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Research Article

The effects of cold stress on stevosides induction and protein electrophoretic pattern of *Stevia rebaudiana* Bertoni

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Abstract

***Stevia rebaudiana* Bertoni, (Asteraceae) is a well-known plant with its natural sweetening compounds named as steviosides. In the present work, Stevia plants was treated with 2 and 4 days of cold stress at 4 °C and then total stevioside level, content of certain steviosides and electrophotetic pattern of proteins were evaluated using spectrophotometry, HPLC and SDS-PAGE methods, respectively. The results indicated that cold treatment (4 days) caused a 40% increase in total sativoside content of *Stevia rebaudiana.* The results of HPLC analysis showed that the amount of Rebaudioside A, a known stevioside, increased significantly after 2 days of cold treatment as 70% more than control. The SDS- PAGE analysis of the plant proteins revealed that some protein bands density were increased in the cold treated groups of Stevia compared with control group. These bands might be related to heat chock proteins. From a practical point of view, cold treatment can elevate Rebaudioside A in Stevia cultivation. This compound has large applications in heart-healthy foods and debating cookies as food additive.**

Key words: *Stevia rebaudiana,* Steviosides, SDS- PAGE, Rebaudioside A

Introduction

Stevia rebaudiana Bertoni, (Asteraceae) is a perennial shrub indigenous to South America. Although, the plant is a sweet herb of Paraguay where it has a long history of use by the Guarani people, but it has cultivated in all over of the world for its valuable secondary metabolites (Hossein *et al.,* 2017). It produces diterpene glycosides known as steviosides or steviol glycosides,that are natural, low calorie and high potency sweeteners about 350 times sweeter than sucrose. These compounds can be used as a substitute of sucrose in food and pharmaceutical products and can be regarded as the best alternative source of sugar for diabetes patients (Lemus-Mondaca *et al.,* 2012). Stevioside and Rebaudioside A are the main sweetening compounds of Stevia. Stevioside with 9% and Rebaudioside A with 3.7% of dry weight are the major stevia glycosides (SGs) in wild Stevia. Other SGs (Rebaudioside A, Dulcoside, Rebaudioside C) totally contain only 5 % of dry weight (Goyal and Goyal, 2010).

It has been shown that stevia glycosides indicated different pharmacological and biological activities as antibacterial, antiseptic, anti-inflammatory, anti-fertility, hypotensive, diuretic and cardiotonic properties (Hossein *et al.,* 2017). However, physiological function of the steviosides in the stevia plant has not been exactly elucidated yet. In recent years, some possible reasons for making these large glycoside compounds in Stevia were proposed. It was assumed that this compounds can be indicated insects deterrent effects, to be carbon reservoir for the plant or regulate cell metabolism (Ceunen and Geuns, 2013). One of the other assumed functions of steviosides in the plant cell is the osmotic regulating role at different stresses conditions.

It is well known that climatic factors and agronomical practices change the quality and quantity of stevia glycosedes. The concentration of stevioside in the plant leaves is elevated when the stevia plants are grown at long photoperiods (long days condition) and when these plants are harvested before flowering. Time of harvesting can be related to habitat type, kind of stevia and growing season. The first harvest can be performed four months after planting and subsequent harvest once after every 3 months (Hossein *et al*., 2017). On the other hand, several previous researches implied on the effects of some abiotic stresses such as salinity and drought promoting osmotic pressure in the plants on de novo production of these compounds (Cantabella, 2017; Karimi *et al*., 2015). Moreover, it was previously reported that some elicitors like salicylic acid and hydrogen peroxide promote steviol glycosides production in the chilling imposed stevia plants (Soufi *et al*., 2016). Even so, it has not been focused on effects of cold stress on stevioside biosynthesis. The changes in leaf yield and accumulation of stevioside in response to different environmental conditions might led us to

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develop some strategies for increasing the productivity of the stevia. In the present study, the changes of steviosides content and SDS-PAGE electrophoretic bands of proteins in Stevia plants at cold conditions was evaluated.

Material and methods

Stevia growth condition and treatments: Stevia seedlings (*Stevia rebaudiana* var. bertoni) were purchased from Nahal Gostar Royan Company (Qazvin, Iran). The seedlings were cultured in pots (three seedling in each pot) contained coco peat and perlite (2:1) and irrigated with Hoagland solution (50%). The Stevia seedlings were grown at 25° C under 16/8 h (L/D) photoperiod with 85 µmol m^{-2} s^{-1} light intensity for 6 weeks and then subjected to cold treatment at 4º C. For cold treatment, the chamber temperature was reduced 5º C per hour until reach to 4º C. The experiment with completely randomized design was carried out with three replications in 3 groups of same age plants: Control (no treatment), 2 and 4 days of cold treatment. Then leaves were harvested and held in -80º C freezer for the analyses.

