

**“Development of bioformulation using  
beneficial plant-associated microorganisms for  
protection from fungal pathogens and yield  
improvement of *Stevia rebaudiana*”**

**Thesis**

**SUBMITTED TO  
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY  
LUCKNOW**

**BABASAHEB  
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ESTABLISHED 1996**

**FOR THE DEGREE OF**

**Doctor of Philosophy  
IN  
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**Submitted By**

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**M.Sc., NET, GATE  
(Enrolment no. 326/11)**

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VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226 025  
UTTAR PRADESH, INDIA**

**2019**

***DEDICATED***

***TO***

***MY FAMILY***

## CERTIFICATE

This is to certify that the thesis titled “**Development of bioformulation using beneficial plant-associated microorganisms for protection from fungal pathogens and yield improvement of *Stevia rebaudiana***” submitted by **Mr. Jai Prakash** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulations – 1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

*Naveen Arora*  
24/01/2020  
Supervisor

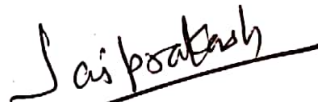
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## DECLARATION

This is to certify that I have worked on the Ph. D thesis entitled “**Development of bioformulation using beneficial plant-associated microorganisms for protection from fungal pathogens and yield improvement of *Stevia rebaudiana***”. In this thesis, all the data were collected and received during the genuine work done by me. Data obtained from other agencies have been duly acknowledged. None of the findings pertaining to the work has been concealed. The result embodied in this report has not been submitted to any other University, Institution or Research Centre for the award of any degree. “The thesis is essential free from all kinds of plagiarism”

Place: Lucknow

Date: 24/01/2020

  
(Mr. Jai Prakash)

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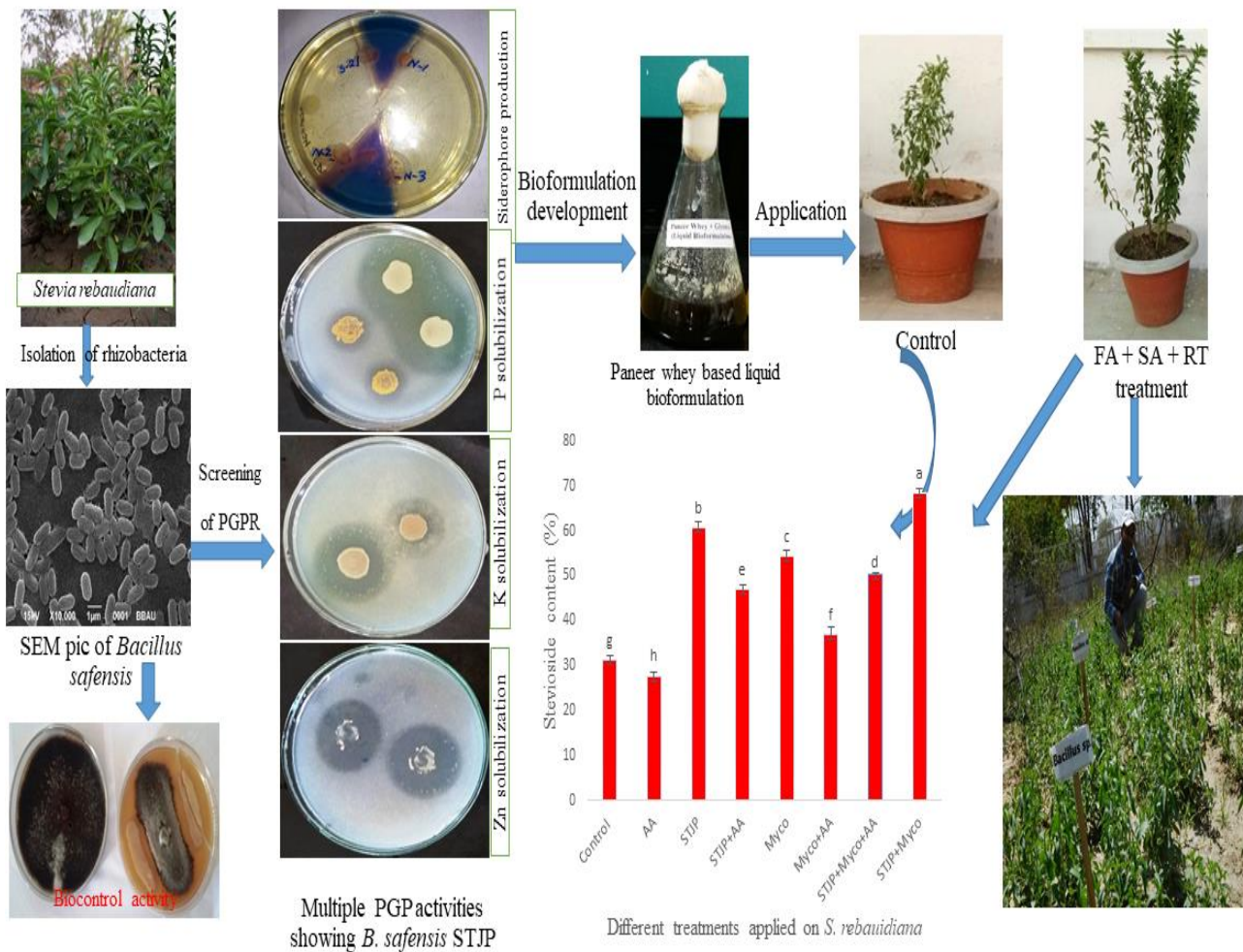
<b>Symbol</b>	<b>Full name</b>
<b>%</b>	Percent
<b>° C</b>	Degree Celsius
<b>° E</b>	Degree East
<b>° N</b>	Degree North
<b>µg</b>	Micro Gram
<b>µL</b>	Micro Liters
<b>ACC</b>	1-Aminocyclopropane-1-Carboxylate
<b>ANOVA</b>	Analysis of Variance
<b>BLAST</b>	Basic Local Alignment Sequence Tool
<b>BNF</b>	Biological Nitrogen Fixation
<b>bp</b>	Base Pairs
<b>BTB</b>	Bromothymol Blue
<b>CAS</b>	Chrome Azurol S
<b>CFU</b>	Colony Forming Unit
<b>cm</b>	Centimeters
<b>CMC</b>	Carboxymethyl cellulose
<b>cDNA</b>	Complementary DNA
<b>DAS</b>	Days After Sowing
<b>DMRT</b>	Duncan's Multiplicity Range Test
<b>DNA</b>	Deoxy Ribonucleic Acid
<b>EC</b>	Electrical Conductivity
<b>EPS</b>	Exopolysaccharides
<b>FT-IR</b>	Fourier Transmission Infra-Red
<b>gm</b>	Gram
<b>GAs</b>	Gibberellins
<b>GPA</b>	Glucose Peptone Agar
<b>GC-MS</b>	Gas chromatography mass spectroscopy
<b>ha</b>	Hectare
<b>HAM</b>	Hofer's Alkaline Media
<b>HCN</b>	Hydrogen Cyanide
<b>HDTMA</b>	Hexadecyltrimethylammonium
<b>HPLC</b>	High performance liquid chromatography
<b>hrs</b>	Hours
<b>IAA</b>	Indole-3-Acetic Acid
<b>IAR</b>	Intrinsic Antibiotic Resistance
<b>K</b>	Potassium
<b>Kg</b>	Kilogram
<b>KNO<sub>3</sub></b>	Potassium Nitrate
<b>LPWG</b>	Legume Phylogeny Working Group

<b>l</b>	Litre
<b>m</b>	Meter
<b>mg</b>	Milligram
<b>Mha</b>	Million Hectares
<b>ml</b>	Mili Liters
<b>MR-VP</b>	Methyl-Red and Voges-Proskauer
<b>N</b>	Nitrogen
<b>NaNO<sub>3</sub></b>	Sodium Nitrate
<b>NH<sub>3</sub></b>	Ammonia
<b>NH<sub>4</sub>Cl</b>	Ammonium Chloride
<b>NH<sub>4</sub>SO<sub>4</sub></b>	Ammonium Sulphate
<b>nm</b>	Nano Meter
<b>P</b>	Phosphorous
<b>PCR</b>	Polymerase chain reaction
<b>PDA</b>	Potato Dextrose Agar
<b>PGP</b>	Plant Growth Promoting
<b>PGPR</b>	Plant Growth Promoting Rhizobacteria
<b>PHB</b>	Poly-P-Hydroxybutyrate
<b>PIPES</b>	Piperazine-N, N'-Bis (Ethane Sulfonic acid)
<b>PSB</b>	Phosphate Solubilizing Bacteria
<b>PSI</b>	Phosphate Solubilization Index
<b>PSU</b>	Percent Siderophore Unit
<b>P-WBF</b>	Paneer whey based bioformulation
<b>rpm</b>	Rotation per Minutes
<b>rRNA</b>	Ribosomal Ribonucleic Acid
<b>RT-PCR</b>	Real time PCR
<b>SGs</b>	Steviol glycosides
<b>SD</b>	Standard Deviation
<b>SEM</b>	Scanning Electron Microscopy
<b>TLC</b>	Thin Layer Chromatography
<b>UV</b>	Ultra Violet
<b>WHO</b>	World Health Organization
<b>Zn</b>	Zinc
<b>α</b>	Alpha
<b>β</b>	Beta
<b>γ</b>	Gama

