

**Effect of different plant beds and fertilizers on Stevia (*Stevia rebaudiana* Bertoni) production**

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**Abstract**

*Stevia rebaudiana* Bertoni is well-known for its high content of steviol glycoside, a diterpene compound (about 4–20%) in dryleaf matter that is responsible for providing the non-caloric value sweet taste. Steviol glycosides are natural sugar substitutes for diabetics and other people on carbohydrate-controlled diets. Studies revealed that the glycosides yield, especially stevioside, greatly depends on the total biomass yield, which in turn depends on agricultural practices for cultivation of stevia. In this study, the effect of five various combinations of soil, cocopeat, perlite, and vermicompost plant beds mixed with four different fertilizers of tea compost, vermiwash, Hoagland and water, on four different physiological (leaf area, stem length, fresh weight and dry weight) and six different biochemical (chlorophyll content, carotenoid, protein, glucose, phenols and rebaudiosides) characteristics of plant were evaluated. To gain the highest content of biochemical compounds, especially all types of rebaudiosides, a combination of vermicompost, perlite and cocopeat with vermiwash or any other fertilizers can be useful.

**Keywords:** Fertilizer; Growth bed; Natural sweetener; Organic fertilizer; Rebaudioside.**Introduction**

*Stevia rebaudiana* Bertoni branched bushy shrub of the Asteraceae family, is native to the north-east region of Paraguay, also grows in the neighboring parts of Brazil and Argentina (Soejarto, 2002). Nowadays, stevia is known for its high content of sweet diterpene (about 4–20%) in the dryleaf matter (Ghanta et al. 2007) and the stevia glycosides are responsible compounds for the sweet taste. From 230 species of *Stevia* genus, only the species *rebaudiana* and *phlebophylla* produce steviol glycosides (Brandle and Telmer, 2007). The analysis by liquid chromatography coupled with UV, MS and ELS detection showed that the Steviol is as common aglycone backbone of the sweet stevia glycosides (Cacciola et al. 2011). Stevioside, reported as most abundant stevia glycoside (4–13%w/w) found mainly in plant leaves followed by stems, seeds and roots, which is about 300 times sweeter than sucrose and can be particularly useful to those suffering from obesity, diabetes mellitus, heart disease and dental caries (Ghanta et al., 2007). *Stevia* stays well in a wide range of soils with a consistent supply of moisture and adequate drainage (Shock, 1982). The longer days are favored to leaf yield and stevioside contents. *Stevia* is sensitive to cold and does not usually tolerate temperatures below 9°C, while, the rapid growth occurs at 20–24°C (Singh and Rao, 2005).

Studies reveal that the glycosides yield, especially stevioside, greatly depends on the total biomass yield, which in turn depends on agricultural practices for cultivation of stevia plants (Geuns, 2003). To achieve high production of glycosides, an adaptation of modern agro-techniques (Das et al., 2008), water management (Fronza and Folengatti, 2003) and fertilizer applications (Mamta et al., 2010) are needed.

In addition, *in vitro* culture techniques and application of bioreactors have also been tested (Madan et al. 2010). Among those, reliable nutrient supply is crucial for plant yield production. The optimum requirement of macro and micro nutrients essential for normal growth are gained from different types of soil composition and fertilizers. However, the main limiting factors are high costs and low on-farm feasibility. For instance, chemical fertilizers constitute a few mineral nutrients and create an imbalance in the whole mineral pattern of the plant body by hindering the uptake of other useful nutrients (Das et al., 2008). Also, there is growing concern over the adverse effects of using chemical fertilizers, pesticides, and herbicides on soil productivity and environment quality. Therefore, organic structure of plant growth fertilizers and soil mixtures, which results in high plant yield, are in demand.

