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Boosting biochemical composition of stevia plant using biochar loaded with beneficial microorganism strains

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Abstract

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The present research was carried out to determine the effect of biochar loaded with either inorganic nitrogen or beneficial microorganism strains (individual and in combination) on biochemical composition and nutritional values of stevia plants (*Stevia rebaudiana*) grown in sandy soil for two continuous seasons (2018–2019) compared with commercial chemical NPK fertilizers (control). Growth parameters (plant height, number of leaves, leaf area and leaf area index), besides chemical constituents symbolized in (total chlorophylls, ash, protein, fiber, total carbohydrates, essential and non-essential amino acids, mineral nutrients (N, P, K, Mg, Fe and Zn), stevioside, fatty acids and vitamins) were recorded at 60, 90 and 120 days after transplanting. The obtained results in response to the different treatments of biochar and microorganism strains indicated that biochemical ingredients and nutritional values of stevia plants were boosted in comparison to control plants. Hence the application of biochar loaded with beneficial microorganisms as organic and biofertilizers can improve plant growth, enhance plant biochemical components, and ensure safety and sustainable production particularly in new reclaimed areas.

Keywords: biochar; microorganisms; growth parameters; chemical composition; stevia plant

Introduction

The worldwide demand for medicinal and aromatic plants has been raised to match the global pharmaceutical and cosmetics industries enlargement. *Stevia rebaudiana Bertoni* is a Paraguayan perennial antidiabetic herb that belongs to Asteraceae family (Ghaheri et al*.,* 2017). It is an industrially and medicinally important herb, mainly due to its steviol glycoside content, which is a calorie-free natural sweetener (Lucho et al., 2019). Furthermore, their leaves contain numerous chemical constituents such as flavonoids, labdanes, chlorophylls, sterols, triterpenoids, mono-disaccharides, organic acids and inorganic salts. Stevia plant is known as an accumulator of diterpenoid steviol glycosides, which are approximately 300 times sweeter than regular sugar (Abdelsalam et al., 2019). *Stevia rebaudiana* possess high antioxidant, anti-inflammatory, and antimicrobial properties (Zen et al., 2019). Furthermore, Giritlioglu and Dizlek (2018) mentioned that *Stevia rebaudiana* is suggested for diabetic patients as it reduces the blood glucose without influencing insulin metabolism, besides its uses in a great deal of food products, particularly in the formulation of bakery products like biscuits.

Biofertilizers are products containing beneficial living microorganisms or natural substances that are able to improve chemical and biological soil properties (Ronga et al*.,* 2019). They are considered a valuable eco-friendly agricultural approach to minimize the negative impacts on the

environment induced by chemical fertilization (Kulkarni et al., 2018). They reduce environmental pollution, increase nutrients availability, improve the physical, chemical & biological properties of the soil and enhance root proliferation due to the release of growth promoting hormones (Yadav and Sarkar, 2019). Some microorganisms are frequently used as biofertilizers such as nitrogen-fixing soil bacteria (Azotobacter and Rhizobium), nitrogen-fixing cyanobacteria (Anabaena), phosphate-solubilizing bacteria (Pseudomonas sp.) and *Arbuscular Mycorrhizal* Fungi (AMF). In addition, phytohormone (auxin)-producing bacteria and cellulolytic microorganisms are also used in biofertilizers production (Umesha et al., 2018).

Biochar is a charcoal-like material produced via pyrolysis of biomass which is widely used for various environmental remediation strategies such as soil amendment because of its intrinsic carbon negativity and porosity (Kwan et al., 2019). The addition of biochar to the soil increases nutrients availability, and improves the conditions for plant growth. Moreover, biochar plays an important role through increasing the soil water-holding capacity as well as being a pollutant sorbent. In addition, it is known for its long-lasting chemical properties, large surface area and carbon-richness, which make it an efficient method for the immobilization of organic and inorganic contaminants such as heavy metals. It also improves crop yield in infertile soils – Rodriguez et al. (2019) and Obour et al. (2019).

The objective of the present experiment is to verify the hypothesis of biochar loaded with beneficial living microorganism strains might enhance morphological characters and chemical composition of *Stevia rebaudiana* plants as well as improving soil physical and chemical properties compared to commercial chemical fertilizers in new reclaimed area of the desert.

Materials and Methods

The current investigation was carried out at the research station farm, Cairo University, Fac., of Agric., Wadi El-Natron, Behera Governorate (Longitude 28°54′ E, Latitude 28°20′ N and Altitude 130 m), Egypt. The seedlings of *Stevia rebaudiana* were purchased from Faculty of Pharmacy, Cairo University. Before planting, the soil was first mechanically ploughed deeply (35–45 cm) and planked twice till the soil surface had been settled; then planted on 10th February 2019 spaced at 60x20 cm between rows and plants that drip irrigated (4.0 L hr^{-1}); the cuts were taken on 10th April, 11th May and 10th Jun 2018 for the first season, same steps and cuts were followed 2019 as the second season. The field experiment was arranged in a completely randomized block design with three replicates. The chemical characteristics of the soil were as follows: coarse sand 13.2%, sand 80.60%, silt 3.2%, clay 3%, organic carbon 0.34%, available S 0.002%, available N 0.005 %, available P 4.2%, available K 117.0 µg/g, pH 7.89 and EC 1.23 dS/m , soil texture (sandy) according to (Jackson 1973). Five treatments were carried out as follows:

1 – Control (chemical NPK fertilizers).

2 – Biochar loaded with nitrogen (Biochar-1).

3 – Biochar loaded with *Azotobcter chrococcoum*+ *Bacillus subtilis*+ *Pseudomonas fluorescens* (Biochar-2).

4 – Biochar loaded with *Splrulina platensis* + *Anabaena azollae* (Biochar-3)

5 – Biochar loaded with all strains of microorganisms (Biochar-4).