# Pictorial Travelogue



# GRAPHICAL ABSTRACT



## सारांश

स्टीविया ररेयबौडियाना एक महत्वपूर्ण औषधीय फसल है जो चीनी, बेहतर स्वाद की रूपरेखा, व्यापक औषधीय गुणों और कृषि संबंधी रुचि के लिए भी विकल्प है। वर्तमान अध्ययन में, एस. रेबाउडियाना के राइजोस्फेरिक मिट्टी से अलग किए गए जीवाणु एसटीजेपी और 16 एस आरएनए जीन अनुक्रमण के आधार पर बैसिलस सेफेन्सिस के रूप में पहचाना गया था। आइसोलेट एसटीजेपी नियंत्रण के मुकाबले एस रीबाउडियाना में एक शानदार संयंत्र विकास को बढ़ावा देने वाला और बायोकेन्ट्रोल एजेंट और उन्नत बायोमास है। बायोकेन्ट्रोल मैकेनिज्म की जांच के बाद, पनीर-मट्टा (एक डेयरी अपशिष्ट) आधारित बायोफॉर्मूलेशन (पी-डब्ल्यूबीएफ) को अलग-अलग जीवाणु एसटीजेपी का उपयोग करके विकसित किया गया था और एस में वृद्धि, पोषक तत्वों के ऊपर उठने और स्टीवियोसाइड सामग्री की गुणवत्ता और क्षमता के लिए निरीक्षण किया गया था। पनीर-मट्टा आधारित बायोफॉर्मूलेशन ने स्टीविया के पौधों को नियंत्रित करने की तुलना में पत्तियों की संख्या में काफी अधिक ताजा और सूखा वजन दिखाया। पी-डब्ल्यूबीएफ के अनुप्रयोग ने नियंत्रण में तुलना में एस और रीबौडियाना में एस और रीबौडियाना में स्टीविओसाइड की काफी उच्च सांद्रता प्रदर्शित की। बाद में, दो जीन (यूजीटी सेवेंटी सिक्स जी वन और यूजीटीएटीफाइवसी वन) ने एस। रेबौडियाना में स्टेविओल ग्लाइकोसिल (एसजीएस) संचय की अभिव्यक्ति और प्रतिलेख के अप-विनियमन में वृद्धि की। इसके अलावा, पौधों के ऊतकों में फॉस्फोरस, के, और जेडएन अपटेक की वृद्धि भी पी-डब्ल्यूबीएफ के आवेदन द्वारा दर्ज की गई थी। यह अध्ययन पोषक तत्वों से जुड़े तंत्र द्वारा स्टीविया संयंत्र में स्टीवियोसाइड सामग्री को बढ़ाने के लिए बी। सफेंसिस एसटीजेपी का उपयोग करके पी-डब्ल्यूबीएफ आधारित जैव उर्वरक का उपयोग करने का सुझाव देता है। यह उपन्यास दृष्टिकोण एक साफ-सुथरी तकनीक हो सकता है और यह जैव-कृषि की तैयारी में अपशिष्ट के उपयोग के लिए और फसल की पैदावार को बढ़ाने के लिए महत्वपूर्ण है जो एक स्थायी रूप से स्थायी कृषि के लिए अग्रणी है।

कुंजी: बैसिलस सफेंसिस, लिक्विड बायोफॉर्मूलेशन, स्टेविओसाइड, द्वितीयक मेटाबोलाइट, डेयरी अपशिष्ट

## **Abstract**

*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 RNA gene sequencing was taken as plant growth promoting microbe for making bioformulation to enhance the growth of *S. rebaudiana*. Isolate STJP is a prolific plant growth promoting and biocontrol agent and enhanced biomass in *S. rebaudiana* as compared to control. After the investigation of biocontrol mechanism, paneer-whey (a dairy waste) based bioformulation (P-WBF) was developed utilizing the isolate bacterium STJP and inspected for the quality and ability to enhance the growth, nutrients uptake, and stevioside content in *S. rebaudiana*. P-WBF treated Stevia plants showed significantly higher fresh and dry weight, number of leaves as compared to control. The application of P-WBF displayed a significantly higher concentration of stevioside in *S. rebaudiana* in pots and field conditions as compared to control. Afterward, two genes (UGT76G1 and UGT85C1) enhanced in the up-regulation of expression and transcript of steviol glycosyl (SGs) accumulation in *S. rebaudiana*. Further, enhancement of phosphorous, K, and Zn uptake in plant tissue and improvement of soil health 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 nutrients linked mechanism. This novel approach can be a clean technology and also important for utilization of waste in preparation of bioformulation and enhancement of crop yield by an ecofriendly manner leading to sustainable agriculture.

**Keywords:** *Bacillus safensis*, Liquid bioformulation, Stevioside, Secondary metabolite, Dairy waste

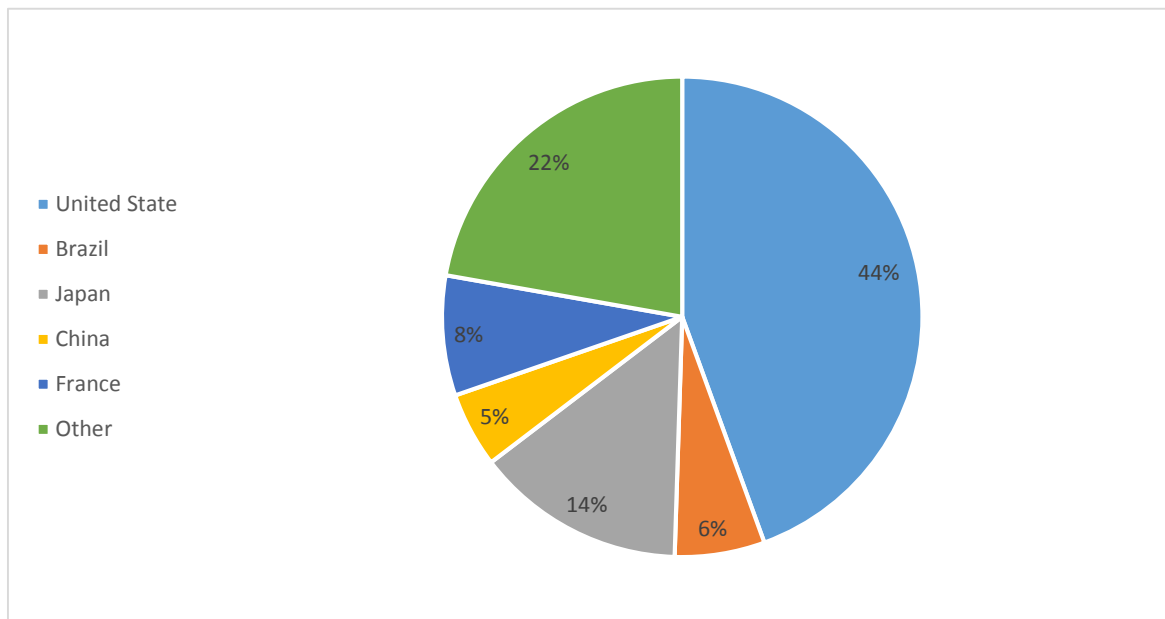
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*Chapter-01*  
*General Introduction*



