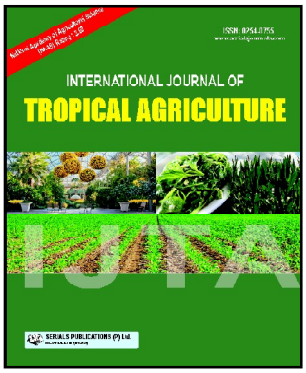

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Total Phenolics and Flavonoids Content, Free Radical Scavenging Potential and Major Chemical Constituents of Tissue Culture Raised *Stevia rebaudiana* Bertoni

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Abstract: *In vitro* propagated plants of *Stevia rebaudiana* Bertoni, a natural sweetener, were evaluated for phenolic compounds, free radical scavenging potential and, rebaudioside A and stevioside content and compared with mother plant to test the reliability of *in vitro* regeneration protocol. Plants were propagated *in vitro* from nodal segments containing axillary buds. Explants were inoculated on Murashige and Skoog's (MS) medium containing 3% (w/v) sucrose, 0.8% (w/v) agar supplemented with 1.0 iM thidiazuron (TDZ) for shoot initiation and multiple shoot formation and further transferred in half strength MS medium without any growth regulator for rooting following Lata *et al.,* (2013). Fully rooted plantlets were successfully established in soil and grown to maturity at the survival rate of 95% in the climatic controlled indoor growing facility. Leaf samples from mother and tissue cultured raised plants were harvested at the peak vegetative stage *i.e.* just before flowering. Air dried and powdered leaf samples were used for further analysis. Our results show that *in vitro* propagated plants (IVPP) were found to be highly comparable to each other and to that of the mother plant (MP) in terms of mean phenolics, flavonoids, antioxidant properties using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and cellular antioxidant (CAA) assays and, rebaudioside A and stevioside content. These results, thereby confirm the clonal fidelity of tissue culture raised *S. rebaudiana* plants.

INTRODUCTION

Plants are well known sources of natural bioactive compounds such as secondary metabolites and antioxidants. Among all, flavonoids and phenolics are the most important groups of secondary metabolites and bioactive compounds in plants (Kim *et al.,* 2003). These are known as natural antioxidant agents capable of scavenging free superoxide radicals, acting as anti-aging and reducing the risk of cancer. Medicinal plants are therefore, continuously being investigated for their antioxidant properties, and the demand for natural antioxidants and food preservatives is increasing (Peschel *et al.,* 2006).

Stevia rebaudiana Bertoni (Asteraceae) a perennial shrub, native to Paraguayan regions and also found in Brazil and Argentina (Lemus-Mondaca *et al.,* 2012), is an important medicinal plant consumed globally as a non-caloric artificial sweetener. The leaves of *S. rebaudiana* are the sources of diterpene glycosides including, steviolbioside, rubsoside, rebaudioside A, B, C, D, E and F, dulcoside and stevioside. Stevia was approved for use as a sweetener by the Joint Food and Agriculture Organization/World Organization Expert Committee on Food Additives (JECFA, 2008; JECFA, 2009) and the European Food Safety Authority have considered the safety of the leaf extract (EFSA, 2010). In addition, the dried leaves also contain high levels of total phenols (Periche *et al.,* 2015), minerals and other compounds (Lemus-Mondaca *et al.,* 2012) with a high antioxidant capacity (Barba *et al.,* 2014; Yildiz-Ozturk *et al.,* 2015) with their potentially beneficial effects on human health. Due to high levels of antioxidant properties, *S. rebaudiana* leaf has been widely consumed as infusions in the food and cosmetic industry with the production of *S. rebaudiana* leaf risen to an estimated 4000 t annually (Barba *et al.,* 2014; Gawe³-Bêben *et al.,* 2015).

