

Influence of biofertilizers on protein, moisture and ash content in relation to swelling property, water absorption capacity, mineral elements, total phenolic level of *Stevia rebaudiana* Bert.) plant grown under acidic soil zone of South India

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ABSTRACT

The field experiment on *Stevia rebaudiana* (SR), (Family: Asteraceae) was carried out in acid soil zone of Shimoga (Karnataka) in the year 2009 and 2010 to investigate the influence of biofertilizers on protein, moisture and ash content in the dried *Stevia* leaf and their correlation with swelling property, water absorption capacity and mineral element contents. Results revealed that second harvested sample (August) in first year (2009) was better for all aspects than other harvested samples and the results showed significant increase in protein content (16.22 %), swelling index (SI) (5.10 % w/w), water absorption capacity (WAC) (4.91 ml/g) with the treatment T₈ where three bio fertilizers were applied togetherly with simultaneous moisture content varied from 6.61 to 7.18 %. The significant higher correlations were observed (significant at 1 %) between protein content with SI ($r = 0.99$), WAC ($r = 0.99$) and SI with WAC ($r = 0.98$) in 2009 harvested samples. The ash content was higher in third harvested sample (13.54 %) in the year 2009 with simultaneous increased amount of Fe (6.19 mg/kg), Mn (1.24 mg/kg), Cu (0.72 mg/kg) and Zn (1.22 mg/kg) contents due to the residual effect of bio fertilizers. Nitrogen (0.74 mg/kg), P (0.40 mg/kg) and K (0.68 mg/kg) contents were higher during second harvested samples and thereafter decreased. Total phenolic content was also recorded highest (64.52 mg/g) with three bio fertilizers applied togetherly during year 2009 compared to the year 2010 (50.12 mg/g). The results suggest that the application of bio-fertilizers either single or more numbers in a balanced way may be beneficial in improving protein, phenol and mineral contents of stevia plant.

Keywords: Atomic Absorption Spectrophotometer; Biofertilizers; *Stevia rebaudiana*; physiochemical properties; elements; phenols

1. INTRODUCTION

S. rebaudiana Bertoni (Family: Asteraceae) is an ancient South American perennial plant (native to Paraguay and Brazil) with great potential as an agricultural crop for the production of a high-potency natural sweetener (Soejarto, 2002). It is the source of a number

of sweet ent-kaurene diterpenoid glycosides namely Stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside E, and dulcoside A. (Prakash et al., 2008). Among that stevioside is mainly present (about 4-20 %) in dry-leaf matter (Ghanta et al., 2007) of Stevia leaf. Stevioside has been used as intensive more energetic sweeteners (have non caloric) in many countries of South America and Asia. The potential medicinal uses of Stevia are hypoglycemic, cardiovascular, antimicrobial, digestive tonic, dental and skin care (Mowrey, 1992). It is grown commercially in many parts of Brazil, Paraguay, Uruguay, Central America, Israel, Thailand and China. It was reported that the amounts of active components are dependent on total biomass yield, climatic feature, method of agro-techniques, water management and also fertilizer applications (Fronza & Folegatti, 2003; Megeji et al., 2005). Even herbal drug industry requires large quantities of biomass for extraction of the sweetening compounds and hence need to enhance its biomass production through improved cultural techniques and application of manures and fertilizers including biofertilizers. Considering the importance, the stevia plant has domesticated in India in late 20th century and commercially cultivated in few states of India especially in Gujarat, Karnataka, Tamil Nadu, West Bengal, and Uttar Pradesh. Recently, *Vesicular Arbuscular Mycorrhiza* (VAM), *Phosphate Solubilizing Bacteria* (PSB) and *Azospirillum* (AZO) are the most widespread bio-fertilizers significantly contributing macronutrients (N, P and K) and micronutrients (Fe, Mn, Cu and Zn) to plants (Smith and Read, 1997) and increased biomass with directly related to the better growth of the plant for commercial cultivation.

Phosphorus-solubilizers are biofertilizers which solubilize the adsorbed or fixed phosphorus in soil and make it available to plants. *Azospirillum*, heterotrophic nitrogen fixing organism, has been reported to be beneficial and economical on several crops. Bio fertilizers improved the growth, yield and protein content as well as the productivity of the crop. While, VAM fungi are the best known for their ability to improve plant growth in low phosphate soils by exploiting large areas of soil and actively transporting the phosphate back to the plants. It also increases the conditions of soil, uptake of Zn and P, improve nitrogen fixation, pH tolerance, increases the uptake of immobilized nutrients and finally improves the crop growth. Along with the biofertilizers, farm yard manures (FYM) are also mixed with the soil to control the deficiency of macro and micronutrient contents in soil and increases the water holding capacity of the soil to stimulate the activity of beneficial microorganisms making unavailable nutrients available to the plants. Bio fertilizer application enhances the protein content and it is evident that proteins increase water holding capacity, when their swelling ability is enhanced. Water holding capacity is an important function of protein in viscous foods such as soups, gravies, doughs, and baked products. Hence, there is a need to develop a suitable low cost technology for better agricultural crop production. Apart from this, bio fertilizer also increases the phenolic content in the crop which are important for normal growth, development and defense against infection and injury. The presence of phenolic compounds in injured plants imparts the oxidative stability, microbial safety and important to human health because of their antioxidant potency (Hollman et al., 1996).

Keeping these in view, the present field investigation was undertaken to evaluate potentiality of Stevia plant about its functional, health promoting properties and raw materials for industry under improved agronomic management practices coupled with bio fertilizer application.

2. MATERIALS AND METHODS

2. 1. Description of experimental sites and its cropping history

This study was conducted at Ripponpet farm, Shimoga (latitudes 13°27' and 14°39' N and between the longitudes 74°38' and 76°04' E at a mean altitude of 640 metres above sea level) during 2009 and 2010. The soil at the Ripponpet farm has a sandy loam texture, with relatively high fertility, pH 6.10. The area usually receives mean annual rainfall of 1815 mm and daily temperature range of 26 to 30 °C, but in summer time it reaches to 40 °C.

2. 2. Details of experimental design and field layout

Cuttings of *Stevia* plants, collected from Shimoga district, Karnataka, India, were used as a test plant for the present study. The field experiments were conducted in the month of February 2009 and data were collected for two years. Experimental fields were divided into beds according to treatments. The results of pre-planting surface (0-15 cm) soil analysis from the experimental plot was analyzed as pH 6.10, organic carbon 0.54 %, cation exchange capacity (CEC) 9.89 cmol (p+)/kg, available N, P and K were 55.89 mg/kg, 6.8 mg/kg and 86.4 mg/kg. DTPA extractable Zn, Cu, Fe and Mn were 0.54 mg/kg, 0.15 mg/kg, 5.2 mg/kg and 3.4 mg/kg. Based on that results, and as per advise of agronomist, the following treatments were used: T₁ – Control, only FYM (farmyard manure) at 10 kg, without application of biofertilizers; T₂ – Soil application of FYM at 10 kg mixed with 250 g of PSB (phosphorus solubilizing bacteria, *Bacillus megatherium*); T₃ – Soil application of FYM at 10 kg mixed with 250 g of AZO (*Azospirillum*); T₄ – Soil application of FYM at 10 kg mixed with 500 g of VAM (*Vesicular arbuscular mycorrhiza*); T₅ – Soil application of FYM at 10 kg mixed with 250 g of VAM and 250 g of AZO; T₆ – Soil application of FYM at 10 kg mixed with 250 g of PSB and 500 g of AZO; T₇ – Soil application of FYM at 10 kg mixed with 250 g of PSB and 500 g of VAM; T₈ – Soil application of FYM at 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 in all the beds. Each bed contains 864 (36 X 24) plants with 6 rows and 6 columns (6X6). The moisture content was maintained at 60-70 % of water holding capacity (WHC) by irrigating as per requirements.