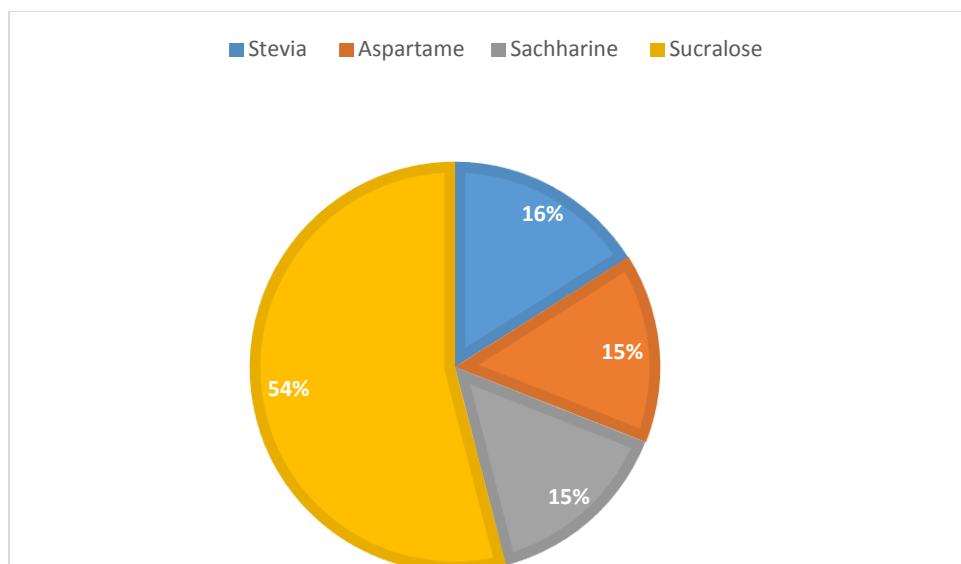
*Stevia rebaudiana* (commonly known as stevia) is a herbaceous plant and has several other common names including “sweet leaf” or “candy leaf” or “honey leaf” or “sweet herb” or “meethi patti”. It belongs to the Asteraceae family and grows up to 1-meter height. It is popular worldwide for its natural non-calorie high sweetener value (Ashwell 2015). The cultivation of *S. rebaudiana* is common in certain regions of South America, mainly in Brazil and Paraguay (Lemus-Mondaca et al. 2012). The plant has a wide range of biomedical applicabilities viz. anti-inflammatory, anti-hyperglycaemic, anti-tumor, hepatoprotective activity, immunomodulation, blood sugar control, hypertension reducer and in treating skin disorders (Gawel-Beben et al. 2015). *S. rebaudiana* also contains essential nutrients such as iron, silica, cobalt, manganese, calcium, magnesium, selenium, tin, zinc, vitamin C, beta-carotene, niacin, thiamine, and riboflavin (Tandel 2011). Beside essential elements, the extracted leaves of stevia contain caffeic acid, isoquercitrin, phenylpropanoids, quercetin, scopoletin, and umbelliferone (Li et al. 2012). In addition to high medicinal value, it is a natural sweetener with zero calories and low glycemic index (Lemus-Mondaca, et al. 2012). Its sweetener property depends on the alkaloid, diterpene glycosides (DGs). The DGs consist of several compounds like stevioside, steviolbioside, rebaudioside A, B, C, D, E and dulcoside, which have been extensively explored (Bhutia and Sharangi 2016). Among them, stevioside is in limelight due to its harmless sweetener quality. Stevioside is slightly bitter in taste, non-fermentable, non-caloric and 300 times sweeter than normal/table sugar with a long shelf life (Yadav et al. 2011; Kregiel 2015). Leaf of *S. rebaudiana* contains the highest content of stevioside (6-18%) and gradually it decreases in flowers (8.2%), stems (~7.54%), seeds (~4.42%), and roots (~3.14%) (ŠicŽlabur et al. 2013; Pal et al. 2015). The European Food Safety Authority (EFSA) along with the Food and Agriculture Organization (FAO) as well as the World Health Organization Expert Food Committee (WHO-EFC) have approved stevioside as safe for human use and an alternative to sugar, especially in case of diabetes patients (EFSA 2010; JECFA 2016). The priceless medicinal properties of stevioside have lead to an upturn in *S. rebaudiana* cultivation all over the world. Till date, China is chief Stevia producer as well as exporter (approx. 80%) and United States and Japan are the largest consumers (Lemus-Mondaca et al. 2012). However, Stevia has been mainly used for beverages and sweet

foods in various countries because of its calorie-free sweetener metabolite and commercial value (Figure 1).



**Fig. 1** Country wise use of Stevia (in food and beverages) Source: Abdullah (2013).

Recently, Stevia has gained a huge response in the Indian market as in last five years the demand of *S. rebaudiana* has increased by more than 300 times (Pal et al. 2015; Debnath et al. 2018). It is estimated that the global market for stevia production has reached 11,000 tons (in terms of dry leaf) by 2014. Among them, total annual production of dry leaf in India is nearly 600 tonnes (Kumari and Chandra 2014). The global sweetener market of stevia is estimated to be USD 60 billion (Barclay et al. 2014). Currently, stevia is the rapidly growing plant (product) as the substitute of sugar and according to World Health Organization (WHO) stevia can replace 20% of the sugar market equivalent to USD 10 billion (Duckett and Swerissen 2016). Stevia is already handsomely contributing to the market of natural sweeteners (Figure 2).



**Fig. 2** Leading sugar substitutes in the global market (Source: Martins et al. 2017)

Stevia crop is cultivated globally in around at 32,000 ha farmland (Das et al. 2010) and approximately 5000 ha agricultural land would be used to grow Stevia by 2021 in India (<http://www.businessworld.in/article/The-Green-Sugar-Substitute/05-03-2018-142354/>). Currently in India, the cultivation of *S. rebaudiana* is growing rapidly in Rajasthan, Maharashtra, Uttar Pradesh and West Bengal (Angelini et al. 2018). It is in high demand in pharmaceutical, food, beverages, and sweet manufacturing industries (Figure 3). Thus, its cultivation can help farmers to generate better revenue and improve their economic and social condition. But, little knowledge and information are available about *S. rebaudiana* in India and so a lot needs to be done on the growth, management of disease, and yield enhancement of stevia in countries like India. It will also be important to cultivate stevia without the chemical inputs because it is directly consumed by the humans. There is a need to motivate farmers for the organic cultivation of *S. rebaudiana* by involving useful technology involving bioinoculants or biofertilizers.

SGs are the most attractive secondary metabolites present in *S. rebaudiana* due to the intense sweetness and therapeutic functions particularly important for diabetics (Gupta et al. 2017). The biosynthesis of SGs show terpenoids pathway and it is similar with the biosynthetic pathway of gibberellic acid ( $GA_3$ ) (Modi et al. 2014). SGs and  $GA_3$  are derived from 2-C-methyl-D-erythrose-4-phosphate (MEP) pathway inside the plastids. Hence, in this way these compounds are synthesized maximum in the leaves (Ceunen and Geuns 2013). However, the amount of SGs in the leaves is 10,000 times higher as compared to  $GA_3$ , which shows perseverance of this plant towards SGs synthesis by the

MEP pathway (Modi et al. 2014). A total of 15 genes are involved in the MEP pathway of SGs biosynthesis. Three genes play a major role in the synthesis of glycoside, UGTs-UGT85C2, UGT74G1, and UGT76G (Hayes and Pietruszka 2017).



**Fig. 3** Stevioside based products in the market

One of the major problems for the cultivation of Stevia is its susceptibility towards several fungal diseases. It affects the growth as well as crop's yield. The most destructive fungal pathogen reported for *S. rebaudiana* is *Alternaria alternata* (which causes leaf spot disease) (Maiti et al. 2007) and *Sclerotium rolfsii* (causes root rot disease) (Koehler and Shew 2014). Among both phytopathogens, *A.alternata* is more common and is responsible for the reduction in crop productivity of *S. rebaudiana* (Maiti et al. 2007; Yan et al. 2018). The symptoms of this disease are recorded as the presence of dark brown color or necrotic lesions on the leaf (Figure 4) (Yan et al. 2018). This disease has been found more commonly during the monsoon season and dry summer months in India. The symptoms of this disease during initial infection are; slightly irregular or round to oval dark brown spots, concentric rings surrounded by pale yellow margins on leaves of the plant (Sen et al. 2012). But later on, all spots merge together and form large dark patches and subsequently

lead to heavy defoliation and decrease in stevioside content (Figure 4) resulting in great loss in terms of market value and to the farmers.



**Fig. 4** Symptoms of leaf spot disease in *S. rebaudiana*

(Source: authors own field trial)

Use of chemical fertilizers and pesticides against fungal diseases for the cultivation of medicinal and aromatic plants (MAPs) is highly restricted, due to the negative impact on the quality of agricultural crop and persistence of the chemicals in the plant tissue (Carrubba et al. 2015) Use of chemicals in stevia cultivation may result in the rejection of the product due to stringent regulations related with human consumption (Clay 2013). Moreover, low seed germination percentage of the *S. rebaudiana* is also a serious issue worldwide, responsible for its low production (Vafadar et al. 2014). In order to achieve high yield and seed germination, scientists are trying to develop new cultivation strategies and in this regard use of plant-associated microorganisms is viewed as a superior alternative that not only can enhance crop yield but also protect the plant from phytopathogens in an eco-friendly manner (Mahanty et al. 2017). In addition, it also can significantly improve seed germination percentage and phytochemical content of MAPs. There is ample literature which describes potential uses of plant growth promoting rhizobacteria (PGPR) in stimulating growth and in improving soil and plant health (Kloepper et al. 1980; Khalid, et al. 2004; Kalam et al. 2017). PGPR inhabit the rhizosphere of plants and perform a variety of functions in growth promotion such as increased nutrient availability to plants by fixing nitrogen (Pérez-Montaña et al. 2014), phytohormone production (Schmelz et al. 2009), siderophore production (Ahmed and Holmström 2014), solubilization of phosphate (Prakash and Arora 2019), zinc (Sultana et

al. 2019) and potassium (Pramanik et al. 2019), and by protecting from destructive phytopathogens (Tewari and Arora 2018).