Recently, the contrasting effect of organic and chemical fertilizers, microorganism's interaction effects on plant growth, mixed fertilizers and their effect on physiological and biochemical characteristics of plant have been studied (Liu et al., 2011; Mandal et al., 2013; Patil, 2010; Vafadar et al., 2014; Yang et al., 2013). While these studies have great value to achieve high yield stevia production, the study of soil structure and its mixed fertilizers followed by knowing of which mixture have the maximum effect, still lacking. The objective of our study was to evaluate the effect of different plant beds mixed with different fertilizers to find out the suitable nutrient source for stevia production.

## Results

Here four different physiological (leaf area, stem length, fresh weight and dry weight) and six different biochemical (chlorophyll content, carotenoid, protein, glucose, phenols, and rebaudiosides) characteristics of the plant were analyzed. The analysis of variance showed significant following differences among all characters ( $P < 0.01$ ) (Table 4):

A. Leaf area: The highest leaf area was found in plant bed G12 ( $16.6 \text{ cm}^2$ ) followed by G15, G11 and G9 with 16.2, 16 and  $14.9 \text{ cm}^2$  respectively, while least leaf area was shown in G1 ( $7.3 \text{ cm}^2$ ) (Table 5 and figure 1).

B. Stem length: Stem length was found to be most effective by bed G11, which reached the peak of 36.0 cm followed by G14 and G3 (33.7 and 31.3 cm). The shortest length was found in G1 with 13.9 cm (Table 5).

C. Fresh weight: The highest fresh weight was in bed G15 (0.33g) which was not significantly different from G11, and G12 (0.29 g) but different with others. Also, G6 (0.14 g) had least fresh weight followed by G1 and G2 (0.15 and 0.16 g) with non-significant different from each other (Table 5).

D. Dry weight: On dry weight examination, the highest weight was found in G11 (0.072 g) followed non-significantly by G7 and G12 (0.071 and 0.068 g), and the lowest dry weight was in G6 (0.035 g) followed non-significantly by G2, G1, and G13 (Table 5 and figure 2).

The analysis of variance in biochemical studies shows significant following differences in those characterizations and the treatments applied ( $P < 0.01$ ) (Table 4):

A. Chlorophyll a: The highest amount of chlorophyll was found in G8 (18.8 mg/gFW) followed by G7 and G13 (18.4 and 18.3 mg/gFW), while the lowest amount calculated in G6 (10.5 mg/gFW) followed by G15 (10.7 mg/gFW).

B. Chlorophyll b and carotenoid: It was almost same in chlorophyll a and carotenoid, which the highest amount of those found in G8, 8.46 and 2.83 mg/gFW respectively. The lowest amount also showed in G6 and G15 in those factors (Table 5).

C. Protein, glucose, and phenols: The protein content found most in G3 (3.26%) and least in G7 (0.92%) (Table 5). The highest amount of glucose and phenols calculated in G6 by 758.2 and 153.2 mg/gDW respectively, while the G16 showed the lowest amount of those factors (Table 5).

D. Rebaudiosides: The sweet responsible agent, rebaudiosides, found the highest amount in G4 (184.3 mg/gDW) and least in G10, G13, and G11 respectively, which were not significantly different (Table 5). There are several reports of higher carbohydrate concentrations on different soil structures (Schaarschmidt et al., 2006; Wu et al. 2011).

## Discussion

The results show that, by using planting bed combination like G11, the highest physiological characterization can be achieved without any additional fertilizer. This can prove the important effect of beds compared to fertilizers in the case of stevia plant cultivation, as the lowest physiological characterization found in the control treatment. The variation among physiological characters was observed by different studies facing biological soil composition

treatments (Mandal et al., 2013). These can be due to changes occurred in the soil structure and could be effective on root surface area for absorption of nutrients and water (Jefferies et al., 2003). Pal et al., (2015) were analyzed the effect of different soil nutrition due to different crop-ecology. They found a significant effect of environmental factors and agronomic management on dry leaf yield. The biomass is also dependent on its ability to improve photosynthesis.

