Volume 14(5). Published February, 01, 2015
www.jnsciences.org
ISSN 2286-5314

Early effects of chilling stress on the morphological and physiological statut of pretreated *Stevia rebaudiana* Bert. seedlings

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Abstract - The effect of exogenous application of hydrogen peroxide (H_2O_2), salicylic acid (SA), calcium chloride ($CaCl_2$) and 6-benzylaminopurine (BAP) on Stevia (*Stevia rebaudiana* Bert.) exposed to day night temperature (10/6°C) in a growth chamber for 120h was investigated. There was a positive physiological effect on growth and development of chilling-stressed seedlings by these compounds as shown by a higher dry weight and leaf mass area, electron transport rate (ETR) and quantum photosynthetic yield (Y) and both chlorophyll and carotenoid contents remains higher during exposure to low temperature in pretreated plants in comparison with control plants suggested that these compounds may improve the chilling tolerance of Stevia.

Keywords: Stevia / cold stress / signaling compounds / physiology / photosynthetic yield.

1. Introduction

Due to the changing climate, temperature stress is one of the significant concerns for plant scientists worldwide (Watanabe et *al*., 2009; Shah et *al*., 2011). Low temperature or cold stress is another major environmental factor that often limits plant growth, damage crop productivity and leads to substantial crop losses (Sanghera et *al.*,2000; Xin et *al.*,2011). Chilling stress results from temperatures cool enough (0-15°C) that may induce injury without forming ice crystals in plant tissues. Generally, tropical and subtropical plant origins are more sensitive to chilling injury and lack this mechanism of cold acclimation (Sanghera et *al.*, 2000). Stevia *(Stevia rebaudiana* Bert.), is a valuable medicinal plant originated from Paraguay and Brazil, and is considered as an alternate substitute for cane and beet sugar, it has a great potential as a new agricultural crop since consumer demand for herbal foods is increasing. It synthesizes a high level of sweetening compounds, known as steviol glycosides, which are about 300 times sweeter than saccharose. The main sweetening compounds of interest among steviol glycosides produced are stevioside and rebaudioside A that offers a therapeutic properties and possess antioxidant, antimicrobial and antifungal activity. Their thermo stability up to 200°C, making them suitable for alimentary uses (Muanda et *al.*, 2010). The optimum growth temperature of Stevia is 23°C. Generally, low temperatures affects plant growth and development at early seedlings stage and accelerates senescence. Previous studies have shown that specific groups of molecules such salicylic acid (SA), hydrogen peroxide (H_2O_2), calcium chloride ($CaCl_2$) and others induced tolerance of creeping bentgrass by reducing oxidative damage.(Larkindale et *al.*, 2003). It is also reported that SA as a naturally signaling molecule played a key role in signaling and establishing a defense response against several pathogenic infections (Malamy et *al*., 1990; Durner et *al*., 1997). Recently, it has been prouved that pretreatment of plant with hydrogen peroxide (H_2O_2) allowed by an up-regulation of defense-related proteins the ginseng seedlings to recover from salt stress (Sathiyaraj et *al.*, 2014). In this study, we tried to evaluate the damage and change in physiological behavior in Stevia induced by chilling stress and pre-treatment with salicylic acid (SA), hydrogen peroxide (H_2O_2), calcium chloride ($CaCl_2$) and 6-benzylaminopurine (6-BA) Stevia seedlings.

In order to evaluate the degree of temperature-related stress influence, the most sensitive component is the photosynthetic apparatus.

2. Materials and methods

2.1. Plant materials and chemical treatments

Rooted seedling of Stevia obtained from mature plants grown in field plot Horticulture at INAT Institute, were transferred into plastic pots filled with peat, clipped and allowed to grow for six weeks in a growth chamber at 23°C (day/night), 70% relative humidity, 2000 lux irradiance and 16 hours photoperiod. During this period plants were watered daily and fertilized weekly with Full-strength Hoagland's nutrient solution. Stevia plants were then sprayed with 10 ml of different concentration of chemical compounds: 0, 5 mM salicylic acid (SA), 10 mM hydrogen peroxide (H_2O_2), 10 mM calcium chloride ($CaCl_2$) and 30 µM 6-benzylaminopurine (BAP). Plants treated with similar volume of distilled water were taken as controls. Then these plants were placed for 120 hour in a growth chamber at temperature 10°C/6°C (Day/night) 11 photoperiod, 70% relative humidity and 2000 lux irradiance.