Among all PGPR, *Bacillus* spp. are excellent rhizobacteria because of predominant presence in the rhizosphere and ability to survive biotic and abiotic stresses (Shameer and Prasad 2018). The multifaceted plant growth promoting properties of *Bacillus* spp. makes them alternative and efficient substitute for chemical fertilizers and pesticides (Hashem et al. 2019). *Bacillus*-based bioinoculants are more successful as compared to other bioformulations in the market due to wide range of metabolites production and spore-forming property (which helps them tide over the harsh conditions and maintaining shelf life in the bio-product (Malusá et al. 2012). Use of *Bacillus*-based bioinoculants for increasing the crop yield is a very important practice for eco-friendly and sustainable agriculture and this is also important for maintaining the agricultural sustainability. *Bacillus* spp. live in close association with roots of the crop and play an important role in the (Shameer and Prasad 2018). soil due to distinct features like biofilm formation, heavy metal tolerance, and various growth promoting activities (Vaishnav et al. 2018).

Arbuscular mycorrhiza (AM) are one of the most important organisms which are associated with plant and microbes (Hildebrandt et al. 2002). AM occur in the majority of natural soils and a range of important agro-ecological systems. AM in particular by enhancing the growth, nutrient uptake, metabolite content of the plant and also improve soil structure and fertility (Bhardwaj et al. 2014). The term mycorrhiza is derived from a Greek words which means “fungus” and “root”. AM develop specialized areas to interact with the host plant called symbiotic interfaces (Bonfante and Genre 2010). Mycorrhiza fungi can be divided into two parts: aseptate endophytes such as Glomeromycota, or septate Asco- and Basidiomycota (Lanfranco et al. 2017). AM are a normal and major components of the rhizosphere microflora, occur worldwide and nearly 70-90% of the land plant species in all ecosystem can become colonized by these fungi (Parniske 2008). There can be of crucial importance for acceptable growth of plant species with a small root surface area when growing in soils low in nutrients. In addition of direct benefits, AM can increase the plant resistance against phytopathogens by indirect mechanisms (Wang et al. 2018).

The chemical-based fertilizers and pesticides damage the natural agro-environment directly or indirectly. PGPR and AM fungi are a good option and can be used directly or through a carrier in the rhizosphere or on the crops (Bhattacharya et al. 2016). Carrier based method of bioinoculant production and application is most effective, and widely

accepted. Hence it becomes important to identify an economical, eco-friendly and sustainable carrier which can support the PGPR and take it to the rhizosphere particularly for the cultivation of MAPs. Bioinoculants based on cheap and reliable carrier and effective PGPR can prove to be a reliable solution particularly for the cultivation of MAPs (Arora and Mishra 2016). Bioformulations are the naturally active gradients 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 growth and yield of plants/crops, improve soil fertility and protect against pathogens related to plants (Kang et al. 2014; Nagachandrabose 2018). Bioformulations can be in liquid or solid forms, but liquid once are considered better than solid as they have longer shelf-life, can easily colonize the roots or plant surface, can perform well in pots or field conditions, and have low formulation cost (Goljanian-Tabrizi et al. 2016; Nagachandrabose 2018).

On the basis of above-mentioned problems and possible solutions the present study was focused on isolating and selecting effective PGPR strains, and develop the bioinoculants (from these strains) by utilizing locally available effective carrier material. The work was aimed to determine the impact of rhizospheric microorganisms associated with *S. rebaudiana* on growth and fitness of the host plant as well as to investigate qualitative and quantitative impact on medicinally important alkaloids of stevia. Moreover, the study also intended to prepare a suitable bioformulation after screening and selection of effective rhizospheric bacterial strains and the application of designed bioformulation on the *S. rebaudiana* for enhancing growth, productivity, and nutrients uptake as well as improving soil health. In addition, selected bioformulation was also checked for the expression of different genes of the steviol glycosides (SGs) biosynthesis pathway in the leaf of *S. rebaudiana* in relation to SGs content, thus confirming the impact of plant-microbe interactions on growth and metabolite synthesis in stevia. This novel approach can be a clean technology, for production of eco-friendly product and for enhancing the livelihood of farmers through enhanced production of *S. rebaudiana*, leading to sustainable agriculture and providing green metabolites to the pharmaceutical and other related industries.

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## *Chapter-02* *Objectives*



- 1 Isolation of rhizobacteria from various medicinal plants including *Stevia rebaudiana* from diverse areas
- 2 Screening of isolated rhizobacteria for their PGP traits as well as biocontrol activity
- 3 Study of biocontrol potential of isolated rhizobacteria against *Alternaria alternata*, a fungal leaf pathogen of *S. rebaudiana*
- 4 Study of mechanisms involved in biocontrol and extraction and identification of bioactive compounds
- 5 *In vitro* and *in vivo* study of selected rhizobacteria on plant growth, disease management and seed germination of *S. rebaudiana*
- 6 Development of suitable bioformulation using selected strain for *Stevia* crop and further field trials to investigate the effect of prepared bioformulation on plant growth and fitness as well as on soil health.

*Chapter-03*  
*Review of Literature*



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**Review of Literature**

Medicinal and aromatic plants (MAPs) have proved as one of the major sources of medicine all over the world. They are extensively used for the treatment of several diseases as well as play significant role in economy and public health (Rates 2001). In the modern era, medicinal plants are in high demand due to better revenue generation for the farmers that helps them to maintain desired living standards (WHO 2013). But nowadays, assurance of the efficacy, quality, and supply of MAPs and their products has become a major challenge around the globe due to decreasing natural resources (Mills 2007). The natural reserves of MAPs are depleting quickly due to degradation or destruction of habitat (Cohen-Shacham et al. 2011). Hence, the need has arisen to cultivate the MAPs in an eco-friendly manner in order to maintain their continuous supply and derivation of useful products. In the cultivation of MAPs, use of biological methods to enhance productivity is proving better and it can also reduce the load of chemicals in agro-ecosystems (Bevilacqua et al. 2012). Hence, the use of plant growth promoting rhizobacteria (PGPR) can provide a sustainable way in the cropping of MAPs. In the modern world, diseases related to over intake of sugar are very common. So, there is an urgent demand of sugar free sweetener products. In this regard *S. rebaudiana* is serving as a very potential candidate to sugar. With increasing demand Stevia production is also rising by the day. However, this medicinal and commercial plant requires to be grown in a sustainable manner in order to reap maximum benefits without harming the ecosystem and human health.

***Stevia rebaudiana***

It is a very important medicinal plant and is considered as boon for diabetic patients (Figure 5). *S. rebaudiana* belongs to Asteraceae family, which is native to regions of South America such as Paraguay and Brazil (Sivaram and Mukundan 2003; Modi et al. 2014). Stevia cultivation has now progressed greatly from its native countries to other regions of the world i.e., from United States, Paraguay to Brazil through Mexico, Central America, Southern American Andes and other countries including in Asia (India as well) (Assaei et al. 2016). Stevia is a perennial herb and it comprises of approximately 150–200 species (Modi et al. 2014). In semidry mountains it grows

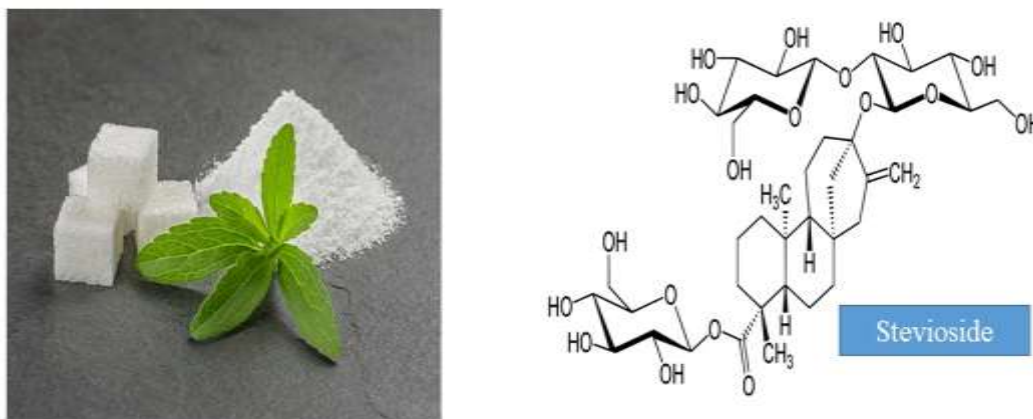
mostly at altitudes of 500–3000 m. Moises S. Bertoni, an Italian botanical scientist, from the College of Agriculture in Asuncion, discovered and described Stevia for the first time (Cantabella et al. 2017) from Paraguay. Hence, Stevia is also known as *S. rebaudiana* Bertoni (after the names of its discoverer). Since centuries, the Guarani people from South America used Stevia as a traditional herbal tea, in other beverages and for treating heartburn (Puri et al. 2011; Lemus-Mondaca et al. 2012). *S. rebaudiana* Bertoni is of great economic, medicinal and scientific interests due to the presence of natural non-caloric sweetener.