Total stevioside measurement: At first, dried leaves of *Stevia rebaudiana* (100 mg) were grounded and boiled in distilled water (5 ml) and then were filtered using Whatman filter paper. Then, the extracts were decolorized by activated charcoal. Finally, the samples UV absorption were measured at 210 nm, common λ max of steviosides, by spectrophotometer. The Rebaudioside A (Sigma) standard was used to draw the calibration curves (Moradi *et al*., 2018).

HPLC analysis of steviosides: For extraction of SGs, 0.1 g of frozen fresh samples were grounded with 1 ml doubled distilled water by mortar and pestle. Samples centrifuged at 8500 rpm for 15 min. Supernatant was collected and filtered through 0.2 µm filter. Then samples were injected to HPLC column. Determination of SGs were carried out using Sykam® HPLC apparatus (Germany) with analytical amino column, (Teknokroma®, Spain) 250 x 4.6 mm, particle size 5 µm, in isocratic mode. Mobile phase was 50: 50 Acetonitrile: Water; with flow rate of 0.8 ml min^{-1}. Temperature of column maintained at 25 ºC and UV detector adjusted at 205 nm. The concentration of glycosides was calculated using Stevioside and Rebaudioside A (Sigma) standard curve.

Extraction of total protein for electrophoresis: Stevia leaves were grounded in mortar and pestle with liquid N_2 and extracted in the extraction buffer (0.5 g homogenized plant matter in 1.5 ml extraction buffer). Extraction buffer contained 5 ml Tris-HCl (50 mM, pH7.5), 200 µL 1M Na_2EDTA and 200 µL of 2-mercaptoethanol (0.04%) in 100 ml distillated H_2O. Samples were vortexed for 15-20 sec. and centrifuged at 15000 rpm for 10 min. at 4º C. 800 µL of supernatant was mixed with 200 µl of sample buffer. For preparation of sample buffer (5X) 10 ml of Tris base buffer (2.42 g Tris-HCl base in 20 ml distillated H_2O, pH: 6.8), 5 ml of glycerol, 1 g SDS, 0.5 ml of Bromophenol blue (0.1% in ethanol) and 1ml of 2-mercaptoethanol were mixed and brought up to 20 ml with distilled water. SDS-PAGE electrophoresis conducted according to Laemmli (1970) method. The concentration of acrylamide stacking gel was 4% and resolving gel was 10%. The electrophoresis was performed for 3 hours at 145 V. The gel was stained with coomassie blue (R-250) for 60 min. Then destained in 10% methanol and 10% acetic acid. Bovain serum albumin (BSA) with molecular weight of 66 KDa, α S2 casein (25 KDa) and β casein (23 KDa) were used as markers. The electrophoretic bands of the gel were analyzed using Lab Works Ver4. Software.

Results

The results indicated that cold treatment (4 days) caused a significant increase (4%) in total sativoside content of *Stevia rebaudiana*. No significant changes in stavioside content were seen after 2 days of cold treatment (Figure 1). The results of HPLC analysis showed that the amount of glycoside Rebaudioside A increased significantly after 4 days of cold treatment, as well as. The elevation rate is approximately 70% than related control. In addition, there was no significant difference in the amount of Stevioside among the studied groups (Figure 2A). Chromatograms of the HPLC samples have also been presented (Figure 2B).

SDS- PAGE analysis of the plant proteins displayed that density of some protein bands were increased in the cold treated groups of Stevia compared with the control. Two electrophoretic bands with molecular weights higher than 66 KD, were strongly found in cold treated groups. This bands might be indicated molecular weights of 70-85 KD and were slightly detected in untreated control plants. Some other protein bands with lower molecular weights than 30 KD have been intensely seen in cold treated plants than control group, as well as (Figure 3, 4).

