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ORIGINAL PAPER

Development of *Bacillus safensis*-based liquid bioformulation to augment growth, stevioside content, and nutrient uptake in *Stevia rebaudiana*

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Abstract

The application of chemical fertilizers to enhance crop production is a major concern due to associated environmental pollution and health hazards. Hence, there is an urgent need to develop an eco-friendly solution to improve crop production and promote sustainable agriculture simultaneously. *Stevia rebaudiana* is an important medicinal crop being substitute for sugar, superior flavor outline, extensive medicinal properties, and also of agronomic interest. In the present study, bacterium STJP isolated from the rhizospheric soil of *S. rebaudiana* and identified as *Bacillus safensis* on the basis of 16S rRNA gene sequencing, showed good amount of zinc (4.4 mg/L) and potassium (5.4 mg/L) solubilization. Paneer-whey (a dairy waste) based bioformulation (P-WBF) was developed utilizing isolate *B. safensis* STJP (accession number NAIMCC TB-2833) and inspected for the quality and ability to enhance the growth, nutrients uptake, and stevioside content in *S. rebaudiana*. The application of P-WBF displayed a significantly higher concentration (153.12%) of stevioside in *S. rebaudiana* as compared to control. P-WBF treated Stevia plants showed significantly higher fresh and dry weight as well (as compared to control). Further, enhancement of phosphorous, nitrogen, potassium, and zinc uptake in plant tissue was also recorded by application of P-WBF. This study suggests the use of P-WBF based biofertilizer using *B. safensis* STJP to increase stevioside content in Stevia plant by a nutrient(s) linked mechanism. This novel approach can also be beneficial for utilization of a dairy waste in preparation of bioformulation and, for enhancement of crop yield by an ecofriendly manner leading to sustainable agriculture.

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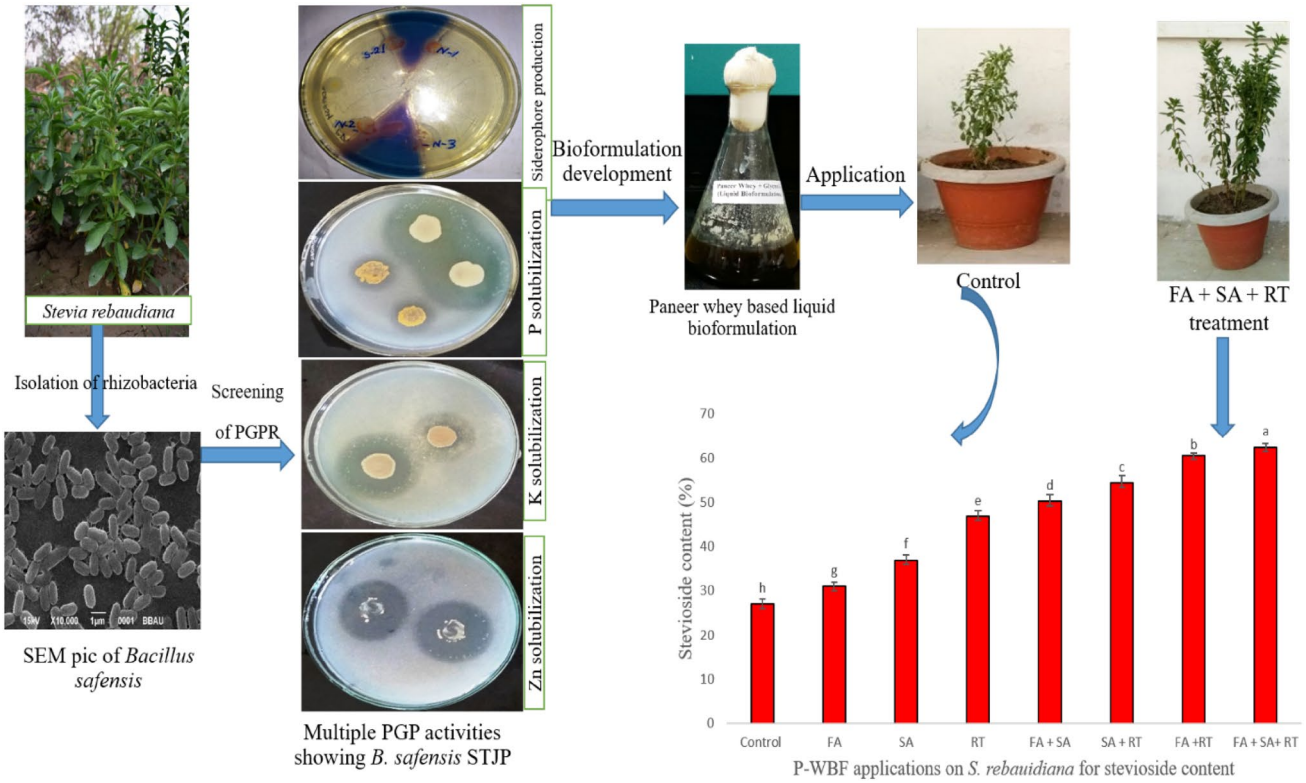
Graphic abstract



Keywords *Bacillus safensis* · Liquid bioformulation · Stevioside · Secondary metabolite · Dairy waste

Introduction

Stevia rebaudiana (Stevia) is an ayurvedic herb of family Asteraceae, native to South America. It is also known as sweet leaf/candy leaf/honey leaf/sweet herb/Meethi Patti (Salazar et al. 2018). The leaf of *S. rebaudiana* contains diverse minerals (Fe, Si, Co, Mn, Ca, Mg, Se, Ti, and Zn) and vitamins (ascorbic acid, beta-carotene, niacin, thiamine, and riboflavin) (Tandel 2011). Due to high vitamin and mineral content, *S. rebaudiana* has been explored for its therapeutic use as anti-inflammatory (Gaweł-Bęben et al. 2015), anti-hyperglycaemic (Assaei et al. 2016), anti-tumor (Gupta et al. 2017), hepatoprotective (Latha et al. 2017) and immunomodulatory (Boonkaewwan and Burodom 2013) and thus, it has high demand in the medical industry. Moreover, it is also used as a natural sweetener due to low glycemic index that depends on the alkaloids, diterpene glycosides (DGs) (Richman et al. 1999; Modi et al. 2014). From the past few decades, the study of structural, chemical and functional aspects of *S. rebaudiana* has been in the limelight in order to discover different DGs. Extensive research has led to the identification of more than 30 DGs from *S. rebaudiana* (Kregiel 2015; Cantabella et al. 2017). Among all the reported DGs, stevioside is most common with properties like slightly bitter taste, non-fermentable, and low calorific value (Modi et al. 2014). However, stevioside is 300 folds sugary with extended shelf life as compared to normal sugar (Yadav et al. 2011). Leaf of *S. rebaudiana* contains highest content of stevioside (6–18%) and gradually it decreases in flowers (8.2%), stems (~7.54%), seeds (~4.42%), and roots (~3.14%) (Šic Žlabur et al. 2013; Pal et al. 2015). The European Food Safety Authority (EFSA) along with the joint report of Food and Agriculture Organization (FAO) and World Health Organization Expert Food Committee (WHOEFC) have approved stevioside as accepted safe for human use and alternative to sugar, especially in case of diabetes patients (EFSA 2010; JECFA 2016). The priceless medicinal properties of stevioside have resulted in upturn of *S. rebaudiana* cultivation, all over the world. At present, China is chief Stevia producer as well as exporter (approx. 80%) and Korea and Japan are the largest consumers in the world (Lemus-Mondaca et al. 2012). Recently, Stevia has gained huge response in the Indian market and in last three years, the demand of *S. rebaudiana* has increased by up to 300 times (Pal et al. 2015; Debnath et al. 2018). The demand is mainly from the pharmaceutical, food, beverages,