**Fig. 5** *Stevia rebaudiana* Bertoni

*S. rebaudiana* is widely used for medical purposes such as anti-hypertensive, anti hyperglycemic, and anti-carcinogenic (Cantabella et al. 2017). The leaves of Stevia contain several amino acids such as aspartate, arginine, histidine, lysine, serine, isoleucine, glutamic acid, leucine, macro and micro-nutrients (phosphorous, calcium, potassium, iron, zinc, magnesium, sodium), lipids (linolenic acid, linoleic acid, oleic acid, stearic acid, palmitic acid, and palmitoleic acid) and vitamins (niacin, thiamine, folic acid, vitamin C, vitamin B2, and vitamin B6) and also has significant amount of protein (Chatsudthipong and Muanprasat 2009; Modi et al. 2014). The leaf of Stevia also contains a complex mixture of eight sweet diterpene glycosides (DGs), including stevioside, steviolbioside, rebaudioside (A, B, C, D and E) and dulcoside (Modi et al. 2014). Among SGs, stevioside and rebaudioside are sweetest metabolites as compared to other DGs (Ceunen and Geuns 2013). Stevioside is the main sweetener component (because of high quantity) in the leaves of *S. rebaudiana* and tastes about 300 times sweeter (Figure 6) than sucrose (Brandle et al. 1998; Lemus-Mondaca et al. 2012;

Ceunen and Geuns 2013). Rebaudioside A, another sweet compound with a sweetening potency is even higher than stevioside and is 400 times sweeter than sucrose but its concentration in the plant is very low (Modi et al. 2014). Stevia also contains significant quantities of chlorogenic acid which has hypoglycemic effects (Gregersen et al. 2004; Lemus-Mondaca et al. 2012).



**Fig. 6** Chemical structure of Stevioside

Stevioside has three different glycosidic bonds i.e., a  $\beta$ - linked sophorose ( $\beta$ 1-2-D-glucopyranosyl D-glucose) on C-13 and an ester  $\beta$ -glucosidic linkage on the C-19 carboxyl group (Lemus-Mondaca et al. 2012). The whole plant of *S. rebaudiana* gives health benefits without any side effects. It is not only the natural sweetener but also cholesterol free (Modi et al. 2014). It can reduce the demand of sugar, particularly in industries involved in making sweets, beverages, biscuits, confectionaries, dairy products and breads (Martins et al. 2017). Stevioside is also becoming very important for the diabetic patients, and it also regulates the blood glucose level by enhancing the insulin secretion and utilization (Ahmad et al. 2017). It can help in preventing diabetic complications and may serve as a good alternative in the present armamentarium of anti-diabetic drugs. Therefore, stress should be on research for its further utilization in pharmacological applications and maintaining stability of products (based on stevia) (Martins et al. 2017). Considering the medicinal potential of this plant and the increasing demand of huge population in countries like India and China, it is going to be a very important futuristic crop.

The priceless medicinal properties of stevioside has led to an upturn in *S. rebaudiana* cultivation all over the world. Recently, Stevia has gained huge response in the Indian market as in last three years, the demand of *S. rebaudiana* has increased by up to 300 times (Pal et al. 2015; Debnath et al. 2018). In India, this commercially

important medicinal plant has been introduced in states of Maharashtra, Tamil Nadu, Karnataka, Rajasthan and West Bengal (Pal et al. 2015). However, looking at the demand, need is to involve other states in cultivation of this important MAP.

#### **Problems associated with *S. rebaudiana***

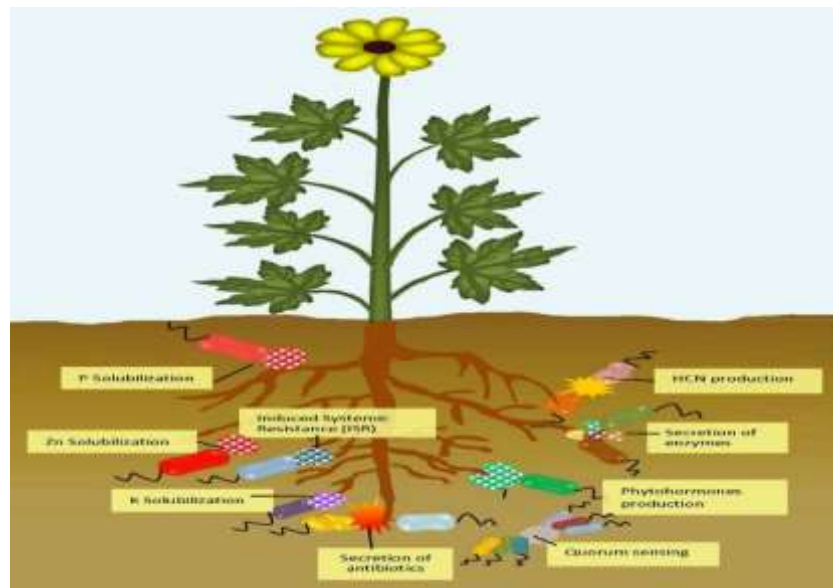
The stevioside biosynthesis and production depends on total biomass of plant which in turn relies on the climatic conditions, agro-techniques, water availability and fertilizer applications (Debnath et al. 2018). Overall, the demand of medicinal plants and their alkaloids are increasing day by day due to the global demand. For the production of medicinal crops, huge amount of chemical fertilizers are being used, although this technique enhances the growth and productivity but has its own disadvantages (Lemus-Mondaca et al. 2012). The use of chemical fertilizers and pesticides to enhance the crop yield has been increased up to an alarming stage since past few decades to fulfil the demand of food for increasing human population with limited cropland area. Farmers are forced to apply synthetic agrochemicals in an indiscriminate way which have posed a serious threat to environment, plants and plants based products as well as to living beings including humans (Glick 2012; Modi et al. 2014; Debnath et al. 2018). Hence, there is urgent need to reduce the load of chemicals, to prevent health hazards, and increase the crop or MAP production by alternative and eco-friendly methods (Singh et al. 2014). Use of PGPR is a good possibility, which can facilitate the plant growth and enhance the productivity either by solubilization of minerals like phosphorous, zinc, potassium or production of metabolites such as siderophores, phytohormones, nitrogen (N<sub>2</sub>) fixation etc (Ahmad et al. 2018). Additionally PGPR have several advantages and their communities are found in rhizosphere of wide variety of crops and are also known to survive stress conditions very well (Abd\_Allah et al. 2018). Use of PGPR has become a very important practice for eco-friendly and sustainable agriculture for enhancing crop production and control phytopathogens and maintain soil fitness even in the presence of biotic and abiotic stresses (Kumar and Gopal 2015; Alori et al. 2017; Tewari and Arora 2018). A lot is still needed to be explored in the area of plant microbe interactions and mechanisms of PGPR in helping the plants. Further details about the PGPR are described in other sections.

***Alternaria alternata*: a common fungal pathogen of *S. rebaudiana***

The genus *Alternaria* is considered as ubiquitous group of fungi. It is an opportunistic plant pathogen that follows different routes to penetrate plant tissues such as wounds, lenticels, stem ends, and pedicels or by breaking of the host cuticle (Pane and Zaccardelli 2015). When the pathogen (*Alternaria*) enters in the plant tissue, it remains inactive for long times until the tissue has started to decay (Jayapradha and Raja 2016). In this way, the fungal pathogen *Alternaria* causes severe effect on the plant and ultimately reduce the crop's productivity. *A. alternata* is one of the best-known fungal pathogen of *S. rebaudiana* (Maiti et al. 2007). This fungus has abundant branched septate, solitary or in short chains, brownish mycelia, conidiophores, olive-brown color, and variable in length with terminal conidia (Pane and Zaccardelli 2015). *A. alternata* produces several toxins such as dibenzo- $\alpha$ -pyrone (alternariol, alternariol monomethyl ether (AME), attenuate, isoaltenuene and altenuisol), tetramic acid metabolites, tenuazonic acid (TzA) and perylene derivatives altertoxin I, II and III and stemphytoxin (Puntscher et al. 2019). However, most of compounds are non-toxic in nature for plants and animals (Dolan et al. 2010). *A. alternata* causes root rot and leaf spot diseases in the *S. rebaudiana* and both diseases are soil borne infecting through root hairs (Maiti et al. 2007).

