I J T A
© Serials Publications
National Academy of Agricultural Science (NAAS) Rating : 3.03

Polyhouse Cultivation of *In vitro* Raised elite *Stevia rebaudiana* Bertoni: An Assessment of Biochemical and Photosynthetic Characteristics

Hemant Lata[1], Suman Chandra[1], Yan-Hong Wang[1], Mahmoud A. ElSohly[1,2] and Ikhlas A. Khan[1,3]

ABSTRACT: *Polyhouse cultivated Stevia rebaudiana Bertoni plants, initially raised from synthetic seeds, were assessed for biochemical and photosynthetic characteristics and compared with their mother plant.Synthetic seeds were produced using nodal segments containing single axillary buds excised from in vitro proliferated shoots with 5% sodium alginate and 50 mM $CaCl_2$. The synthetic seeds were stored at 25°C for 4 months and re-generated under the tissue culture conditions (16-h photoperiod, 25°C) on Murashige and Skoog (MS) medium supplemented with thidiazuron (TDZ 0.2 mgL^{-1}). Well-developed in vitro regenerated plantlets were successfully acclimatized inside the climatic controlled growing room and finally cultivated under the polyhouse conditions. Fully developed plants were compared with each other and with mother plant for their chlorophyll (Chl) and carotenoids (Car) contents, photosynthetic and water vapor exchange characteristics, and dulcoside A, rebaudioside A, Rebaudioside D, steviobioside and stevioside content. Our data shows that regenerated plants were highly comparable to mother plants in terms of their photosynthetic pigment contents and gas and water vapour exchange characteristics. High-performance liquid chromatography (HPLC), was used to analyze major secondary metabolites in Stevia plants. Dulcoside A, Rebaudioside D and steviobioside content was found below the level of detection, whereas, no significant differences ($p< 0.05$) in rebaudiosideA and steviosidecontent between the mother and regrown plants following four months of in vitro storage was observed. These results confirm clonal fidelity of S. rebaudiana plants derived from synthetic seeds following in vitro storage.*

Keywords: *Stevia rebaudiana, Stevioside, Rebaudioside A, In vitro propagation*

INTRODUCTION

Stevia rebaudiana Bertoni is a herbaceous perennial plant belonging to the familyAsteraceae. It is native to Paraguay, where it grows wild in sandy soils (Katayama *et al.*, 1976). Today its cultivation has spread to other regions of the world, including Canada and some parts of Asia and Europe (Amzad-Hossain *et al.*, 2010; Gardana *et al.*, 2003). Presently, Stevia is well-known for its high content of sweet diterpene (about 4–20%) in dry-leaf matter (Ghanta *et al.*, 2007). It is the source of a number of diterpenoid glycosides (Prakash *et al.*, 2008), the compounds responsible for the sweet taste. These glycosides are up to 300 times sweeter than sugar that can be extracted for use as a nonnutritive sweetener (Soejarto *et al.*, 1982; Chalapathi and Thimmegowda 1997; Liu and Li 1995). Among the 230 species in the genus Stevia, only the species *rebaudiana* and *phlebophylla* areknown to produce steviol glycosides (Brandle and Telmer 2007).

Stevia is a rapidly emerging crop in the US and other parts of the world as more and more companies look to find improved Sugar substitutes. The main producers of Stevia are Japan, China, Taiwan, Thailand, Korea, Brazil, Malaysia and Paraguay. Currently, Stevia is consumed in Japan, Brazil, Korea, Israel, the United States of America, Argentina, China, Canada, Paraguay and Indonesia (Crammer and Ikan 1986; Singh and Rao 2005) and to date there have been no reports of adverse effects from its use (Kinghorn and Soejarto 1985; Brandle and Rosa 1992). The US is the largest single market for high purity stevia extracts at approximately 300 metric tons of finished product annually. This was approximately 65% of the global market for high purity Stevia extracts in 2011.

[1] National Center for Natural Product Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, MS 38677, USA.

[2] Department of Pharmaceutics, School of Pharmacy, The University of Mississippi, University, MS 38677, USA.

[3] Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, University, MS 38677, USA.

The health benefits of *Stevia* are evident from the fact that they are approved as a dietary supplement by the Food and Drug Administration (FDA). Also other major global regulatory organizations, including the Food and Agriculture Organization/ World Health organization's Joint Expert Committee on Food Additive (JECFA), the European Food Safety Authority (EFSA), and Food Standards Australia New Zealand (FSANZ), have determined high purity stevia extract to be safe for use.

Propagation of Stevia by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition (Nakamura and Tamura 1985). Moreover, seed germination is verypoor, commonly due to infertile seed (Felippe and Lucas 1971). Vegetative propagation by stem cutting is limited and requires enough stocks of stem cuttings (Sakaguchi and Kan 1982; Carneiro *et al.*, 1997). Thus, the development of an efficient alternative method for mass micropropagation of *S. rebaudiana* Bertoni is important for large-scale plant production. A number of protocols for *in vitro* propagation of this species have been described during recent years (Ahmed *et al.*, 2007; Debnath 2008; Satpathy and Das 2010). In our laboratory, we have successfully developed an efficient high frequency *in vitro* regeneration protocol of elite *Stevia rebaudiana* using Thidiazuron-TDZ (Lata *et al.,* 2013) and have further developed an efficient conservation protocol to store high yielding *S. rebaudiana* germplasm using synthetic seed technology (Lata *et al.,* 2014). Since *S. rebaudiana* is considered as an important medicinal plant of large industrial and therapeutical value and also based on horticultural and pharmacological use, it is very important to confirm the quality of the plantlets for its commercial utility. The present work reports assessing the clonal fidelity of polyhouse cultivated *S. rebaudiana* plants derived from synthetic seeds following *in vitro* storage, based on their gas and water vapour exchange characteristics, pigments content and quantitative analysis of rebaudiosideA and stevioside using HPLC.

