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Influence of biofertilizers on stevioside content in *Stevia rebaudiana* grown in acidic soil condition

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ABSTRACT

The present study was carried out to evaluate the influence of biofertilizers on stevioside content (Main active principle) in the leaves of Stevia rebaudiana grown in acidic soil condition. The six month field experiment study was revealed the combined application of three biofertilizers showed that the total yield of fresh biomass has been recorded an increase up to sixth month, being highest in the combined application of biofertilizers over that of their corresponding sole applications. The percent increase of bio-mass yield was recorded highest (22.14%) in the treatment when all the bio-fertilizers were applied together. Further the results envisaged the content of stevioside were recorded significantly higher (20.17 %) with the same treatments (T_8) as compared to control by applied HPLC chromatogram. The chromatographic separations were carried out using a C_{18} column using the mobile phase consisting of methanol and water, and with UV detection at 210 nm. The limits of determination of stevioside were 4 μg/ml for the leaf extracts.

Key words: Biomass yield, Biofertilizers, HPLC, Stevioside, Stevia.

INTRODUCTION

Stevia rebaudiana, the nature's sweetest gift belongs to the family asteraceae. It is an amazing plant from the rain forest of Amazone. The other names of *Stevia* are sweet leaf, honey leaf, sweet herb, honey yerba. *Stevia* is a native to South America (Paraguay, Brazil) [1] but extensively grown in places like Central America, Israel, Australia, Japan and China [2]. *Stevia* is distinguished by the presence of the sweet diterpene glycosides: rebaudioside A, rebaudioside C, stevioside and dulcoside in its leaf tissue [3]. Stevioside, a white crystalline compound

isolated from *Stevia* is 100 to 300 times sweeter and rebaudioside A is 150–320 times sweeter than sucrose [4]. *Stevia* have versatile medicinal uses without any side effects that focus the interest towards *Stevia* in World wide. It is used for the treatment of various conditions such as cancer [5], diabetes [6], obesity, cavities, hypertension [7], fatigue, depression, and in cosmetic and dental preparations [8]. It possesses hypoglycemic, hypotensive, vasodilating, taste improving, sweetening, anti-fungal, anti viral, anti inflammatory, anti bacterial [9] properties and increases urination function of the body.

Literature survey revealed active constituents especially stevioside content in *Stevia* plant is greatly depends on the package of practices for the cultivation of *Stevia* and adoption of modern agro- techniques [10, 11] and water management [12]. In view of the above fact, cultivation of *Stevia* is gradually coming into focus in Indian agric-field due to having no optimum agronomic management practices. Biofertilizers can increase the amount of deficient nutrients and enrich the soil nutrients where deficiency in the soil was observed. In combination it helps biological nitrogen fixation for the benefit of plants. Along with the biofertilizers farm yard manures (FYM) are also mixed to the soil to control the deficiency of phosphate content in soil and increase the water holding capacity of the soil to stimulate the activity of beneficial microorganisms that makes the plant food elements in the soil readily available to the plants. Looking at that the present Ph.D research was carried out to determine the stevioside content in Stevia leaves by applied biofertilizers and this was the first report by the Indian researchers.

MATERIALS AND METHODS

Cutting of *Stevia* plants, collected from Ankur Nursery, Ripponpet (Shimoga, Karnataka), India, were used as a test plant for the present study. A field experiment was conducted in the month of November 2005 at the Ripponpet, Shimoga on acidic soil reaction. Before start of the experiment, initial soil pH, org carbon, Cationic Exchange Capacity (CEC), available N, P and K were determined separately. Soil pH were determined by pH meter (Elico, India) by preparing solution with ration of soil: water, 1: 2.5 w/v, organic carbon by wet digestion with mixing of potassium dichromate, CEC by Kjeldahl method after extracting the soil with ammonium acetate. The relevant physicochemical properties of soils were: pH 6.10; organic carbon, 0.54%; CEC, 9.89 Cmol (p+) kg^{-1}. Fields were divided into beds according to treatments. The following treatments were used.