**Plant growth promoting rhizobacteria (PGPR)**

The soil is a living system where a vast array of microbes reside and interact with each other. However, there is an area surrounding the plant roots which influences microbial interactions to a maximum level and is known as "rhizosphere" (Hiltner 1904). The rhizosphere is habitat for several beneficial rhizobacteria also named as PGPR (Kloepper and Schroth 1978). These bacteria aggressively colonize plant roots and have an enormous role in plant growth promotion (PGP) (Antoun and Kloepper 2001). PGPR are potential microbes that facilitate plant growth either directly or indirectly by colonizing the plant root (Figure 7) (Kloepper 1978; Lugtenberg and Kamilova 2009; Ahemad and Kibret 2014; Arora and Mishra 2016). These soil bacteria have the ability to colonize the roots and stimulate plant growth. PGPR belong to different genera such as *Azoarcus*, *Azospirillum*, *Rhizobium*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconoacetobacter*, *Pseudomonas*, and *Serratia* (Sivasakth et al. 2014; Suman et al. 2016). During recent past, much research has been carried out to reveal the mechanisms of plant-microbe interactions, particularly exchanges in the rhizosphere region (Beneduzi et al. 2012). PGPR are known to affect plant growth directly or indirectly.



**Fig. 7** Mechanisms of plant growth promoting rhizobacteria

Direct growth promotion involves the production of phytohormones, fixation of atmospheric nitrogen ( $N_2$ ), synthesizing iron chelators known as siderophores, solubilizing inorganic minerals such as phosphorus (P), potassium (K) and zinc (Zn), making them more readily available for plant growth (Ahemad and Kibret 2014). Indirect role can be seen in the form of their biocontrol activity against phytopathogens by one of the several mechanisms such as antibiotics or antifungal metabolite production, depletion of iron from the rhizosphere, induced systemic resistance (ISR), production of fungal cell wall lytic enzymes, and competition for binding sites on the roots (Cheng 2009). Moreover, PGPR are also accepted as potential microbes that can protect the plant from various environmental stresses and help in the improvement of plant growth and development in normal as well as stress environment (Khare and Arora 2011; Kang et al. 2014; Prakash and Arora 2019). There is an ample literature which describes the effective role of PGPR in sustainable agriculture (Ahemad and Kibret 2014). Although, initially the role of PGPR was explored only for enhancing the crop productivity, but several studies are now confirming that PGPR play a pivotal role in proper functioning of the agroecosystems (Cheng 2009). Research shows that they can be used in the restoration of degraded land, improving the quality of soil, reducing the environmental pollutants from the soil and also combating climate change (Kuiper and Pesut 2004). The rhizobacteria which are found in rhizosphere or on the rhizoplane are known as extracellular plant

growth promoting rhizobacteria (ePGPR) (Mishra et al. 2016). The examples of ePGPR are *Agrobacterium*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Flavobacterium*, *Micrococcus*, and *Pseudomonas*. On the other hand some rhizobacteria which exist inside root cells or cortex, generally form specialized nodular structures are called intracellular plant growth promoting rhizobacteria (iPGPR) (Kuiper and Pesut 2004) for example, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*.

### **Direct mechanisms of PGPR**

#### **Phytohormones production**

Indole-3-acetic acid (IAA) is a major plant hormone which helps in several stages of plant growth such as cell division and enlargement, pigment formation, responses to light and gravity, resistance to stressful conditions and tissue differentiation (Myo et al. 2019). Many rhizobacteria are able to synthesize IAA and influence plant growth such as increasing branching number, size, and weight of plants, improve the soil quality (Ahemad and Kibret 2014).

Plant hormones such as cytokinins, gibberellic acid, IAA, salicylic acid, and jasmonic acid released by PGPR, help in the root formation, cell elongation, cell division, and cell differentiation in the plants under biotic and abiotic stress conditions through involvement of bacterial species (Parray et al. 2016). In abiotic/stress conditions, these phytohormones also improve the increment of root surface area by which plants can easily absorb nutrients under stress condition (Onaga and Wydra 2016). Plants can also respond to exogenous IAA, and can dismiss the effect of biotic stress (Calvo et al. 2014). According to a study, IAA producing strain of *Bacillus* spp. enhanced the wheat growth even under nutrient deficient condition (Dodd and Perez-Alfocea 2012). In addition, IAA produced by PGPR stimulates growth and productivity of agricultural crop by increased root weight and shoot weight (Dodd and Perez-Alfocea 2012). IAA also recorded to enhance the defense of bacterial cell against environmental stress conditions (Bianco et al. 2006). Among all PGPR, *Bacillus* spp. can enhance plant growth by changing of hormonal balance inside the plants and maintain the soil fitness (Prakash and Arora 2019). Gibberellic acid is another type of phytohormone which plays a key role in enhancement of plant growth by upregulation of cell elongation, cell division, growth of hypocotyl and stem, size of leaf and root meristic tissue (Steffens et al. 2006).

### **Siderophore production**

Iron is the fourth important element of the soil. In soil, most of the iron is present in the insoluble form as ferrous ( $\text{Fe}^{2+}$ ) or ferric ( $\text{Fe}^{3+}$ ). Microorganisms and plants need a high level of iron for normal physiological activities (Saha et al. 2016). Some rhizobacteria are capable to solubilize and chelate iron from soils by siderophore production (Ahmed and Holmström 2014). Siderophore is a low molecular weight compound with a very specific and high affinity to chelate iron and is produced by bacteria, fungi and plants. Approximately 500 different types of siderophores are now well known (Saha et al. 2016). On the basis of the functional group, siderophore are classified as hydroxycarboxylate, catecholate and hydroxamate types. However, mostly siderophore-producing bacteria belong to genus *Pseudomonas* and *Bacillus* (Saha et al. 2016).

### **Mineral solubilization**

#### **Phosphate solubilization**

Phosphorus (P) is an important macronutrient (apart from nitrogen) which is also an essential factor for growth promotion and development of crop (Ahmad et al. 2017). Most of the P is found in an insoluble form in the soil such as aluminium phosphate ( $\text{AlPO}_4$ ), ferric phosphate ( $\text{FePO}_4$ ) which cannot be taken up by the plant (Chen et al. 2017). PGPR, also known as phosphate solubilizing bacteria (PSB) solubilize insoluble phosphate present in the soil and make availability of P to plant by one of the several mechanisms, such as production of organic acids (gluconic, oxalic and succinic acids), alkaline phosphatase enzyme, and ion exchange or chelation (Wei et al. 2018). Among all PSB, *Bacillus* spp. are recorded good phosphate solubilizers present even in stress conditions (Radhakrishnan et al. 2017; Ahmad et al. 2017). Hussain et al. (2013) reported that PGPR release organic acids into the soil resulting in decrease of soil pH. In the presence of acidic soil, insoluble form of phosphorous is converted into soluble form and hence plants can easily uptake it. Goswami et al. (2014) observed that *B. licheniformis* A2 enhances the vegetative parameters such as root length, shoot length, fresh biomass of *Arachis hypogaea* (Groundnut) by solubilization of inorganic phosphate. Mukhtar et al. (2017) reported that rhizobacteria (*B. safensis* and *Bacillus megaterium*) as good phosphate solubilizers, and their roles were recorded in enhancement of plant growth and productivity. Phosphate solubilizing bacteria *Acinetobacter* sp. RC04 and *Sinorhizobium* sp. RC02

showed the ability to improve safflower seed germination and, when co-inoculated, improved seedling growth and plant growth and productivity under pot and field conditions (Zhang et al. 2019). Another phosphate solubilizing *Bacillus* sp. STJP along with tricalcium phosphate (TCP<sup>200</sup>) showed significant increase in the plant growth parameters, oil yield and P uptake in medicinal plant *Mentha arvensis* as compared to the control plants (Prakash and Arora 2019). This approach of using fertilizer along with phosphate solubilizing *Bacillus* sp. worked very well and was more effective in comparison with individual treatment of fertilizer or PGPR. Phosphate solubilizing bacteria (PSB) and AMF are reported for a long time to solubilize insoluble phosphates and help in increasing yield of several crops (Khan et al. 2013; Sharma et al. 2013). Studies indicate that application of rock phosphate in conjunction with PSB could reduce 50% cost of addition of chemical fertilizers (Wei et al. 2013). There are several publications demonstrating that PSB based inoculation increase P content of sugarcane (Sundara et al. 2002), mung bean (Bansal 2009), maize (Shankar et al. 2013), Chilli (Shankar et al. 2013), rice (Khan et al. 2003) , and wheat (Egamberdiyeva et al. 2004). PGPR are known to have the capability of iron uptake in low iron condition and enhance plant productivity (Gupta et al. 2015).