Discussion

The results of present works showed that cold treatment can trigger the biosynthesis of total steviosides, in particular Rebaduside A. There is some reports in the literature on possible roles of steviosides in *Stevia rebaudiana* Bertoni. This compounds may represent as an osmolyte for the plant at stressful conditions. It was previously shown that exposure of stevia to drought, salinity increased steviosides content in the plant cells (Cantabella, 2017; Karimi *et al*., 2015). Badran *et al.* (2015) used polyethylene glycol (PEG) for drought stress induction and reported increased production of stevioside sugars at 30000 ppm of PEG. In addition, Karimi *et al*. 2015 observed that reducing soil moisture to 60% field capacity, increased total steviosides significantly. Also, salt stress by NaCl or Na_2CO_3 promoted significant increase in Stevioside and

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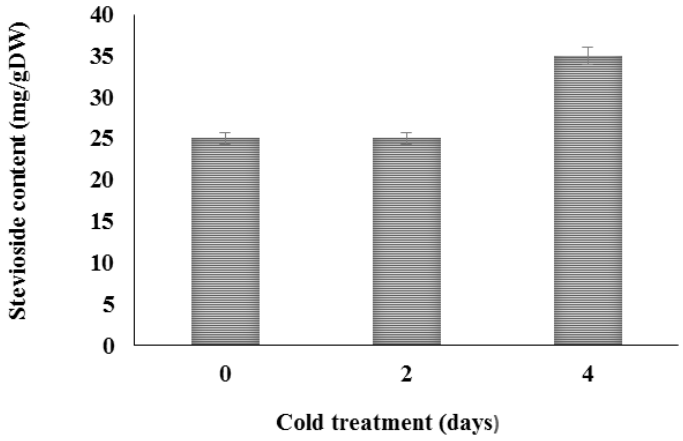

Figure 1. Change of total stevioside under cold treatment (2 and 4 days).

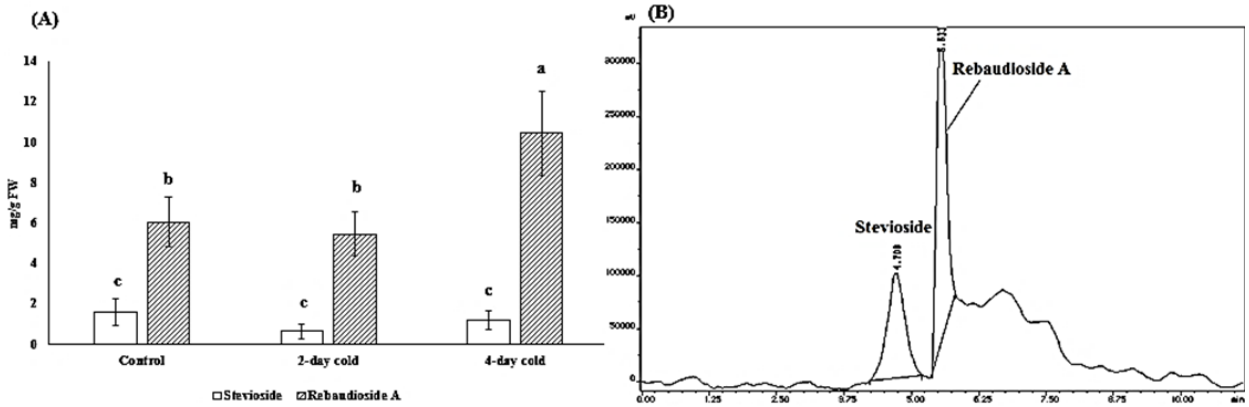

Figure 2. The changes of Steviol glycosides under cold treatment (2 and 4 days). Different letters represent a significant difference at P ≤ .05 (A). HPLC chromatogram of samples is also presented (B).