2. 3. Plant sampling

Fresh leaf samples at an interval of three months were collected (May, August and November) for continuous two years, dried and was analyzed separately for various physicochemical parameters. Year wise pooled biomass was collected separately (as per treatment wise) and analyzed for SI, WAC, element contents and total phenol content.

2. 4. Preparation of *Stevia* leaves

The *Stevia* plants that reached (the maximum growth stage every after 3 months intervals) were harvested by cutting the plant at 5 cm from the cultivated plots (flowering was avoided by cutting the tops every 15 days alternate). The brown and yellow leaves were removed from the plants then, washed in clean water and spread on trays covered with cheese-cloth to remove the excess of water. The plants were dried in an oven temperature at 35-40 °C for 4-8 h. Dried leaf samples were coarsely powdered, weighed and stored in

moisture free sealed plastic cover in a dust proof cupboard for the further investigations. The yields of the pooled dried leaf biomass in the year 2009 and 2010 were presented in Table 7.

2. 5. Preparation of extract

An aqueous extraction was performed for dried powdered *Stevia* leaves. 100 g dried leaves were soaked with 2.5 g of CaCO_3 at pH 10 for 8 hours and removed the impurities. All samples were refluxed with water for 4hrs after standardized the method. Finally crude extract was collected after filtration followed by rotary flash evaporation at 45 °C and were stored in well closed glass bottles in refrigeration condition at 4 °C. The crude extracts were used for further investigation.

2. 6. Physicochemical assessment

Chemical analysis to determine proximate composition of sample was carried out using standard procedure. Moisture content was determined by air drying at 105 °C until a constant weight was attained (AOAC, 1990), proteins content was determined from the nitrogen content by Kjeldahl method (Regal's 234, supplied by Popular Science Apparatus Workshops Pvt Ltd, India) (AOAC, 978.04) using factor 6.25 and calculated as $\text{N} \times 6.25$ (AOAC, 1995), swelling property was determined by measuring the volume difference between before and after swelling by soaking in water for overnight (Mukherjee, 2007), water absorption capacity was determined by method described by Rosario and Hores (1981), ash contents by incineration method at a temperature 450 °C, mineral elements composition estimated using the atomic absorption spectrophotometer (AAS) after acid digestion of the samples. All the results were expressed as % on dry weight basis.

2. 7. Elemental analysis

Essential and non essential element contents, i.e. nitrogen (N), copper (Cu), manganese (Mn), iron (Fe) and zinc (Zn) were determined from aliquots of the ash solutions (after digestion with acid mixtures) according to the method of AOAC (2000) using Kjeldahl method (for N) and AAS. Further Determination of phosphorus (P) content in plant was done by using a spectrophotometer (ECIL, made in India) after development of yellow color with vanado-molybdo phosphoric acid and then the intensity of color was determined at 420 nm (blue filter) and potassium (K) content in plant samples was determined by using Flame Photometer (Elico, made in India) after plant samples (0.5 g) were digested with a di-acid mixture (H_2SO_4 : HClO_4 : 6: 4).

2. 8. Atomic absorption spectrophotometer

Content of the mineral elements in the dried leaf sample of cultivated *Stevia* were measured with double-beam atomic absorption spectrophotometer (Model-Aanalyst 100, supplied by Perkin Elmer, USA). The main source used for AA was the hollow cathode lamp (HCL). The dual option burner system was used with flow spoiler in a spray chamber. The common oxidant/fuel combination used in AA was air-acetylene. The best wavelengths were selected for the analysis based on the concentration ranges of sample. The slit width (0.2 nm) was the optimum for the elements which are determined. The following conditions were selected for said metals analysis:

Elements	Wavelength (nm)	Ash Temperature (°C)	Atomization Temperature (°C)
Mn	279.5	800	2400

Flame Atomic Absorption System

Elements	Wave length	Gas mixture
Cu	327.4	Air-acetylene
Fe	372.0	Air-acetylene
Zn	213.9	Air-acetylene

The concentration of the above said elements was determined by using the standard conditions. The method was based upon the linear relation between the absorbance (AU) and concentration of the determined element. Samples were made in triplicate. The obtained results are expressed in mg/kg on a dry mass weight in sample.

2. 9. Determination of total phenolic content

Phenolic content in the leaf extract (aqueous extract) of SR were determined with Folin-Ciocalteu reagent according to the method described by Slinkard and Singleton (1977). Gallic acid was used as a standard. About 1.0 ml of extract solution containing 1.0 g extract in a volumetric flask was diluted with 46 ml of distilled water. About 1.0 ml of Folin–Ciocalteu reagent was added and mixed. After few minutes 3.0 ml of 2 % sodium carbonate was added and then the mixture was allowed to stand for 3 h with occasional shaking. The absorbance of the blue color that developed was measured at 760 nm. The concentration of total phenols was expressed as mg/g of dry extract (Kim et al., 2003) that was determined as mg of Gallic acid equivalent per g using the equation, taken from prepared standard Gallic acid calibration curve $y = 15.27x + 12.44$, $R^2 = 0.997$.

2. 10. Statistical Analysis

The results of the field experiment were expressed as mean $\pm$ standard error of mean (SEM) of three replicates. The data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by using Graph Prism Pad 5 software. The values showing $p < 0.05$ were regarded as significant. All results further analyzed by post test (Dunnett comparison test) for the significant results.

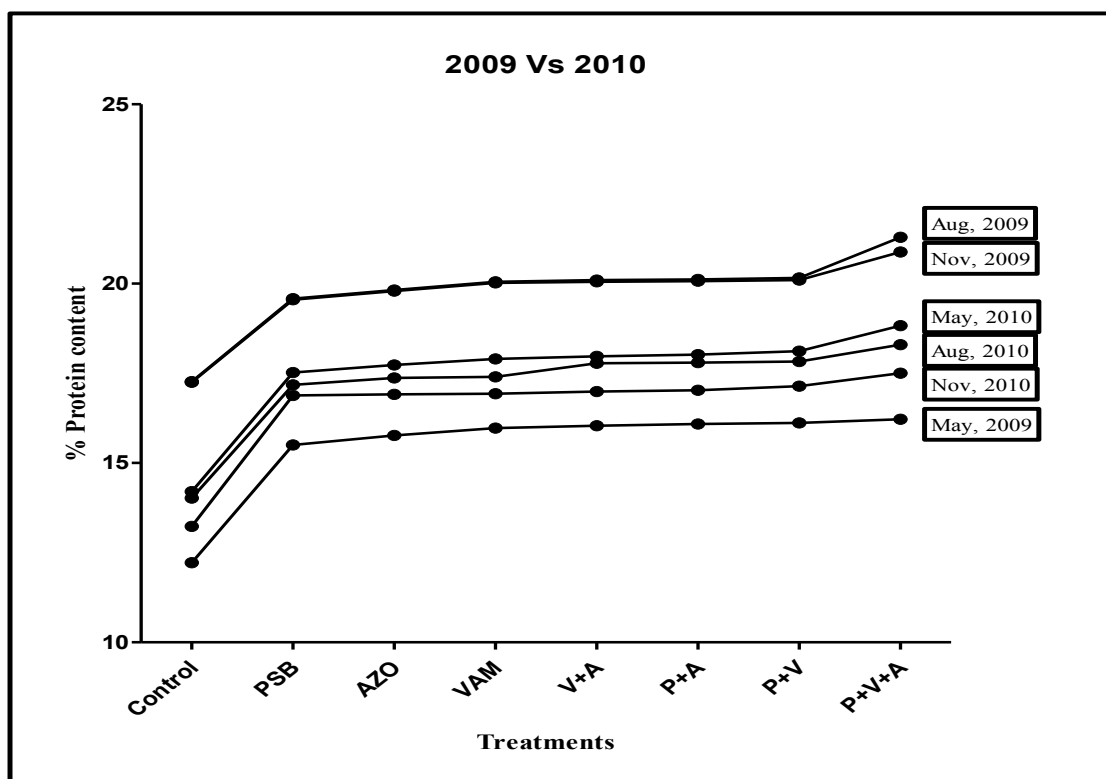
3. RESULTS

3. 1. Physicochemical assessment

The First harvested sample in May, 2009, where crude protein content was higher with the treatment eight (16.22 %) followed by treatment seven (16.12 %) where combinations of three and two biofertilizers were applied respectively. All the values were even higher than