and sweet manufacturing industries. Hence, there is motivation in farmers for cultivation of *S. rebaudiana* by utilizing appropriate nutrients and fertilizers.

For the cultivation and yield enhancement of *S. rebaudiana* chemical fertilizers are being used commonly (Liu et al. 2011). However, these fertilizers although increase plant's growth and productivity but also adversely affect beneficial macro and micro-organisms of the soil and ultimately emerge in the form of biomagnification via food chain, causing eutrophication in water bodies and several other impacts on the environment (Savci 2012; Mishra et al. 2016; Mukherjee et al. 2017; Arora et al. 2018). Moreover, it has been proved that for the cultivation of medicinal and aromatic plants (MAPs)/crops, chemical fertilizers should be avoided, as MAPs or their products are directly used/consumed by humans (Smith-Hall et al. 2012). In order to overcome the hitch, an economical, eco-friendly and sustainable method should be developed for the cultivation of MAPs. In this regard, bioformulations or bioinoculants can prove to be a reliable solution particularly for the cultivation of MAPs. As mentioned earlier by the author (of this manuscript) and other researchers, bioformulations are the naturally active gradient containing potent strain(s) of endophytic, rhizo or phyllospheric microbes or metabolites, embedded in economically cheap or waste materials which can be used to enhance the growth and yield of plants/crops, improve soil fertility and protect against pathogens related to plants (Arora et al. 2010; Arora and Mishra 2016; Nagachandrabose 2018). Bioformulations may be in liquid or solid forms, but liquid are considered better in comparision to solid, as they have longer shelf-life, can easily colonize on the root or plant surface, perform well in pots or in field conditions, and have low cost (Goljanian-Tabrizi et al. 2016; Nagachandrabose 2018). The present study was focused on the development of liquid bioformulation to increase the stevioside content in *S. rebaudiana* by utilizing plant growth promoting bacteria. In this regard, the impact of liquid bioformulation on various growth parameters like root and shoot length, fresh and dry weight along with nutrient uptake (in plants) was determined. The developed liquid bioformulation may prove to be a noble asset for the farmers involved in the cropping of *S. rebaudiana*.

Materials and methods

Rhizobacteria and growth conditions

Bacillus safensis STJP (Gene bank accession number: KX372540.1) isolated and identified in the Laboratory for Rhizospheric Microbiology, School for Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow was used in the current experiment. Isolate STJP was submitted in the National Bureau of Agriculturally Important Microorganisms Culture Collection (NAIMCC), Uttar Pradesh, India (a national culture collection center) with the accession number NAIMCC-TB-2833. Isolate STJP showed plant growth promoting (PGP) traits viz. production of siderophore, indole acetic acid (IAA) and phosphate solubilization as reported in an earlier study by the authors (Prakash and Arora 2019) (data presented in supplementary Table 1). Isolate STJP was purified and kept on the nutrient agar medium at 4 °C and renewed periodically for further experiments.

K and Zn solubilization

To check potassium (K) and zinc (Zn) solubilization by the isolate *B. safensis* strain STJP, Aleksandrov broth ($g\text{-}^{L}$; $K_2HPO_4.3H_2O$: 0.2, $CaPO_4$: 0.2, $CaCO_3$: 0.01, $MgSO_4.7H_2O$: 0.05, $C_6H_{12}O_6$: 0.5, $FeCl_3$: 0.0005, pH 7.2) supplemented with mica powder (0.1 g/L) (Sigma, USA) as the sole source of insoluble K was taken (Hu et al. 2006). Briefly, log phase of STJP culture was inoculated in Erlenmeyer flask (EF) containing 100 mL Aleksandrov broth and incubated (120 rpm, 30 °C, 5 days); for control, uninoculated broth was taken. After incubation, broth was centrifuged (Model: REMI CM-12 PLUS, England) for 10 min at 10, 000 rpm and collected supernatant was used for examination of soluble K by atomic absorption spectrometry (AAS) (Model: AA240FS Fast Sequential AAS, USA) at 766.5 nm (Parmar and Sindhu 2013).

For solubilization of Zn, the fresh culture of STJP was inoculated in minimum salt broth [containing (g^{-L}); KCl: 0.2, K_2HPO_4: 0.1, $MgSO_4$: 0.2, $(NH_4)_2SO_4$: 1, dextrose: 10], and inorganic Zn compound (ZnO: 0.1 g/L) (Sigma, USA) was added and the pH was maintained at 7 (Saravanan et al. 2003). The broth was incubated at 120 rpm; 28 °C for 5 days. After incubation, the broth was centrifuged (rpm: 10, 000; time: 10 min) and supernatant taken. Finally, the obtained supernatant was injected to AAS column to check the solubilization of available Zn content (Fuwa et al. 1964). During the experiment of Zn solubilization, the absorbance of AAS was read at 213.9 nm as per Fuwa et al. (1964).