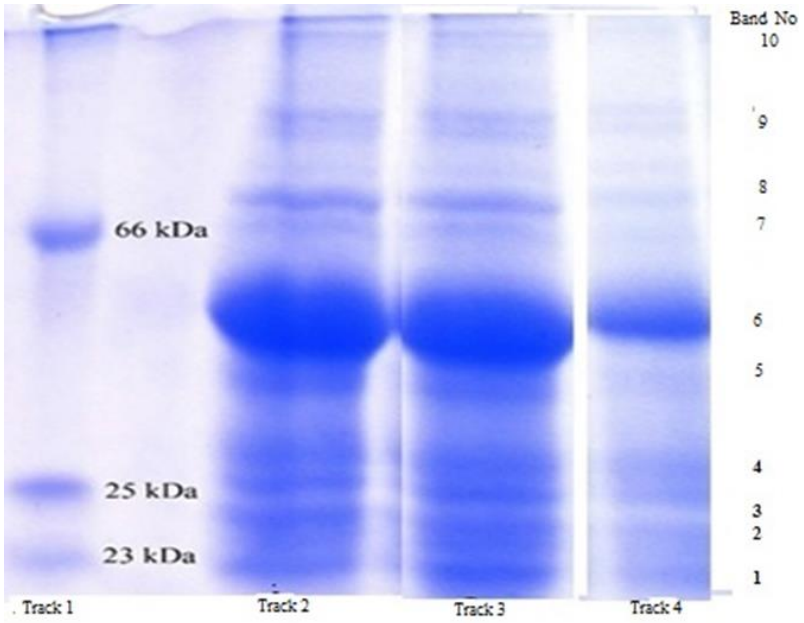

Figure 3. SDS PAGE of Stevia proteins under cold treatment. Track1: Markers: BSA (66 KDa), α S2 casein (25 KDa) and β casein (23 KDa), Track 2: Control, Track 3: 2 days cold treatment, Track 4: 4 days cold treatment.

Rebaudioside A in Stevia suspension culture (Gupta *et al*., 2014). The result of Cantabella *et al*. (2017) showed that salinity (2 g/L NaCl) caused an increase in Stevioside concentration by about 25% as well. The

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findings of the present study showed that cold stress significantly increased steviosides by 40%. Although cold conditions don't promote osmotic stress for plants, they do promote dehydration to plant cells in any way. Drought, salt or cold stresses cause many common physiological damages in plants by reducing the plants cells water potential. It was assumed that steviosides in the Stevia cells with many hydroxyl groups may elevate the cells osmotic pressure to maintain the cells water content under stressful conditions. It is well documented that under stressful conditions, plants maintain turgor pressure through biosynthesis and the formation of compounds such as proline, glycine betaine, free sugars, amino acids and other compatible osmolytes (Szabados and Savoure, 2010). Our previous work demonstrated that cold treatment on Stevia plants has no effects on the elevation of proline and glycine betain as common plants osmolytes (Moradi *et al*., 2018a). It led us to conclude that steviosides may carry osmoregulation duties, instead of proline or glycinbetaine in Stevia plants. Further investigation is needed to confirm this hypothesis. Pure Steviosides form hydrate composition with an indefinite number of H_2O molecules (Wood *et al*., 1955). These compounds have several glucoses on the Steviol structure with many hydroxyl groups making them strong water absorbents. Because more than 10% of the Stevia plant dry weight is dedicated to Steviosides, these compounds might adsorb a considerable amount of cell water and prevent water lost from the cytoplasm.

On the other hand, it was proposed that steviosides may have antioxidant activity in Stevia plants under stressful conditions. This mechanism can be regarded as another way to combat cold stress by scavenging reactive oxygen species in the Stevia plants. Some reports in the literature have shown the antioxidant capacity of stevia leaf or callus extract (Rao *et al*., 2014) measured by DPPH, ferric reducing ability and BHT methods. The DPPH radical scavenging activity of total stevioside was found to be 47.64% for 100 µg. Free radicle scavenging activity may protect the plant functional membranes like thylakoids from ROS induced damages at cold conditions. The steviosides molecular structure with many hydroxyl groups make predicts their antioxidant potential. In a previous work, we described that cold stress intensified antioxidant potential of Stevia plant extract along with increasing the plant stevioside content (Moradi *et al*., 2018b).

Moreover, Steviosides were also found to have ecological importance for Stevia plants. These compounds may act as a feeding deterrent agent to protect the plants from herbivorous insects (Urban *et al.*, 2013; Lowery, 2017). Another possible biological function of stevia glycosides in the Stevia plant could be attributed to the absorption of UV radiation (Chester *et al*., 2012; Jaworska *et al*., 2012; Schulz, 2015; Verdaguer, 2017).