MATERIALS AND METHODS

Plant Material and Explant Source

Nodal segments from a one-year-old stevia plant were used to establish *in vitro* cultures. Explants were surface disinfected using 0.5% NaOCl (15% v/v bleach) and 0.1% tween 20 for 20 min prior to inoculation on the culture medium. High-frequency shoot regeneration was achieved on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3 % (w/v) sucrose and 0.8 % (w/v) type-E agar supplemented with 0.2 mg/l thidiazuron (TDZ) and adjusted to pH 5.7 (Lata et al., 2013). The sub-culturing was done every three weeks on sterile medium dispensed (25 ml) in glass culture vessels (4 cm diameter × 9.5 cm high, glass jars with magenta B caps). All cultures were incubated at 25 ± 2°C with 16 h photoperiod under fluorescent light with a photon flux of ~ 52 μmol $m^{-2} s^{-1}$.

Encapsulation/Formation of Synthetic Seeds

Encapsulation of explants was done using hydrogel (sodium alginate 5%) and complexing agent ($CaCl_2.2H_2O$ 50 mM). Sodium alginate was prepared by dissolving in full strength Murashige and Skoog's medium (MS). The complexing agent was prepared in distilled water. Both the solutions were autoclaved separately for 15 min at a pressure of 1.1 kg cm^{-2} and temperature of 121 °C. The beads were formed by dropping explants mixed with sodium alginate solution into $CaCl_2.2H_2O$ in a flask, placed on an orbital shaker at 80 rpm. The resulting beads (0.5-0.8 cm in diameter) containing the entrapped nodal segments were left in the calcium chloride solution for 30 min for complexation. These were retrieved using a nylon mesh and the traces of calcium chloride were removed by washing with sterilized distilled water. The encapsulated nodal explant is now called as synthetic seeds.

Storage and Re-growth

The encapsulated (synthetic seeds) nodal segments were stored at 25°C for 4 month storage period. After 4 months of storage, the 'encapsulated' segments were inoculated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with thidiazuron (TDZ 0.2 mg L^{-1}, Lata *et al.*, 2014) and monitored for the re-growth and survival frequency under the tissue culture conditions (~52 μmol $m^{-2} s^{-1}$ Photosynthetic photon flux density, 16-h photoperiod, 25°C). Following Lata *et al.*, 2013, proliferated shoots were rooted on ½ MS without any growth regulator.

Transfer of Plants to Soil for Acclimatization and Growth

Well rooted *in vitro* propagated plants were carefully taken out of the medium and washed thoroughly in running tap water to remove all traces of medium attached to the roots without damaging the roots.

Plantlets were preincubated in coco natural growth medium (Canna Continental, Los Angeles, CA) in thermocol cups for 10 days. The cups were covered with poly ethylene bags to maintain humidity and kept in growroom and later acclimatized in sterile potting mix- fertilome (Canna Continental, Los Angeles, CA) in large pots. All these plantlets were kept under similar environmental conditions grown in an indoor cultivation facility at The Coy Waller laboratory, University of Mississippi. Light, 16 hour photoperiod, was provided with full spectrum 1000 watts HID (high density discharge) lamps (Sun Systems, CA) hung 3-4 feet above the plants. The grow room temperature and relative humidity were kept nearly constant at 25°C and 60% respectively. Plants were watered regularly and individually to maintain sufficient moisture content in the pots. Fully acclimatized plantlets were finally transferred to a polyhouse for further cultivation.

Photosynthetic Measurement

After a desirable growth in polyhouse, fully developed synthetic seed raised plants and the mother plant were compared for their gas and water vapour exchange characteristics. All the photosynthetic measurements were made on intact leaves at their peak vegetative stage (just before initiation of flowers). Measurements were carried out on three upper undamaged, fully expanded and healthy leaves of five randomly selected tissue culture raised plants and their mother plant with the help of a closed-circuit portable infrared gas analyzer (IRGA, Model LI-6400; LI-COR, Lincoln, Nebraska, USA). Leaves were exposed to 1600 mmol $m^{-2}s^{-1}$ photon flux densities (PPFD) level under the controlled temperature (25 ± 1 °C), humidity (55 ± 5 %) and CO_2 (360 ± 5 mmol mol^{-1}) conditions. Light was provided with the help of an artificial light source (Model LI-6400-02; light emitting silicon diode; LI-COR), fixed on top of the leaf chamber and recorded with the help of quantum sensor kept in the range of 660-675 nm, mounted at the leaf level. Temperature of the cuvette was controlled by integrated peltier coolers, which is controlled by the microprocessor. Control CO_2 concentration was supplied to the cuvette of climatic unit (LI-6400-01, LI-COR Inc., USA) by mixing pure CO_2 with CO_2 free air and were measured by infrared gas analyzer. Air flow rate (500 mmol s^{-1}), temperature, humidity, CO_2 concentration and relative humidity were kept constant throughout the experiment. Since steady state photosynthesis is reached within 30–45 min, the leaves were kept for about 45–60 min. under each set of light conditions before the observations were recorded.

Four gas exchange parameters viz., photosynthetic rate (P_N), transpirational water loss (E), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were measured simultaneously at steady state condition under the each light level. Water use efficiency (*WUE*) was calculated as a ratio of the rate of photosynthesis and transpiration. Mean values (± SD) of each parameters for mother plant (n = 9) and *in vitro* propagated plant (n = 15) were used for comparison.

Pigments Content

Leaves used for the photosynthetic gas exchange measurements were later harvested for the determination of pigments content. Chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (total Chl), chlorophyll a/Chlorophyll b (Chl a/Chl b ratio), carotenoid (Car) and total chlorophyll/Carotenoid (Chl/Car ratio) were measured following the method of Hiscox and Israelstam (1979).