2.2. Estimation of plant growth, relative water content and leaf mass area

At the end of the treatment period and as an estimation of plant growth under stress conditions number of leaves per plant and fresh weight of the whole aerial part was measured. In addition, three leaves (upper, middle and bottom) of each plant were weighed and leaf area was immediately photographed and measured using an image-processing program Environment for Visualizing Images (*ENVI 4.5, ITT Corporation,* NY) .The latter is based on counting the pixels of each color of the digital image and then converting them into cm^2 by referring to an already known surface (reference).. The leaf mass per area ratio (LMA) was calculated as follows: DW/leaf area.

2.3. Photosystem II activity

Chlorophyll a fluorescence parameters; quantum yield of PSII (Y) and electron transport rate (ETR) were assessed by determining fluorescence with a pulse-modulated fluorometer (*OS5p, Opti-Sciences, Hudson, NH,* USA).at 0, 24, 48,72 and 120 hours under chilling conditions

For Y and ETR, plants placed under a steady state of photosynthesis, a prerequisite for measuring Y and ETR. A photosynthetically active radiation (PAR) clip (OS5p PAR Clip, Opti-Sciences, Hudson, NH, USA) provides the PAR measurements while measuring Y and ETR. While measuring the Y and ETR the range of PAR was 600-700 µmol m^{-2-1}.

2.4. Photosynthetic pigments

The content of chlorophyll a, b, and carotenoids were estimated at 0, 24, 48,72and 120 hours of chilling following the method of Torrecillas et al., (1984). Fresh leaf samples (100mg) were homogenized and incubated overnight in 80% (v/v) acetone solution (5ml) at 4°C. The extract was centrifuged at 14000 x g for 5 minutes, and the absorbance of the supernatant was read at 460, 645 and 663 nm using a spectrophotometer (*Optizen 3220UV*, Korea).

The chlorophyll a and b were calculated according to the formulae developed by Mc Kinney et al., (1941) and Arnon et al., (1949):

$$Chlorophyll\ a\ (mg/g\ FW) = 12{,}7 \times OD\ (645\ nm) - 2{,}69 \times OD(663\ nm)) \times \frac{V}{1000} \times W$$

$$Chlorophyll\ b\ (mg/g\ FW) = 22{,}9 \times DO\ (645\ nm) - 8{,}02 \times DO\ (663\ nm)) \times \frac{V}{1000} \times W$$

$$Carotenoids\ (mg/g\ FW) = 5 \times OD\ (460\ nm) \times \frac{[(Chloro\ a \times 8.19) + (Chloro\ b \times 30.8)]}{200} \times \frac{V}{1000} \times W$$

2.5. Statistical analysis

Effects of the chemical treatments on the morphological and physiological parameters were assessed with proc ANOVA of SAS (9.0) using t-test. When the interaction was significant a subroutine PDMix800 of the proc mixed was used to compare means at ($p<0.05\%$).

3. Results and discussion

3.1. Morphological responses of pretreated Stevia under subsequent chilling stress

All of the pretreated plants showed a significantly higher LMA ($p<0.01$) from those of control plants (Figure.1A). LMA pretreated plants with BA expressed in (g/m^2) was relatively higher (53.11± 4.4) then control plants and other pretreated plants that maintained a high LMA but with significant differences between treatment. Aerial biomass appeared to be promoted significantly ($p<0.01$) when signal molecules were applied. Great fresh biomasses were obtained in plants pretreated with $CaCl_2$ and SA with 86% and 66% of increase (Figure 1B) compared to control plants. Also a slight increase ($p>0.05$) by 10.44% and 9.76% was noted in pretreated plants with SA and H_2O_2. At the end of the experiment, number of leaves per plant was enhanced ($p<0.05$) by respectively 56.14%, 48.82% and 46.41% in pretreated plant with SA, H_2O_2 and $CaCl_2$ relative to control(Figure1C).