Stable nutrient status has also been reported to contribute to a net increase in photosynthesis in plants (Giri et al. 2003). By studying the results, we can realize, the presence of vermicompost in planting beds is most important in comparing with the other factors in the case of chlorophyll and carotenoid content of the plant. Also, the fertilizer composition does not have a significant effect on these factors, while, the suggested nutrient agent can be vermiwash and teacompost. In contrast, the nutrient composition (Hoagland) has shown essential to the accumulation of proteins, as its effect alone was significant. Study of glucose, phenols and rebaudiosides results shows the important effect of cocopeat and its amount on stevia planting beds, as the highest amount of cocopeat combined with perlite results an accumulation of those important chemicals. To achieve the highest amount of biochemical compound especially rebaudiosides, a combination of vermicompost, perlite and cocopeat combined with vermiwash or any other fertilizer can be useful. Here, adding vermicompost to the G4 combination may achieve the results. Pal et al., (2015) also suggested, the proper nutritional management and environmental factors increase secondary metabolites significantly.

## Materials and methods

The fertilizers and soils used in this study were made and analyzed for nutrient components in plant production department (Table 2&3). The Plant samples of *Stevia rebaudiana* (Berton) were obtained from biotechnology department of Jihad-e-daneshgahi Institute, Karaj, Iran, and grown in 30 cm wide diameter (Volume 3 L) earthen pot on the terrace at the greenhouse to reach normal day and night light condition with 25/17°C temperature.

### Morphological measurement

For growth observation, total fresh leaves area and stem length were quantified. After recording the fresh weight of above and below ground parts of plants, green-killing treatment was done at 105°C and the samples were oven dried at  $70 \pm 2^\circ\text{C}$  until a constant weight was attained to calculate the percentage of dry matter accumulation.

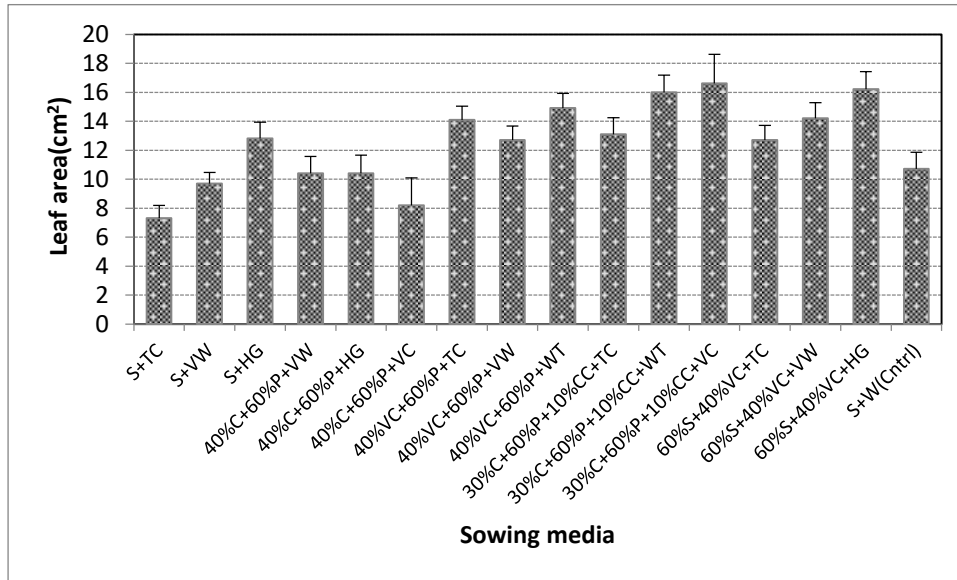
### Chlorophyll and carotenoid content

Leaves were collected from the experimental unit to chlorophyll content estimation. The fractions of pigments (chlorophyll a, chlorophyll b, and total chlorophyll) were estimated using the spectrophotometric method recommended by Arnon (1949). 200 mg fresh leaf sample was separated from each sample. Chlorophyll was extracted in a solution of 80% acetone (v/v). Subsequently, the