T_1-- Control, only FYM @ 10 kg, without application of bio-fertilizers. T_2 – soil application of FYM @ 10 kg mixed with 250 g of Phosphorus Solubilizing Bacteria (PSB) (*Bacillus megatheriam*). T_3 – soil application of FYM @ 10 kg mixed with 250 g of Azospirillum (AZO). T_4 – soil application of FYM @ 10 kg mixed with 500 g of *Vesicular Arbuscular Mycorrhiza* (VAM). T_5 – soil application of FYM @ 10 kg mixed with 250 g of PSB and 250 g of AZO. T_6 – soil application of FYM @ 10 kg mixed with 250 g of PSB and 500 g of VAM. T_7 – soil application of FYM @ 10 kg mixed with 250 g of AZO and 500 g of VAM. T_8 – soil application of FYM @ 10 kg mixed with 250 g of PSB, 250 g of AZO and 500 g of VAM. Each treatment was replicated thrice in a completely randomized design (CRD). Altogether there were 24 (8 X 3) beds, with each bed size of 1 M X 1.25 M. Cutting of *Stevia* plants were planted at the same time in all the beds. Each bed contains 6 rows and 6 columns that contain 36 plants (6X6), with total of 864 plants (36 X 24). The moisture content was maintained to 60 % of water holding

capacity (WHC) by irrigating three times in a week. The periodic collection of leaves samples were made and analyzed for stevioside content in month wise intervals up to six months by HPLC method described by Bovanova et al., 1998 [13].

Conditions for HPLC study: Column used C-18, Mobile Phase used Methanol: water (80:20) with the flow rate of 1.5 ml/min (injection: 10µl). Peak detection was made at 210 nm at the room temp of 25^0C.

Standard Preparation: 1mg of stevioside sample was dissolve in 10 ml of methanol. Then 10 µl was applied to HPLC chromatogram.

Sample Preparation: 1 mg of each extracted samples were dissolved in 50 ml of methanol and 10 µl was applied to the HPLC chromatogram.

RESULTS AND DISCUSSION

Biomass yield:

The results (Table-1) show that the amount of fresh biomass yield has been found to increase progressively irrespective of treatments over control. However the magnitude of such changes varied with treatments, being recorded highest (928 g) in the treatment T_8 at 6 months of plant growth which might be due to combined application of biofertilizers which caused maximum fixation of atmospheric nitrogen, increased uptake of soil P and K by the *Stevia* plant. The percent response of microbial inoculant towards the total biomass yield was recorded highest (22.14%) in the treatment where VAM+PSB+AZO was inoculated altogether which was closely followed by PSB+AZO (18.61%) and VAM+AZO (17.12%).

The results also show that the percent increase in the bio-mass yield of *Stevia* was recorded highest in the treatment receiving FYM + PSB + AZO + VAM simultaneously followed by the treatment T_6 where FYM + PSB +VAM were applied together. Sood and Kumar (1994) [14] also reported that green and dry foliage yield increased with increasing levels of N and P, which also confirmed the results obtained in the present study.

Table-1. Fresh biomass (g) of *Stevia* in acidic soil zone

Treatments	1 month	2 month	3 month	4 month	5 month	6 month	Mean(kg)	%Response
Control	400	560	648	694	720	740	3.76	----
PSB	460	610	720	756	798	810	4.15	10.42
AZO	470	670	750	750	765	783	4.18	11.32
VAM	380	612	690	798	822	859	4.16	10.61
V + A	473	720	765	790	822	836	4.40	17.12
P + A	495	690	770	816	836	855	4.46	18.61
P + V	468	620	780	800	828	848	4.34	15.47
P + A + V	350	735	815	865	902	928	4.59	22.14
CD value(p= 0.05)	54.78	39.45	37.32	41.68	59.44	65.27		