Chemical fertilizers:

Inorganic NPK fertilizers were applied as the control treatment according to the recommended dose by the Ministry of Agriculture and Land Reclamation; nitrogen in the form of ammonium sulphate (20.5% N) at the rate of 40 kg/fed (one Fadden equal 4,200 m^2) was divided into two doses; the first was added 2 weeks after planting and the second four weeks later. While both calcium superphosphate (15.5% P) at the rate of 30 kg/fed and potassium sulphate (48% K) at the rate of 25 kg/fed were added during land preparation before planting.

Organic matter (compost)

Compost at the rate of 2.50 ton/ fadden was incorporated into the soil 14 days before planting (Table 1).

Table 1. Chemical properties of applied compost

Property	Value
Moisture content (%)	25
pH (1:5)	7.5
EC (1: 5 extract) dSm^{-1}	3.1
Organic-C (%)	33.11
Organic matter (%)	70
Total-N (%)	1.82
Total-K (%)	1.25
C/N ratio	14:1
Total-P (%)	1.29
Fe (ppm)	1019
Mn (ppm)	111
Cu (ppm)	180
Zn (ppm)	280
Total content of Bacteria (cfu.g^{-1})	2.5 x 10^7
Phosphate dissolving Bacteria (cfu.g^{-1})	2.5 x 10^6
Weed seeds	0

Preparation of biochar and its properties:

The rice husk was cut into small fragments (4–5 mm) and was pyrolyzed in oven at 350°C for 24 hours to produce (derive) biochar. The chemical properties and composition of rice husk derived biochar are presented in Table 2. The contents of ash, carbon, nitrogen and hydrogen were determined according to Kinney et al. (2012). Biochar-pH (in 1:1, w: v) water suspension was determined by pH meter. EC value was estimated by EC-meter. The contents of Si, Ca, K, Mg and S were measured by Atomic Adsorption Spectrophotometer with air-acetylene, fuel (Pye Unicam, model SP-1900, US). The C: N ratios after soaking in ammonium sulphate and after inoculation by microorganism were calculated. Zeta potential (ZP) was measured for Bio-char by Zeta-Meter 3.0+ system (Zeta Meter Inc., VA) at National Research Center. Giza, Egypt.

Table 2. Chemical properties and composition of prepared rice husk derived Biochar

Property	Rice husk derived biochar
Si, mg/kg	179
Ca, mg/kg	213
K, mg/kg	199
Mg, mg/kg	179
Water, %	3.88
Ash, %	47.90
pH	7.65
Fixed C (mg)	46.35
H (mg)	2.64
N (mg) as N_2O after inoculation	2.4
N (mg) after soaking in ammonium sulphate	3.65
S (mg)	0.22
O (mg)	2.74
Volatile matter %	–
H : C	0.05
C:N after inoculation by microorganism	18.85
C:N after soaking in ammonium sulphate	12.92
EC (dS/m)	0.14
Zeta potential	-26.6 mV

Preparation of biochar loaded with nitrogen and microorganism strains:

Biochar loaded with nitrogen was prepared by soaking it in ammonium sulphate solution (1M) for 4 days at 25°C according to Li et al. (2013), total N content was analyzed using the Kjeldahl method (Peach and Tracy, 1956). The biochar was then air dried for 2 days under shaded area. The biochar loaded with microorganism strains was prepared using *Azotobacter chrococcum, Bacillus subtilis*, *Pseudomonas fluorescence, Splrulina platensis* and *Anabaena azollae* strains. *Azotobacter chrococcoum, Anabaena azollae and Spirulina platensis* strains were obtained from the Microbiology Department, Soil, Water and Environment Research Institute, Agric. Res. Center (ARC), Giza, Egypt. *Azotobacter chrococcoum* was cultured in conical flasks contained adapted Ashbys media for five days at 28-30 C (Abd El-Malak and Ishac, 1968). The *A. chrococcoum* in the liquid cultures accounted for 10^7 CFU/ml. in 250 ml. *Both Bacillus Subtilis* and *Pseudomonas fluorescence* active strain were cultured on King Media (King et al.,1954), then they were gently shaken on a rotary shaker incubator at 30 C ± 2 C up to attain the long phase (10^7cfu m^{-1}). *Azotobacter chroococcum, Bacillus subtilis and Pseudomonas fluorescence* colonies strains growth were monitored in a solution for 24 h, 48 h and for three days.

Ananbaena azollae strain which was isolated from *A. pinnata*, according to Abd El-Aal (2013), was grown on BG11 medium (Rippka et al., 1979), while Zarrouk medium was used for *Spirulina platensis* culture (Zarrouk, 1966), then the cultures were kept warm in a growth cavity under permanent illumination (2000 lux), at 25 C ± 2 C and 35 C ± 2 C for both *Anabaena azollae* and the mesophilic alga *Spirulina platensis,* respectively (Table 3). The microorganism strains were applied to the seedlings after 30, 60 and 90 days from planting at the rate of:

47 L/ hectare of *Azotobcter chrococcoum, Bacillus subtilis and Pseudomonas fluorescens*

115 L/hectare of *Splrulina platensis* and *Anabaena azollae and*

35.0 L/hectare of the mixture of bacteria and algae.

Table 3. Some characteristics of used microorganism strains

Strains	N_2-ase M.mole C_2H_4/m/h	Phytohormons µg/l culture		
		IAA	GA_3	C.K
Azotobcter chrococcoum	285.55	67.36	102.20	149.00
Bacillus subtilis	–	192.00	861.09	200.60
Pseudomonas fluorescens	–	163.00	510.20	217.50
Splrulina platensis	–	15.13	97.65	-196.2
Anabaena azollae	186.15	19.85	87.80	186.16

IAA; indole acetic acid, GA3; gibberellic acid, C.K: cytokinins

Cytokinins were measured according to Plamer et al., 1981. Gibberlic acid (GA) and indole-acetic acid (IAA) were measured according to Fales and Jaouni (1973). Nitrogenase was measured by a colorimetric method according to Larue and Kurz (1973).