Development of bioformulation and analysis of its physiochemical properties

For the development of liquid bioformulation, four liquid carriers were taken viz, potato broth (water left after boiling potato), rice broth, paneer-whey, and molasses. The carriers were selected on the basis of ease of availability. All the four carriers being waste products can be cost-effective as well. For preparation of liquid bioformulation, bacterial isolate STJP was grown in nutrient broth (temperature: 30 °C, incubation time: 48 h). After the growth period, bacterial

population was maintained at 10^8 cells/mL with the help of deionized water as per method described by Singh et al. (2014). Further, 10% bacterial inoculum and 5% glycerol were mixed in each carrier (potato broth, rice broth, paneer-whey, and molasses) for preparation of bioformulation (Goljanian-Tabrizi et al. 2016). The pH (Model: Manti Lab MT-103 M Digital pH meter, India) of all the carriers was adjusted by adding 1 N HCl or 1 N NaOH and prepared bioformulations were stored at 30 °C for six months. After storage, insurance of quality of all bioformulations; pH, electric conductivity (EC) (Model: Cyberscan PCD 6500, India), shelf life, and presence of pathogenic microorganisms (*Salmonella*, *Escherichia coli* and *Shigella*) was checked as per Pandey and Maheshwari (2007) and Tanih et al. (2015), whereas total P and K were analyzed through digestion method as per Page et al. (1982). Finally, the ratio of carbon/nitrogen (C/N) was analyzed for all carriers according to the procedure given by Jimenez and Ladha (1993).

Effect of bioformulation on growth promotion of *S. rebaudiana*

On the basis of availability, maintaining good bacterial population, being non-pathogenic, physio-chemical characteristics and eco-friendly, paneer whey based bioformulation (P-WBF) was selected for further study. P-WBF was applied on the *S. rebaudiana* for pot study in the greenhouse of Horticulture Research Farmhouse (HRF) during February to April, 2017 (90 days) and repeated in February to April, 2018 for 90 days and July to September, 2019 (60 days) as per the method suggested by the corresponding author of this manuscript (Arora et al. 2001). Briefly, the unsterilized soil was collected from the agricultural field of HRF. After doing moist heat sterilization (121 °C, 15 PSI, 1 h), 6 kg of sterilized soil and similarly non-sterilized (soil) was filled into each pot (30 cm × 25 cm × 25 cm). Subsequently, the seedling of *S. rebaudiana* were sterilized externally for removal of surface microbes with 70% ethyl alcohol (time: 2 min) followed by sodium hypochlorite (percentage: 2%; time: 5 min) and finally rinsed with deionized water (time: 10 min) (Arora et al. 2001). The decontaminated seedling roots were treated by P-WBF (cell population: 10^8 cells/mL) as per following 8 sets: (1) control (*S. rebaudiana*); (2) foliar application (FA); (3) soil application (SA); (4) root treatment (RT); (5) FA + SA; (6) FA + RT; (7) SA + RT; (8) FA + SA + RT. The liquid formulation P-WBF was applied in SA as per Berger et al. (2013), dose (2 mL/pot); FA (2–3 mL/plant) (https://www.krishisewa.com/articles/organic-agriculture/115-biofertilizers.html) and RT of Stevia plant was done by root dip method (Mamta et al. 2010). The equal amount of water was used for irrigation as per requirement (two times in a week). The plants were harvested after 90 days from sterilized soils and 60 days from non-sterilized soils and checked for fresh weight and dry weight. No chemical fertilizers nor any pesticides were used in this experiment.

Extraction and estimation of stevioside

The harvested shoots of *S. rebaudiana* from sterilized and non-sterilized soils were dried separately in the hot air oven (Model: Thermostatic RSTI-101, India) (temp: 50 °C; incubation time: 4 days). The extraction of stevioside was done by hot water method (Rai et al. 2012). Briefly, the complete dried shoots were crushed into powder form and dissolved in the water and boiled (temperature: 78 ± 2 °C; crushed dry shoots: 1 gm; water: 14 mL; heating timing: 56 min). After boiling, sample was tickled through Whatman filter paper No. 42 and the obtained sample was filled in the rotatory evaporator (Model: R-100 Thermo Fisher Scientific, USA) (temp: 45 C;; pressure: 75 mbar; rpm: 55 rev min^{-1}) for complete water vaporization (Megeji et al. 2005).

Obtained dry sample from rotatory evaporator was dissolved and mixed with water and acetonitrile (HPLC grade, Merck) in a ratio of 20:80. Additionally, the sample was further filtered (0.45 Millipore membrane, England) in HPLC vial and the collected filtrate was analyzed for stevioside content by high performance liquid chromatography (HPLC; Model No: Waters 2489 UV/Visible Detector, USA) consisting of a mixed-mode wax-1 column, pore size: 5 µm. During the experiment, 20 µL sample was injected into the sample loop and the mobile phase was fixed in a mixture of water and acetonitrile (20:80 v/v; HPLC Grade, Merck) (flow rate; 1.0 mL/min, pressure; 1100 psi, temperature; 40 °C, and run timing; 10 min) (Bovanova et al. 1998; Mamta et al. 2010). Further, sample was injected in the analytical column and the result was inspected at 210 nm. The obtained result was matched with standard stevioside (purity 98%) procured from Sigma-Aldrich, USA.

Effect of bioformulation on nutrients uptake in *S. rebaudiana*

After tilling of *S. rebaudiana* from both soils (sterilized and non-sterilized), all plants were dried in the hot air oven (Model no: Thermostatic RSTI-101 Series, India). For analysis of P, 100 mg dried sample was grinded and digested with 6 mL of H_2SO_4 and $HClO_4$ (ratio: 9:1) on the hot plate for 10 min. Digested colourless samples were diluted into 100 mL distilled water. Finally, the prepared sample was examined for P as per Vanadomolybdophosphoric yellow color method (Koenig and Johnson 1942). Available Zn uptake in the plants was determined after the digestion process by using AAS (Model: AA240FS Fast Sequential AAS, USA) (Horwitz et al. 1970). Subsequently, K uptake

in *S. rebaudiana* plants was evaluated by tri-acid digestion method according to Jackson (1967).

Statistical analysis

The pot trial was planned as completely randomized block design (CRD) with five replica and data was represented as the mean ± standard deviation (SD). To check the result validity, an analysis of variance (ANOVA) (Gomez and Gomez 1984) was done and finally Duncan's multiple range test ($p < 0.05$) was implemented to check the significant differences of various sets.

Results

K and Zn solubilization traits

In this study, the ability of *B. safensis* STJP was checked for solubilization of K (Fig. 1) and Zn at different time intervals. Isolate STJP showed maximum K solubilization (4.4 mg/L) after 5 days of incubation. *B. safensis* STJP maximally solubilized Zn (5.4 mg/L) after 5 days of incubation (Fig. 2). However, further increment of the incubation period brought reduction in solubilization of both K and Zn.