control (12.22 %) treatment. Ash content, SI and WAC were also followed the same trend as like protein, being higher in T₈ treatment with ash, SI, and WAC contents of 9.13 %, 5.10 % w/w and WAC 4.91 mg/ml respectively. Moisture content was varied with the treatments being higher with treatment four (7.18 %) where VAM biofertilizers was applied followed by 7.15 % with the T₅ and T₇ respectively. Second harvested sample in 2009 were also showed the same trend as first harvested sample with respect to applied biofertilizers.

Fig. 1. Comparative effect biofertilizers on protein content during in two different years.



ANOVA Table

	<u>SS</u>	<u>df</u>	<u>MS</u>
Treatment (between columns)	67.20	7	9.599
Residual (within columns)	120.6	40	3.015
Total	187.8	47	

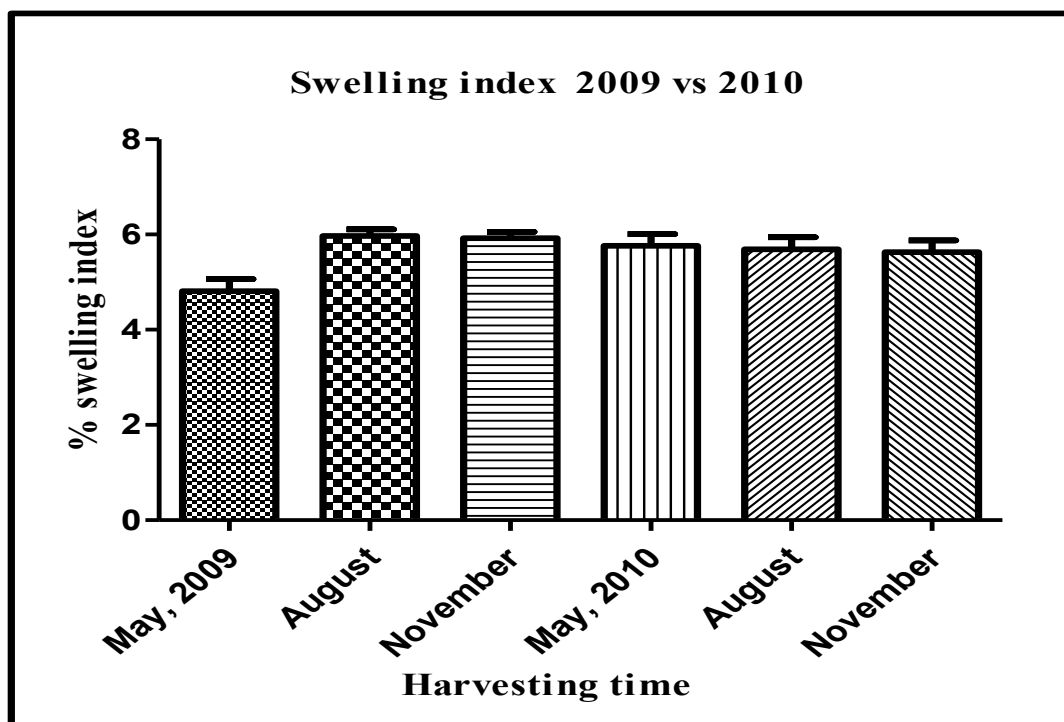
Post test for linear trend

Slope	0.1944
R squared	0.2029
P value	0.0010
P value summary	***

The highest protein content (21.29 %), SI (6.42 %), WAC (5.12 ml/g), ash content (13.43 %) were reported with T₈ from second harvested sample in August, 2009 whereas same has been reported with 20.88 %, 6.22 %, 4.99 ml/g and 13.54 % in third harvested sample collected in November, 2009 which were higher than control sample but moisture contents were varied with the applied biofertilizers in both the dried leaf samples in the range

of 6.91 to 7.00 % from both the harvested crops. In case of harvested sample in 2010, there were significant changes observed for protein content, ash value, SI and WAC whereas no significant changes observed with moisture content. The fourth harvested sample in May 2010, resulted protein content (18.83 %), ash value (13.43 %), SI (6.18 %) and WAC (4.97 ml/g) were higher with same treatments. Fifth and sixth harvested samples in 2010 were also followed the same trend, being high protein content 18.30 and 17.50 %; ash content 12.99 and 10.29 %; SI 6.11 and 6.04 %; and WAC 4.94 and 4.86 ml/g in fifth (August) and sixth (November) harvested samples respectively with treatment eight in the year 2010 but the results were lesser than that of former results. Effect of biofertilizers on protein content during two different years was depicted in Figure 1 and clearly showed higher protein content during year 2009 with second harvested samples. SI and WAC of dried Stevia leaves were also reported higher than other harvested samples (Figure 2 & 3).

Fig. 2. Determination of swelling index from all the harvested Stevia leaf in 2009 and 2010.



ANOVA Table

Treatment (between columns)

SS

df

MS

15.14

7

2.163

Residual (within columns)

8.486

40

0.2122

Total

23.63

47

Post test for linear trend

Slope

0.08680

R squared

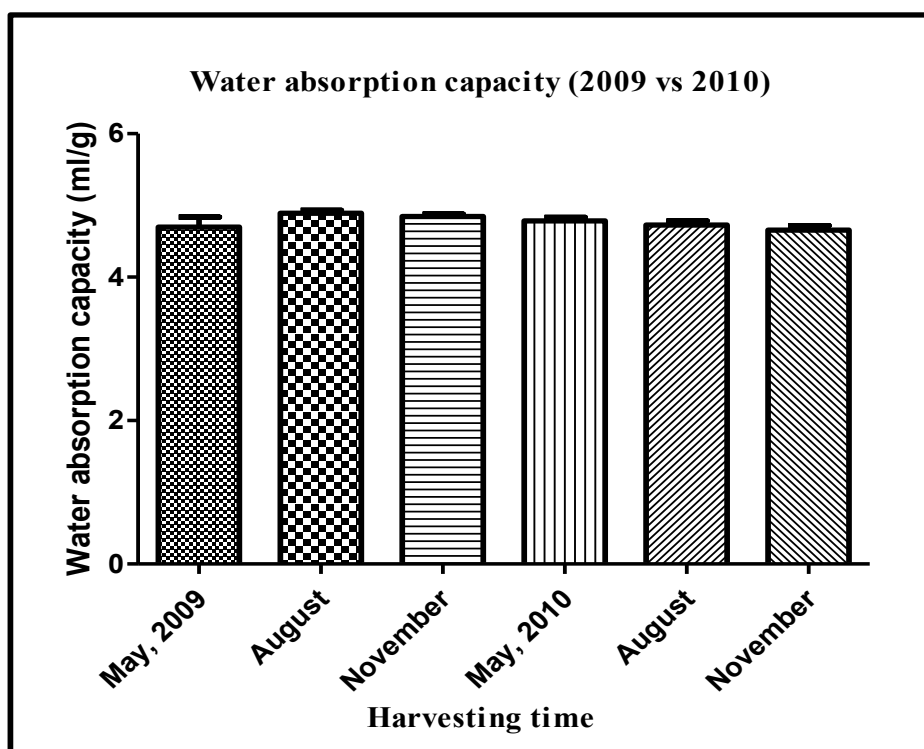
0.3214

P value

P < 0.0001

P value summary

Fig. 3. Determination of water absorption capacity from all the harvested Stevia leaf in 2009 and 2010.