Mixing microorganism strains with biochar:

Biochar loaded with microorganism strains were soaked in strains media for two days, then measured by high resolution scan microscope FE-SEM (Field Emission Scanning Electron Microscope), quanta FEG 250. The electron microscope unit at National Research Center, Giza, Egypt, was used to prove the growth strains inside macro, meso and micro pores of biochar (Figure1 a, b, c and d).

Application of biochar

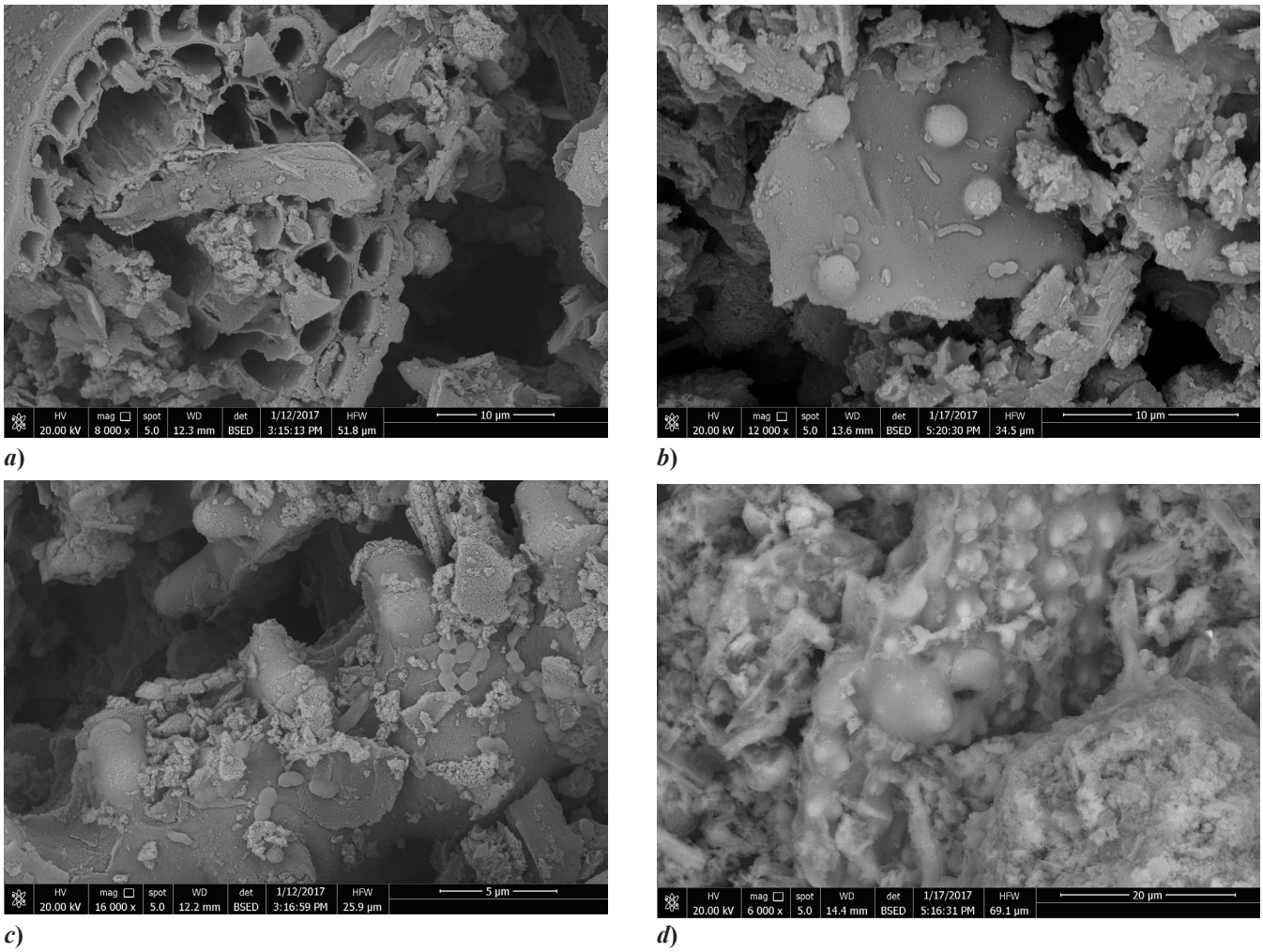
Both prepared biochars (loaded with nitrogen and loaded with strains) were added to sandy soil 5 days before planting and mixed well at the rate of 10 tons/ hectare.

Harvesting

The plants were cut above the surface ground by 5 cm and divided into groups (fresh and sun dried according to analysis target and procedure).

Data Recorded

1 – Plant height (cm)
2 – Number of leaves/plant
3 – Leaves area (cm^2)



a) ***b***) ***c***) ***d***)

Fig. 1. spongy structure of biochar (a), biochar loaded with *Azotobcter chrococcoum* + *Bacillus subtilis* (b), biochar loaded with *Pseudomonas fluorescens* (c) and biochar loaded with algae *Splrulina platensis* + *Anabaena azollae* (d)

4 – Leaves area index
5 – The total nitrogen content was determined using the modified- micro-Kjeldahel method of the dried leaves according to Helrich (1990), the nitrogen percentage was multiplied by 6.25 to estimate the crude protein percentages (Clifton and Pomeranz, 1987).
6 – Phosphorus was determined calorimetrically using the chloro-stannous molybdo-phosphoric blue color method in sulphuric acid according to Jackson (1973).
7 – Potassium content was determined by flame photometer apparatus (CORNING M 410, Germany).
8 – Mg, Fe and Zn contents were determined using Atomic Absorption Spectrophotometer with air-acetylene, fuel (Pie Unicom, model SP-1900, US).
9 – Total carbohydrates (DW %) were determined in leaves by phosphomolybdic acid method according to Helrich (1990).
10 – Total chlorophylls in fresh leaves were measured by Spectrophotometer and calculated according to the equation described by Moran (1982).
11 – Crude fiber was measured by loss on ignition of dried residue remaining after digestion of sample with 1.25% H_2SO_4 , NaOH 40%, perchloric acid and 400 C° (Helrich, 1990).
12 – Ash was determined by combustion of the samples in a muffle furnace at 550°C for 12 h (Singh et al., 2010).
13 – Determination of amino acids composition was done using capillary electrophoresis "KAPEL- 105" of the manufacturer "Lumex" (Tarasenko et al., 2015)
14 – Ascorbic acid in fresh leaves was determined and estimated per100 ml fresh leave juice, according to Helrich (1990).
15 – Stevioside content was determined using high performance liquid chromatography (HPLC) according to Nishiyama et al. (1992).
16 – Vitamin B2 and folic were estimated according to Kim *et al*, (2011).
17 – The fat content of the samples was extracted with hexane in a Soxhlet apparatus and then Gas- liquid chromatographic analysis of fatty acids was done on methyl ester which was prepared and purified by the method of according to Kinsella (1966).