The seeds of Stevia have low germination percentage (Mitra and Pal 2007) with a heterogeneous population, leading to the variability of important traits like sweetening levels and composition (Nakamura and Tamura 1985; Sivaram and Mukundan 2003; Shatnawi *et al.,* 2011) Vegetative propagation has been reported too slow (Sakaguchi and Kan 1982; Debnath 2008; Mishra et al. 2010) Seeing the limitations, tissue culture is the biotechnological tool for regenerating and conserving elite, valuable plant genetic resources of Stevia. It has also been successfully used for the large scale propagation of the clonal propagules for the commercial planting of the desired genotype. We have established an efficient high frequency plant regeneration protocol for *S. rebaudiana* using tissue culture technology (Lata *et al.,* 2013). The objective of the present study was to further investigate fully developed *in vitro* raised plants for phenolic compounds, free radical scavenging potential and major chemical constituents (rebaudioside A and stevioside), and to compare with mother plant to test the reliability of *in vitro* regeneration protocol.

MATERIALS AND METHODS

Micropropagation

S. rebaudiana plants were grown from the cuttings. These were obtained from screened and selected high yielding stevia mother plants cultivated at climatic controlled indoor growing facility at the Coy Waller research laboratory at The National Center for Natural Product Research, School of Pharmacy, University of Mississippi. For the initiation of shoot cultures, nodal segments containing axillary buds (~1 cm in length) were used as an explant following Lata *et al.,* (2013).

Explants were surface disinfected using 0.5% NaOCl (15% v/v bleach) and 0.1% Tween 20 for 20 min. The explants were washed in sterile distilled water 3 times for ~5 min prior to inoculation on the culture medium. Disinfected explants were inoculated on Murashige and Skoog's medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 0.8% (w/v) Type E Agar (Sigma Chemical

Co., St. Louis) supplemented with 1.0 ìM thidiazuron (TDZ) for shoot formation and half strength MS medium without any growth regulator for rooting (Lata *et al.,* 2013). All cultures were incubated at 25 ± 2°C with 16 h photoperiod under fluorescent light with a photon flux of ~ 60 µmol m^{-2} s^{-1}.

Rooted plants were carefully taken out of the medium and washed thoroughly in running tap water to remove all traces of medium attached. Plantlets were pre-incubated in coco natural growth medium in thermocol cups under 16 hour photoperiod provided by fluorescent bulbs for 10 days. The cups were covered with polythene bags to maintain humidity and kept in a growing room and later acclimatized in sterile potting mix- fertilome in large pots. Fully acclimatized *in vitro* propagated plants were then moved under full spectrum 1000 watts HID (high density discharge) lamps (Sun Systems, CA) hung on the top of plants for further growth with mother plants. All the plants were kept under similar environmental conditions in an indoor cultivation facility at Coy-Waller laboratory, University of Mississippi. Using an automatic electric timer artificial day/night cycle was regulated with a 16 h photoperiod. Growing room temperature and relative humidity was kept nearly 25-30°C and 55-60%, respectively. Plants were watered regularly and individually to maintain sufficient moisture content in the pots.

Collection of Samples for Total Phenolics, Flavonoids, and Antioxidant Properties

Biomass samples from seven randomly selected tissue culture raised plants and mother plant were collected for antioxidant analysis, and determination of total phenolic and flavonoids content. Freshly harvested plant material was collected and placed in the container containing dry ice. Immediately after the collection, plant material was brought to the laboratory and stored at –80°C until further use. All the samples were then freeze-dried and ground using a planetary ball mill (PM-400, Retsch, GmbH, Germany) at a low temperature. Out of nine, three randomly selected freeze-dried, powdered samples were used for further extraction.

Extract Preparation

Dried leaves (10 g) from each sample were used for the preparation of extract for total phenolics, flavonoids, and antioxidant properties. Samples were extracted with 75 mL (95% v/v) ethanol at 40°C for 10 min. The solvent was evaporated at 40°C under a reduced pressure. The dried extract was used for further analysis.