Bioformulation and its physiochemical properties

After six months storage, pH of potato broth and P-WBF was recorded to be approximately 7.0 (Table 1). However, the pH of rice broth and molasses based bioformulations were found to be 6.1 and 8.9, respectively. Low or high pH can affect the bacterial count. Bacterial cell viability is another serious matter for assessing the quality of a bioformulation. In this regard, the minimum bacterial cells were found in the potato broth. Further, rice broth liquid bioformulation could not maintain cell population as per Bureau of Indian Standards

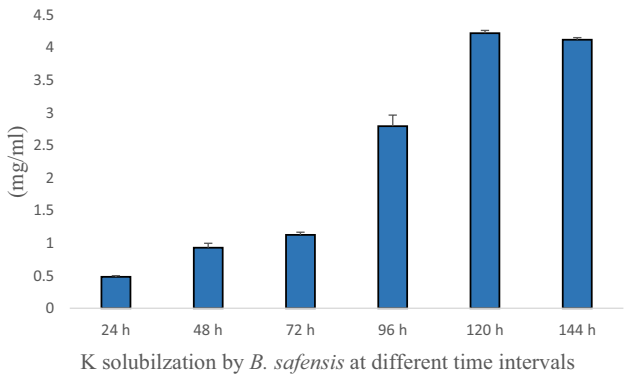


Fig. 1 K solubilization by *B. safensis* STJP at different time intervals. Data are mean of five replica ± standard error of means

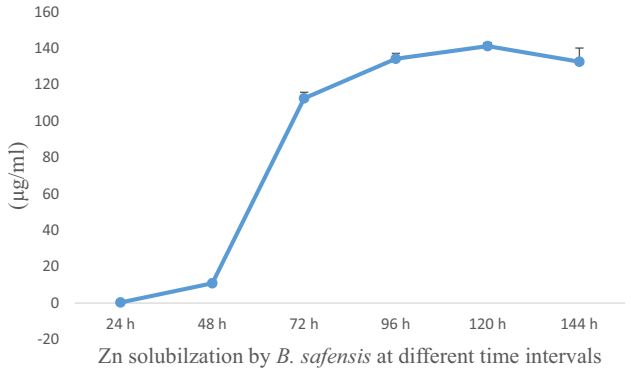


Fig. 2 Zn solubilization by *B. safensis* STJP at different time intervals. Data are mean of five replica ± standard error of means

(BIS) (2017). However, molasses based liquid bioformulation was found to be good in population (10^8 cell/mL) even at high pH (8.9). In P-WBF also bacterial population was 10^8 cells/mL, after six months storage. The EC of all bioformulations was recorded between 0.26 and 0.81 mho (Table 1). In the present study, the pathogenic microorganisms such as *E. coli*, *Shigella* and *Salmonella* were not observed in any of the bioformulations. Nutrient composition of a carrier is another important issue for survival of bacterial cells. In this regard, P-WBF and molasses recorded rich amount of K and P, while in other carriers, low nutrients level (except for rice broth, where P content was almost equivalent to P-WBF) were observed. Potato, rice and molasses based bioformulation showed high C/N ratio of 189:1, 186:1 and 172:1, respectively. Overall paneer-whey was found to be an excellent liquid carrier on the basis of bacterial shelf-life, ease of availability, and presence of nutrients. On the basis of above mentioned qualities, P-WBF was taken for further studies.

Effect of bioformulation on growth promotion of *S. rebaudiana*

Fresh weight of *S. rebaudiana* improved when treated with P-WBF in comparison to control. Fresh weight increased by 58.12%, 27.37% and 9.60% by the RT, SA and FA treatments respectively, when compared with control under sterilized soil (after 90 days) (Table 2). However, under non-sterilized conditions, there was 31.85%, 22.76% and 5.63% enhancement of fresh weight by single treatments (RT, SA and FA) as compared with control plants (after 60 days) (Table 3). Whereas in dual application (RT + SA, RT + FA, SA + FA), there was 79.93%, 63.32%, and 31.72% (sterilized soil) and 62.20%, 42.82% and 26.89% (non-sterilized soil) (Table 3) enhancement in the fresh weight of Stevia, respectively. The maximum fresh weight (116.98% and 105.80%) was observed with triple application (RT + SA + FA) as compared to without application (control) in case of sterilized

Table 1 Characteristics of carriers used for the development of bioformulation

Physiochemical properties	Potato broth	Rice broth	Molasses	Paneer whey
pH	7.1	6.1	8.9	6.8
Electric conductivity (mho)	0.81	0.18	0.56	0.26
Salmonella	–	–	–	–
E. coli	–	–	–	–
Shigella	–	–	–	–
Total K (%)	0.19	0.136	0.68	2.54
Total P (%)	0.79	2.56	1.54	2.58
C/N	189:1	186:1	172.2	26:1
Shelf life in cfu/mL (after 180 days)	10^4	10^6	10^8	10^8

Table 2 Plant growth parameters of *S. rebaudiana* after application of P-WBF under sterile soil conditions (after 90 days)

Treatments	Fresh weight of plant (gm)			Dry weight of plant (gm)		
	Roots (gm)	Stem (gm)	Leaves (gm)	Roots (gm)	Stem (gm)	Leaves (gm)
Control	4.32 ± 0.38e	06.51 ± 0.27f	05.72 ± 0.21f	0.62 ± 0.18b	1.39 ± 0.19c	1.44 ± 0.30d
FA	4.80 ± 0.17d	07.32 ± 0.38e	06.02 ± 0.30e	0.66 ± 0.15b	1.46 ± 0.17c	1.49 ± 0.29cd
SA	5.63 ± 0.19c	08.28 ± 0.40d	07.17 ± 0.20de	0.74 ± 0.18b	1.66 ± 0.09c	1.62 ± 0.20cd
SA + FA	5.78 ± 0.14c	08.69 ± 0.39d	07.33 ± 0.29d	0.69 ± 0.25b	1.69 ± 0.07c	1.68 ± 0.18bcd
RT	6.59 ± 0.27b	09.83 ± 0.35c	09.75 ± 0.24c	0.83 ± 0.11ab	2.19 ± 0.19b	2.14 ± 0.29abc
RT + FA	6.44 ± 0.31b	10.21 ± 0.25c	10.38 ± 0.41bc	0.93 ± 0.19ab	2.33 ± 0.31ab	2.31 ± 0.41ab
RT + SA	6.85 ± 0.27b	12.46 ± 0.37b	10.47 ± 0.29b	1.01 ± 0.14ab	2.51 ± 0.26ab	2.45 ± 0.30a
RT + SA + FA	7.57 ± 0.35a	14.18 ± 0.47a	12.31 ± 0.39a	1.21 ± 0.25a	2.76 ± 0.21a	2.76 ± 0.23a