GLC of fatty acid methyl esters:

Separation of fatty acid methyl esters were carried out using capillary column which contained 15% diethyl glycol succinate DEGS. The injector port and flame ionization detector were set at 240 C°. The flow rate of carrier gas, nitrogen, was 10 mL/ minute. The gas chromatograph (Perkin-Elemar model 8310) had a temperature program from 100 to 190 C° with interment rate of 7 C° / minute. The initial and final time were identified according to their retention time compared to those of authentic samples.

Results and Discussion

Vegetative parameters:

Growth parameters of stevia plants in response to different biochar treatments at 60, 90 and 120 days after transplanting (DAT) are presented in Table 4. The results revealed significant increases in plant height as a consequence of biochar-4 treatment which donated 12%, 13% and 6% at 60, 90 and 120 DAT respectively in comparison to commercial chemical NPK fertilizers (control). Meanwhile it was remarkable that biochar-1 recorded insignificant difference in plant height compared to the control at 60, 90 and 120 DAT, respectively. Similar results were obtained by Lin et al. whom found that biochar significantly increased soybean plant height.

Focus on the same data in Table (4) disclosed that leaves number per plant were significantly affected by biochar-4 treatment implementation since increased by 21%, 5.3% and 2.3% at 60, 90 and 120 DAT, respectively compared with chemical fertilizers NPK (control). While biochar-1 treatment recorded insignificant difference in leaves number contrast to control at 60, 90 and 120 DAT, respectively. Ji et al. (2018) stated that biochar treatments increased number of leaves, leaf length, and leaf width of Chinese cabbage.

Going with leaf area parameter which recorded significant augments 17.5% and 2.5% respectively at 60 and 90 DAT with biochar-4 treatment over control (NPK fertilizers), while at the last period (120 days) insignificant increase was recorded between both biochar-4 treatment and control plants (Table 4). Similarly, Lin et al. (2020) stated that soybean leaf area increased by the addition of biochar.

Application of biochar-4 treatment resulted in significant increments of leaf area index (2% and 5%) at 90 and 120 days from transplanting, respectively compared to the control plants. Whereas in the first cut (60 days from transplanting), there was insignificant difference between both mentioned treatments. In this respect, Bonin et al. (2018) found that biochar significantly increased leaf area index of some high-yielding grass species.

The profound effects of loaded biochar by either bacteria and\or algae on improving vegetative parameters over control may due to their beneficial effects on plant growth represented in hormones like secretion from microorganism strains, available and retention of nutrients represented in nitrogen content within the rhizosphere as a result of nitrogen

Table 4. Vegetative parameters of stevia plant in response to different biochar treatments after 60, 90 and 120 days from transplanting

Treatments	Plant height (cm)	leaves number /plant	Leaf area cm^2	Leaf area Index
60 DAT				
Control(NPK)	15.31[b]	12.98[b]	89.35[b]	1.52[a]
Biochar-1	14.45[b]	12.75[b]	78.61[c]	1.26[b]
Biochar-2	13.59[c]	12.65[b]	67.90[d]	1.21[b]
Biochar-3	14.15[b]	11.17[b]	75.65[c]	1.19[b]
Biochar-4	17.19[a]	15.73[a]	105.00[a]	1.58[a]
90 DAT				
Control(NPK)	25.70[b]	48.52[b]	308.00[b]	2.10[b]
Biochar-1	24.13[b]	47.23[b]	302.00[b]	2.06[b]
Biochar-2	22.51[c]	34.25[c]	204.00[d]	1.81[c]
Biochar-3	23.45[c]	31.32[c]	190.45[c]	1.79[c]
Biochar-4	29.00[a]	51.10[a]	316.00[a]	2.14[a]
120 DAT				
Control(NPK)	55.16[b]	255.19[b]	641.27[a]	1.26[b]
Biochar-1	54.10[b]	253.00[b]	635.15[b]	1.24[b]
Biochar-2	41.60[c]	112.95[c]	430.18[b]	0.67[c]
Biochar-3	39.00[c]	110.79[c]	420.12[c]	0.63[c]
Biochar-4	58.69[a]	260.90[a]	643.87[a]	1.32[a]

Data represented average of two growing seasons 2018–2019
Means with the same letters in a column are not significantly different by DMRT 5%
DAT; days from transplanting

fixation and enhance macro and micronutrients uptake (Hassan et al., 2020). Moreover, it was found that incorporated nutrient management using chemical and biofertilizers increased the vegetative growth of stevia plant (Aguirre-Medina et al., 2018) and gaining the highest content of biochemical compounds which in turn resulted in increasing biomass production of stevia plant (Asghari, 2018). Islas-Valdez et al. (2017) stated that biofertilizers stimulate physiological or natural processes that elevate nutrient absorption, since such bacteria initiate the production of metabolites, related directly to the growth of the plant such as auxins, gibberellins, and cytokinins. They also synthesize antibiotics, siderophores and hydrocinnamic acid that reduce pathogen activities.