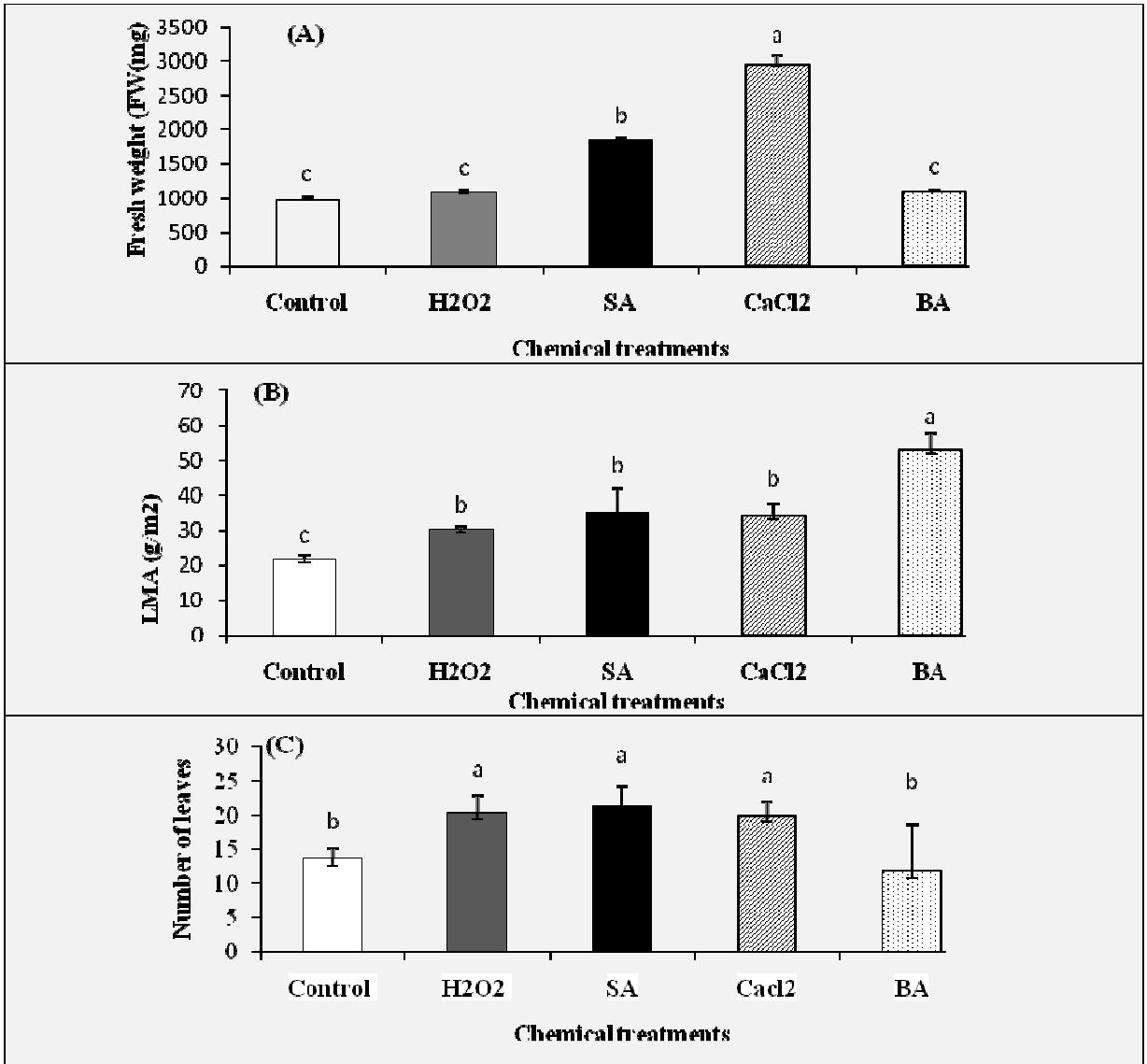

Figure 1. Dry aerial biomass, leaf mass area (LMA) and number of leaves of *Stevia rebaudiana* Bert. after 6 days exposure to chilling stress. Plants have been previously pretreated to either one of the following treatments: Control, non pre-treated plants; H_2O_2,10mM; SA,0.5mM; $CaCl_2$,10mM; BA,30µM. Data represented as the mean ± SE (n = 3). The values with different superscripts for three samples in each column are significantly different ($p < 0.05$, t-test).

3.2. Effects of chilling and chemical pretreatment on electron transport rate (ETR) and quantum photosynthetic yield (Y)

While the plant is undergoing the photosynthetic process at steady-state photosynthesis lighting conditions, Yield and ETR was measured as a light adapted test that allows to measure plant stress. As shown in Figure 2, Y and ETR in control plants exhibited a progressive decrease under chilling conditions. In pretreated plants Yield and ETR followed a non significant similar pattern of increase then decrease within the first 48h of exposure to chilling conditions. It's after 72h that significant differences among treatments were observed. ETR increased significantly ($P<0.05$) by 24.6%, 18.2% in plants respectively pretreated by 10 mM H_2O_2, and 30µM BA compared to control, pretreated plants with 0.5 mM SA, 10 mM CaCl2 showed a slight non significant decrease. At the end of experiment time pretreated plants kept a higher ETR than control. Yield in previously pretreated plants increased significantly ($P<0.05\%$) (Fig. 2) after 72h with respectively 24.59% , 14.75%, 14.75% and 18.03% in pretreated plants with 10mM H_2O_2, 0.5mM SA, 10mM $CaCl_2$ and 30 BA compared to the control but with no significant differences between pretreatments. Photosynthesis of leaves is directly determined by the efficiency of photochemical reaction. Among chlorophyll fluorescence variables, Crop yield correlates positively with activities of PSII and evaluate the transfer rate of photosynthesis electrons between PSI and PSII. It estimates the efficiency of transformation of light energy in PSII, which indicates the efficiency of primary light capture when the PSII reaction center was partially shut down. (Genty, 1989 Maxwel & Johonson, 2000). Electron transport rate (ETR), expresses the relative rate of electron transport through PSII, estimated from the product of PSII efficiency and absorbed light (Krall & Edwards, 1992). Differences between control and previously pretreated plants appears after 48h of exposure to chilling conditions showing an increase in Y and ETR in all pretreated plants. This result suggests that PSII reaction centers remains intact, it can open, capture the light energy for photochemical reactions, and start photosynthesis normally.