**ANOVA Table**

	<u>SS</u>	<u>df</u>	<u>MS</u>
Treatment (between columns)	1.183	7	0.1690
Residual (within columns)	0.9197	40	0.02299
Total	2.103	47	

Post test for linear trend

Slope	0.03029
R squared	0.4398
P value	P < 0.0001
P value summary	***

Table 1. Correlation matrix between mean % protein content, swelling index and water absorption capacity in both the years.

Parameters	2009			2010		
	Protein	Swelling Index	Water absorption capacity	Protein	Swelling Index	Water absorption capacity
Protein	1.00			1.00		
Swelling Index	0.99**	1.00		0.99**	1.00	
Water absorption capacity	0.99**	0.98**	1.00	0.93*	0.91*	1.00

**Significant at 1 %; *Significant at 5 %

Correlation matrices among protein content, SI and WAC were showed in Table 1, and showed highly significant positive correlation (Significant at 1 %) in year 2009 than in year 2010.

3. 2. Elemental analysis

Table 2 revealed the various microelements contents in the dried Stevia plant in year 2009.

Table 2. Elemental analysis (Micro) in dried Stevia leaf in 2009 (Mean $\pm$ SEM, n = 3, from three replicated plots).

Ts	After first harvest (May) (mg kg ⁻¹ , dry wt basis)				After second harvest (August) (mg kg ⁻¹ , dry wt basis)				After third harvest (November) (mg kg ⁻¹ , dry wt basis)			
	Fe	Mn	Cu	Zn	Fe	Mn	Cu	Zn	Fe	Mn	Cu	Zn
T ₁	3.09 $\pm$ 0.017	0.54 $\pm$ 0.008	0.46 $\pm$ 0.008	0.58 $\pm$ 0.003	3.13 $\pm$ 0.006	0.55 $\pm$ 0.005	0.45 $\pm$ 0.006	0.59 $\pm$ 0.003	3.09 $\pm$ 0.012	0.59 $\pm$ 0.003	0.43 $\pm$ 0.003	0.58 $\pm$ 0.005
T ₂	3.08 $\pm$ 0.011 ^{ns}	0.58 $\pm$ 0.005*	0.47 $\pm$ 0.003 ^{ns}	0.61 $\pm$ 0.006*	4.19 $\pm$ 0.008***	0.67 $\pm$ 0.008***	0.50 $\pm$ 0.003***	0.65 $\pm$ 0.006***	4.21 $\pm$ 0.008***	0.70 $\pm$ 0.005***	0.54 $\pm$ 0.008***	0.72 $\pm$ 0.033***
T ₃	3.17 $\pm$ 0.008 ^{ns}	0.62 $\pm$ 0.008***	0.50 $\pm$ 0.008**	0.65 $\pm$ 0.010***	4.27 $\pm$ 0.008***	0.74 $\pm$ 0.012***	0.50 $\pm$ 0.005***	0.69 $\pm$ 0.005***	4.30 $\pm$ 0.008***	0.77 $\pm$ 0.006***	0.59 $\pm$ 0.005***	0.73 $\pm$ 0.003***
T ₄	3.13 $\pm$ 0.014 ^{ns}	0.57 $\pm$ 0.008 ^{ns}	0.51 $\pm$ 0.003***	0.64 $\pm$ 0.005 ^{ns}	4.20 $\pm$ 0.012***	0.71 $\pm$ 0.008***	0.51 $\pm$ 0.006***	0.67 $\pm$ 0.005***	4.25 $\pm$ 0.006***	0.74 $\pm$ 0.008***	0.55 $\pm$ 0.003***	0.73 $\pm$ 0.005***
T ₅	3.22 $\pm$ 0.043**	0.68 $\pm$ 0.008***	0.57 $\pm$ 0.003***	0.66 $\pm$ 0.008***	4.85 $\pm$ 0.014***	0.85 $\pm$ 0.010***	0.59 $\pm$ 0.006***	0.80 $\pm$ 0.008***	4.87 $\pm$ 0.003***	0.87 $\pm$ 0.006***	0.61 $\pm$ 0.005***	0.90 $\pm$ 0.014***
T ₆	3.15 $\pm$ 0.012 ^{ns}	0.69 $\pm$ 0.003***	0.56 $\pm$ 0.012***	0.63 $\pm$ 0.005***	4.81 $\pm$ 0.005***	0.86 $\pm$ 0.003***	0.57 $\pm$ 0.006***	0.88 $\pm$ 0.008***	4.84 $\pm$ 0.003***	0.90 $\pm$ 0.005***	0.64 $\pm$ 0.008***	0.97 $\pm$ 0.011***
T ₇	3.18 $\pm$ 0.008*	0.66 $\pm$ 0.006***	0.54 $\pm$ 0.005***	0.65 $\pm$ 0.008***	4.81 $\pm$ 0.006***	0.89 $\pm$ 0.003***	0.55 $\pm$ 0.003***	0.87 $\pm$ 0.008***	4.83 $\pm$ 0.013***	0.91 $\pm$ 0.006***	0.62 $\pm$ 0.015***	1.00 $\pm$ 0.005***
T ₈	3.24 $\pm$ 0.011***	0.72 $\pm$ 0.008***	0.60 $\pm$ 0.003***	0.69 $\pm$ 0.005***	6.13 $\pm$ 0.006***	1.21 $\pm$ 0.008***	0.58 $\pm$ 0.008***	1.15 $\pm$ 0.006***	6.19 $\pm$ 0.010***	1.24 $\pm$ 0.010***	0.72 $\pm$ 0.012***	1.22 $\pm$ 0.012***

- Ts = Treatments; T₁ = Control (FYM), T₂ = FYM + PSB, T₃ = FYM + AZO, T₄ = FYM + VAM, T₅ = FYM+VAM+AZO, T₆ = FYM + PSB + AZO, T₇ = FYM + PSB + VAM, T₈ = FYM + PSB + AZO + VAM;
- *P < 0.05, **P < 0.01 and ***P < 0.001; ns = Non significant (compared with control treatment).

During third harvest of the samples in November showed higher accumulation of the elements than other treatments, being higher Fe content 6.19 mg/kg, Mn content 1.24 mg/kg, Cu content 0.72 mg/kg and 1.22 mg/kg with the treatment eight where combination of three biofertilizers were applied.

The values were even higher than control samples. The same trend were also followed in year 2010 where accumulation of Fe (5.77 mg/kg), Mn (1.18 mg/kg), Cu (0.66 mg/kg) and

Zn (1.16 mg/kg) content were higher with the treatment eight in fifth harvested samples in May (Table 3) and further decreased drastically with rest of the harvested samples.

Table 3. Elemental analysis (Micro) in dried Stevia leaf in 2010 (Mean $\pm$ SEM, n = 3, from three replicated plots).