Analysis of Secondary Metabolite Content

Leaves of *S. rebaudiana* were harvested just before flowering. Leaves were air dried and powdered for the chemical analysis. Two hundred milligram dry powdered samples were weighed and sonicated in 2.5 mL of 80% methanol (MeOH/water = 80:20, v/v) at room temperature for 30 min followed by centrifugation for 10 min at 4000 rpm. The supernatant was transferred to a 10 mL volumetric flask. The procedure was repeated three times and respective supernatants combined. The final volume was adjusted to 10 mL with 80% methanol and mixed thoroughly. Prior to injection, an adequate volume (ca. 2 mL) was passed through a 0.45 µm nylon membrane filter. The first 1.0 mL was discarded and the remaining volume was collected in a LC sample vial.

Samples were analyzed for five different compounds, dulcoside A, rebaudioside A, Rebaudioside D, steviobioside and stevioside content. Standards of dulcoside A, rebaudioside A, Rebaudioside D, steviobioside and stevioside contentwere isolated at NCNPR, the identity and purity was confirmed by chromatographic (TLC, HPLC) methods and by the analysis of the spectroscopic data (IR, 1D- and 2D-NMR, HR-ESI-MS). All HPLC analyses were applied on Waters Alliance 2695 HPLC system (Waters Corp., Milford, MA, USA) and a computerized data station using

Waters Empower 2 software. A reverse phase Gemini C18 column (250 × 4.6 mm i.d., 5 μm particle size) from Phenomenex (Torrance, CA, USA) was used as the stationary phase and the temperature was maintained at 40 °C. The column was equipped with a 2 cm LC-18 guard column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of water (A), acetonitrile/methanol (B), both containing 0.1% phosphoric acid. Analysis was performed using the following linear gradient elution at a flow rate of 0.7 mL/minute: 0-15 min, 58% A/42% B to 42% A/52% B; 15-20, 42% A/52% B to 100% B; 20-25 min, 100% B. The total run time was 25 min. Each run was followed by a 5 min wash with 100% acetonitrile and an equilibration period of 15 min. Ten microliters of sample was injected and detective wavelength was 205 nm. Peaks were assigned by spiking the samples with standard compounds, comparison of UV spectra and the retention times.

Statistical Analysis

Statistical analysis of the data with the level of significance set at 5% was done by agricolae module using statistical software "R" version 2.2.1 (2005).

RESULTS AND DISCUSSION

Fig. 1 shows a schematic diagram of experimental design for the assessment of biochemical and photosynthetic characteristics of polyhouse grown, synthetic seed raised plants of *S. rebaudiana*and their comparison with the mother plant. Synthetic seeds were formed using nodal explants from established Stevia cultures, stored up to 4 months at 25°C at culture condition, regrown and transferred to polyhouse with mother plant for further cultivation. Reliability of *in vitro* propagation protocol and storage was tested by comparing *in vitro* raised plants with the mother plant in terms of their gas and water vapour exchange characteristics, pigments content and useful secondary metabolites.

As the popularity of *Stevia* as an alternative sweetener depends on the quality of products, selection of superior genotypes based on chemical activity is of interest. In our laboratory, we have masspropagated and conserved elite Stevia cultivars using micropropagation techniques (Fig. 2). For the present study, *in vitro* propagated *S. rebaudiana* plantlets were produced according to a protocol described by Lata *et al.,* (2013). Nodal segments (3-5 mm) excised from *in vitro* proliferated shoots were used as explants for making synthetic seeds (Lata *et al.,* 2014). The encapsulated (synthetic seeds) nodal segments were stored at 25°C for 4 months and monitored for the re-growth and survival frequency under the tissue culture conditions (16-h photoperiod, 25°C) on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with thidiazuron (TDZ 0.2 mg L^{-1}). After 4 months of storage at 25°C about 88% plant survival was observed in MS supplemented with 0.2 mg L^{-1} TDZ medium. The regrowth from encapsulated cultures in MS medium with TDZ was higher than those produced in MS basal medium without TDZ. Proliferated shoots were rooted on ½ MS without any growth regulator. The rooted plantlets were successfully established in soil and grown to maturity at the survival rate of 98% in the climate controlled indoor grow room conditions. Well-developed rooted plants were transferred to polyhouse for further mass scale cultivation (Fig. 3).

Safety and efficacy are the most important factors in production of food or food ingredient. Therefore, consistency in the starting material is of utmost importance. The aim of the present study was to assess