**Table 1.** Sowing bed compositions (G1 – G16) and different organic fertilizers.

Name	Sowing bed	Fertilizer	Name	Sowing bed	fertilizer
G1	100%Soil	Tea compost	G9	40%vearmicompost+60%pearlite	Water
G2	100%Soil	Vermiwash	G10	30%vermicompost+60%perlite+10%Cocopeat	Tea compost
G3	100%Soil	Hoagland	G11	30%vermicompost+60%perlite+10%Cocopeat	Water
G4	40%Cocopeat+60%perlite	Vearmiwash	G12	30%vermicompost+60%perlite+10%Cocopeat	Vermiwash
G5	40%Cocopeat+60%perlite	Hoagland	G13	60%Soil+40%vermicompost	Tea compost
G6	40%cocopeat+60%perlite	Tea compost	G14	60%Soil+40%vermicompost	Vermiwash
G7	40%vermicompost+60%perlite	Tea compost	G15	60%Soil+40vermicompost	Hoagland
G8	40%vearmicompost+60%perlite	Vearmiwash	G16	100%Soil	Water

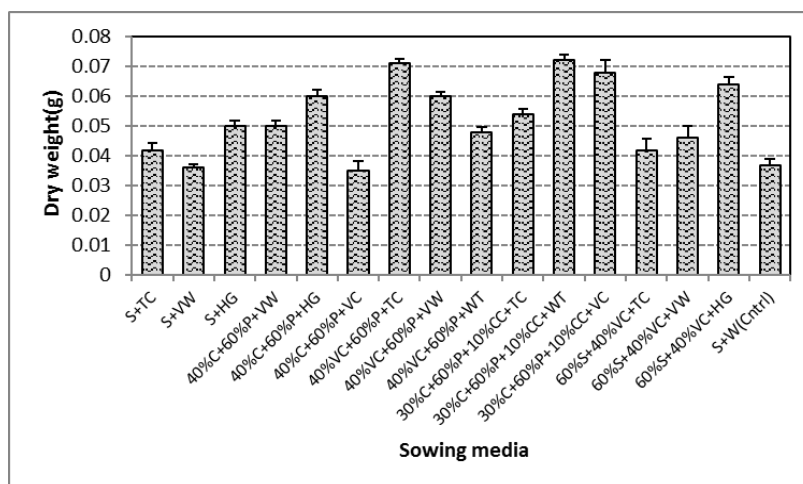
Note: Water without any amount of fertilizers is used as a control



**Fig 1.** The leaf area in different sowing media.

**Table 2.** Chemical analyses of organic fertilizers.

Fertilizer component	Mg (%)	Ca (%)	N (%)	P (%)	Fe (ppm)	Zn (ppm)	Mn (ppm)	K (%)	Na (%)	EC ( $\mu\text{s}/\text{cm}^2$ )	pH
Vermiwash	0.104	0.256	0.046	0.029	6.676	163.5	56.44	0.504	0.047	470	7.58
Teacompost	0.413	0.453	0.046	0.331	82.988	320.46	56.44	6.334	0.071	2300	6.77
Vermicompost	0.334	0.44	0.046	0.298	89.045	343.35	67.38	6.125	0.072	2200	6.95



**Fig 2.** The plant dry weight in different sowing media.

**Table 3.** Chemical analyses of soil.

Soil	P(ppm)	N (%)	K(ppm)	OC (%)	pH	EC( $\text{ms}/\text{cm}^2$ )
	260.7685	2.776667	210.4931	24.91043	8.45	5.9