Determination of Total Phenolic and Flavonoids Content

Reagents and Chemicals

Folin-Ciocalteu reagent, gallic acid, and quercetin standards were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Aluminum chloride hexahydrate, methanol, and sodium carbonate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified using a Milli-Q system (Millipore).

Sample Preparation

About 5 mg of the extract was dissolved in 5 mL methanol and sonicated for 45 minutes at 40°C followed by centrifugation at 1,000 ×g for 10 min. The clear supernatant was collected and stored in an amber bottle for analysis.

Total Phenolic Content

The total phenolic comtent of the extracts was determined using the Folin and Ciocalteu reagent, following the method described by Singleton *et al.* 1999, with slight modifications. Sample and standard readings were made using a spectrophotometer (Cary 50 Bio UV-Vis Spectrophotometer, Varian) at 765 nm against the reagent blank.

For total phenolic determination, gallic aid was used to make the standard calibration curve. Stock solution was prepared by dissolving 5.0 mg gallic acid in 1.0 mL methanol, then the standard solutions were prepared by serial dilutions using methanol (5–265 µg/mL). The test sample (0.2 mL) and gallic acid solution (0.2 mL) was mixed separately with 0.6 mL of water and 0.2 mL of Folin-Ciocalteu's phenol reagent (1:1). After 5 min, 1 mL of saturated sodium carbonate solution (8% w/v in water) was added to the mixture and the volume was made up to 3 mL with distilled water. The reaction was kept in the dark for 30 min and after centrifuging the absorbance of blue color from different samples was measured at 765 nm. The phenolic content was calculated as gallic acid equivalents (GAE)/g of dry plant material on the basis of a standard curve of gallic acid [5–265 mg/L, $Y = 0.00325x - 0.0058$, $R^2 > 0.9$]. All determinations were carried out in triplicate.

Total Flavonoids Content

The aluminum chloride colorimetric method was used for the determination of the total flavonoid content of the sample (Chang *et al.*, 2002; Miliauskasa *et al.*, 2004; Marinova *et al.*, 2005; Pourmorad 2006). For total flavonoid determination, quercetin was used to make the standard calibration curve. Stock quercetin solution was prepared by dissolving 5.0 mg quercetin in 1.0 mL methanol, then the standard solutions of quercetin were prepared by serial dilutions using methanol (5–265 µg/mL). An amount of 0.6 mL diluted standard quercetin solutions or sample extracts was separately mixed with 0.6 mL of 2% aluminum chloride. After mixing, the solution was incubated for 60 min at room temperature. The absorbance of the reaction mixtures was measured against blank at 440 nm wavelength with a Varian UV-Vis spectrophotometer (Cary 50 Bio UV-Vis Spectrophotometer, Varian). The concentration of total flavonoid content in the test samples was calculated from the calibration plot ($Y = 0.01055x - 0.0194$, $R^2 > 0.9$) and expressed as mg quercetin equivalent (QE)/g of dried plant material. All the determinations were carried out in triplicate.

Determination of Antioxidant Activity

The extracts were dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 20 mg/mL. The antioxidant activity of the extracts was measured by following two methods.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The capacity of plant extracts (1005µg/mL) to directly react with and quench free radicals was evaluated as described earlier (Cheng *et al.*, 2006) A stock solution of DPPH (200 5ØßM) was prepared in ethanol. The assay was performed in 96-well plates. The reaction mixture, containing 100 µL of DPPH and 100 5µL of the diluted test sample, was incubated at 37 °C for 30 min. The absorbance was measured at 515 nm. Gallic acid was used as a positive control. Percent DPPH radical scavenging activity was calculated as follows:

Percent radical scavenging activity =

{1-(sample - blank)/(control - blank)} × 100

Gallic acid showed 78% radical scavenging activity at 20 µM.