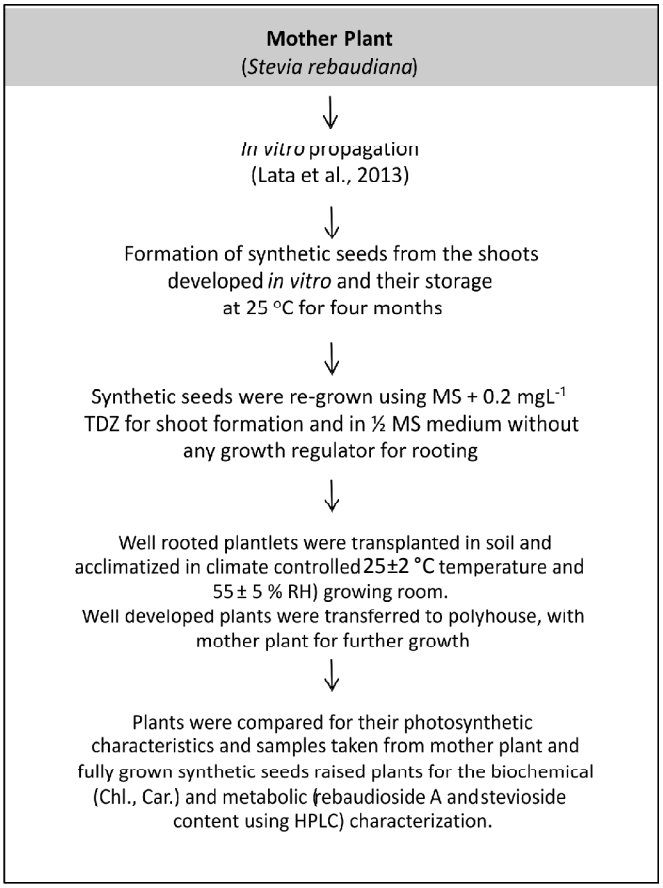

Figure 1: Schematic diagram of biochemical and metabolic analysis of the mother plant and synthetic seeds raised *S. rebaudiana* plants following *in vitro* storage

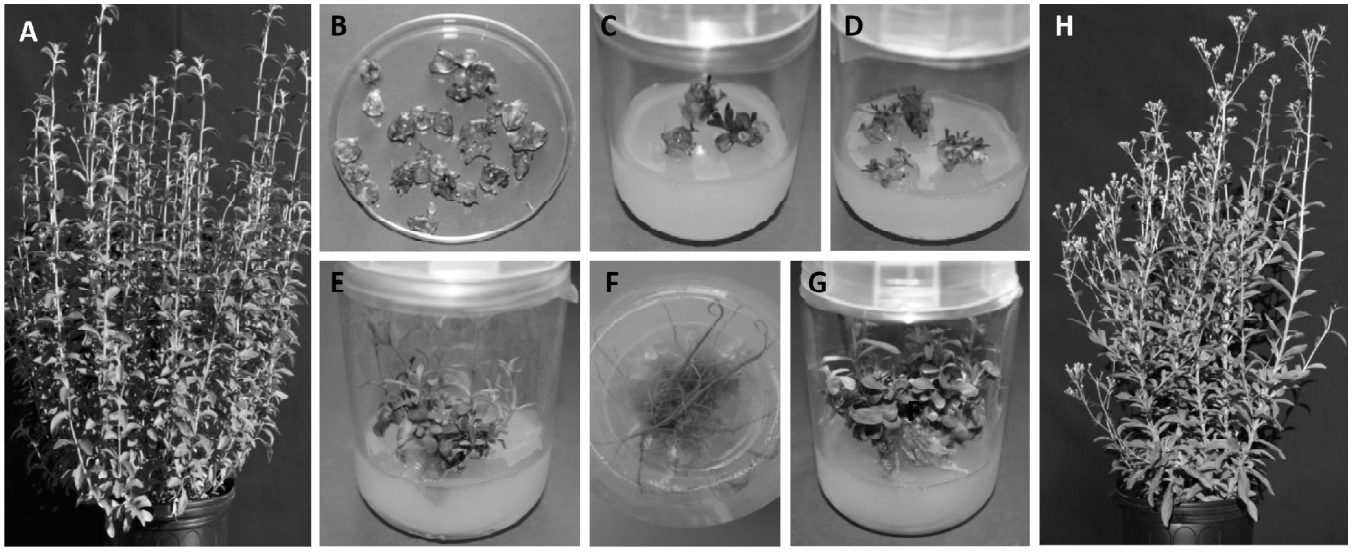

Figure 2: Propagation of *S. rebaudiana* from synthetic seeds following *in vitro* storage. A: Mother plant, B: synthetic seeds, C, D and E: formation of multiple shoots, F: roots formation, G: well rooted plant and H: a fully acclimatized synthetic seed raised *S. rebaudiana* plant