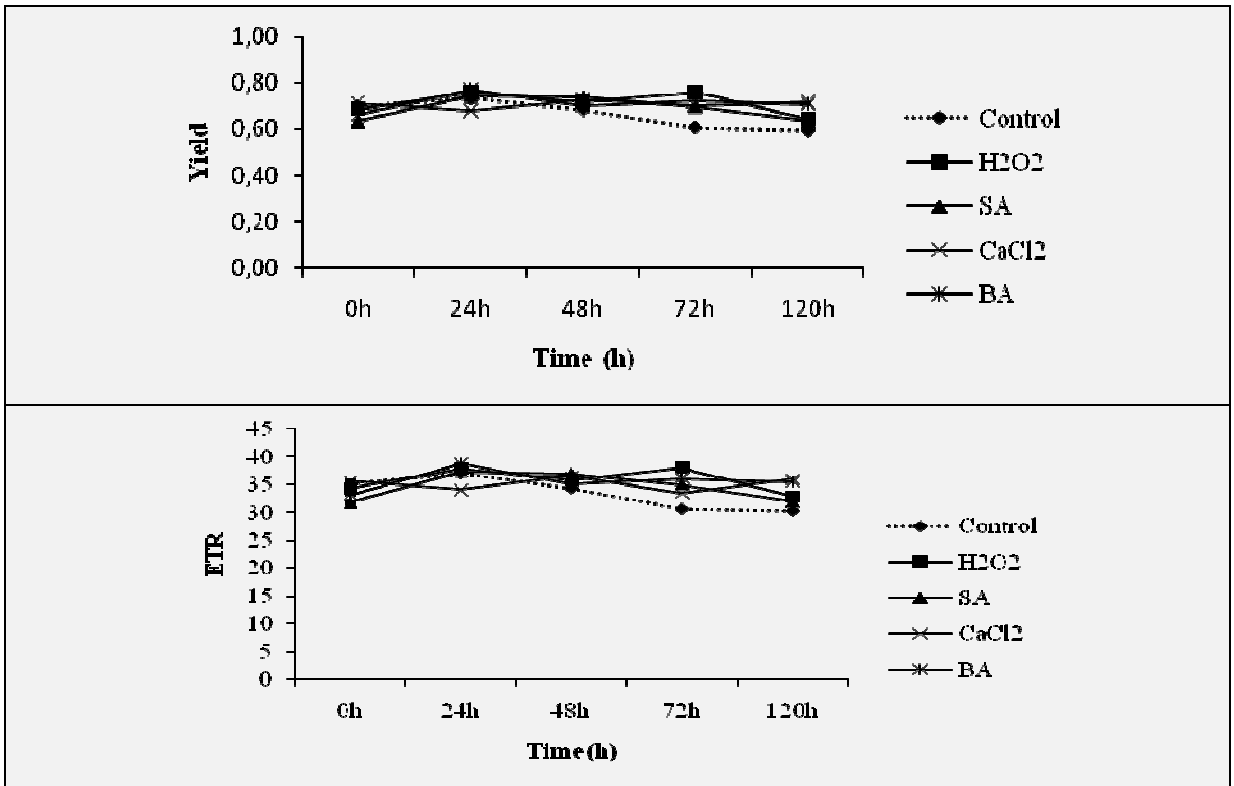

Figure 2. Effect of pre-treatment with SA, H_2O_2, $CaCl_2$ and 6-BA on electron transport rate (ETR) and quantum photosynthetic yield (Y) at 0, 24, 48, 72 and 120 h of chilling (10/6 °C, day/night) in *Stevia rebaudiana* Bert. Control plants were pre-treated with distilled water and sampled at the same time. Values expressed as means ± SE (n = 3).

3.5. Effects of chilling and chemical pretreatment on Photosynthesis pigments contents

As shown in Table 1, the contents of *Chl. a*, *Chl. b*, and carotenoids of *Stevia* sprayed leaves with H_2O_2, SA, $CaCl_2$ and BA were concomitantly affected ($p<0.05$) by exogenous pulverization of signal molecules and time of exposure. *Chl. a* content in leaves of *Stevia* pretreated plants with 10mM H_2O_2, 0.5 mM SA and 30µM BA prior to chilling was practically increased respectively with 29.17%, 33.6%, 58.36% in comparison to control plants. As chilling stress continued, *Chl.a* content in controls and pre-treated leaves was gradually decreased then increased. However, all pre-treated samples showed higher contents of *Chl.a* than controls during the cold stress (Table 1). Carotenoids content was also promoted using of signal molecules during stress span with an increase of 28.87% (H_2O_2), 5.63% (SA), 8.45% ($CaCl_2$) and 7.04 % (BA).

Table 1. Effects of pre-treatment with H2O2, SA, CaCl2, and BA on contents of photosynthetic pigments at 0, 24, 72 under chilling (10/6 °C, day/night) in*Stevia rebaudiana* Bert. Control plants were pre-treated with distilled water and sampled at the same time. Means ± SE (n = 3).