Cellular Antioxidant Activity Assay (CAA Assay)

The cellular antioxidant activity was measured in $HepG_2$ cells as described by Wolfe and Rui (2007). The method measures the ability of phytochemicals in the plant extracts to prevent intracellular generation of peroxy radicals in response to ABAP (used as a generator of peroxyl radicals). The CAA assay is a more biologically relevant method than a chemical assay because it represents the complexity of biological system and accounts for cellular uptake, bioavailability, and metabolism of the antioxidant agent.

$HepG_2$ cells (acquired from American type culture collection, ATTC, Rockville, MD) were

grown in DMEM supplemented with 10% FBS and antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin). For the assay, cells were seeded in the wells of a 96-well plate at a density of 60,000 cells/well and incubated for 24 hrs. The medium was removed and cells were washed with PBS before treating with the test sample (500 µg/mL) diluted in the medium containing 25 µM DCFH-DA for 1 hr. After removing the medium, the cells were treated with 600 µM ABAP and the plate was immediately placed on a Spectra Max plate reader for kinetic measurement every 5 min for 1 hr (37°C, emission at 538 and excitation at 485 nm). Quercetin was used as the positive control. The antioxidant activity was expressed in terms of CAA units. The area under the curve (AUC) of fluorescence versus time plot was used to calculate CAA units as described by Wolfe and Rui (2007):

$$\text{CAA unit} = 100 - \{(\text{AUC sample})/(\text{AUC control}) \times 100$$

Quercetin showed CAA unit of 65 at 25 µM. This indicates that quercetin (at 25 µM) caused 65% inhibition of cellular generation of peroxyl radicals in $HepG_2$ cells.

Analysis of Rebaudioside A, Stevioside Content

Biomass samples of same plants that were used for the determination of total phenolics, flavonoids contents, and antioxidant properties were harvested and used for the analysis rebaudioside A, stevioside content. 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 rebaudioside A and stevioside content. Standards of rebaudioside A and stevioside were 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-ESIMS). 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 detection wavelength was 205 nm. Peaks were assigned by spiking the samples with standard compounds, comparison of UV spectra and the retention times.

RESULTS AND DISCUSSION

Fig. 1 shows the chemical structure of rebaudioside A and stevioside, the major compounds in stevia responsible for the property as natural sweetener. An efficient protocol to *in vitro* propagate stevia was developed by our group using thidiazuron-TDZ (Lata *et al.,* 2013). Seven, *in vitro* propagated (following Lata *et al.,* 2013, Fig. 2), acclimatized and fully grown

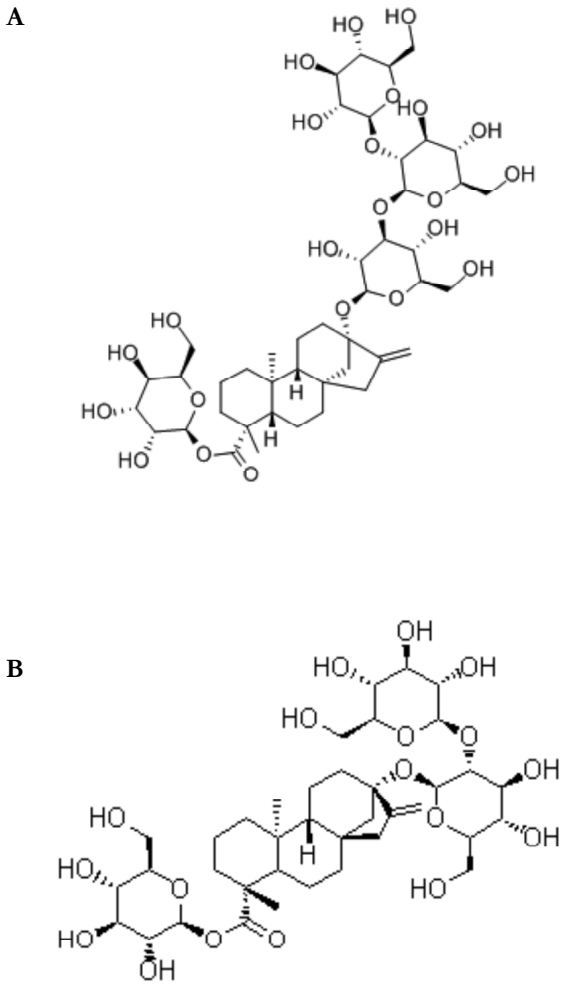

Figure 1: (A) Rebaudioside A: MF- $C_{44}H_{70}O_{23}$ and MW- 967.01 and Stevioside: MF- $C_{38}H_{60}O_{18}$, MW- 804.87.