Data are mean of five replica ± standard error of means

Means, followed by the same letter in a column are not significantly different by Duncan's multivariate test (DMRT)

Table 3 Plant growth parameters of *S. rebaudiana* after application of P-WBF under non-sterile soil conditions (after 60 days)

Treatments	Fresh weight of plant (gm)			Dry weight of plant (gm)		
	Roots (gm)	Stem (gm)	Leaves (gm)	Roots (gm)	Stem (gm)	Leaves (gm)
Control	3.52 ± 0.39e	5.30 ± 0.13g	4.49 ± 0.27e	0.45 ± 0.08e	0.97 ± 0.17e	1.21 ± 0.08e
FA	3.96 ± 0.10de	5.40 ± 0.38f	4.70 ± 0.33e	0.47 ± 0.04de	1.00 ± 0.30de	1.24 ± 0.07e
SA	4.20 ± 0.39c	7.00 ± 0.19e	5.13 ± 0.37d	0.51 ± 0.04d	1.17 ± 0.17c	1.31 ± 0.15de
SA + FA	4.34 ± 0.34b	7.30 ± 0.21de	5.24 ± 0.17d	0.58 ± 0.05c	1.04 ± 0.11d	1.24 ± 0.10d
RT	4.32 ± 0.08b	7.34 ± 0.16d	5.89 ± 0.16c	0.59 ± 0.03c	1.21 ± 0.06c	1.52 ± 0.013c
RT + FA	4.55 ± 0.07b	8.00 ± 0.37c	6.46 ± 0.79b	0.70 ± 0.04bc	1.48 ± 0.08b	1.66 ± 0.06b
RT + SA	5.22 ± 0.35ab	9.14 ± 0.31b	7.23 ± 0.16b	0.78 ± 0.02b	1.98 ± 0.01ab	1.68 ± 0.02ab
RT + SA + FA	6.10 ± 0.15a	9.89 ± 0.78a	7.96 ± 1.42a	0.98 ± 0.02a	2.12 ± 0.07a	1.98 ± 0.08a

Data are mean of five replica ± standard error of means

Means, followed by the same letter in a column are not significantly different by Duncan's multivariate test (DMRT)

as well as non-sterilized soil (Table 2; supplementary Fig. 1). Dry weight is an effective parameter to determine the growth and development of a plant. In this regard, the single application of RT, SA and FA showed pronounced effect in terms of dry weight, as an increment of 49.56%, 16.52% and 4.63%, respectively, was observed as compared to untreated plants (sterilized soils). Further, experiment was also repeated in non-sterile (natural) soil conditions (for 60 days) and in this case, the single treatment of RT, SA and FA on *S. rebaudiana* increased dry weight by 26.23%, 13.68% and 3.04%, respectively (Table 3). When RT + SA, RT + FA and SA + FA treatments were applied 73.04%, 61.44%, and 17.68% enhancement in dry weight was observed as compared to without treated plants (in sterile soil), respectively. Similarly, in case of non-sterile soil there was an increase of 68.82%, 46%, and 8.74% in

dry weight, when dual treatments (RT + SA, RT + FA and SA + FA respectively) were given to Stevia plants (Table 3). However, best results were obtained when RT, FA, and ST were given in combination, and increment in dry weight was 95.07% and 93.15% as compared to control in sterilized and non-sterilized soil, respectively. Overall the triple treatment (RA + SA + FA) gave best results in the enhancement of fresh weight and dry weight of *S. rebaudiana* plants under sterile and non-sterile (soil) conditions.

Stevioside content

The treatment with RT + SA + FA showed significantly high stevioside content (153.12%, and 107.10% respectively) in *S. rebaudiana* as compared to control when treated with P-WBF under sterilized and unsterilized soils (Fig. 3). RT + SA treatment resulted in better stevioside quantity in Stevia plants when compared with other dual applications (FA + RT and FA + SA) under both conditions (sterilized and non-sterilize soils). However, significant difference was not observed in stevioside content of plants when treated with FA + RT and FA + SA (Fig. 3). Among all the single treatments, RT treated plants showed greater amount of stevioside followed by SA and FA in both sterilized and non-sterilized soils (Fig. 3). Overall, results suggested that RT + SA + FA treatment was a more effective method in augmentation of stevioside concentration in the *S. rebaudiana*. This results were confirmed by comparing peaks of chromatogram generated by HPLC for pure (Sigma Aldrich) and extracted stevioside (from developed plants) (Fig. 4).

Bioformulation enhances nutrients uptake in *S. rebaudiana*

Application with RT + SA + FA treatment resulted in ~ 2.8 (sterilized soil) and ~ 2.37 (non-sterilized soil) folds enhancement of P in the plant tissue when compared with control (Table 3). RT + FA and RT did not show statistical difference in uptake of P, however result was much better than without treated plants. Zn and K nutrients uptaken in *S. rebaudiana* were significantly enhanced when applied with RT + SA + FA after 90 days (for sterile soil) and 60 days (for non-sterile soil) (Table 3). The quantity of Zn and K was enhanced by 245.88% and 224.48% respectively, by RT + SA + FA treated *S. rebaudiana*, than that of without inoculated plants, under sterilized soil conditions; whereas in the un-sterilized soils, increment of 235.29 and 181.50% of Zn and K respectively, was recorded (as compared to control) by the same treatment. RT + FA and RT treated plants showed significantly similar results for uptake of K and Zn, but were significantly higher than that of control plants. Overall, RT + SA + FA was found to be a reliable and better method in uptake of nutrients (P, K and Zn) in *S. rebaudiana* under sterilized and non-sterilized soil experiments (Table 4).