Analyzing the SDS-PAGE gel of Stevia leaves proteins detected protein bands with a molecular weight below 100 KD that might be attributed to heat shock proteins (HSPs). Heat shock proteins play a role in protecting plant functional molecules against stress caused injuries. These proteins protect enzymes, proteins or biomembranes from the harmful effects of cold stress by acting as intra-cellular chaperones or by interfering with cellular protein–protein interactions. Our SDS-PAGE analysis revealed two electrophoretic bands with a molecular weight of 70-85 KD strongly appeared in the cold-treated Stevia groups but had slight intensity in control group. These bands might be related to class 3 of heat shock proteins, HSP70, which was found to have chaperon function in all the plants. The role of HSP 70 proteins as chaperones in chilling acclimation was previously documented in some other plants such as Tobbaco, Wheat and Ardabidopsis (Khan *et al*., 2021). On the other hand, some bands with a molecular weight lower than 30 KD might be related to class 5 of HSPs named as small heat shock proteins. This HSPs binds to partially folded or denatured substrate proteins and performs the degradation of the proteins that do not have the ability to refold. This process prevents irreversible unfolding or incorrect protein aggregation (Al-Whaibi, 2011). It was previously shown that small molecules HSPs indicated a key role in cold tolerance in some plants such as carrot and that their related genes were upregulated under cold conditions (Song and Ahn, 2010). The protective role of small HSPs against cold of stress has been rapidly studied in some crops such as wheat (Khan *et al*., 2021).

In spite of the fact that *Stevia rebaudiana* is native to warm climates, our previous reports demonstrated that the plant exhibit some degree of tolerance to chilling temperature (Moradi *et al*., 2018). This chilling tolerance might be attributed to presence of steviosides proposed to act as osmo-resultant or play antioxidant role for protection of functional photosynthetic membranes against harmful reactive oxygen species under cold condition. The tolerance may be concerned to heat shock proteins that support biomolecules as chaperones under cold stress, as well as.

Conclusion

It was concluded that cold stress intensifies the biosynthesis of some stevioside diterpens such as Rebaudioside A. Although Stevia is indigenous to warm climates, its cultivation at temperate regions with chilly temperatures in some seasons can tend to overproduction of steviosides with large applications in heart-healthy and debating cooking as food additives. Stevia plants with some mechanisms such as the synthesis of heat shock proteins may indicate some degree of tolerance against chilling condition.

References

[DOR: 20.1001.1.23222727.1402.12.55.7.9]

Al-Whaibi, M. (2011). Plant heat-shock proteins: A mini review. *Journal of King Saud University* - Science *23*(2), 139-150. https://doi.org/10.1016/j.jksus.2010.06.022.

Badran, A.E., Alhady, M.R.A., & Hassan, W.A. (2015) In vitro evaluation of some traits in Stevia rebaudiana (Bertoni) under drought stress and their relationship on stevioside content. *American Journal of Plant Sciences* 6*(5)*, 746-750. https://doi.org/10. 4236/ajps.2015.65080

Cantabella, D., Piqueras, A., Acosta-Motos, J.R., Bernal-Vicente, A., Hernández, J.A., & Díaz-Vivancos P. (2017). Salt-tolerance mechanisms induced in Stevia rebaudiana Bertoni: Effects on mineral nutrition, antioxidative metabolism and steviol glycoside content. *Plant Physiology and Biochemistry 115*(3), 484-496. https:// doi: 10.1016/j.plaphy.2017.04.023.

Ceunen, S., & Geuns, J.M.C. (2013). Glucose, sucrose, and steviol glycoside accumulation in Stevia rebaudiana grown under different photoperiods. *Biologia plantarum 57*(2), 390-394. https:// doi: 10.1007/s10535-012-0289-6.

Chester, K., Tamboli, E.T., Singh, M., & Ahmad, S. (2012). Simultaneous quantification of stevioside and rebaudioside A in different stevia samples collected from the Indian subcontinent. *Journal of Pharmacy & Bioallied Sciences 4*(4), 276-282. https:// doi: 10.4103/0975-7406.103236

Goyal S.K., & Goyal R.K. (2010). Stevia (Stevia rebaudiana) a bio-sweetener: a review. *International journal of food sciences and nutrition 61*(1), 1-10. https:// doi: 10.3109/09637480903193049.

Gupta, P., Sharma, S., & Saxena, S. (2014). Effect of salts (NaCl and Na 2 CO 3) on callus and suspension culture of Stevia rebaudiana for steviol glycoside production. *Applied biochemistry and biotechnology 172*(6), 2894-2906. https:// doi: 10.1007/s12010-014-0736-2.

Hossein, F., Islam, M. T. Islam M. A., & Akhtar, S. (2017). CULTIVATION AND USES OF STEVIA (Stevia rebaudiana Bertoni): A REVIEW. *African Journal of Food, Agriculture, Nutrition and Deveplment 17*(4), 12745-12757. https:// doi: 10.18697/ajfand.80.16595

Jaworska, K., Krynitsky, A. J., & Rader, J. I., (2012). Simultaneous analysis of steviol and steviol glycosides by liquid chromatography with ultraviolet detection on a mixed-mode column: application to Stevia plant material and Stevia-containing dietary supplements. *Journal of AOAC International 95*(6), 1588-1596. https:// doi: 10.5740/jaoacint.11-435.