Ts	After first harvest (May) (mg kg ⁻¹ , dry wt basis)				After second harvest (August) (mg kg ⁻¹ , dry wt basis)				After third harvest (November) (mg kg ⁻¹ , dry wt basis)			
	Fe	Mn	Cu	Zn	Fe	Mn	Cu	Zn	Fe	Mn	Cu	Zn
T ₁	2.74 $\pm$ 0.008	0.49 $\pm$ 0.011	0.36 $\pm$ 0.003	0.52 $\pm$ 0.033	2.60 $\pm$ 0.004	0.40 $\pm$ 0.004	0.30 $\pm$ 0.005	0.46 $\pm$ 0.004	2.50 $\pm$ 0.003	0.30 $\pm$ 0.008	0.27 $\pm$ 0.005	0.40 $\pm$ 0.006
T ₂	4.02 $\pm$ 0.012***	0.62 $\pm$ 0.006***	0.50 $\pm$ 0.005***	0.64 $\pm$ 0.003***	4.00 $\pm$ 0.010***	0.54 $\pm$ 0.012***	0.42 $\pm$ 0.010***	0.56 $\pm$ 0.000***	3.10 $\pm$ 0.011***	0.43 $\pm$ 0.014***	0.36 $\pm$ 0.003***	0.46 $\pm$ 0.012***
T ₃	4.13 $\pm$ 0.005***	0.72 $\pm$ 0.004***	0.52 $\pm$ 0.014***	0.65 $\pm$ 0.004***	4.01 $\pm$ 0.006***	0.58 $\pm$ 0.008***	0.44 $\pm$ 0.012***	0.57 $\pm$ 0.003***	3.13 $\pm$ 0.004***	0.45 $\pm$ 0.012***	0.38 $\pm$ 0.003***	0.48 $\pm$ 0.012***
T ₄	4.19 $\pm$ 0.005***	0.67 $\pm$ 0.012***	0.49 $\pm$ 0.003***	0.68 $\pm$ 0.012***	4.04 $\pm$ 0.012***	0.59 $\pm$ 0.000***	0.47 $\pm$ 0.012***	0.59 $\pm$ 0.011***	3.18 $\pm$ 0.008***	0.47 $\pm$ 0.004***	0.42 $\pm$ 0.011***	0.50 $\pm$ 0.010***
T ₅	4.16 $\pm$ 0.008***	0.76 $\pm$ 0.005***	0.53 $\pm$ 0.004***	0.84 $\pm$ 0.010***	4.06 $\pm$ 0.012***	0.63 $\pm$ 0.012***	0.48 $\pm$ 0.003***	0.68 $\pm$ 0.006***	3.24 $\pm$ 0.010***	0.49 $\pm$ 0.010***	0.43 $\pm$ 0.012***	0.54 $\pm$ 0.008***
T ₆	4.57 $\pm$ 0.015***	0.85 $\pm$ 0.003***	0.57 $\pm$ 0.012***	0.87 $\pm$ 0.06***	4.12 $\pm$ 0.006***	0.72 $\pm$ 0.004***	0.50 $\pm$ 0.008***	0.69 $\pm$ 0.004***	3.26 $\pm$ 0.003***	0.53 $\pm$ 0.005***	0.46 $\pm$ 0.004***	0.57 $\pm$ 0.006***
T ₇	4.71 $\pm$ 0.006***	0.85 $\pm$ 0.012***	0.59 $\pm$ 0.006***	0.98 $\pm$ 0.011***	4.52 $\pm$ 0.012***	0.74 $\pm$ 0.006***	0.51 $\pm$ 0.005***	0.79 $\pm$ 0.010***	3.32 $\pm$ 0.012***	0.57 $\pm$ 0.004***	0.47 $\pm$ 0.008***	0.67 $\pm$ 0.011***
T ₈	5.77 $\pm$ 0.015***	1.18 $\pm$ 0.010***	0.66 $\pm$ 0.010***	1.16 $\pm$ 0.008***	5.17 $\pm$ 0.008***	1.00 $\pm$ 0.011***	0.56 $\pm$ 0.012***	1.00 $\pm$ 0.012***	4.28 $\pm$ 0.003***	0.86 $\pm$ 0.011***	0.49 $\pm$ 0.010***	0.96 $\pm$ 0.003***

- Ts = Treatments; T₁ = Control (FYM), T₂ = FYM + PSB, T₃ = FYM + AZO, T₄ = FYM + VAM, T₅ = FYM+VAM+AZO, T₆ = FYM + PSB + AZO, T₇ = FYM + PSB + VAM, T₈ = FYM + PSB + AZO + VAM;
- *P < 0.05, **P < 0.01 and ***P < 0.001; ns = Non significant (compared with control treatment).

In case of macro nutrient content, Table 7 and 8 showed N, P and K contents were higher with the treatment eight as same trend followed with micronutrient contents in leaves. Table 4 revealed second harvested (August) samples were higher accumulation of N (0.74 mg/kg), P (0.40 mg/kg) and K (0.68 mg/kg) than other two harvested samples in 2009.

The amounts of the same were significantly lower with the fourth, fifth and sixth harvested samples in the year 2010 (Table 5) but treatment wise higher accumulation of N, P and K was observed where three biofertilizers were applied in combinations.

Further linear correlation matrices of macro and micro elements were analyzed for the year 2009 and 2010 with the ash content and revealed positive significant correlation with the ash content, being higher with the harvested samples in year 2009 than the year 2010, significant at 1 % (Table 6).

Table 4. Elemental analysis (Macro) in dried Stevia leaf in 2009
(Mean $\pm$ SEM, n = 3, from three replicated plots).

Ts	First harvest in May (mg kg ⁻¹ , dry wt basis)			Second harvest in August (mg kg ⁻¹ , dry wt basis)			Third harvest in November (mg kg ⁻¹ , dry wt basis)		
	N	P	K	N	P	K	N	P	K
T ₁	0.42 $\pm$ 0.002	0.10 $\pm$ 0.002	0.50 $\pm$ 0.002	0.44 $\pm$ 0.012	0.12 $\pm$ 0.004	0.52 $\pm$ 0.006	0.42 $\pm$ 0.004	0.10 $\pm$ 0.012	0.50 $\pm$ 0.004
T ₂	0.46 $\pm$ 0.004**	0.12 $\pm$ 0.004*	0.52 $\pm$ 0.003*	0.50 $\pm$ 0.014***	0.16 $\pm$ 0.012**	0.54 $\pm$ 0.004**	0.48 $\pm$ 0.004***	0.14 $\pm$ 0.006***	0.52 $\pm$ 0.012**
T ₃	0.58 $\pm$ 0.012***	0.15 $\pm$ 0.005**	0.57 $\pm$ 0.014***	0.62 $\pm$ 0.002***	0.18 $\pm$ 0.012***	0.58 $\pm$ 0.012***	0.60 $\pm$ 0.012***	0.15 $\pm$ 0.004***	0.56 $\pm$ 0.001***
T ₄	0.47 $\pm$ 0.006**	0.17 $\pm$ 0.011***	0.59 $\pm$ 0.011***	0.54 $\pm$ 0.004***	0.19 $\pm$ 0.014***	0.60 $\pm$ 0.012***	0.53 $\pm$ 0.003***	0.16 $\pm$ 0.012***	0.58 $\pm$ 0.006***
T ₅	0.56 $\pm$ 0.014***	0.22 $\pm$ 0.012***	0.53 $\pm$ 0.003**	0.67 $\pm$ 0.012***	0.28 $\pm$ 0.016***	0.59 $\pm$ 0.012***	0.64 $\pm$ 0.013***	0.25 $\pm$ 0.003***	0.57 $\pm$ 0.003***
T ₆	0.58 $\pm$ 0.012***	0.28 $\pm$ 0.006***	0.56 $\pm$ 0.006**	0.70 $\pm$ 0.013***	0.30 $\pm$ 0.004***	0.62 $\pm$ 0.004***	0.68 $\pm$ 0.001***	0.28 $\pm$ 0.002***	0.59 $\pm$ 0.012***
T ₇	0.54 $\pm$ 0.003***	0.31 $\pm$ 0.004***	0.54 $\pm$ 0.012**	0.71 $\pm$ 0.013***	0.37 $\pm$ 0.002***	0.64 $\pm$ 0.014***	0.69 $\pm$ 0.014***	0.32 $\pm$ 0.012***	0.60 $\pm$ 0.004***
T ₈	0.59 $\pm$ 0.003***	0.37 $\pm$ 0.012***	0.60 $\pm$ 0.003***	0.74 $\pm$ 0.012***	0.40 $\pm$ 0.002***	0.68 $\pm$ 0.016***	0.71 $\pm$ 0.006***	0.37 $\pm$ 0.012***	0.63 $\pm$ 0.014***