PSB= Phosphate solubilising bacteria, AZO= Azospirillum, VAM= Vesicular arbuscular mycorrhiza, V+P+A= VAM+ PSB+ AZO

HPLC Study:
The HPLC study of Stevioside in the aqueous extracts of dried *Stevia* leaves (collected from acidic soil field, up to 6 month), was determined and were tabulated in table 2 (Graph-1& 2 respectively). In Table-1, all the individual *Stevia* extracts (Month wise collected from acidic soil zone) showed the presence of Stevioside when compared with that of standard Stevioside (Purity = 97%) with the retention time at 6.01 minutes with the R^2= 0.9998. Results were triplicates tabulated with its significant data. Results showed that content of Stevioside have been found to be increased significantly with the increase of biomass content up to sixth month of study irrespective of treatments. However, the results further envisaged that the percentage content of Stevioside has been recorded highest in the combined application of three biofertilizers (20.17%) over that of corresponding sole applications and that of control treatment. It has been proved by research trials conducted at New Delhi, India, indicated attainable yield in the order of 2000 to 3000 kg/ha dry leaf and stevioside content ranged 15-20%, which was double than that of other parts of the world where *Stevia* has been already been grown and processed commercially [15]. It was also stated that cultivation of *Stevia* will allow a 7-fold increase in production of sugar equivalent [16]. The same trend was followed in this present study.

Statistical Analysis: Results were repeated thrice and the Standard Error Mean were calculated by using Instat Graph Pad, Version 3. Further Critical differences were find out for three field trial readings to find out biomass content in Stevia plant.

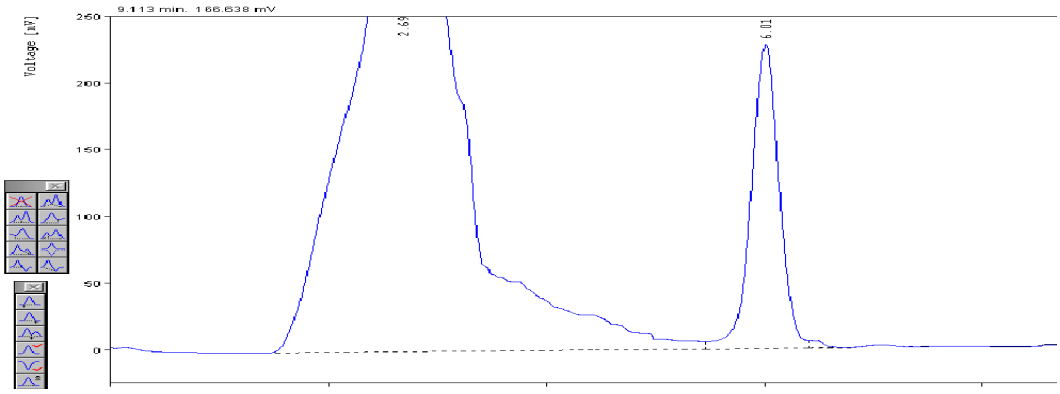
Table-2. Stevioside content (%) in *Stevia* collected from acidic soil zone
(Mean of three readings ± SEM)

Treatments	1 month	2 month	3 month	4 month	5 month	6 month
Control	2.90±0.013	6.55±0.017	9.50±0.023	11.20±0.033	11.05±0.023	11.64±0.014
PSB	2.77±0.021	7.06±0.025	10.47±0.019	10.91±0.013	11.78±0.012	12.22±0.003
AZO	3.02±0.021	7.87±0.027	9.91±0.034	9.50±0.032	9.91±0.023	11.93±0.018
VAM	1.62±0.014	5.58±0.018	7.91±0.012	9.96±0.005	10.08±0.007	11.27±0.023
V + A	2.90±0.042	10.33±0.023	11.34±0.021	11.93±0.016	12.07±0.012	12.36±0.011
P + A	3.80±0.018	10.18±0.012	11.78±0.010	12.80±0.027	12.94±0.014	13.38±0.010
P + V	2.77±0.003	7.60±0.033	11.78±0.018	12.51±0.017	13.09±0.012	13.09±0.010
P + A + V	2.26±0.020	11.93±0.019	13.38±0.006	19.08±0.013	19.40±0.012	20.17±0.017