**Table 4.** Analysis of variance for different biological and biochemical factors.

Changes sources	Treatment	Error	cv
Leaf area (cm <sup>2</sup> )	22.24*	5.78	7.1
Stem length (cm)	85.58*	17.530	7.4
Fresh weight (g)	0.00861*	0.001	19.3
Dry weight (g)	0.000449*	0.000083	14.1
df	15	31	
Chlorophyll a (mg/gFW)	21.332*	12.93	7.4
Chlorophyll b (mg/gFW)	6.693*	0.009	2.1
Carotenoid (mg/gFW)	0.996*	0.002	3.1
Protein %	1.286*	0.002	2.5
df	15	31	
Glucose (mg/gDW)	9031.1*	45.4	15.3
Phenols (mg/gDW)	3459.3*	5.6	2.6
Rebaudiosides (mg/gDW)	7871.2*	4.4	3.5
df	7	16	

\*Significant at 1%; df: Degrees of freedom.

**Table 5.** Comparative analysis of different physiological and biochemical factors. The values in the column with similar alphabets are non-significant with each other ( $P \leq 0.05$ ).

Treatment	Leaf area (cm <sup>2</sup> )	Stem length (cm)	Fresh weight (g)	Dry weight (g)	Chlorophylla (mg/gFW)	Chlorophyllb (mg/gFW)	Carotenoid (mg/gFW)	Protein %	Glucose (mg/gDW)	Phenols (mg/gDW)	Rebaudiosides (mg/gDW)
G1	7.3 <sup>d</sup>	13.9 <sup>g</sup>	0.15 <sup>e</sup>	0.042 <sup>def</sup>	15.7 <sup>de</sup>	4.50 <sup>g</sup>	1.44 <sup>h</sup>	1.90 <sup>f</sup>	398.2 <sup>h</sup>	80.7 <sup>i</sup>	57.8 <sup>h</sup>
G2	9.7 <sup>cd</sup>	23.5 <sup>def</sup>	0.16 <sup>e</sup>	0.036 <sup>ef</sup>	16.7 <sup>cd</sup>	5.18 <sup>e</sup>	1.63 <sup>f</sup>	2.26 <sup>d</sup>	429.7 <sup>f</sup>	86.2 <sup>gh</sup>	77.8 <sup>g</sup>
G3	12.8 <sup>abc</sup>	31.3 <sup>abc</sup>	0.20 <sup>de</sup>	0.050 <sup>cd</sup>	17.4 <sup>bc</sup>	5.79 <sup>d</sup>	1.74 <sup>e</sup>	3.26 <sup>a</sup>	454.5 <sup>e</sup>	92.0 <sup>ef</sup>	58.1 <sup>h</sup>
G4	10.4 <sup>bcd</sup>	20.2 <sup>ef</sup>	0.19 <sup>de</sup>	0.050 <sup>cd</sup>	13.3 <sup>f</sup>	3.81 <sup>i</sup>	1.06 <sup>k</sup>	1.42 <sup>h</sup>	625.6 <sup>c</sup>	126.3 <sup>c</sup>	184.3 <sup>a</sup>
G5	10.4 <sup>bcd</sup>	25.7 <sup>cde</sup>	0.21 <sup>de</sup>	0.060 <sup>abc</sup>	17.2 <sup>bc</sup>	5.22 <sup>e</sup>	1.67 <sup>ef</sup>	1.23 <sup>j</sup>	728.9 <sup>b</sup>	147.2 <sup>b</sup>	180.3 <sup>b</sup>
G6	8.2 <sup>d</sup>	25.3 <sup>cde</sup>	0.14 <sup>e</sup>	0.035 <sup>f</sup>	10.5 <sup>g</sup>	2.89 <sup>f</sup>	0.84 <sup>i</sup>	1.00 <sup>j</sup>	758.2 <sup>a</sup>	153.2 <sup>a</sup>	158.9 <sup>c</sup>