- Ts = Treatments; T₁ = Control (FYM), T₂ = FYM + PSB, T₃ = FYM + AZO, T₄ = FYM + VAM, T₅ = FYM+VAM+AZO, T₆ = FYM + PSB + AZO, T₇ = FYM + PSB + VAM, T₈ = FYM + PSB + AZO + VAM;
- *P < 0.05, **P < 0.01 and ***P < 0.001; ns = Non significant (compared with control treatment).

Table 5. Elemental analysis (Macro) in dried Stevia leaf in 2010 (Mean $\pm$ SEM, n = 3, from three replicated plots).

Ts	First harvest in May (mg kg ⁻¹ , dry wt basis)			Second harvest in August (mg kg ⁻¹ , dry wt basis)			Third harvest in November (mg kg ⁻¹ , dry wt basis)		
	N	P	K	N	P	K	N	P	K
T ₁	0.41 $\pm$ 0.012	0.10 $\pm$ 0.004	0.48 $\pm$ 0.003	0.40 $\pm$ 0.002	0.10 $\pm$ 0.012	0.45 $\pm$ 0.012	0.35 $\pm$ 0.002	0.08 $\pm$ 0.002	0.41 $\pm$ 0.006
T ₂	0.45 $\pm$ 0.006***	0.12 $\pm$ 0.014**	0.50 $\pm$ 0.006**	0.42 $\pm$ 0.016**	0.10 $\pm$ 0.003 ^{ns}	0.49 $\pm$ 0.003***	0.40 $\pm$ 0.016***	0.09 $\pm$ 0.001*	0.45 $\pm$ 0.012***
T ₃	0.59 $\pm$ 0.001***	0.14 $\pm$ 0.003***	0.54 $\pm$ 0.012***	0.54 $\pm$ 0.012***	0.12 $\pm$ 0.014**	0.51 $\pm$ 0.002***	0.43 $\pm$ 0.012***	0.10 $\pm$ 0.012**	0.47 $\pm$ 0.012***

T ₄	0.54 ± 0.002***	0.15 ± 0.002***	0.56 ± 0.001***	0.50 ± 0.012***	0.13 ± 0.012***	0.52 ± 0.004***	0.46 ± 0.012***	0.11 ± 0.006***	0.48 ± 0.006***
T ₅	0.63 ± 0.012***	0.23 ± 0.002***	0.54 ± 0.001***	0.57 ± 0.001***	0.19 ± 0.012***	0.52 ± 0.006***	0.47 ± 0.001***	0.14 ± 0.003***	0.49 ± 0.014***
T ₆	0.62 ± 0.003***	0.27 ± 0.016***	0.58 ± 0.001***	0.60 ± 0.004***	0.25 ± 0.006***	0.56 ± 0.012***	0.49 ± 0.004***	0.18 ± 0.012***	0.51 ± 0.013***
T ₇	0.67 ± 0.004***	0.30 ± 0.014***	0.58 ± 0.002***	0.62 ± 0.002***	0.28 ± 0.001***	0.57 ± 0.003***	0.50 ± 0.002***	0.20 ± 0.006***	0.52 ± 0.002***
T ₈	0.70 ± 0.004***	0.35 ± 0.002***	0.60 ± 0.004***	0.64 ± 0.002***	0.30 ± 0.001***	0.58 ± 0.012***	0.51 ± 0.002***	0.24 ± 0.012***	0.54 ± 0.006***

- Ts = Treatments; T₁ = Control (FYM), T₂ = FYM + PSB, T₃ = FYM + AZO, T₄ = FYM + VAM, T₅ = FYM+VAM+AZO, T₆ = FYM + PSB + AZO, T₇ = FYM + PSB + VAM, T₈ = FYM + PSB + AZO + VAM;
- *P < 0.05, **P < 0.01 and ***P < 0.001; ns = Non significant (compared with control treatment).

Table 6. Correlation matrix between mean % Ash content and other elements in the year 2009 and 2010.

	Year 2009							
	Ash content	Fe	Mn	Cu	Zn	N	P	K
Ash content	1.00							
Fe	0.91**	1.00						
Mn	0.87**	0.98**	1.00					
Cu	0.93**	0.95**	0.94**	1.00				
Zn	0.87**	0.97**	0.99**	0.95**	1.00			
N	0.88**	0.87**	0.88**	0.95**	0.89**	1.00		
P	0.86**	0.90**	0.94**	0.92**	0.97**	0.90**	1.00	
K	0.94**	0.88*	0.88**	0.84*	0.88**	0.81*	0.84*	1.00

	Year 2010							
	Ash content	Fe	Mn	Cu	Zn	N	P	K
Ash content	1.00							
Fe	0.89**	1.00						
Mn	0.84*	0.96**	1.00					
Cu	0.96**	0.96**	0.91**	1.00				
Zn	0.82*	0.86**	0.98**	0.88**	1.00			
N	0.94**	0.85*	0.86**	0.93**	0.81*	1.00		
P	0.83*	0.83*	0.92**	0.87**	0.95**	0.92**	1.00	
K	0.95**	0.89**	0.89**	0.96**	0.88**	0.97**	0.92**	1.00

Significant at 1 %; * Significant at 5 %.

Table 7. Pooled biomass and respective total phenolic content in SR leaf in 2009 and 2010.

Ts	Year 2009		Year 2010	
	Total pooled dried biomass (g)	Total phenolic content (mg of Gallic acid per g of extract)	Total pooled dried biomass (g)	Total phenolic content (mg of Gallic acid per g of extract)
T ₁	102.0 ± 2.082	40.76 ± 0.023	87 ± 1.203	36.55 ± 0.017
T ₂	154.3 ± 1.155***	44.56 ± 0.011***	94 ± 1.320***	39.78 ± 0.011***
T ₃	168.3 ± 1.202***	46.81 ± 0.014***	104 ± 2.031***	41.03 ± 0.011***
T ₄	187.7 ± 2.903***	52.96 ± 0.011***	112 ± 1.420***	43.80 ± 0.011***
T ₅	253.3 ± 2.208***	53.53 ± 0.012***	123 ± 1.202***	48.42 ± 0.012***
T ₆	257.2 ± 1.092***	56.85 ± 0.017***	125 ± 2.104***	48.95 ± 0.017***
T ₇	262.9 ± 1.023***	57.07 ± 0.017***	127 ± 1.089***	49.94 ± 0.014***
T ₈	271.4 ± 1.003***	64.52 ± 0.014***	140 ± 2.110***	50.12 ± 0.011***

- Ts = Treatments; T₁ = Control (FYM), T₂ = FYM + PSB, T₃ = FYM + AZO, T₄ = FYM + VAM, T₅ = FYM+VAM+AZO, T₆ = FYM + PSB + AZO, T₇ = FYM + PSB + VAM, T₈ = FYM + PSB + AZO + VAM;
- *P < 0.05, **P < 0.01 and ***P < 0.001; ns = Non significant (compared with control treatment).