Figure 2: *In vitro* propagation of *S. rebaudiana*. A-B: Formation of multiple shoots, C: Root formation, D: Rooted plant ready to go to soil and E: Fully grown *in vitro* raised *S. rebaudiana* plants.

plants were analyzed for phenolics, flavonoids, antioxidant activities using DPPH and CAA assays, and rebaudioside A and stevioside Content, and were compared with mother plant.

Variation in phenolic content of tissue culture raised plants and mother plant of *S. rebaudiana* is shown in Figure 3. Polyphenols are plant secondary metabolites which have been linked with a variety of health beneûts including prevention of cancer, type II diabetes and cardiovascular disease (Hertog *et al.,* 1993, Kroon *et al.,* 2004, Crozier *et al.,* 2009, Del Rio *et al.,* 2010). In Stevia, phenolic content is reported to vary with varieties, plant parts and irrigation, fertilizer and environmental conditions. In the present study, phenolic content varied with highest in P5 (111 ± 1.29 mg GAE/g dry wt.) to minimum in P1 (103.11 ± 2.96 mg GAE/g dry wt.) among tissue culture raised plants. Whereas, it was 109.81 ± 3.21 (mg GAE/g dry wt.) in mother plant of Stevia. These minor variations in phenolic content in tissue culture raised Stevia plants and mother plant were found statistically insignificant.

Figure 4 shows a comparison of total flavonoid content between mother plant and tissue culture raised plants of *S. rebaudiana*. Among tissue culture raised plants, the lowest amount of flavonoid content is observed in clone P5 (45.44 ± 1.31 mg QE/g dry wt.) and the highest was found in P1 (59.92 mg QE/g dry wt.). Whereas, in mother plant total flavonoid content is observed as 52.12 ± 0.09 mg QE/g dry wt. The data shows that tissue culture raised plants of *S. rebaudiana* were highly comparable to the mother plant in terms of total flavonoid content.

In general, the measurement of antioxidant activity using biologically relevant assays is very important to screen plant biomass, fruits, vegetables, natural products, and dietary supplements for potential health benefits. Furthermore, batch to batch consistency in useful secondary metabolites and their biological activity of any biomass product is utmost important if it is grown to be used as food or

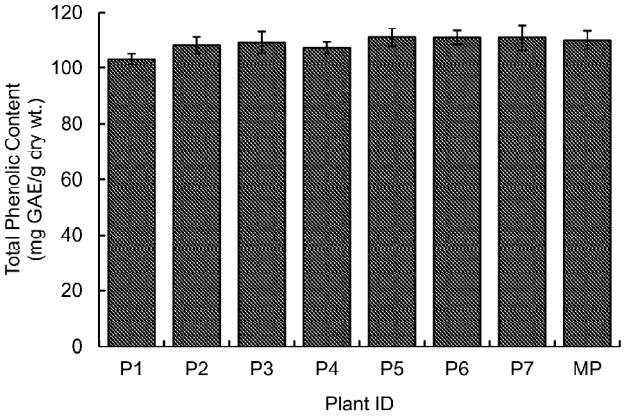

Figure 3: Assessment of total phenolic content (mg GAE/g dry wt.) in different tissue culture raised plants (P1-P7) of *Stevia rebaudiana* and their comparison with mother plant (MP)