### **Zinc solubilization**

Zinc (Zn) is an essential micronutrient, required for normal plant growth and productivity by imperative role in physiological process such as carbohydrate metabolism, regulation of auxin synthesis, regulation of IAA, immune system, repair of cellular membrane, components of protein and several enzymatic reactions (Mumtaz et al. 2018). Approximately, 50% of the world's soil has deficient Zn that causes reduction in growth and yield of crops both qualitatively and quantitatively (Welch and Graham 2004). *Bacillus* spp. are well known to solubilize Zn and its uptake in plants resulting in the improved growth, productivity and nutrients quality (Mumtaz et al. 2018). Phosphate-zinc-solubilizing bacterial strains *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 shown beneficial inoculation effect on uptake of Zn and P in chickpea grain yield (Zaheer et al. 2019). Further strains of *Pseudomonas* and *Bacillus* spp. significantly enhanced the dry mass of root and shoots (12.96 g) and uptake of nitrogen (2.268%), potassium (2.0%), manganese (60 ppm), and zinc (278.8 ppm) in maize crop as observed by Goteti et al. (2013). Previous studies also supported that *Bacillus* spp. are involved in solubilization of Zn

mineral in *in-vitro* as well as in the soil system (Dinesh et al. 2015; Etesami et al. 2017). Jorjani et al. (2011) reported that *Bacillus coagulans* based bioformulation applied on sugar beet 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 the wheat, super Basmati rice and Basmati-385 and showed increase in the fresh and dry weight of crop under pot trails containing sterilized soils by solubilization of Zn and other minerals (Shakeel et al. 2015)

### **Potassium solubilization**

Potassium (K) is the third most essential nutrient in the soil, eighth most abundant element on earth's crust, most abundant cation in plant cells and most ample nutrient in leaves after nitrogen (N) (White and Buss 2003; Bhattacharya et al. 2016) that takes part in the plant growth metabolism, rate of assimilation, accumulation of sugars, proper seed germination, regulation of stomata, improving resistance to drought, cold tolerance, transpiration, water uptake, formation of adenosine triphosphate (ATP), opening and closing of stomata, increase root formation, and also helps in the growth regulation of crop (Wang et al. 2013). K is generally found in different forms such as feldspar and mica (90-98%), non-exchangeable K (1-10%), and exchangeable K (1-2%) (Etesami et al. 2017). The presence and solubility of K in the organic matter is usually recorded to be very low (Wang et al. 2013). Application of PGPR can stimulate solubilization of K and may enhance the growth and yield of crop. Among all PGPR, *Bacillus* spp. are most significant in the rhizosphere for solubilization of K even under nutrient deficient environment. K plays a positive role in the physiological status and enzymatic activities of the plant and the carbohydrate metabolism (Bräsen et al. 2014). Potassium solubilizing *B. circulans*, XD-K-2 improved the total biomass and uptake of nutrient (K) in kiwifruit (*Actinidia chinensis*), but when this isolate combined with N<sub>2</sub>-fixation (*Bacillus amyloliquefaciens* XD-N-3), P-solubilization (*B. pumilus* XD-P-1) bacteria as consortial inoculant, showed pronounced effect in terms of nutrients uptake (N, P, and K) and plant growth, productivity and soil fertility (Shen et al. 2016). The application of potassium solubilizing bacteria significantly influenced the availability of K, growth and yield (plant height, number of leaves per plant, stem girth, chlorophyll content, grain and stubble) of maize (Bräsen et al. 2014). K solubilisation shown by *Bacillus* strains, is known to play a positive role in the physiological status and enzymatic activities of the plant (Bräsen et al. 2014). In addition, K promotes the

uptake of sucrose molecule in the sieve cells of Stevia plant as reported by Fakhrul et al. (2014). *Bacillus* sp. solubilized K in the rhizosphere and improved plant growth and yield as proved by several researchers (Wang et al. 2013; Kalita et al. 2015; Etesami et al. 2017).

### **Nitrogen fixation**

Nitrogen is one of the most important nutrient for plant growth and productivity and plays several roles in the crops such as enhancement of proteins, nucleic acids, enzymes, chlorophyll content and growth regulators. However, its scarcity causes limited growth via leaf yellowing, reduced branching and small trifoliate leaves in plants impacting overall growth and survival of the plant (Arora and Mishra 2016). Plants cannot uptake nitrogen directly from environment. Hence, rhizospheric microbes are the only viable and eco-friendly solutions for fixing of nitrogen and enhancement of growth and productivity of plants. There are two types of nitrogen fixers; 1) symbiotic (e.g. rhizobia, *Frankia* etc.) and (b) non-symbiotic (*Anabaena*, *Nostoc*, *Azospirillum*, *Azotobacter*, *Gluconoacetobacter diazotrophicus* and *Azocarus* etc.). Nitrogenase enzyme produced by rhizobacteria helps in the fixing of nitrogen from atmosphere (Ahemad and Kibret 2014). Microbes contribute about 100 million tons and 30 to 300 million tons of nitrogen in the terrestrial and marine ecosystems, respectively (Pedraza 2008). Nitrogen fixing *Pseudomonas* sp. RFNB3 and *Serratia* sp. RFNB14 with multiple plant growth-promoting activities enhanced the height, dry biomass, macro-and micro-nutrients uptake, and chlorophyll content of tomato and red pepper (Islam et al. 2013). In addition, increased indigenous microbial population especially heterotrophic PGPR was also noticed in the soil (Islam et al. 2013). Inoculation of N-fixing bacteria resulted in higher root nodules, and grain yields (22%) of soybean crop after harvesting as compared to control field conditions (dos Santos Cordeiro and Echer 2019). Overall, nitrogen fixers are amongst the most important PGPR which need to be further utilized to increase the productivity of agro-ecosystems in a sustainable manner.

### **Indirect mechanisms of PGPR**

#### **PGPR as biocontrol agents**

Biocontrol is a method in which one microorganism inhibits the growth of other pathogenic microorganisms. Several PGPR are known as excellent biocontrol agents and among them *Pseudomonas* and *Bacillus* are very good examples (Kumari et al.

2016). These microbes have proved to be effective in the suppression of plant diseases by secretion of metabolites such as hydrogen cyanide, lytic enzymes including chitinases,  $\beta$ -1, 3 glucanases, antibiotics, siderophores etc (Abdelmoteleb et al. 2017). Fluorescent pseudomonads are widely used as biocontrol agents to inhibit the growth of fungal pathogens by secretion of metabolites. *Bacillus* spp. are another ubiquitous group of PGPR, good root colonizers, secretes several metabolites, enhance growth and productivity and maintain soil health by inhibiting the phytopathogens. Among all, *Bacillus safensis* is excellent plant growth promoting agent which can work even under stress conditions (Santoyo et al. 2012; Prakash and Arora 2019). Abdelmoteleb et al. (2017) observed that *Bacillus* sp. significantly reduced the growth of *A. alternata* by production of volatile compounds. Several PGPR strains produced biocontrol activity and minimized the soil-borne diseases related with fungi under pot and field conditions (Harbut et al. 2015; Fira et al. 2018). *Bacillus* isolate W19 showed antagonistic activity against fungal pathogen and reduced wilt disease of banana plants and increased plant productivity (Bubici et al. 2019).

#### **Hydrogen cyanide production**

Hydrogen cyanide (HCN) is a volatile compound produced by some rhizobacteria and this compound plays an important role in biocontrol activity against many fungal pathogens (Arora et al. 2001; Babalola 2010). HCN producing rhizobacteria mainly belong to *Pseudomonas* and *Bacillus* spp. and it is strongly dependent on nutrient availability (Gupta et al. 2015). Some PGPR strains exhibit antagonistic activity against *A. alternata*, a phytopathogen of *S. rebaudiana* and promote the plant growth and productivity. PGPR strains (*Bacillus* sp. *Serratia* sp. and *Pseudomonas* sp.) are known to produce significant amount of HCN and results suggest the possibility to use these rhizobacteria as biopesticides to increase the yield of crops (Agbodjato et al. 2015). HCN is recognized as a biocontrol agent, based on its ascribed toxicity against plant pathogens. In addition, it is involved in chelation of metals, indirectly increasing the availability of phosphate and enhanced plant growth (Rijavec and Lapanje 2016).

#### **Salicylic acid production**

Salicylic acid is a natural phenolic compound present in many plants and it is an important component in the signal transduction pathway. It is a chief plant growth hormone which can prevent the entry of fungal phytopathogens inside the plants

(Dieryckx et al. 2015) and results in induced systemic resistance (ISR) against phytopathogens (Beneduzi et al. 2012; Zhang et al. 2013). Salicylic acid produced by PGPR such as *P. fluorescens* plays an important role in growth inhibition of *Rhizoctonia solani*, a fungal pathogen (Nagachandrabose 2018). Mandal and Mallick (2009) demonstrated that exogenous application of salicylic acid on tomato plants enhanced ISR against *Fusarium oxysporum* sp. *lycopersici* and promoted growth. Salicylic acid triggers localized or systematic acquired resistance in plants associate by enhancement of immunity, growth, supply of nutrients, and induced resistance against fungal pathogens.

### **Lytic enzymes production**

Chitinase is a cell wall degrading enzyme, belonging to glycosyl hydrolase family, breakdowns the glycosidic bond present in the fungal cell wall chitin and is well known to be secreted by PGPR (Kumar et al. 2016). In addition, this enzyme can augment the plant's defense system by inhibition of fungal phytopathogen *A. alternata* due to degradation of chitin present in the fungal cell wall and their role is further reported in the enhancement of plant growth and productivity (Pasonen et al. 2004; Kumar et al. 2018). Many rhizobacteria use chitin as an energy source by secreting chitinase.  $\beta$ -1, 3-glucanase is another enzyme produced by rhizobacteria including *Bacillus* spp. and *Pseudomonas* spp. which also helps in degradation of fungal pathogen by acting on  $\beta$ -1, 3-glucan, a major component of cell wall (Veliz et al. 2017).