- Correlation matrix between dried biomass and phenolic content is $r = 0.96$; in year 2009.
- Correlation matrix between dried biomass and phenolic content is $r = 0.94$; in year 2010.
- Correlation matrix of phenolic content is $r = 0.92$, between year 2009 and 2010.

3. 3. Determination of total phenolic content

Results of the present study revealed that the level of the phenolic compounds in pooled dried leaves of SR (treatment wise) were found to be 64.52 mg/g and 50.12 mg/g which were higher with the treatment eight where three combinations of biofertilizers were applied in the year 2009 and 2010 respectively (Table 7). From the harvested samples in the year 2009, the results indicate that SR leaves contained very high amount of phenolic compound which was significant positively correlated with the increased dried biomass yield ($r = 0.96$) than harvested samples in year 2010 ($r = 0.94$) and also was higher than that of control sample but lesser than that of standard gallic acid (87.92 mg/g). Table 7 also revealed the linear correlation (r) of phenolic content between two years harvested sample was 0.92.

4. DISCUSSION

4. 1. Effect of biofertilizers on physicochemical assessment

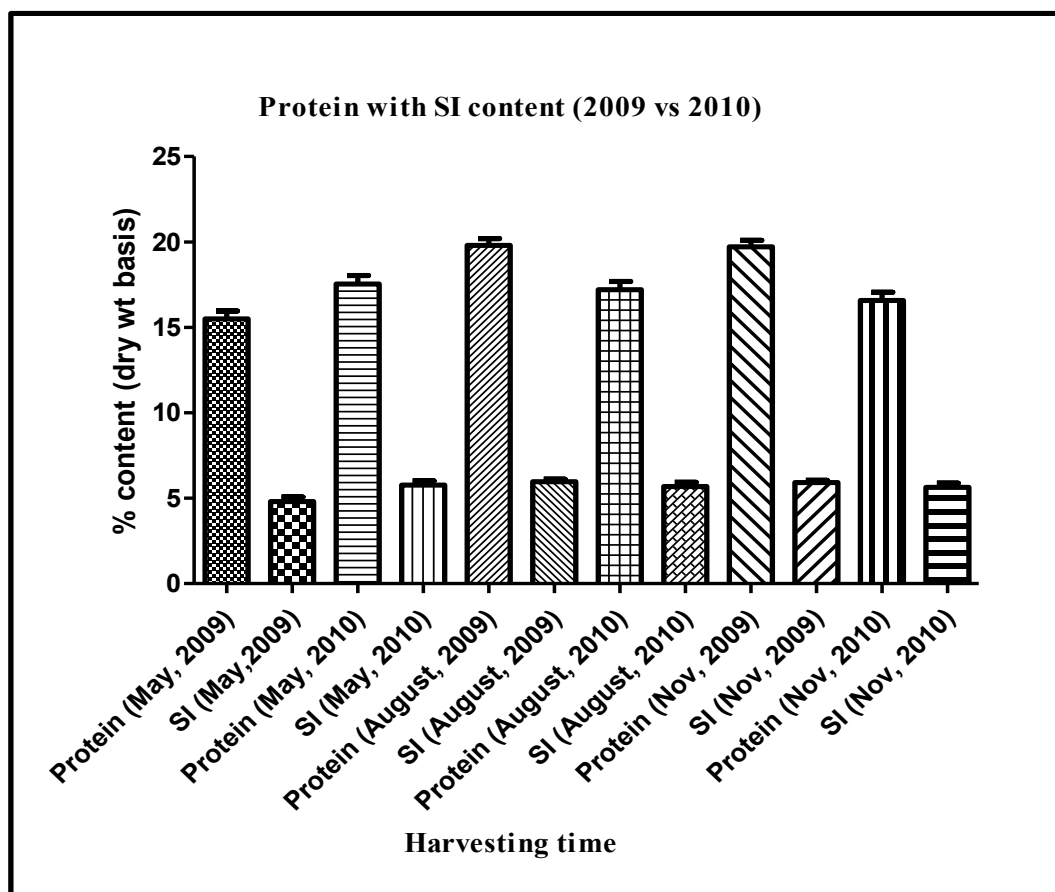
Different physicochemical parameters like % protein content, moisture content, ash value, SI and WAC were determined from all harvested Stevia crop, cultivated in acid soil zone of South India in the year 2009 and 2010. The results revealed second moth of harvested sample showed higher accumulation of % protein, SI and WAC than the other harvested samples from both the years. Of late, protein is a basic component of metabolic regulation in plants which provides the path of varying enzymatic complement during the response to the environmental conditions (Huffakar and Peterson, 1974). One of the environmental conditions is application of biofertilizers. Biofertilizers are used to increase the yield of the biomass by uptaking the nutrients and accumulation in the leaf.

The increased effect was due to accumulation of the nutrients supplied by biofertilizers in the soil and hence increased the protein content in the leaf of SR during second time harvested samples in the year 2009, and the same content was decreased due to dilution effect of the nutrients in the plant. The result also confirmed the findings of Rakshit et al. (2002), who reported that the interaction between rhizobia and VAM fungi has received considerable attention because of the relatively high phosphorus demand for nitrogen fixation. It was reported that second harvest of plant resulted higher protein content than first harvested samples (Cupic et al., 2001). Further the effect of biofertilizers on Stevia plant cultivation and their effects on biomass yield were reported higher during pilot study in various soil environments (Das et al., 2009). It was also reported that biofertilizers had a favorable effect on growth and chemical composition of *Jatropha curcus* L. seedlings when studied in two different seasons (El-Quesni et al., 2013) and even it was reported that protein content increased after 45 days while at 30 and 60 days the content was least and in the middle phase of life cycle of plant protein content was highest (Patil, 2010) with applied biofertilizers.

The higher percent protein content of green pods of Pan 311 variety of cowpea in 2005/06 was due to its early maturity status and better nutrients uptake ability including nitrogen (Sebetha et al., 2010). The earlier studies were reported that % protein in SR about 10 % (Mishra et al., 2010) and even 20 % (Tadhani and subhash, 2006). The present study was also followed the same trend and improved the protein content upto 21.29 % due to

maturity status and better nutrients uptake ability especially nitrogen with applied biofertilizers in combinations. The present study also showed that SI and WAC was dependent on protein content in the leaf of the plant. The content of SI increased with the increased protein content (Figure 2). It was reported that protein increased water holding capacity, when their swelling ability is enhanced (Mishra et al., 2010). Water absorption capacity of Stevia was recorded higher (Figure 3) with the second harvested samples in the year 2009 which might be due to higher protein content in SR leaf. Earlier study was revealed that WAC and SI in dried SR leaf were 4.7 ml/g and 5.01 % respectively (Savita et al., 2004) whereas the results of the present investigation increased content of the same to the level of 5.12 ml/g and 6.42 % which might be explained by higher amount of nitrogen accumulation in SR leaf with combined application of biofertilizers (Figure 4 & 5).

Fig. 4. Effect of biofertilizers on % protein content and swelling index (SI) in Stevia leaf (dry wt basis) in 2009 and 2010.