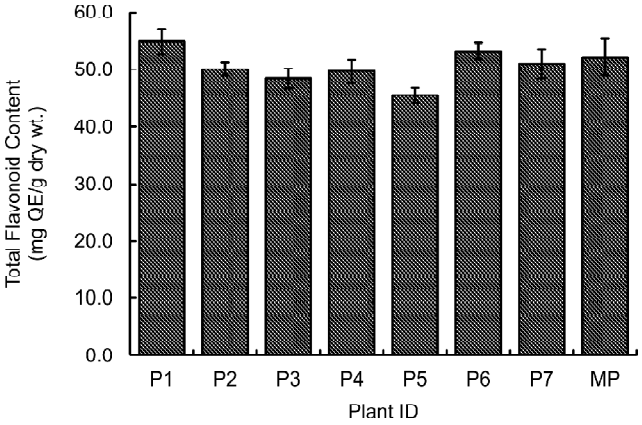

Figure 4: Total flavonoid content (mg QE/g dry wt.) in different tissue culture raised plants of *Stevia rebaudiana* (P1-P7) and their comparison with mother plant (MP).

phytopharmaceuticals. In the present study, tissue culture raised plants of *S. rebaudiana* were evaluated for percent DPPH radical scavenging activity and cellular antioxidant activity using $HepG_2$ cells and compared with the mother plant (Fig. 5A and 5B). Our results show that tissue culture raised plants were highly comparable to each other and to mother plant in their antioxidant properties using DPPH assays. Among tissue culture raised plants, antioxidant activity in terms of radical scavenging activity, using DPPH assay, ranged between 61.30% (P3) to 66.8% (P6), whereas it was 65.4% in the mother plant (MP). Similarly, using the cellular antioxidant assay (CAA), the maximum activity among tissue culture raised plants was found in clone # P1 (48.0 CAA units) and minimum was in P4 and P5 (30 CAA units) with a mean value of 36.96 ± 6.32 (CAA units). Whereas, antioxidant activity in mother plant using cellular antioxidant assay was found 39.00 (CAA units), which is comparable to tissue culture raised plants. In general, a positive correlation was observed between total phenolic content and DPPH radical scavenging activity (Fig. 3 and Fig. 5A). A pattern of minor variations in flavonoid content found in tissue culture raised plants was also noticed in antioxidant activity of those plants using cellular antioxidant assay (Fig. 4 and Fig. 5B). Similarly, Rawat *et al.* (2011),

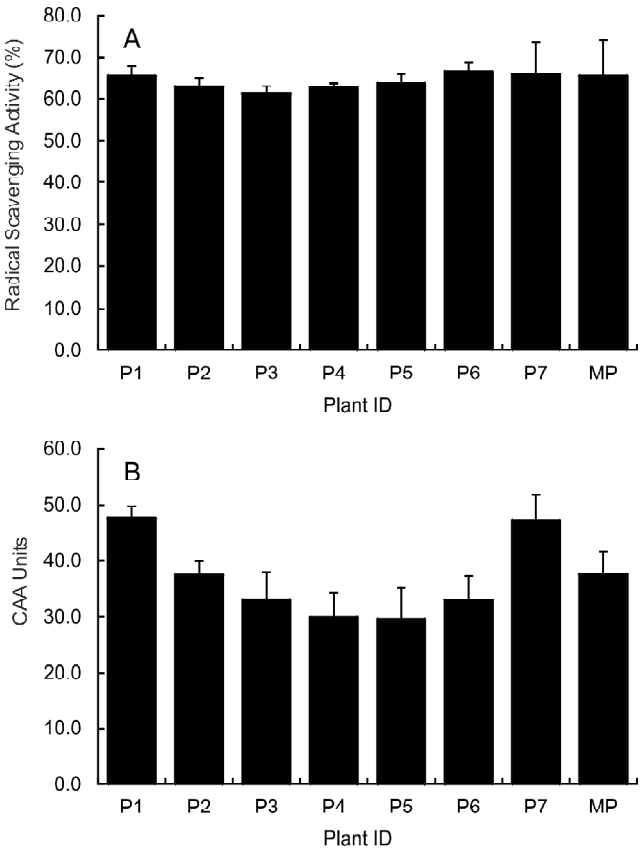

Figure 5: Assessment of antioxidant activity of seven tissue culture raised plants (P1-P7) and mother plant (MP) of *Stevia rebaudiana* (A) at 100 µg/mL by DPPH assay and (B) at 500 µg/mL by Cellular antioxidant assay (CAA)

