	12.90 $\pm$ 0.43
1.0 μM EBR	12.43 $\pm$ 0.37	14.53 $\pm$ 0.29	12.40 $\pm$ 0.11	12.79 $\pm$ 0.15	12.30 $\pm$ 0.12	14.80 $\pm$ 0.058
10.0 μM EBR	12.17 $\pm$ 0.43	12.33 $\pm$ 0.17	12.00 $\pm$ 0.08	12.37 $\pm$ 0.07	12.60 $\pm$ 0.07	12.30 $\pm$ 0.033
Glycine betaine (mM/g Fresh weight)						
0.0 μM EBR	20.7 $\pm$ 0.19	21.3 $\pm$ 0.21	20.0 $\pm$ 0.19	21.0 $\pm$ 0.21	20.7 $\pm$ 0.17	20.0 $\pm$ 0.25
0.1 μM EBR	33.0 $\pm$ 0.47	31.3 $\pm$ 0.38	32.0 $\pm$ 0.23	33.0 $\pm$ 0.19	33.0 $\pm$ 0.23	32.0 $\pm$ 0.19
1.0 μM EBR	37.7 $\pm$ 0.34	37.7 $\pm$ 0.27	37.0 $\pm$ 0.33	37.0 $\pm$ 0.31	37.7 $\pm$ 0.33	37.0 $\pm$ 0.31
10.0 μM EBR	30.7 $\pm$ 0.39	30.3 $\pm$ 0.19	30.0 $\pm$ 0.27	35.7 $\pm$ 0.09	35.7 $\pm$ 0.27	36.0 $\pm$ 0.41

Table 5 Correlation coefficient of traits among different growth, physiological and biochemical parameters

	CT	RWC	MII	Chlorophyll	Protein	Starch	Glucose	Sucrose	Glycine betaine
CT	1.000								
RWC	0.515	1.000							
MII	-0.593	-0.923	1.000						
Chlorophyll	0.141	0.230	-0.326	1.000					
Protein	0.131	0.452	-0.505	0.719	1.000				
Starch	0.031	0.108	-0.167	0.740	0.840	1.000			
Glucose	0.167	0.544	-0.546	0.716	0.833	0.803	1.000		
Sucrose	0.167	0.450	-0.491	0.817	0.784	0.729	0.890	1.000	
Glycine betaine	-0.236	-0.327	0.308	-0.340	-0.229	-0.261	-0.209	-0.069	1.000

** Significant at $P=0.01$

The correlation coefficient analysis between cold stress and morpho-physiological and biochemical attributes (Table 5) revealed that growth characters has positive correlation with morpho-physiological and biochemical traits. Canopy temperature is significantly correlated with Relative water content, Glucose and Sucrose, while other parameters have showed non significant relation with canopy temperature. Relative water content is significantly correlated with Chlorophyll, protein, starch, glucose and sucrose content. Membrane injury

index is significantly correlated with glycine betaine, while other parameters have showed non-significant relation with Membrane injury index. Total Chlorophyll is significantly correlated with Canopy temperature, Relative water content, Starch, Glucose and Sucrose content. Protein content is found significantly correlated with canopy temperature, Relative water content, Starch, Glucose and Sucrose. Glycine betaine is only significantly correlated with Membrane injury index while other parameters showed a non significant correlation.

Fig. 2 (a) Protein as function of total chlorophyll content in maize leave treated with EBR in controlled and cold stress condition. (b) Membrane injury index (MII) as function of canopy temperature (CT) in maize leave treated with EBR in controlled and natural field condition. (c) Relative water content (RWC) as function of MII in maize leave treated with EBR in controlled and natural field condition. The Figure was prepared using MS Office Excel 2007

Correlation and regression analysis showed significant relationship between various morpho-physiological traits (Fig. 2a, b and c). Among all traits, protein is highly significantly correlated with total chlorophyll, total chlorophyll with canopy temperature, Relative water content with Membrane injury index and Membrane injury index with canopy temperature.

In conclusion, application of EBR at seedling stage can improve low temperature tolerance in LM-17 maize. The performance of maize was also improved at normal temperature by EBR treatment, 1.0 μM concentration being the most effective under both conditions. Maintenance of high tissue water content, reduced membrane injury index, increased total chlorophyll, soluble sugar, glycine betaine and protein content are possible indicators of EBR induced chilling tolerance in maize.

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