Chemical analysis

Chlorophylls, Carbohydrates, Ash, Protein and Crude fiber

It appears from data in Table 5 that, the application of biochar-4 treatment significantly raised total chlorophyll by14 and 13% at 60 and 90 DAT, respectively compared to NPK fertilizes (control). While at the last cut (after 120 days from transplanting) insignificant differences between biochar-4 treatment and chemical fertilizers were recorded. After 60 days, biochar-4 caused insignificant increment of total carbohydrates compared to control plants. However, after 90 and 120 days, significant increments were emerged (5.2% and 4.5%, respectively) judged against control plants, albeit the increment was in favor of biochar-4 treatment.

The results were in accordance with those obtained by Vafadar et al. (2014) whom found that the dual use of biofertilizers (including Azotobacter and Bacillus bacteria) increased plant height, fresh & dry weight of shoots and roots, chlorophyll a, b, and total content of Stevia plants. Ucar et al. (2017) mentioned that chlorophyll contents increased with increasing nitrogen doses from organic and biofertilizers. Aguirre et al. (2018) stated that chlorophyll content of the shoots increased by using biofertilizers. Tavarini et al. (2019) stated that stevia plants inoculated with biofertilizers showed an increase in harvest index, total soluble sugars and total monosaccharides in their roots. Also, Al-Erwy et al. (2016) found that treating plants with PGPR (Azotobacter and Rhizobium) caused a significant increase in total carbohydrates and mineral contents, and application of *P. fluorescence* and *B. subtilis* strains improved seed quality and nutritional quality such as increasing carbohydrate contents of plants. Regarding ash content, it was noticed that biochar-4 treatment realized significantly had the upper hand over the control since it recorded 6.5, 15 and 17% after the three cuts, respectively over the control treatment (Table 5). Furthermore it was mentioned that high ash content implies from stevia leaves are a good source of inor-

Table 5. Analysis of some components of stevia leaves (g 100 g^{-1} dry weight basis) after 60, 90 and 120 days from transplanting in response to biochar treatments

Components	Control (NPK)	Biochar-1	Biochar-2	Biochar-3	Biochar-4
60 DAT					
Total chlorophylls (mg g^{-1} f. w.)	3.40^{b}	3.22^{c}	2.95^{d}	3.06^{e}	3.89^{a}
Carbohydrates g\100 g^{-1}\ D.W	54.65^{a}	52.25^{b}	47.12^{c}	53.56^{b}	55.78^{a}
Ash g\100 g^{-1}\ D.W	11.98^{b}	11.10^{b}	9.84^{c}	12.31^{a}	12.75^{a}
Protein g\100 g^{-1}\ D.W	3.05^{a}	2.26^{b}	1.94^{c}	2.81^{b}	3.62^{a}
Crude fiber g\100 g^{-1}\ D.W	15.43^{a}	14.65^{b}	10.45^{d}	14.50^{b}	12.10^{c}
90 DAT					
Total chlorophylls (mg g^{-1} f. w.)	4.52^{b}	4.38^{b}	3.98^{c}	3.78^{c}	5.10^{a}
Carbohydrates g\100 g^{-1}\ D.W	58.71^{b}	56.44^{c}	51.39^{d}	56.19^{c}	61.81^{a}
Ash g\100 g^{-1}\ D.W	13.79^{c}	12.51^{d}	10.24^{e}	14.22^{b}	15.86^{a}
Protein g\100 g^{-1}\ D.W	3.74^{b}	2.84^{c}	2.68^{c}	3.57^{b}	4.01^{a}
Crude fiber g\100 g^{-1}\ D.W	18.71^{a}	17.81^{b}	12.64^{d}	17.72^{b}	14.83^{c}
120 DAT					
Total chlorophylls (mg g^{-1} f. w.)	5.10^{a}	4.90^{b}	4.56^{b}	4.10^{b}	5.65^{a}
Carbohydrates g\100 g^{-1}\ D.W	60.17^{b}	59.47^{b}	54.58^{c}	60.78^{b}	62.92^{a}
Ash g\100 g^{-1}\ D.W	14.76^{c}	14.08^{c}	12.61^{d}	15.13^{b}	17.33^{a}
Protein g\100 g^{-1}\ D.W	4.28^{b}	4.93^{b}	3.98^{c}	3.65^{c}	5.93^{a}
Crude fiber g\100 g^{-1}\ D.W	19.71^{a}	18.77^{b}	13.88^{d}	19.61^{a}	16.94^{c}

Data represented average of two growing seasons 2018-2019
Means with the same letters in a column are not significantly different by DMRT 5%
DAT; days from transplanting

ganic minerals and the content of ash in dried *Stevia rebaudiana* leaves was increased due to biofertilizers application Das and Dang (2014).

At the first cut (60 days), there was insignificant difference in protein content as shown in Table 5 between biochar-4 treatment and NPK fertilizers. However, significant increases were recorded in protein content at 90 and 120 days as a result of biochar-4 treatment (7.2 and 38%, respectively). Kraska et al. (2018) stated that soil amended with biochar increased the total protein content of winter rye grain. Pandey et al. (2018) found that addition of plant growth bacteria (Bacilli sp.) increased fat and protein content of wheat plants.

As for crude fiber, it was found that chemical NPK fertilizers (control) treatment surpassed significantly all other biochar treatments, particularly when compared to biochar-4 treatment as it recorded 21%, 20% and 14% after 60, 90 and 120 days from transplanting, respectively. Iliemene and Atawodi (2019) found that crude fiber plays an important role in cancer prevention, presumably through mechanisms that involve limiting the extent of oxidative stress and preventing or delaying pro-carcinogenic inflammatory processes.

The elevated amount of total chlorophylls, carbohydrates, ash and protein maybe due to synergetic beneficial effects of biochar and microorganism strains to liberate more nutrients from the unavailable reserves. Also, they corrected iron and zinc deficiency in sandy soil which restores photosynthesis process efficiency by increasing photosynthetic pigments content of leaves. While the positive role of biochar might be referred to its components of available macro and micro nutrients besides their role in increasing root surface per unit of soil volume as well being involved in carbohydrate metabolism and photosynthesis (Taiz and Zeiger, 2006 and Hassan et al., 2020).