have reported a significant positive correlation between total phenolic and flavonoids content, and antioxidant activity of *Myrica esculenta,* a popular wild edible fruit in Indian Himalayan region.

Tissue culture raised plants of *S. rebaudiana* were also evaluated for rebaudioside A and stevioside content, and compared with mother plant (Fig. 6). Our results show a high level of homogeneity in the chemical profile, and rebaudioside A (Fig. 6A) and stevioside content (Fig. 6B) of the mother plant and the randomly selected fully hardened micropropagated clones of *S. rebaudiana*. The results reveal that *in vitro* propagated and hardened plants of *S. rebaudiana* are biochemically as well as functionally comparable to each other and to the mother plant.

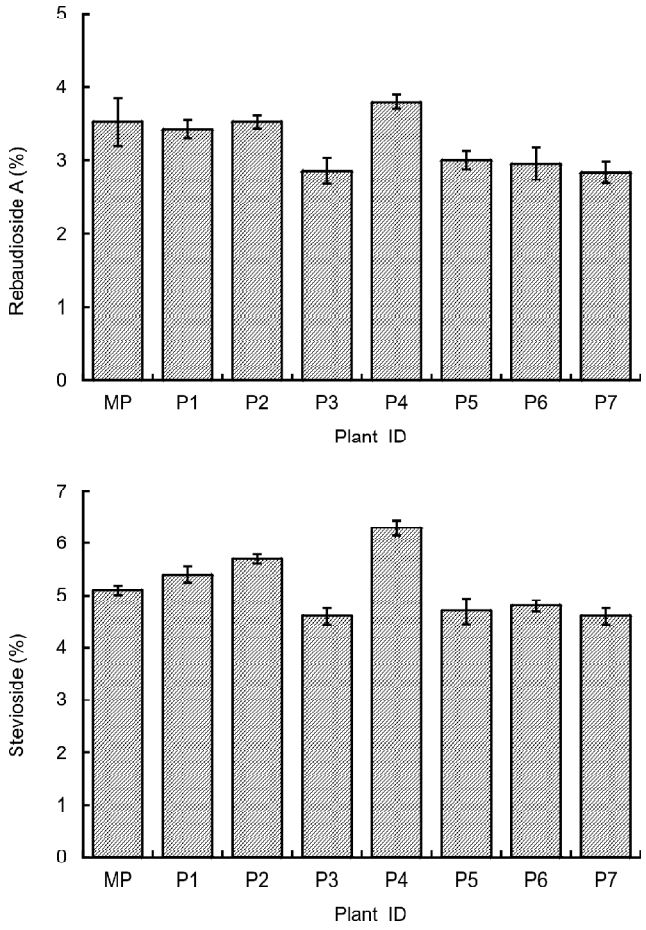

Figure 6: Rebaudioside A (A) and stevioside (B) content in the leaves of mother plant and randomly selected tissue culture raised plants (P1-P7) of *Stevia rebaudiana*. *n* = 3.

In conclusion, the micropropagation protocol followed in the study is a useful supplement to the conventional and vegetative propagation of *S. rebaudiana* with no significant variations in phenolic and flavonoids contents antioxidant activity and, rebaudioside A and stevioside content between mother plant and tissue culture raised plants and therefore, can be used for mass propagation *S. rebaudiana* to fulfill ever growing demand of natural sweetener in global market.

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