Discussion

Bacillus safensis is a Gram-positive, spore-forming ubiquitous microorganism, known to show excellent plant growth promoting (PGP) activities and displays imperative character in growth promotion and productivity of plants including *Mentha arvensis*, after root colonization (Lateef

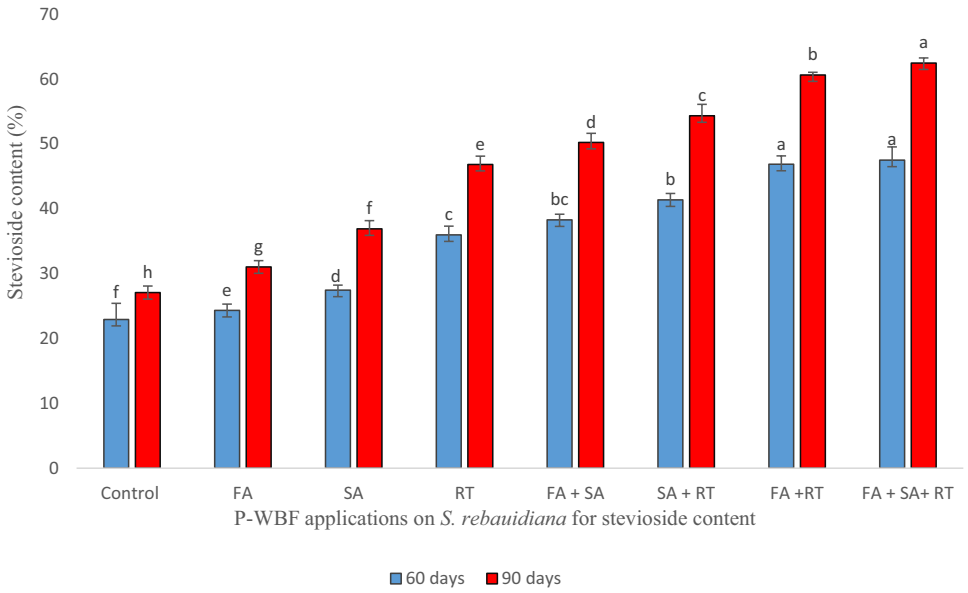


Fig. 3 Effect of P-WBF on stevioside content of *S. rebaudiana* under sterilized (90 days) and non-sterilized soil conditions (60 days). Data are mean of five replica ± standard error of means. Means, followed by the same letter in a column are not significantly different by Duncan's multivariate test (DMRT)

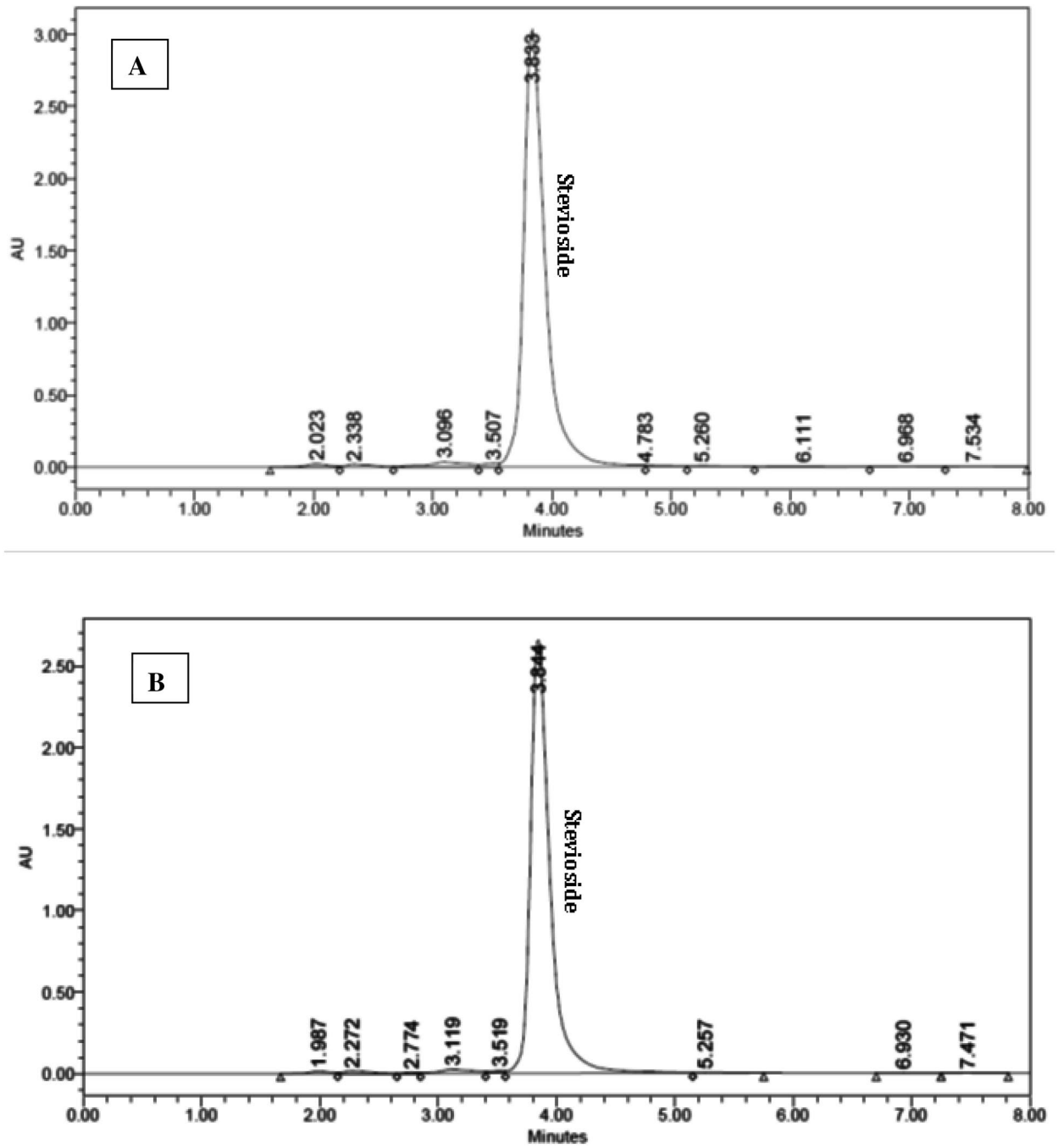


Fig. 4 HPLC chromatogram **a** showing stevioside content of control *S. rebaudiana* and **b** plants inoculated with RA + SA + FA treatments of P-WBF based bioformulation

et al. 2015; Mayer and Kronstad 2017; Prakash and Arora 2019). *B. safensis* STJP produced significant quantity of siderophore, IAA and showed solubilization of inorganic phosphate as reported in a previous study by the authors (Prakash and Arora 2019). Until now, its pathogenic activity has never been detected, thus *B. safensis* is a safe candidate to use as biofertilizer and secure food security in a sustainable manner (Kavamura et al. 2013).