Essential and non-essential amino acids content

Data in Table 6 discerned that, the content of amino acids (essential and non-essential) at 120 days from transplanting in the leaves of stevia plants were affected by all treatments. However the superiority of biochar-4 treatment was outstanding since it gave the highest amount of essential amino acids represented in Arginine, Lysine, Histidine, Phenylalanine, Leucine, Methinine and Isoleucine. While the highest amount of Valine and Threonine were recorded from control treatment. As for non-essential amino acids, it was noticed that the maximum amount of Aspartate, Glutamic, Proline, Alanine, Cysteine and Tyrosine were donated from biochar-4 treatment whereas the utmost quantity of Serine and Glycine were recorded from NPK treatment. It was unequivocal that both total essential and non-essential amino acids as shown in Figure 2 were resulted from biochar-4 treatment as an aftereffect. Similar results were found by Pandey et al. (2018)

Table 6. Essential and non-essential amino acids content of stevia leaves (g/ 100 g dry) after 120 days from transplanting in response to biochar treatments

Amino acids	Control(NPK)	Biochar-1	Biochar-2	Biochar-3	Biochar-4
Essential amino acids					
Arginine	0.80	0.78	0.43	0.67	0.84
Lysine	0.70	0.42	0.23	0.47	0.69
Histidine	1.03	0.48	0.32	0.97	1.10
Phenyl alanine	0.79	0.67	0.72	0.80	0.89
Leucine	0.96	0.89	0.83	1.12	1.21
Methinine	1.35	1.17	0.93	1.25	1.60
Valine	0.95	0.87	0.61	0.81	0.91
Threonine	1.18	1.05	0.74	1.02	1.12
Isoleucine	0.74	0.71	0.46	0.57	0.76
Non-essential amino acids					
Aspartate	1.65	0.89	0.38	1.45	1.70
Serine	1.09	0.80	0.45	0.89	1.05
Glutamic	1.79	1.12	0.53	1.78	1.94
Proline	1.56	1.35	0.24	1.48	1.60
Glycine	0.83	0.78	0.26	0.76	0.80
Alanine	0.89	0.82	0.50	0.78	0.98
Cysteine	0.55	0.51	0.43	0.52	0.61
Tyrosine	0.98	0.89	0.46	0.90	1.03

Data represented average of two growing seasons 2018–2019

whom mentioned that addition of plant growth bacteria (Bacilli sp.) increased amino acids content in plants.

Nitrogen, phosphorus, potassium and magnesium

Data presented in Table 7 indicated that the highest values of nitrogen at 60, 90 and 120 days (18, 7.5 and 12%, respectively) were recorded as a result of biochar-4 treatment and significantly surpassed NPK fertilizers (control). Whereas phosphorus amount was significant raised over control treatment after first and second cut since it recorded 7.5 and 7%, respectively while after the third cut (120 days) insignificant increment was detected between biochar-4 treatment and control plants, although the increment was in favour of biochar-4 treatment. Regarding potassium, it was found that at 60 and 120 days from transplanting, biochar-4 significantly increased potassium content by 2.3 and 2.5% compared to the control. Whilst after 90 days, insignificant increment was recorded between both mentioned treatments. As for magnesium, it recorded significant increases at 60, 90 and 120 DAT (21, 26 and 12%, respectively), in favour of biochar-4 compared to the control.

These results were in harmony with those obtained by Thomas et al. (2019) whom found that biochar used as a soil amendment, has a variety of properties in particular its potential to increase soil C sequestration and enhancing yields by increasing retention of soil mineral and increased nutrients content within plant tissues. Liao et al. (2019) stated that the bacteria stimulated by the biochar amendment are known for their ability to fix nitrogen and solubilizing phosphorus, which may potentially contribute to the "biochar effect" in the rhizosphere. In addition, Aguirre et al. (2018) found that application of biofertilizers increased N as well as P contents of stevia plants and improved nutrients and water transport to the plant.

Iron and zinc

Micronutrients represented in iron and zinc were significantly affected by different biochar treatments as shown in Table (7). Biochar-4 treatment caused significant increase of iron and zinc compared to the control. Regarding iron, the elevation reached 15.5, 16 and 16.2% and for zinc, the increments reached 20, 42 and 34% after 60, 90 and 120 days from transplanting, respectively.

The increments of macro-nutrients may be attributed to several reasons. Nitrogen increased due to both, its existence in biochar and nitrogen fixation by N fixing bacteria. In addition, the increase of available phosphorus in the soil provides adenosine triphosphate (ATP) as a source of energy which is important for nitrogen fixation process. Additionally, many investigators explained the role of *Bacillus sp.* as phosphate dissolving bacteria (PDB) which increases the availability of phosphorus in the soil. This is attained by the secretion of organic acids that leads to the transfer of fixed phosphate to

Table 7. Nutrients (N, P, K, Fe, Mg and Zn) concentration (mg 100 g^{-1} D.W) in stevia leaves after 60, 90 and 120 days from transplanting in response to biochar treatments

Minerals contents (mg 100 g^{-1})	Control (NPK)	Biochar-1	Biochar-2	Biochar-3	Biochar-4
60 DAT					
Nitrogen	488.3^{b}	361.7^{c}	311.8^{c}	451.13^{b}	579.5^{a}
Phosphorus	284.5^{b}	247.3^{c}	203.6^{d}	173.8^{e}	305.1^{a}
Potassium	1381.6^{b}	939.2^{c}	677.8^{d}	460.3^{e}	1411.0^{a}
Magnesium	295.5^{b}	226.3^{c}	168.6^{d}	122.5^{e}	358.4^{a}
Iron	60.46^{b}	53.51^{c}	46.69^{d}	49.81^{d}	69.75^{a}
Zinc	3.81^{b}	2.16^{c}	1.61^{d}	1.25^{d}	4.57^{a}
90 DAT					
Nitrogen	598.5^{b}	455.5^{c}	429.8^{c}	571.3^{b}	642.5^{a}
Phosphorus	334.8^{b}	298.5^{c}	254.8^{d}	223.6^{e}	358.5^{a}
Potassium	1504.5^{a}	1062.3^{b}	813.9^{c}	587.8^{d}	1534.2^{a}
Magnesium	336.8^{b}	295.2^{c}	237.5^{d}	191.6^{d}	425.1^{a}
Iron	63.88^{b}	57.29^{c}	50.11^{d}	55.89^{c}	74.15^{a}
Zinc	4.35^{b}	2.82^{c}	2.25^{c}	1.89^{d}	6.21^{a}
120 DAT					
Nitrogen	845.0^{b}	789.0^{b}	638.0^{c}	584.1^{d}	950.0^{a}
Phosphorus	434.0^{a}	397.0^{b}	353.0^{c}	323.0^{c}	453.0^{a}
Potassium	1663.0^{b}	1244.0^{c}	970.45^{d}	756.0^{e}	1703.0^{a}
Magnesium	489.0^{b}	415.0^{c}	356.0^{d}	312.0^{e}	548.6^{a}
Iron	65.07^{b}	58.0^{c}	54.0^{c}	50.0^{d}	75.58^{a}
Zinc	5.44^{b}	3.89^{c}	3.34^{c}	2.98^{d}	7.30^{a}