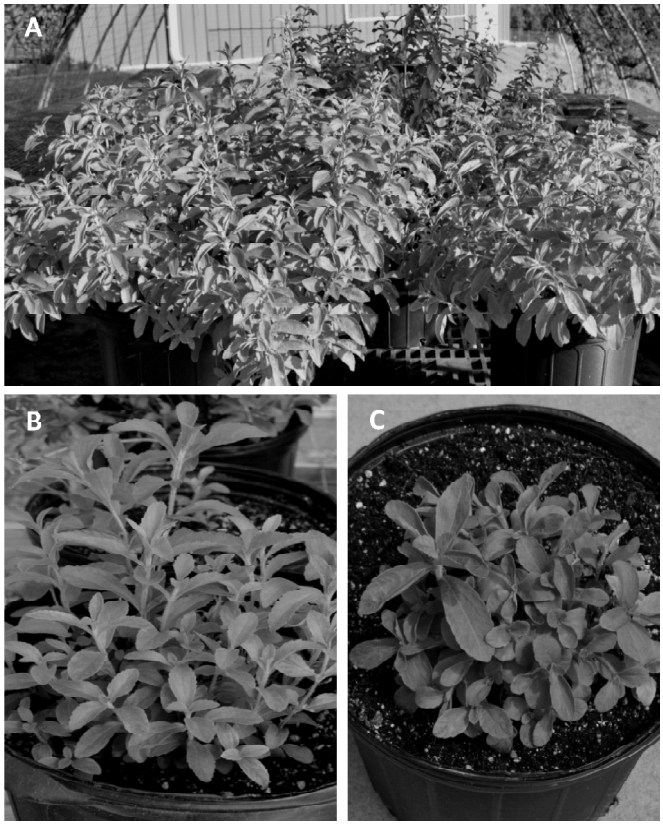

Figure 3: Polyhouse cultivation of *Stevia rebaudiana* Plant

the eco-physiological characteristics and useful secondary metabolites in *invitro* raised plants and their comparison with mother plant for the quality assurance (Fig. 4).The rate of photosynthesis (P_N) was measured on three healthy fully developed upper leaves of five randomly selected *in vitro* raised plants and the mother plant at peak vegetative stage, before the plants were harvested. The average rate of photosynthesis in *in vitro* raised plants was observed 16.18± 0.98 whereas, it was 15.89 ± 1.20 in the mother plant. Similarly, *E* and gCO_2 in *in vitro* raised plants (6.32 ±0.80 and 104.00 ± 6.98, respectively) and the mother plant (6.1 ± 0.60 and108 ± 5.20, respectively) were found highly comparable to each other. Gas and water vapour exchange characteristics provides valuable information on the functioning of plants and, their growth and productivity in a particular environment (Chandra et al., 2008; 2011a; 2011b; 2015).Variation in photosynthetic characteristics of two different species or two different varieties of a particular species in a similar environmental condition may be due to their different genetic makeup (Rawat and Purohit 1991). In this study, *in vitro* raised plants show a highly comparable gas and water vapour exchange characteristics to their mother plant, thereby reflecting their genetic fidelity.The rate of diffusion of CO_2 between leaf tissues and environment is largely controlled by (a) stomatal conductance and (b) CO_2 concentration gradient between carboxylation site and environment. The CO_2 concentration gradient at a given conductance and ambient CO_2 concentration (C_a) is stabilized predominantly by 'inter cellular CO_2concentration (C_i)' which reflects the intrinsic mesophyll efficiency of a plant (Joshi and Palini 1998). Our data on mother plant and *in vitro* raised plants on C_i (224 ± 5.21 and 228 ± 7.98, respectively) and C_i/C_a ratio (0.62 ± 0.05

and 0.61 ± 0.06, respectively) were found highly comparable to each other. Water use efficiency (*WUE*) was calculated as a ratio of $P_N$and *E*. In the present study, average *WUE* was found comparable among *in vitro* raised plants (2.70 ± 0.30) and to the mother plant (2.56 ± 0.40), no significant differences were found between two types of plants ($p < 0.05$).

Leaves used for the measurement of P_N were harvested and used for analysis of pigment content (Fig. 5). Values of Chl a, Chl b, total Chl and Chl a/b ratio were found slightly higher in mother plant whereas, the values of Carotenoids content were found slightly higher in *in vitro* raised plants. The differences however, were found statistically significant ($p < 0.05$).

Fig. 6 shows the Chemical structures of major diterpene glycosides, rebaudioside A and stevioside, in *Stevia rebaudiana* Bertoni. Quantitative analysis of rebaudioside A and stevioside in the samples of *in vitro* raised stevia plants and the mother plant was performed using HPLC method following Wang *et al.*, 2015 (Fig. 7). In Stevia, different plant parts contain different amount of diterpene glycoside i.e. stevioside and rebaudioside (Yadav *et al.* 2011). According to the report by Moraes *et al.*, 2013, the highest amount of diterpene glycoside content is present in leaves followed by flowers, stem and roots. Furthermore, glycoside content is highest at peak vegetative stage, just before flowering. Therefore, in the present study biomass samples (leaves) were harvested just before flowering stage. Samples were taken from nine randomly selected *in vitro* propagated plants and mother plant at peak vegetative growth stage. Among the *in vitro* propagated plants highest stevioside content was found in clone ID: S-8 (5.68%) whereas, the lowest stevioside content was detected in clone ID: S-2 (5.53%). The average stevioside content in the samples from mother plant was 5.59%. On other hand, highest the Rebaudioside content varied between 0.65 and 0.57% (Clone ID: S-6 and S-2, respectively) among *in vitro* propagated plants whereas, it was 0.65 ± 0.02% in the samples taken from the mother plant. However, these minor differences among *in vitro* raised plants and the mother plant were found statistically insignificant ($p<0.05$).

In conclusion, based on our results, micropropagated plants were found to be highly comparable to the mother plant thus, confirming the clonal fidelity of *in vitro* propagated plants of *S. rebaudiana*. This suggests that the biochemical mechanism used to produce the micropropagated plants does not affect the ecophysiological functioning and the metabolic content of these plants. The protocol therefore can be used for propagation of true to type plants of *Stevia* for conservation and mass cultivation for commercial use.