	Time (h)	Control	H_2O_2	SA	$CaCl_2$	BA
Chlorophyll a (mg g^{-1} FW)	0h	0.634	0.819	0847	0.616	1.004
	24h	0.494	0.615	0.501	0.375	0.636
	48h	0.773	0.516	0.775	0.499	1.034
	72h	0.889	1.216	1.145	1.131	0.633
	120h	1.161	1.524	1.254	1.253	1.164
Chlorophyll b (mg g^{-1} FW)	0h	0.378	0.209	0.363	0.631	0.428
	24h	0.324	0.364	0.358	0.255	0.377
	48h	0.373	0.334	0.368	0.368	0.409
	72h	0.373	0.605	0.445	0.468	0.261
	120h	0.432	0.540	0.473	0.486	0.537
Carotenoids (mg g^{-1} FW)	0h	0.090	0.077	0.116	0.117	0.129
	24h	0.084	0.087	0.084	0.060	0.098
	48h	0.104	0.082	0,107	0.080	0.117
	72h	0.114	0.164	0.144	0.155	0.078
	120h	0.142	0.183	0.150	0.154	0.152

4. Conclusion

In conclusion, our results have demonstrated that H2O2, SA, CaCl2, and 6-BA pre-treatments protected Stevia from cold stress. The enhanced chilling tolerance was associated in part to the increase of ETR and Y levels that played a primary role in protecting plants from adverse effects chilling stress. This result suggests that PSII reaction centers remains intact, it can open, capture the light energy for photochemical reactions, and start photosynthesis normally.

5. References

Arnon. D (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase Beta vulgaris. Plant Physiol 24:1-15.

Durner J, Shah J, Klessig D.F (1997) Salicylic acid and disease resistance in plants. Trends Plant Sci 2 : 266–274.

Franck F, Juneau P, Popovic R (2002) Resolution of Photosystem I and Photosystem II contributions to chlorophyll fluorescence of intact leaves at room temperature Biochim Biophys Acta 162: 239–246.

Krall J.P., Edward G.E (1992) Relationship between photosystem II activity and CO_2 fixation in leaves. Physiologia Plantarum. 86(1):180-187.

Genty B, Briantais J.M, Baker N.R (1992) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. -Biochim Biophys Acta 990:87–92.

Larkindale J, Huang B (2004) Thermotolerance and antioxidant systems in *Agrostis stolonifera*: involvement of salicylic acid, abscisic acid, calcium, hydrogen peroxide, and ethylene J Plant Physiol 161: 405–413.

Maxwell K, Johnson G.N (2000) Chlorophyll fluorescence practical guide J Exp Bot, 51: 659–668.

Shah F, Huang J, Cui K, Nie L, Shah T, Chen C, Wang K (2011) Impact of high temperature stress on rice plant and its traits related to tolerance. Journal of Agricultural Science Cambridge, 149: 545-556.

Malamy, J., Carr, J.P., Klessig, D.F (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection Science 250:1002–1004.

Sanghera, G. S., Wani, S. H., Hussain, W., Singh, N. B (2011) Engineering cold stress tolerance crop plants Current Genomics. 12: 30-43.

Sathiyaraj, G., Srinivasan, S., Kim, Y.J., Lee, O.R., Parvin, S., Balusamy, S.R., Khorolragchaa, A., Yang, D.C (2014) Acclimation of hydrogen peroxide enhances salt tolerance by activating defense-related proteins in Panax ginseng Molecular Biology Reports. 41(6):3761-71.

Torrecilas, A., Leon, A., Del Amor, F., Martinez-Mompean, M.C (1984) Determinación rápida de clorofila en discos foliares de limonero. Fruits. 39:617-622.

Mc Kinney, G (1941) Absorption light by chlorophyll solutions J. Biol. Chem. 140: 315-322.

Xin, Z., Browse, J (2000) Cold comfort farm: the acclimation of plants to freezing temperatures. Plant Cell and Environment. 23: 893-902.

Watanabe, T., Kume, T (2009) A general adaptation strategy for climate change impacts on paddy cultivation: special reference to the Japanese context. Paddy Water Environment. 7: 313-320.