Data represented average of two growing seasons 2018-2019
Means with the same letters in a column are not significantly different by DMRT 5%
DAT; days from transplanting

available phosphate and consequently increasing phosphorus absorption as well as phosphorus accumulation in plant tissues (Hasan et al., 2016). The increment in potassium may be due to the increase in organic acids within the soil leading to the decrease of soil pH, which has an important role in potassium absorption from the soil.

Increments of iron contents may be due to the favorable effects of the combination of biochar and microorganism strains on the reduction of Fe^{+3} to Fe^{+2}, making iron chelates readily available to plants. Additionally, Satish (2018) mentioned the role of biofertilizers in preventing the formation of insoluble complexes of zinc and hence facilitating their uptake by the plant, besides their role in the slow release of available zinc and other nutrients. Also, they mentioned that biofertilizers considered as a source of hormones secretion such as IAA, IBA and GA_3 which play an important role in growth promoting substances.

Stevioside, Vitamins C, B2 and folic acid:

It is obvious from data in Table 8 that, treatments of biochar had undisputed effect on stevioside percentage, vitamins C, B2 and folic acid concentrations since insignificant differences were found at first cut (60 days) between biochar-4, biochar-3, biochar-1 and control plants in stevioside percentage, whereas the lowest percentage of stevioside (9.5%) resulted from biochar-2 compared to NPK fertilizers. After the second cut (90 days), it was outstanding that biochar-4 significantly raised stevioside percentage over all the other treatments, mainly over the control by 9%. The same drift was obtained subsequent last cut (120 days) where both biochar-4 and biochar-3 significant donated high percentage of stevioside by 8.5 and 5%, respectively in comparison with NPK fertilizers. Pertaining to other compounds in Table (8), similar tendency was obtained with vitamins C, B2 and folic acid at 60 days as insignificant increases were recorded between biochar-4 treatment and control. While the second cut (90 days) recorded significant increases in vitamins C, B2 and folic acid concentrations by 27.5, 33 and 9.6% respectively as a sequence of biochar-4 treatment application in comparison to control plants. However, at the third cut (120 days), biochar-4 caused insignificant increments of both vitamin C and folic acid concentrations compared to

Table 8. Stevioside (%), water soluble vitamins of stevia leaves (mg 100 g^{-1} dry weight) after 60, 90 and 120 days from transplanting in response to biochar treatments

Vitamins	Control (NPK)	Biochar-1	Biochar-2	Biochar-3	Biochar-4
60 DAT					
Stevioside %	11.61^{a}	11.22^{a}	10.50^{b}	11.54^{a}	11.68^{a}
Vitamin C	10.75^{a}	10.12^{b}	10.36^{b}	10.06^{c}	10.77^{a}
Vitamin B2	0.19^{a}	0.06^{b}	0.09^{b}	0.19^{a}	0.18^{a}
Folic acid	47.82^{a}	40.48^{c}	43.6^{b}	47.29^{a}	48.04^{a}
90 DAT					
Stevioside %	14.25^{b}	14.15^{b}	13.75^{c}	14.37^{b}	15.56^{a}
Vitamin C	11.20^{d}	12.71^{c}	12.82^{c}	13.02^{b}	14.29^{a}
Vitamin B2	0.36^{d}	0.40^{c}	0.41^{c}	0.45^{b}	0.48^{a}
Folic acid	48.31^{b}	50.04^{b}	50.11^{b}	48.68^{b}	52.95^{a}
120 DAT					
Stevioside %	16.46^{b}	16.33^{b}	15.81^{c}	17.25^{a}	17.88^{a}
Vitamin C	14.58^{a}	14.29^{a}	13.56^{b}	13.32^{b}	14.90^{a}
Vitamin B2	0.40^{b}	0.42^{b}	0.34^{c}	0.41^{b}	0.46^{a}
Folic acid	53.34^{a}	52.59^{a}	48.90^{b}	45.78^{c}	54.12^{a}

Data represented average of two growing seasons 2018-2019
Means with the same letters in a column are not significantly different by DMRT 5%
DAT; days from transplanting

the control and significantly increased in the vitamin B2 concentration by 15% in contrast to the control.

The prior data are in reliability with the results obtained by Krasina and Tarasenko (2016) that found that stevia leaves contain water-soluble and fat-soluble vitamins in a sufficient quantity that contribute to its antioxidant activity. Yadav and Sarkar (2019) stated that biofertilizers provide vitamins, nutrients (N, P & K), antibiotics (which serves as nutritional food properties) and hormones like auxins & cytokinins which enrich root rhizosphere and contribute them to the plant.