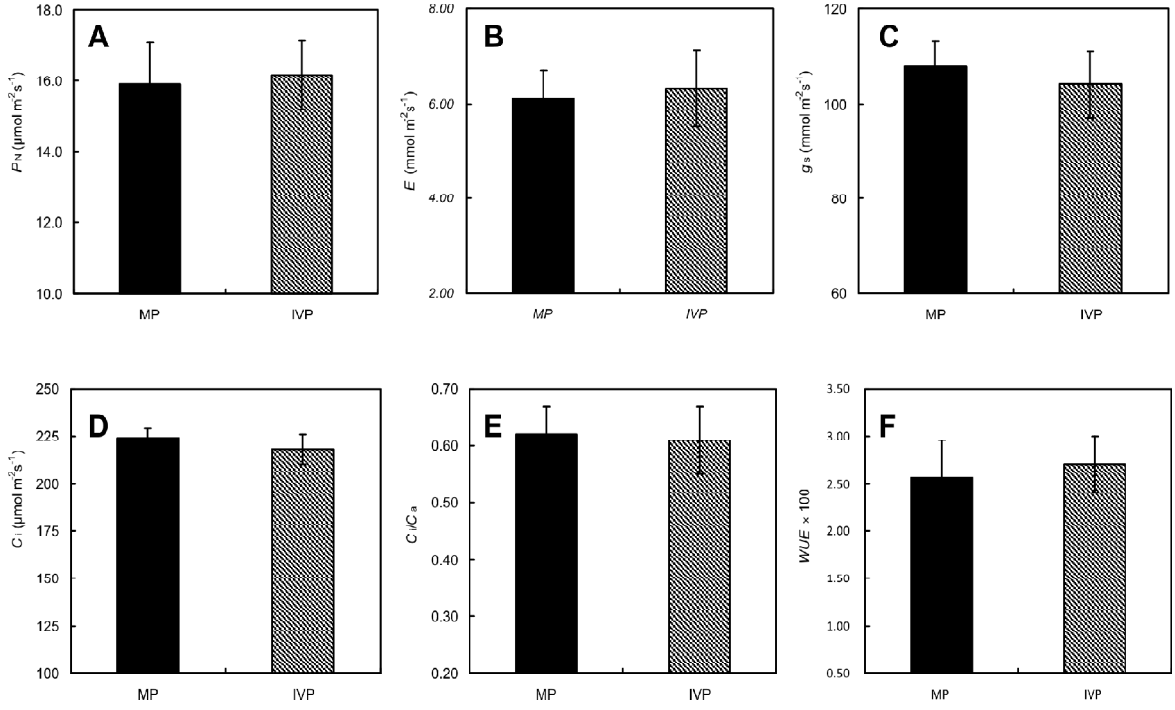

Figure 4: Comparison of eco-physiological characteristics (A-F) between mother plant and *in vitro* propagated plants. P_N, Net photosynthesis; *E*, Transpiration; C_i, intercellular CO_2 concentration; C_i/C_a, the ratio of intercellular to ambient CO_2 concentration; *WUE*, Water use efficiency; Data represents mean ± SD, *n* = 9

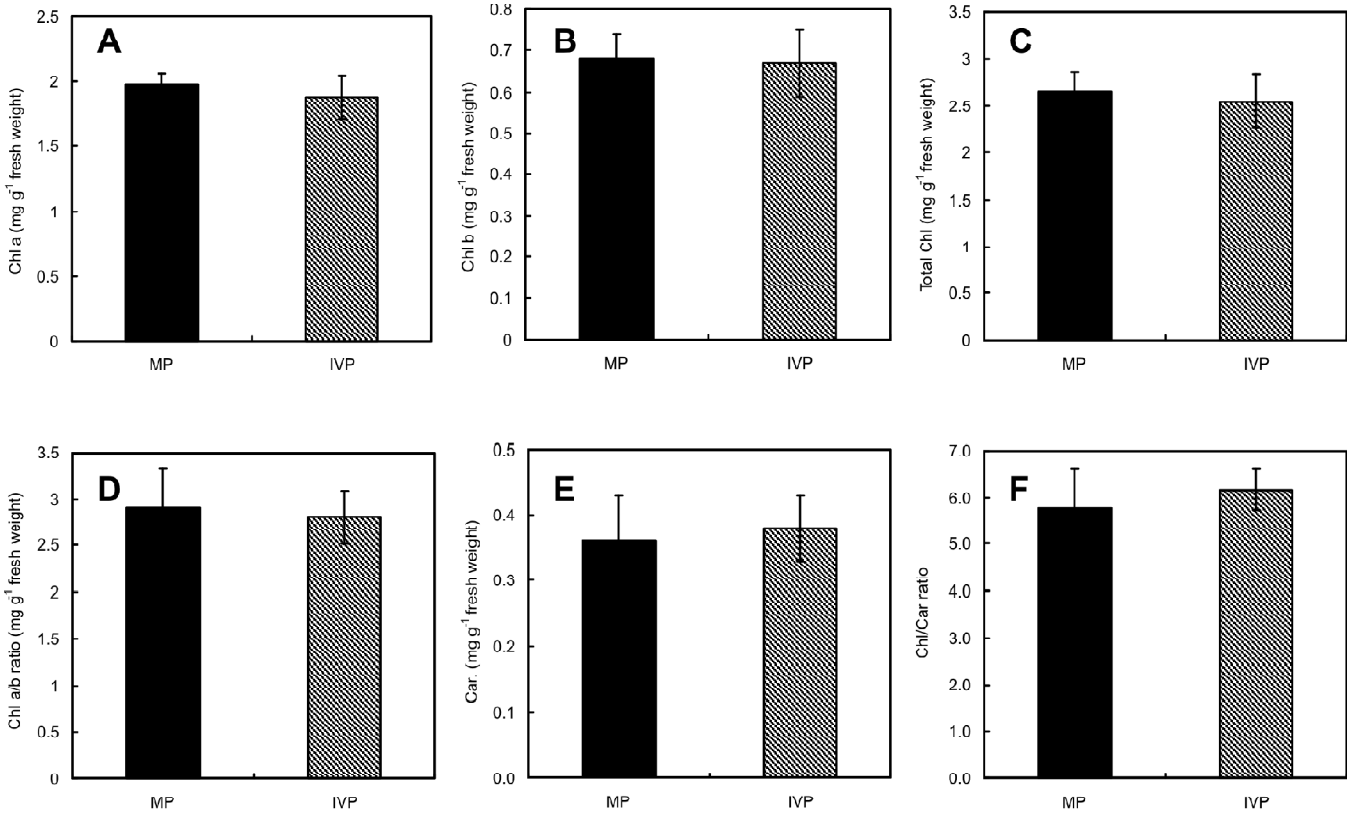

Figure 5: Comparison of photosynthetic pigments content (A-F) between mother plant and *in vitro* propagated plants of Stevia. Data represents mean ± SD, *n* = 9, Chl = Chlorophyll and Car. = Carotenoid