G7	14.1 <sup>ab</sup>	21.4 <sup>ef</sup>	0.28 <sup>abc</sup>	0.071 <sup>a</sup>	18.4 <sup>ab</sup>	6.80 <sup>b</sup>	2.70 <sup>b</sup>	0.92 <sup>k</sup>	248.1 <sup>k</sup>	50.1 <sup>i</sup>	117.3 <sup>d</sup>
G8	12.7 <sup>abc</sup>	18.3 <sup>fg</sup>	0.23 <sup>bcd</sup>	0.060 <sup>abc</sup>	18.8 <sup>a</sup>	8.46 <sup>a</sup>	2.83 <sup>a</sup>	1.92 <sup>ef</sup>	263.3 <sup>j</sup>	76.0 <sup>j</sup>	106.2 <sup>e</sup>
G9	14.9 <sup>a</sup>	28.4 <sup>bcd</sup>	0.18 <sup>de</sup>	0.048 <sup>cde</sup>	14.2 <sup>ef</sup>	4.65 <sup>f</sup>	1.54 <sup>g</sup>	2.65 <sup>b</sup>	435.0 <sup>f</sup>	88.1 <sup>g</sup>	91.3 <sup>f</sup>
G10	13.1 <sup>abc</sup>	26.2 <sup>cde</sup>	0.24 <sup>abcd</sup>	0.054 <sup>bcd</sup>	14.3 <sup>ef</sup>	4.16 <sup>g</sup>	1.33 <sup>j</sup>	1.95 <sup>ef</sup>	439.1 <sup>f</sup>	89.7 <sup>fg</sup>	35.2 <sup>k</sup>
G11	16.0 <sup>a</sup>	36.0 <sup>g</sup>	0.29 <sup>a</sup>	0.072 <sup>a</sup>	12.9 <sup>f</sup>	3.70 <sup>f</sup>	1.17 <sup>j</sup>	1.57 <sup>g</sup>	588.1 <sup>d</sup>	119.0 <sup>d</sup>	38.6 <sup>k</sup>
G12	16.6 <sup>a</sup>	26.5 <sup>cde</sup>	0.29 <sup>a</sup>	0.068 <sup>a</sup>	13.0 <sup>f</sup>	3.78 <sup>f</sup>	1.21 <sup>j</sup>	2.57 <sup>c</sup>	411.1 <sup>g</sup>	83.1 <sup>hi</sup>	52.1 <sup>i</sup>
G13	12.7 <sup>abc</sup>	21.5 <sup>ef</sup>	0.20 <sup>de</sup>	0.042 <sup>def</sup>	18.3 <sup>ab</sup>	6.45 <sup>c</sup>	2.17 <sup>c</sup>	1.53 <sup>g</sup>	280.1 <sup>i</sup>	56.3 <sup>k</sup>	38.0 <sup>k</sup>
G14	14.2 <sup>ab</sup>	33.7 <sup>ab</sup>	0.22 <sup>cd</sup>	0.046 <sup>def</sup>	15.0 <sup>e</sup>	4.39 <sup>g</sup>	1.38 <sup>hi</sup>	2.22 <sup>d</sup>	395.3 <sup>h</sup>	80.1 <sup>i</sup>	43.6 <sup>j</sup>
G15	16.2 <sup>a</sup>	25.5 <sup>cde</sup>	0.33 <sup>a</sup>	0.064 <sup>ab</sup>	10.7 <sup>g</sup>	2.82 <sup>f</sup>	0.89 <sup>i</sup>	1.99 <sup>e</sup>	464.3 <sup>e</sup>	94.1 <sup>e</sup>	48.5 <sup>j</sup>
G16	10.7 <sup>bcd</sup>	25.5 <sup>cde</sup>	0.19 <sup>de</sup>	0.037 <sup>ef</sup>	17.6 <sup>bc</sup>	5.66 <sup>d</sup>	1.83 <sup>d</sup>	1.07 <sup>j</sup>	105.3 <sup>k</sup>	21.5 <sup>m</sup>	50.4 <sup>i</sup>

absorbance of samples at 645 and 663 nm was recorded by spectrophotometer. A pinch of CaCO<sub>3</sub> was added to avoid destruction of chlorophyll and other pigments. Chlorophyll contents concentrations were calculated as mg g<sup>-1</sup> FW.