Apart from the above mentioned PGP characters, *B. safensis* STJP also solubilized K and Zn *in-vitro* conditions. Previous studies also show that *Bacillus* spp. are involved in solubilization of K and Zn minerals *in-vitro* as well as in the soil system (Shakeel et al. 2015; Etesami et al. 2017). K

Table 4 Nutrients content in *S. rebaudiana* after application of P-WBF

Treatments	Phosphorous content (mg/plant)		Potassium content (mg/plant)		Zinc content (mg/plant)	
	Sterilized soils	Non-sterilized soils	Sterilized soils	Non-sterilized soils	Sterilized soils	Non-sterilized soils
Control	2.95 ± 0.02 f	2.21 ± 0.07 f	96.65 ± 5.58 e	89.01 ± 6.84 h	0.98 ± 0.10 d	0.68 ± 0.08 g
FA	4.14 ± 0.05 e	3.22 ± 0.13 e	218.30 ± 2.77 d	160.48 ± 5.68 g	2.12 ± 0.05 c	1.60 ± 0.10 f
SA	5.45 ± 0.10 d	4.32 ± 0.63 d	235.65 ± 3.25 c	171.98 ± 8.77 f	2.46 ± 0.03 b	1.72 ± 0.04 e
SA + FA	5.37 ± 0.06 d	5.23 ± 0.28 c	232.46 ± 5.07 c	175.21 ± 4.76 ef	2.42 ± 0.06 bc	1.81 ± 0.05 d
RT	6.04 ± 0.13 c	5.60 ± 0.22 bc	275.31 ± 6.40 b	205.80 ± 8.61 d	2.64 ± 0.08 b	1.96 ± 0.12 c
RT + FA	6.14 ± 0.11 c	5.97 ± 0.11 b	288.25 ± 6.10 b	211.51 ± 8.27 c	2.53 ± 0.05 b	1.98 ± 0.08 c
RT + SA	7.14 ± 0.11 b	6.01 ± 0.41 b	302.00 ± 6.55 b	221.28 ± 5.76 b	2.74 ± 0.07 b	2.12 ± 0.10 b
RT + SA + FA	8.27 ± 0.06 a	7.46 ± 0.30 a	334.30 ± 2.06 a	250.57 ± 12.79 a	3.18 ± 0.06 a	2.28 ± 0.04 a

Data are mean of five replica ± standard error of means. Means, followed by the same letter in a column are not significantly different by Duncan's multivariate test (DMRT)

is the third most essential nutrient in the soil, eighth most abundant element in earth's crust, most abundant cation in plant cells and most ample nutrient in leaves after nitrogen (Wang et al. 2013; Hasanuzzaman et al. 2018). It is involved in the plant growth metabolism, accumulation of sugars, seed germination, regulation of stomata, improving resistance to drought and cold tolerance, transpiration, water uptake, formation of adenosine triphosphate (ATP), opening and closing of stomata, increased root formation, and growth regulation of crops (Wang et al. 2013). K plays a positive role in the physiological status and enzymatic activities of the plant and the carbohydrate metabolism (Bräsen et al. 2014). Stevioside is a carbohydrate obtained from steviol glycoside (SGs) and hence K is also reported to be involved in its metabolism (Bräsen et al. 2014). In addition, K promotes the uptake of sucrose in the sieve cells of Stevia plant as reported by Fakhrul et al. (2014) suggesting its role in stevioside metabolism. Zn is another essential micronutrient, mandatory for normal growth and yield of plant by playing an imperative part in physiological processes such as regulation of IAA, role in plant immune system, repair of cellular membranes, carbohydrate metabolism, regulation of auxin synthesis, component of protein and several enzymatic reactions (Dinesh et al. 2015). *Bacillus* spp. are well known to solubilize Zn and its uptake in plants resulting in improved growth, productivity and quality of crop (Mumtaz et al. 2017).

Bacillus safensis STJP was further taken for the development of liquid bioformulation. Four types of liquid carriers (paneer whey, molasses, potato and rice broth) were chosen on the basis of economy, availability and ecofriendly properties. Excess of C/N ratio and presence of other nutrients is a problem associated with the carriers used in production of bioinoculants (Arora et al. 2010; Kalita et al. 2015). The pH of bioformulation is also a key factor in survival for any microorganisms. The low or high pH can affect the bacterial population. Minimum bacterial count was recorded in rice broth and molasses based formulation, may be due to very high pH. However, slightly acidic or basic pH is known to be beneficial for bacterial liquid formulations (Kalita et al. 2015).

Bacterial cell viability in bioformulation is a serious issue and in present study as well rice and potato broth based bioformulations could not maintain cell population as per Bureau of Indian Standards (BIS) (2017). Although, molasses based bioformulation maintained good bacterial population (10^8 cfu/mL) but being alkaline its neutralization can increase the cost of the final product (Pandey and Maheshwari 2007). In P-WBF, bacterial population was maintained at 10^8 cfu/mL even after six months storage, which might be associated with rich nutrient characteristics of the carrier, neutral pH and being contamination free. EC of all bioformulations was recorded between 0.26 and 0.81 mho (Table 1). According to Biofertilizers and Organic Fertilizers in Fertilizer (Control) Order (1985), EC of bioformulations should not be more than 0.37 mho, because above this can decrease the bacterial population. Pathogenic microorganisms such as *E. coli*, *Shigella* and *Salmonella* were not observed in prepared bioformulations, which is also an important point for their use in fields and getting the product registered. Non-pathogenic property of bioformulation is good for handling and storage and for end users. Among all the tested bioformulations, P-WBF showed better quantities of K and P. Carrier containing good quantity of nutrients are very essential for soil health, growth and productivity of the crops and maintaining the population of introduced microbe. In addition, carriers had an adequate C/N ratio, thus resulting in better maintenance of microbial count, soil microflora, and improved crop productivity (Whalen). C/N ratio2014 of 20:1 is considered as ideal for rhizobacterial growth, but excess or lesser can create difficulty for bacterial population. An excess C/N ratio was detected in potato, rice and molasses based bioformulations. Overall the paneer-whey was found to be an excellent liquid carrier as proved by

better bacterial shelf-life, viability, availability of nutrients and being eco-friendly.

According to Panghal et al. (2017) worldwide production of paneer whey is 3,625,200 tonnes per year. It is expected that approximately 100,000 tonnes of whey is annually produced as byproduct in India from the dairy industries (Baba et al. 2016). Paneer whey constitutes 70% of milk sugar (lactose), 45–50% of total milk solids, 70–90% of minerals, 20% of milk proteins and almost many water soluble vitamins (Das et al. 2010). The large quantity of whey disposal in water bodies can cause serious environmental pollution and health hazards, toxicity to fish and algae, due to high biological oxygen demand (BOD) and chemical oxygen demand (COD) (Baba et al. 2016). Hence, the accessibility of this waste product and its exploitation as a carrier has tremendous prospects in developing countries such as India (Jayasinghe 2012). According to estimates, the dose of liquid bioformulation is 5–10 mL/kg of seeds or 500–625 mL/hectare (Pal et al. 2015). Hence, the exploitation of paneer whey as bioformulation can cultivate about 166,666 hectares land for improvement of growth and productivity of agriculture crops in India. Thus, paneer whey based bioformulation can be incorporated for cultivation of Stevia crop throughout India and even globally as 32,000 ha farmland is used for its cultivation worldwide (Das et al. 2010) and approximately 5000 ha land in India would be used to grow Stevia till 2021 (https://www.businessworld.in/article/The-Green-Sugar-Substitute/05-03-2018-142354/). Paneer whey being a waste of dairy industry, only the transportation cost will be important for development of the bioformulation from it. Small biofertilizer units can be established as ancillary or nearby to the dairy industries to cut the cost of the formulation. This technology will also be very effective in enhancing the earning of farmers involved in cultivation of Stevia and will encourage others as well. On the basis of the beneficial quality of P-WBF it was selected for further study.