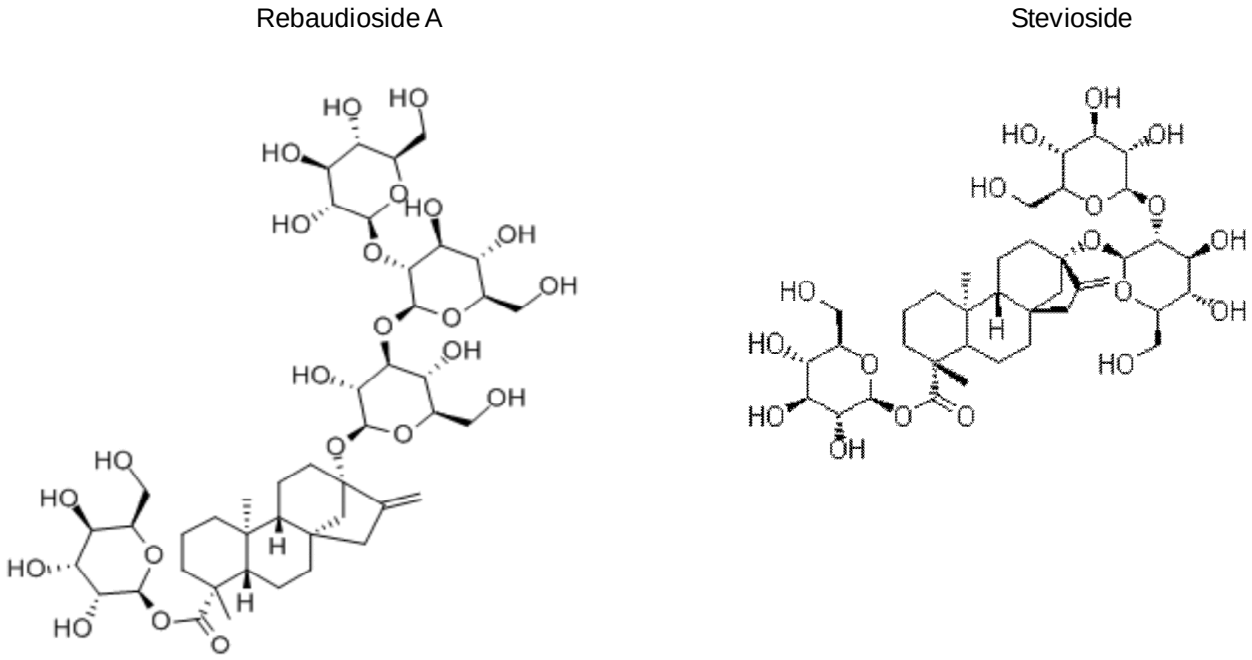

Figure 6: Chemical structures of major diterpene glycosides, rebaudioside A (MF- $C_{44}H_{70}O_{23,}$ MW- 967.01) and stevioside (MF- $C_{38}H_{60}O_{18,}$ MW- 804.87), in *Stevia rebaudiana* Bertoni

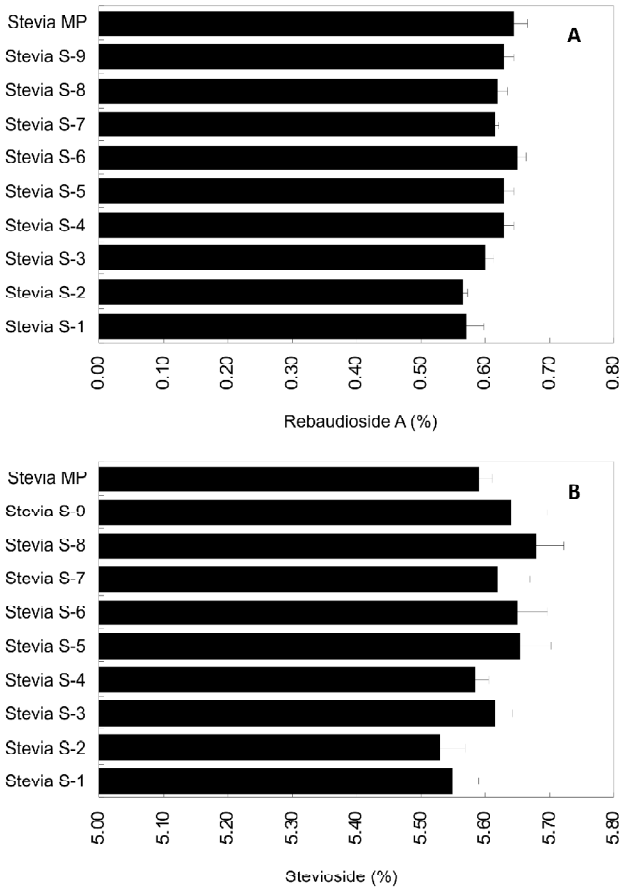

Figure 7: Assessment of rebaudioside A and stevioside content in the mother plant and *in vitro* propagated plants of *S. rebaudiana*

ACKNOWLEDGEMENTS

The work was supported in part by United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Specific Cooperative Agreement No. 58-6408-6-067.

REFERENCES

Ahmed MB, Salahin M, Karim R. (2007), An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana*) in Bangladesh. Am-Eu J Sci Res 2: 121–125.

Amzad-Hossain M, Siddique A, Mizanur-Rahman S, Amzad-Hossain M. (2010), Chemical composition of the essential oils of *Stevia rebaudiana* Bertoni leaves. Asian Journal of Traditional Medicines 5: 56–61.

Brandle, JE, Rosa N. (1992), Heritability for yield, leaf-stem ratio and stevioside content estimated from a landrace cultivar of *Stevia rebaudiana*. Can. J. Plant Sci. 72:1263–1266.

Brandle J, Telmer P. (2007), Steviol glycoside biosynthesis. Phytochemistry 68: 1855–1863.

Carneiro JWP, Muniz AS, Guedes TA. (1997), Greenhouse bedding plant production of *Stevia rebaudiana* (Bert) Bertoni. Can J Plant Sci 77: 473–474.