ANOVA Table

Treatment (between columns)	3634
Residual (within columns)	85.23
Total	3719

SS

3634
85.23
3719

df

11
84
95

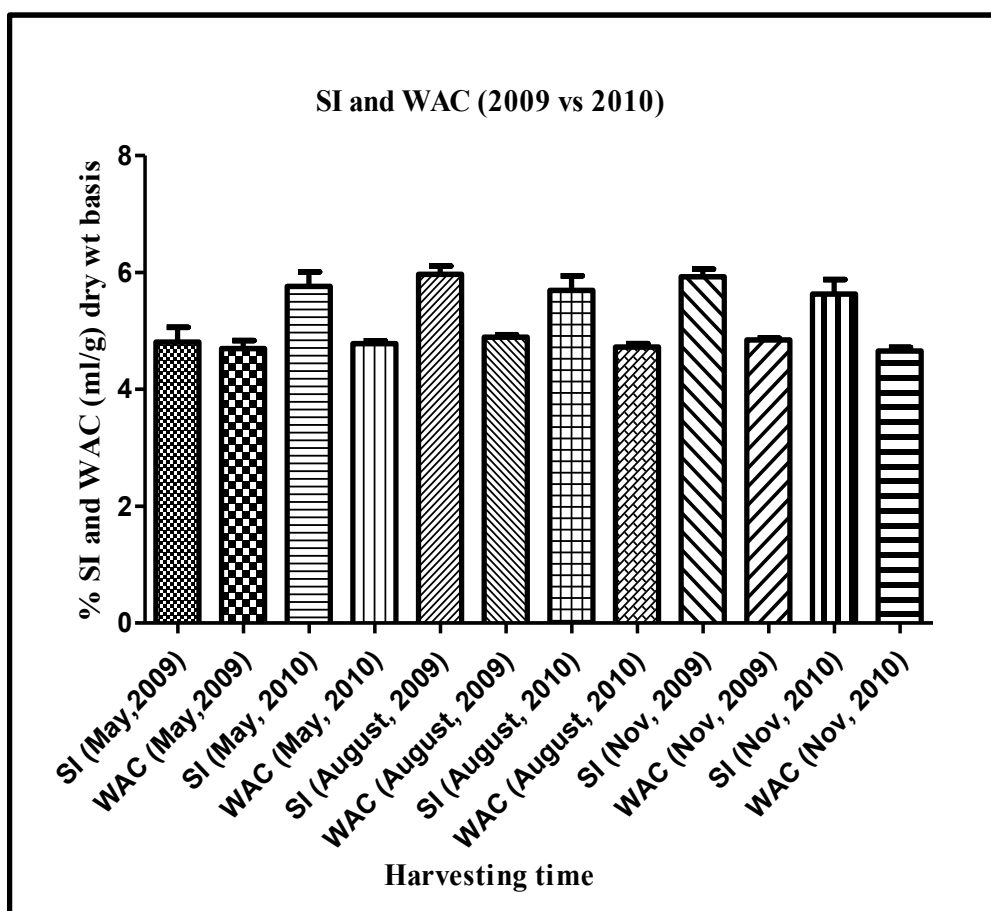
MS

330.4
1.015

Post test for linear trend

Slope	-0.078
R squared	0.007
P value	P < 0.0001
P value summary	***

Fig. 5. Effect of biofertilizers on % swelling index (SI) and water absorption capacity (WAC) in Stevia leaf (dry wt basis) in 2009 and 2010.



ANOVA Table

	<u>SS</u>	<u>df</u>	<u>MS</u>
Treatment (between columns)	25.41	11	2.310
Residual (within columns)	18.12	84	0.2157
Total	43.53	95	

Post test for linear trend

Slope	0.005500
R squared	0.003181
P value	0.4253
P value summary	ns

Interestingly, ash value content was higher up to third harvested samples than the other harvested samples, whereas moisture content was varied with the treatments in all the harvested samples of both the years. The ash content was higher than that of other treatments including control treatment during third harvest of the SR sample in the year 2009. The same trend was followed with the micro elements accumulation in SR leaf (Table 2). It was reported that ash content was dependent on the elemental content in the leaf (Dhiman et al., 2011) and its content were reported as 6.3 % (Goyal et al., 2010), 7.41 % (Abou-Arab et al.,

2010), 13.1 % (Tadhani and Subhash, 2006) whereas increased amount of ash content was investigated in the recent study with 13.54 %. Such increased ash content may be due to residual effect of the biofertilizers and also slow release of nutrients from fixed or adsorbed pools to the plant available pools into soil resulting more accumulation of micro elements in the leaf.

4. 2. Effect of biofertilizers on elemental analysis

The content of microelements was greatly influenced by the applied biofertilizers. There were significant differences ($p < 0.05$) in the micronutrient composition of the SR leaf when compared with the control treatment and followed the same trend for all the harvested samples during both the years. The present study was recorded higher accumulation of Fe, Mn, Cu and Zn in the leaf where combinations of three biofertilizers were applied simultaneously and being highest with third harvested sample. Such results were due to residual effect of the nutrients in the soil. The micronutrient content in the leaf of SR showed a high concentration of Fe (Table 2). Generally, iron is an essential element for synthesis of hemoglobin. Higher amount of iron in *Stevia* leaves accumulation is an additional benefit and importance for the maintenance of normal hemoglobin level in the body and hence, SR leaf can be used in various food preparations. A study was also reported that zinc and manganese are considered as antioxidant micro nutrients and their presence in leaf is essential to boost up the immune system (Jimoh and Oladiji, 2005) and in prevention of free radical mediated diseases. In case of Cu content in leaf, the same was also higher in the third harvested samples which showed an antagonistic action with protein content. It was reported that total protein content in sunflower seedlings decreased with increasing of Cu concentrations compared to the control seedlings, respectively (Zengin and Kirbag, 2007) which also confirmed the results of the present study where Cu concentration increased and decreased content of protein (20.88%) with third harvested sample in year 2009 with the treatment T₈ where three bio fertilizers were applied togetherly.

The content of macro elements (N, P and K) were significantly influenced by applied biofertilizers. The results revealed the higher accumulation of macronutrients in leaf of SR was in the second harvested samples during the year 2009 (Table 4) where combination of three biofertilizers was applied simultaneously. The results of the present study are in agreement with findings of Anjanappa et al., 2011.

4. 3. Effect of biofertilizers and elemental accumulation in dried biomass on total phenolic content

The total phenolic content in leaf of SR was significantly increased with the increased dried biomass as per treatment and was depicted in Table 7. It was seen that higher phenolic content was also followed the same trend with the treatment eight where three biofertilizers were applied in combination with respective higher pooled dried biomass. Overall results revealed that the harvested biomass content was higher during the year 2009 than harvested in 2010. SR already has its antioxidant nature as per earlier literatures (Tadhani et al., 2007; Shukla et al., 2009). Phenolic compounds have been shown to be responsible for the antioxidant activity of plant extracts (Rice-Evans et al., 1996; Gao et al., 2000). Phenols are one of the most important plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano et al., 1989) and its contribute directly to antioxidant action (Duh et al., 1999). The present study showed the positive response as the earlier literature described by Javed and Panwar, (2013). Osuagwu and Edeoga (2012) were reported the effect of

inorganic fertilizers (N, P and K) on total phenolic content in *Ocimum gratissimum* leaves in dose dependent manner. The same trend followed in the present study with applied three biofertilizers resulted higher phenolic content over control sample.

5. CONCLUSION

The results may be concluded that second harvest during first year of the cultivation significantly increases ($p < 0.05$) the percentage protein and macro nutrient contents (N, P and K) with simultaneous increase in the SI and WAC properties of the SR plant. Content of ash in dried SR leaf was also increased due to high accumulation of micro elements (Fe, Mn and Zn) with the nutrients supplied by the biofertilizers. The results further reveal that with an increased content of physiochemical parameters, macro and micro elements in the dried leaf of SR plant the total phenolic contents in the SR plant also increased. The overall results suggest that Stevia can be used as an accessible source of natural antioxidants with consequent health benefits, preparation of various food products like different drinks, beverages and bakery products under balance use of bio-fertilizers.

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