Better fresh weight observed in *S. rebaudiana* by RT, SA and FA, might be associated with solubilization of minerals by *B. safensis*. These nutrients are translocated from root system to shoot by mechanism of nutrients-transpiration xylem pathway as suggested by Atkins et al. (2007). Jorjani et al. (2011) reported that *Bacillus coagulans* based bioformulation applied on sugar beet by RT method recorded high fresh biomass due to solubilization of minerals such as K and Zn in the natural soil conditions. *Bacillus* spp. (including *B. safensis* strain RA-3) applied on wheat showed increase in the fresh and dry weight of plants in pot trials by RT method (Sheirdil et al. 2019). Nutrients such as Zn, K, and P directly help in the increment of fresh biomass and productivity of super Basmati rice and Basmati-385 by RT application (Shakeel et al. 2015). However, FA is also an effective method for increasing the growth of plants. Kalita et al. (2015) developed bioformulation utilizing *Bacillus* sp. and sprayed (FA) over the tomato (*Solanum lycopersicum*), cauliflower (*Brassica oleracea*), chilli (*Capsicum annum*), and brinjal (*Solanum melongena*) crops resulting in increase of biomass and yield. Beside the growth of plant, *Bacillus* OSU-142 also enhanced the nutrient uptake (K, P, and Zn) in apple under pot conditions (Pirlak et al. 2007). However, FA is not as good for increasing biomass of plants as compared to RT as proved in the present study. Solubilization of K and Zn occurs in rhizosphere, hence RT proved to be better than FA as also ascertained by other researchers (Kalita et al. 2015; Teixeira et al. 2017). Overall, RT is considered as better method for improvement of biomass (dry and fresh) and yield in several crops (Wang et al. 2013; Tewari and Arora 2016). However, till now, there is no report available on liquid bioformulation for growth enhancement and secondary metabolite augmentation of *S. rebaudiana*. This is the first report on the growth and stevioside content enhancement of *S. rebaudiana* by application of bioformulation on root, soil, and foliar parts of the plant. It is also for the first time that paneer whey amended with glycerol is being used as carrier for developing plant growth promoting rhizobacteria (PGPR) based bioformulation for enhancing stevioside content and growth enhancement of *S. rebaudiana.* Pandey and Maheshwari (2007) only suggested the possibilities of using paneer whey as an efficient carrier for bacterial bioformulations, but did not apply or tested it on any crop.

Stevioside is a major sweetener component of Stevia plant and an excellent substitute of sugar for diabetics. Mamta et al. (2010) studied that high stevioside concentration (91%) in the *S. rebaudiana* plants was observed due to RT treatment with *Bacillus gladioli* strain 10,216. They reported high stevioside concentration due to increase in P nutrient uptake in Stevia plant. However, in the present study combined treatment (RT + SA + FA) showed the best increment in stevioside content (153% and 107.10% as compared to control in sterilized as well as non-sterile soil respectively) may be due to P, K, Fe and Zn solubilization and availability to the plants. Replication of results in un-sterilized soil confirmed the role of *B. safensis* STJP in improving the growth and stevioside content in Stevia. *Bacillus* based bioformulations containing multiple PGP traits applied on several crops including medicinal, vegetables and fruits are reported to enhance growth and metabolites in plants (Mahanty et al. 2017; Sheirdil et al. 2019). Kumar et al. (2016) reported that the use of PGPR (*Pseudomonas fluorescens* CL12) on *Curcuma longa* increased the growth and curcumin content due to solubilization of inorganic phosphate, production of IAA and siderophore. According to Zaman et al. (2018), the combined application of chemical and organic fertilizers enhanced the growth, nutrients and stevioside content in Stevia. However, *B. safensis* based bioformulation with P, K, Fe and Zn solubilization properties significantly enhanced

the growth, nutrient uptake and stevioside content in Stevia plant without any addition of chemical fertilizers.

The fertility of soil mainly depends on nutrient availability and microbial population (Chowdhary et al. 2018). The soluble nutrients are easily uptaken by the plants and enhance growth and biomass (Masclaux-Daubresse et al. 2010). Nutrients such as P, K, Fe and Zn are generally unavailable to the plant because of their insoluble salts in the soil, although they play several important roles in the physiology of plant (Nieves-Cordones et al. 2016). Overall multifaceted strain such as *B. safensis* STJP, with PGP traits including phosphate, Zn and K solubilization along with ability to chelate Fe (through siderophore) can be a big boon for the sustainable agriculture, as also proved in the present study, by enhancing the biomass and stevioside content of a very important medicinal and commercial plant, *S. rebaudiana*. The application of P-WBF in soil, root and foliar parts resulted in boosted nutrient uptake (in plants) and present a chance to elevate the plant growth in an eco-friendly and sustainable manner.

Conclusion

Bacillus safensis is an eco-friendly tool for promoting plant growth and use as biofertilizer. Paneer whey based *B. safensis* bioformulation showed pronounced increase in the growth parameters and metabolites/bioactive components of *S. rebaudiana* and proved to be an alternative option for chemical fertilizers. *B. safensis* STJP possessing a bouquet of PGP characters, applied as novel (paneer whey based) bioformulation not only enhanced the growth but also the stevioside content in Stevia plants by providing an array of nutrients. The result of present study proposes that *B. safensis* with PGP properties could be a promising tool for enhancing the livelihood of farmers through organic farming of *S. rebaudiana*. The exploitation of such PGP strains as a cheap and effective bioformulation (based on organic waste) could be a better option for sustainable agriculture simultaneously leading to production of healthy food, reducing health problems related with chemical fertilizers, and providing green metabolites to pharmaceutical industry.

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Compliance with ethical standards

Conflict of interest All authors declare that there is no conflict of interest in this original article.

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