Fatty acids

Concerning the effect of different treatments on fatty acids as shown in Table (9), it noticed that at the first cut (60 days), biochar-4 caused a significant increase of palmitic acid (2%) and insignificant increase of oleic acid, linolic acid as well as linolenic acid in comparison to the control. While, NPK fertilizers (control) were significantly increased oleopalmitic and stearic acids by 26 and 11%, respectively compared to biochar-4. At 90 days, biochar-3 recorded significant increase of palmitic acid (17.5%) judged against to control plants. Meanwhile, biochar-4 resulted in significant increase of oleopalmitic acid (7.5%) and insignificant increases of stearic, oleic and linolenic acids compared to NPK fertilizers.

On the other hand, the control treatment resulted in significant increments (2%) of linolic acid compared to biochar-4 treatment. Concerning the last cut (120 days) biochar-4 resulted in significant increases of palmitic, linolic and linolenic acids by 10.5, 5.7 and 5.6%, respectively contrast to the control as well as insignificant increases in oleopalmitic, stearic and oleic acids judged against control. The obtained results are in agreement with those obtained by Chughtai et al. (2019) who stated that saturated, mono and polyunsaturated fatty acids like Palmitic, oleopalmitic, Stearic, Linoleic, Linolenic and Oleic acids were found in considerable quantities in *Stevia rebaudiana*, Sharifi (2016) found that saturated fatty acids (palmitic and stearic acids) were reduced in soybean seeds inoculated with biofertilizers, while unsaturated fatty acids (li-

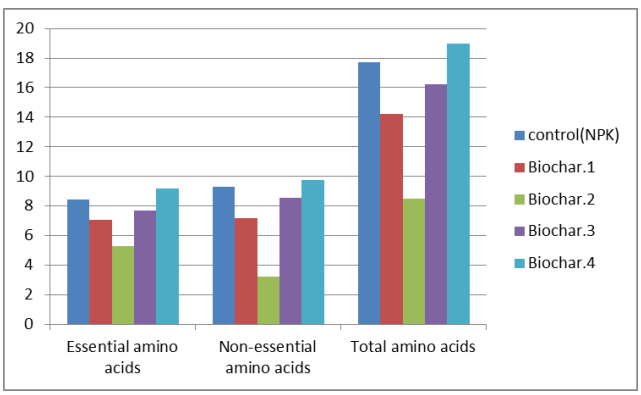


Fig. 2. the content of total essential and non-essential amino acids of stevia leaves (g/ 100 g dry) after 120 days from transplanting in response to biochar treatments

Table 9. The composition of fatty acids contents (g 100 g^{-1}) of stevia oil in response to biochar treatments

Fatty acids	Control (NPK)	Biochar.1	Biochar.2	Biochar.3	Biochar.4
60 DAT					
Palmitic acid	16.82^{b}	11.58^{d}	14.51^{c}	16.43^{b}	17.18^{a}
Oleopalmitic acid	1.52^{a}	0.21^{c}	0.07^{c}	1.01^{b}	1.12^{b}
Stearic acid	2.17^{a}	1.63^{c}	0.51^{d}	1.73^{b}	1.93^{b}
Oleic acid	6.12^{a}	4.66^{b}	2.08^{c}	6.27^{a}	6.78^{a}
Linolic acid	11.62^{a}	7.88^{c}	8.69^{c}	10.55^{b}	11.92^{a}
Linolenic acid	24.11^{a}	23.65^{b}	14.57^{c}	12.67^{d}	25.17^{a}
90 DAT					
Palmitic acid	19.37^{b}	16.62^{c}	18.22^{c}	22.78^{a}	20.45^{b}
Oleopalmitic acid	1.96^{b}	1.77^{c}	0.98^{d}	2.01^{a}	2.11^{a}
Stearic acid	2.79^{a}	2.80^{a}	0.97^{c}	1.54^{b}	2.66^{a}
Oleic acid	7.43^{a}	6.11^{b}	3.17^{d}	5.22^{c}	7.84^{a}
Linolic acid	13.08^{a}	9.92^{e}	10.50^{d}	11.07^{c}	12.85^{b}
Linolenic acid	27.05^{a}	28.16^{a}	20.73^{b}	15.63^{c}	28.79^{a}
120 DAT					
Palmitic acid	26.43^{b}	25.90^{b}	20.76^{d}	23.91^{c}	29.21^{a}
Oleopalmitic acid	2.45^{a}	2.34^{b}	1.26^{c}	1.12^{c}	2.95^{a}
Stearic acid	3.65^{a}	3.45^{a}	1.20^{b}	1.10^{b}	3.90^{a}
Oleic acid	9.12^{a}	8.90^{b}	4.35^{c}	4.75^{c}	9.80^{a}
Linolic acid	15.98^{b}	14.55^{c}	12.67^{d}	11.89^{e}	16.90^{a}
Linolenic acid	30.67^{b}	30.84^{b}	21.80^{c}	19.90^{d}	32.40^{a}

Data represented average of two growing seasons 2018-2019
Means with the same letters in a column are not significantly different by DMRT 5%
DAT; days from transplanting

noleic, linolenic and oleic acids) were increased. Sharifi et al. (2017) also indicated that biofertilizers inoculation enhanced fatty acids composition of soybean seed. Saturated fatty acids were reduced while unsaturated fatty acids were increased in response to biofertilizers treatment.

The increment of fatty acids content in response to biochar-4 treatment could be explained on the basis of available elements, vitamins, gibberellins, cytokines, hormone like substances, amino acids and sugars that led to an increase in biochemical processes within the plant tissues (luxury of metabolism theory), consequently increasing fatty acids content (Taiz & Zeiger 2006).

Conclusion

On the basis of earlier stated data, application of biochar loaded with both inorganic nitrogen and beneficial microorganism strains (*Azotobcter chrococcoum*, *Bacillus subtilis*, *Pseudomonas fluorescens, Splrulina platensis* and *Anabaena azollae*) gave eminent outcome on plant under investigation stevia (*Stevia rebaudiana*) which reflected on high growth traits and chemical composition as well in comparison with results derived from commercial chemical fertilizers NPK. Hence, the integrated use of biochar with different beneficial microorganism strains as biofertilizers is a promising strategy to obtain higher and sustained productivity of *Stevia rebaudiana* plants which is used in medical and pharmaceutical industries.

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