#### Total soluble sugars

The amount of total soluble sugars was estimated using anthrone reagents as given by Thimmaiah (2004). 100 mg sample from the tissue culture derived from plants 6 months after transplanting to the pots, was taken in a boiling tube and hydrolyzed with 5 ml 2.5 N HCl in a water bath for 3 h. It was then neutralized with solid sodium carbonate until effervescence ceased. The volume was made to 100 ml followed by centrifuge at 5000 rpm for 10 min. The supernatant was collected and 1 ml sample was taken for analysis. 4 ml anthrone reagent was added to aliquot and heated in a water bath (70°C) for 1 min. The sample rapidly cooled and the change of green to dark green color was read at 630 nm against blank.

#### Total Phenols

Total phenolic compounds were determined by the Folin-Ciocalteu method. The extract samples (20 µl) were mixed with Folin-Ciocalteu reagent (100 µl) for 5 minutes and then aqueous Na<sub>2</sub>CO<sub>3</sub> (100 µl, 1 M) were added. The mixture was

allowed to stand for 30 minutes. The phenols were determined by the colorimetric method at 765 nm. Total phenol values are expressed in terms of Gallic acid equivalent (µg/g of fresh weight). Total phenol contents were calculated as a Gallic acid from a calibration curve.

#### Total Protein

Biuret method of Racusen and Johnstone (1961) was adopted for the estimation of total soluble protein contents. 2.0 g of plant material was crushed in the ice chilled pestle and mortar containing 0.2 g of PVP (polyvinylpyrrolidone) with 8 ml of 0.1 M phosphate buffer at 4°C. For the extraction of total soluble protein contents, slurry was centrifuged at 10,000 rpm for 10 mins at 4°C. The supernatant was used for further analysis.

#### Steviol Glycosides Assay

Sweet glycosides were extracted from the leaves when the plants were 6 months old. The metabolites were extracted according to the method of Kolb et al. (2001) with slight modifications. 1 g of dried and powdered leaves was immersed in 100 ml of 70% (v/v) ethanol in a conical flask. The sample was incubated for 30 min in a water bath at 70°C with continuous shaking. After cooling, a 10 ml aliquot was filtered (Whatman No. 42). Finally, the sample was passed

through a nylon syringe filter with a pore size of 0.22 µm, and then 20 µl of sample was analyzed using a Shimadzu CLASS-VP V6.13 SP1.A, 250 × 4.6 mm, 5 µm in diameter, reverse phase C-18 silica column HPLC system. The mobile phase for rebaudioside-A consisted of acetonitrile and water in a ratio of 60:40 (by volume) at pH 5.6. The flow rate was adjusted to 1 ml min<sup>-1</sup>. The peaks of steviol glycosides were detected at a wavelength of 210 nm. Chromatographic peaks were confirmed and quantified by comparing their retention times with rebaudioside A standard after obtaining a calibration curve. All chromatographic analyses were performed isocratically at ambient temperature. The standards for rebaudioside A, (Code No. 183-02361) used for the quantification were purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). Water, acetonitrile, and ethanol were of chromatographic grade (Fischer) and all other chemicals were of analytical grade.

### Statistical analysis

Data were analyzed for significance using one-way analysis of variance (ANOVA) and the differences contrasted using Duncan's multiple range test (DMRT) at  $p \leq 0.05$ . All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 11.5).

### Conclusion

The effects of different nutritional components on stevia plant are variable and the ongoing research by different mixtures will be open. In above study, according to physiological and biochemical findings, we can conclude the highest effect can be earned by using G11 planting bed with some modification on cocopeat percentage, which may raise the amounts of rebaudiosides.

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