Khan, S., Jabeen, R., Deeba, F., Waheed, U., Khanum, P., & Iqbal, N., (2021). Heat shock proteins: classification, functions and expressions in plants during environmental stresses. *Journal of Bioresource Management 8* (2), 85-97. https://doi.org/10.35691/JBM.1202.0183

Karimi, M., Ahmadi, A., Hashemi, J., Abbasi, A., Tavarini, S., Guglielminetti, L., & Angelini, L.G. (2015). The effect of soil moisture depletion on stevia (*Stevia rebaudiana* Bertoni) grown in greenhouse conditions: Growth, steviol glycosides content, soluble sugars and total antioxidant capacity. *Scientia Horticulturae 183*(1), 93-99.

Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L., Ah-Hen, K. (2012). Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry 132*(3), 1121–1132. https:// doi: 10.1016/j.foodchem.2011.11.140.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259), 680-5.

Lowery, H. (2017). Insects Associated with *Stevia rebaudiana* Bertoni (Bertoni), and Toxicity of Compounds from *S. rebaudiana* against Spodoptera frugiperda (JE Smith) Larvae. A MSc thesis, University of Tennessee, Knoxville.

Moradi Peynevandi, K., Razavi, S.M., & Zahri, S. (2018). The ameliorating effects of polyamine supplement on physiological and biochemical parameters of Stevia rebaudiana Bertoni under cold stress. *Plant Production Science 21*(2), 123-131. https:// 10.1080/1343943X.2018.1437756.

Moradi Peynevandi, K., Razavi, S.M., & Zahri, S. (2018). The investigation on effects of polyamine supplement and cold on some phytochemical parameters of Stevia, a medicinal plants. *Ecophytochemistry of medicinal plants 20*(4), 55-64.

Rao, G.N., Rao, P.P., Balaswamy, K., & Satyanarayana, A. (2014). Antioxidant Activity of Stevia (*Stevia rebaudiana* L.) Leaf Powder and A Commercial Stevioside Powder. *Journal of Food and Pharmaceutical Science 2*(1), 32-38. https://doi.org/10.14499/jfps.

Schulz, E., Tohge, T., Zuther, E., Fernie, A.R., & Hincha, D.K. (2015). Natural variation in flavonol and anthocyanin metabolism during cold acclimation in Arabidopsis thaliana accessions. *Plant, cell & environment 38*(8), 1658-1672. https://doi: 10.1111/pce.12518.

Song, N.H., & Ahn, Y.J. (2010). cHsp17.7, a small heat shock proteinfrom carrot, Is Upregulated under cold stress and enhances cold tolerance by functioning as a molecular chaperone. *Horticulture Science 45*(3), 469–474.

Soufi, S., ، Urso، G.D., Pizza, C., ، Rezgui, S., ،Bettaieb, T., & Montoro, P. (2016). Steviol glycosides targeted analysis in leaves of *Stevia rebaudiana* (Bertoni) from plants cultivated under chilling stress conditions. *Food chemistry 190*(1), 572-580. https**:**//doi: 10.1016/j.foodchem.2015.05.116.

Szabados, L., & Savoure, A. (2010). Proline: a multifunctional amino acid. *Trends in Plant Science 15*(2), 89-97. https://doi: 10.1016/j.tplants.2009.11.009.

[DOR: 20.1001.1.23222727.1402.12.55.7.9]

Verdaguer, D., Jansen, M.A., Llorens, L., Morales, L.O., & Neugart, S. (2017). UV-A radiation effects on higher plants: Exploring the known unknown. *Plant Science* 255(1), 72-81. https:// doi: 10.1016/j.plantsci.2016.11.014.

Urban, J.D., Carakostas, M.C., & Brusick, D.J. (2013). Steviol glycoside safety: Is the genotoxicity database sufficient. *Food and Chemical Toxicology* 51(1), 386–390. https://doi.org/10.1016/j.fct.2012.10.016.

Wood, J.R., Allerton, R., Diehl, H.W., Hewitt, G., & Fletcher, J.R. (1955). Stevioside. I. The structure of the glucose moieties. *The Journal of Organic Chemistry* 20(7), 875-883. https://doi.org/10.1021/jo01125a012.

[DOR: 20.1001.1.23222727.1402.12.55.7.9]