Chalapathi MV, Thimmegowda S. (1997), Natural non calorie sweetener *Stevia* (*Stevia rebaudiana* Bertoni): A future crop of India. Crop Research Hisar 14: 347 350.

Crammer B, Ikan R. (1986), Sweet glycosides from the stevia plant. Chem. Britain 22: 915-916.

Chandra S, Lata H, Khan IA, ElSohly MA. (2008), Photosynthetic response of *C. sativa* L. to variations in photosynthetic photon flux densities, temperature and CO_2 conditions. Physiol Mol Biol Plants 14: 299–306.

Chandra S, Lata H, Khan IA, ElSohly MA. (2011a), Temperature response of photosynthesis in different drug and fiber varieties of *Cannabis sativa* L. Physiol Mol Biol Plants 17(3):297–303.

Chandra S, Lata H, Khan IA, ElSohly MA. (2011b), Photosynthetic response of *Cannabis sativa* L., an important medicinal plant, to elevated levels of CO_2. Physiol Mol Biol Plants 17(3): 291–295.

Chandra S, Lata H, Khan IA, Mehmedic Z, ElSohly MA. (2015), Light Dependence of Photosynthesis and Water Vapour Exchange Characteristics in Different High Δ^9-THC Yielding Varieties of *Cannabis sativa* L. Journal of Applied Research on Medicinal and Aromatic Plants 2(2): 39-47.

Debnath M. (2008), Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*. J Med Plants Res 2: 45–51.

Felippe GM, Lucas NMC. (1971), Estudo da viabilidabe dos frutos de *Stevia rebaudiana*. Hoehnea 1: 95–105.

Gardana C, Simonetti P, Canzi E, Zanchi R, Pietta P. (2003), Metabolism of stevioside and rebaudioside A from *Stevia rebaudiana* extracts by human microflora. Journal of agricultural Food Chemistry 51: 6618–6622.

Ghanta S, Banerjee A, Poddar A, Chattopadhyay S. (2007), Oxidative DNA damage preventive activity and antioxidant potential of *Stevia rebaudiana* Bertoni, a natural sweetener. Journal of Agricultural Food Chemistry 55: 10962–10967.

Hiscox JD, Israelstam GF. (1979), A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Botany 57:1332–133.

Joshi SC, Palni LMS. (1998), Clonal variation in temperature response of photosynthesis in tea. Plant Science 13:225–232.

Katayama O, Sumida T, Hayashi H , Mitsuhashi H. (1976), The practical application of Stevia and research and development data (English translation) I.S.U. Company, Japan, p. 747.

Kinghorn AD, Soejarto DD. (1985), Current status of stevioside as a sweetening agent forhuman use. In H. Wagner, H. Hikino, and N. R. Farnsworth, eds.

Economic and medical plant research. Academic Press, London, UK.

Lata H, Chandra S, Wang Y, Raman V, Khan IA. (2013), TDZ-Induced High Frequency Plant Regeneration through Direct Shoot Organogenesis in *Stevia rebaudiana* Bertoni: An Important Medicinal Plant and a Natural Sweetener, American Journal of Plant Sciences 4: 117-128.

Lata H, Chandra S, Wang Y, Mahmoud ElSohly, Khan IA. (2014), *In vitro* Germplasm Conservation of Elite *Stevia rebaudiana* Bertoni, Acta Horticulture 1039: 303-308.

Liu J, Li SFY. (1995), Separation and determination of *Stevia* sweeteners by capillary electrophoresis and high performance liquid chromatography. J. Liquid Chromatography18: 1703 1719.

Moraes RM, Donega MA, Cantrell CL, Mello SC, Mcchesney JD. (2013), Effect of harvest timing on leaf production and yield of diterpene glycosides in *Stevia rebaudiana* Bert: a specialty perennial crop for Mississippi. Industrial Crops and Products 51: 385-389.

Murashige T, Skoog F. (1962), A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497.

Nakamura S, Tamura Y. (1985), Variation in the main glycosides of Stevia (*Stevia rebaudiana* Bertoni). Japanese Journal of Tropical Agriculture 29: 109–116.

Prakash I, Dubois G, Clos J, Wilkens K, Fosdick L. (2008), Development of rebiana, a natural, non-caloric sweetener. Food and Chemical Toxicology 46: S75–S82.

Rawat AS, Purohit AN. (1991), CO_2 and water vapour exchange in four alpine herbs at two altitudes and under varying light and temperature conditions. Photosynthesis Research 28: 99-108.

R-Development-Core-Team. (2005), R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Sakaguchi M, Kan T. (1982), Japanese researches on *Stevia rebaudiana*. Ci Cult 34: 235–248.

Satpathy S, Das M. (2010), *In vitro* shoot multiplication in *Stevia rebaudiana* Bert., a medicinally important plant. Gen Appl Plant Physiol 36: 167–175.

Singh SD, Rao GP. (2005), Stevia: The herbal sugar of the 21st century. Sugar Technol. 7: 17-24.

Soejarto DD, Kinghorn AD, Farnsworth NR. (1982), Potential sweetening agents of plant origin. III. Organoleptic evaluation of Stevia leaf herbarium samples for sweetness. J. Nat. Prod. 45: 590-599.

Yadav AK, Singh S, Dhyani D, Ahuja PS. (2011), A review on the improvement of stevia [*Stevia rebaudiana* (Bertoni)]. Canadian Journal of Plant Sciences 91:1–27.

Wang YH, Avula B, Tang W, Wang M, Elsohly MA, Khan IA. (2015), Ultra-HPLC method for quality and adulterant assessment of steviol glycosides sweeteners – *Stevia rebaudiana* and stevia products, Food Additives and Contaminants: Part A 32 (5): 674-685.