PSB= Phosphate solubilising bacteria, AZO= Azospirillum, VAM= Vesicular arbuscular mycorrhiza, V+P+A= VAM+ PSB+ AZO

Graph-1 Standard Stevioside

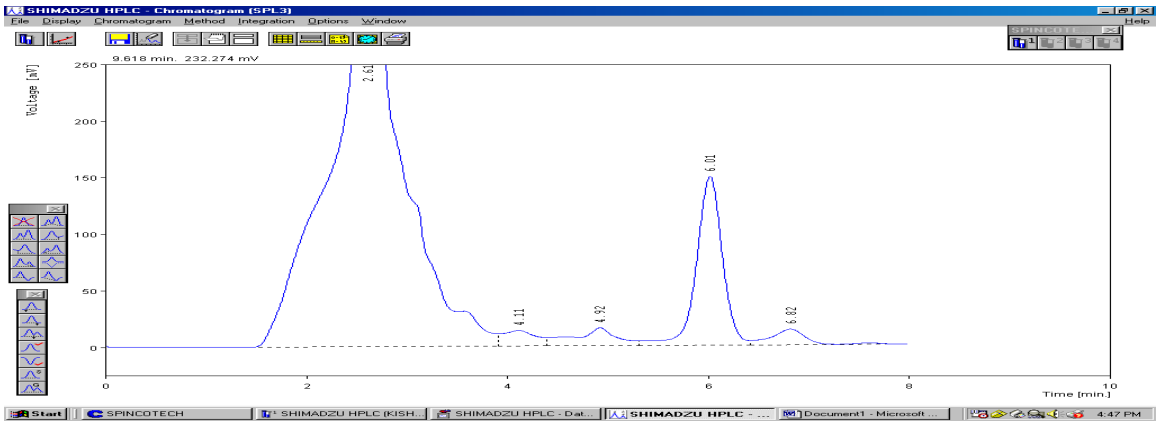
Peak No.	RT	Peak area mV.s	Height mV	W05 (min)	Area %	height %
1	2.687	36967.6019	1003.5278	0.4267	87.5950	70.9677
2	6.013	3108.5727	228.7439	0.2733	9.7353	15.9999
-	Total	40076.1746	1232.2717			



Standard Stevioside

Graph-2 VAM + PSB + AZO Sample (Acid soil)

Peak No.	RT	Peak area mV.s	Height mV	W05 (min)	Area %	height %
1	2.607	20722.4855	785.3084	0.2133	84.4262	80.2729
2	4.107	317.9981	13.7677	0.4867	1.2956	1.4073
3	4.920	462.1775	15.9422	0.3000	1.8830	1.6296
4	6.013	1304.4530	149.5035	0.2733	11.0203	15.2820
5	6.820	337.4743	13.7770	0.3400	1.3749	1.4082
-	Total	23144.588	978.2989			



Presence of stevioside in VAM+ PSB+ AZO applied sample (Sixth month study)

CONCLUSION

The results suggested that the biomass yield and nutrient content in stevia plant has been markedly increased due to bio-fertilizer application with either as single or in combination. However, the overall results suggested that the combined application of bio-fertilizers was always superior as compared to sole applied biofertilizers which enhanced significant growth of

foliage growth of Stevia leaves. In other way, the HPLC study revealed the presence of Stevioside in the Stevia extract in acidic soil and interestingly, the magnitude of increased Stevioside content was higher for Stevia extracts collected from treatment 8 where combination of three biofertilizers were applied and that was due to increased amount of organic carbon and other macro and micronutrient contents in the acidic soil with the biofertilizers.

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