### **Mycorrhiza**

The term mycorrhiza was given by Frank (1885) defined as a symbiotic association of a fungus and the roots of a plant that have not pathogenic property. Mycorrhiza form mycelium inside the plant roots and penetrate it in between cells and inside the cells. They are divided into two types; 1) ectomycorrhiza, 2) endomycorrhiza. Ectomycorrhiza play a very important role in the protection of root from the plant pathogens, whereas endomycorrhizal fungi reach inside the cortical cells, develops vesicules, arbuscules and coils, which help in the nutrient exchange between the plant root and the fungus (Borkowska 2002). Mycorrhiza occur in almost all plant species such as Thallophytes, Pteridophytes, Gymnosperms, and Angiosperms and form arbuscular mycorrhizal fungal (AMF) associations (Smith and Smith 1997). The role

of mycorrhizal association with plants are considered in uptake of nutrients, resistance to attack of certain root pathogens, enhance drought tolerance and photosynthetic activity in plants (Bharat and Mehta 2014). The AMF also called as endomycorrhizae, belong to genus *Glomus* and order Glomales in the taxonomic group (Walker 1992). It is one of the main component of rhizospheric microflora and noticeably interacts with other microorganisms in the rhizosphere of plants (Azcón-Aguilar and Barea 1992). The microbial populations and other soil nutrients of plant roots are influenced in the presence of AMF (St-Arnaud et al. 1997). The positive relationship between root biomass and root shoot ratio with mycorrhizal population has been commonly observed. In the virgin soil, natural conditions do not show AMF spores, but the agricultural practices increase the mycorrhizae population qualitatively and quantitatively ((Douds et al. 1993). Beside these, the changing of cropping order also impacts the population of AMF (Douds et al. 1993). The density of mycorrhizal spores decline when soils are kept uncultivated for long time (McGee et al. 1997). AMF are now days used as biofertilizers as it has been understood that they play vital role in life cycle of plant and in soil ecosystem which are essential for sustainable development (Bhardwaj, et al. 2014). AM fungi solubilise minerals (zinc, phosphate, and potassium) and increase the uptake in plant under nutrient deficient conditions (Miransari 2013). AMF (*Glomus coronatum*) are well known to stimulate plant growth and management of plant diseases (Gianinazzi, et al. 2010). These fungi can inhibit the growth of fungal pathogens related with host plant (Heath 2000). The entry of fungal pathogens are restricted to the epidermis and exodermis in the presence of AMF. Production of PGP attributes by rhizospheric microbes play an important role in enhancement of AMF papulation in the soil. AMF can enhance the rhizobacterial population and promote plant growth and yield. The application of AMF may contribute in minimizing chemical based fertilizer contributions and sustaining growth and productivity of plant in agriculture (Bhardwaj et al. 2014). AMF thus have a wide range of application in sustainable low input agricultural systems which are beneficial for food security and biofortification (Berruti et al. 2016). According to study, it has been observed that inoculation with *Glomus* sp. and *Azotobacter chroococcum*, either alone or in combination positively affected on the crop (rice) growth by nutrient linked mechanisms (Bhardwaj et al. 2014).

### PGPR based bioinoculants

Bioformulations or bioinoculants can prove to be a reliable solution particularly for the cultivation of MAPs. 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). The first patent “Nitragin” was registered in United States of America (USA), on *Rhizobium* sp. for providing nitrogen to the plant (Nobbe and Hiltner 1896). The cyanobacteria occurs in rice fields was first identified by Fritsch (1907), but their major role in soil to supply nitrogen was demonstrated by De (1939). The use of cyanobacterial inoculant for N<sub>2</sub> fixation was successful done by De (1939). Phosphorus deficiency problem in crops is common, however, inoculation of rhizobacteria to solubilize phosphates was reported by Sperber (1957), and later other workers also confirmed microbe-mediated phosphates solubilization (Mulder (1967; Subbarao 1988). During the 1960s the role of mycorrhiza was recognized in nutrient uptake and very soon this organism was used as biofertilizers commercially (Subbarao 1988). Although these reports are of major importance, a holistic approach of soil-inhabiting beneficial bacteria by identifying the potential of PGPR was reported by Kloepper and Schroth (1978). PGPR are capable of affecting plant growth both directly and indirectly, and further research showed that various crops treated by these microbes not only enhanced productivity of crop but also minimized the use of synthetic chemicals based fertilizers/pesticides (Zahra et al.1984; Vandevivere et al. 1994; Sheng and Lin 2006). Later for large-scale use of PGPR, their mass production in the form of appropriate bioformulations started, which included the development of formulations which should be stable, have a long shelf life, and give the desired results when applied in the field with an appropriate delivery system at the lowest effective dose (Arora et al. 2016).

Historical trends that opened opportunities in developing biopesticides was first started by Italian scientist “Agostino Bassi”, who discovered a fungus, *Beauveria bassiana*, causing infectious disease in the silkworm (Bassi 1835). After this discovery, some researchers also started to think that PGPR might prove to be effective agents for controlling plant pests (LeConte 1874; Steinhaus 1975). A fungus referred to as green muscardine and named *Entomophthora anisopliae* (now known as

*Metarhizium anisopliae*) was able to kill beetles (Aronson et al. 1986). A Japanese biologist Shigetane Ishiwata (1901) discovered *Bacillus thuringiensis* (Bt) and gave a new aspect to microbial biocontrol. Since then Bt has been used routinely in a variety of biocontrol products and after its commercial success in 1938, it was sold as a biopesticide named “Sporeine” for the first time in France (Aronson et al. 1986). In the 1950s this biopesticide entered in the United States (Aronson et al. 1986). Afterward, *Bacillus popilliae* was used as another bacterial based biopesticide (Lord 2005). The first genetically engineered Bt crop, Bt field corn, was registered with the United States Environmental Protection Agency in 1995 (USEPA 1999). In 1965 the first fungal product “Boverin” was developed in the former USSR. This product was based on *B. bassiana*, discovered by Agostino Bassi, and used to control the Colorado potato beetle and the codling moth (de Faria and Wraight 2007). Commercial use of Pseudomonads as biocontrol agents was also initiated in the 1970s (Burr et al. 1998).

Biofertilizers are those substances that contain live microorganisms, colonizing the rhizosphere of the plant and increase the supply or availability of primary nutrients and/or growth stimulus to the target crop (Bhattacharjee and Dey 2014). Bioinoculants are applied in the agricultural fields as replacement to conventional fertilizers. Biofertilizer use is increasing to maintain soil health, minimizing environmental pollution and cut down on the use of the chemical load in agriculture (Mishra et al. 2016). Various beneficial microbes have been used as biofertilizers for different crops (Table 1). The use of PGPR, particularly N<sub>2</sub> fixing, phosphate and potassium solubilizers are recommended as a sustainable solution to improve plant nutrient uptake and crop production (Bhattacharjee and Dey 2014). According to an estimate farmers usually need to apply at least 100 kg of N<sub>2</sub> per hectare (Ondersteijn et al. 2003), whereas the use efficiency is typically below 40%, meaning that most applied fertilizer either washes out or is lost to the atmosphere. Biological nitrogen fixation (BNF) in terrestrial ecosystems is in the order of 100 and 290 million tons of N<sub>2</sub>/ year and provide N without any cost out of which 40–48 million tons /year is fixed by crops (Zahran 1999). According to an estimate, cyanobacteria in symbiotic association contribute 7–80 kg N<sub>2</sub>/ha/year, free-living 15 kgN<sub>2</sub>/ha/year and associative (endophytic) bacteria 36 kg N<sub>2</sub>/ha/year (Akinrinlola et al. 2018). It has been observed that cereal crops may obtain up to 30% of their N<sub>2</sub> from BNF when fertilized with high rates of P and K and with micro elements (Pedraza 2008; Mmbaga et al. 2014). Rhizobial inoculants have been applied to legume crops for over 120 years as

biofertilizers (Marks et al. 2013). Many countries are promoting the use of rhizobial inoculants for nitrogen uptake for example in Brazil, application of rhizobial inoculants in soybean crop provides N<sub>2</sub> with a value equivalent to US\$ 7 billion in fertilizers. Rennie and Hynes (1993) also stated that Canada has a substantial market of *Rhizobium* inoculant products. In some studies, *Rhizobium* inoculation has also showed biocontrol potential against soil-borne phytopathogenic fungi (Arora et al. 2001). Among the non-symbiotic N<sub>2</sub>-fixing bacteria, the most extensively studied genus is *Azospirillum*. In addition to increasing plant nitrogen content, it also improves plant growth by the production of phytohormones such as auxins, cytokinins, and gibberellins (Hayat et al. 2010). Recently the legumes and their association with rhizobia and AMF have also been recognized for better nitrogen and phosphate uptake to plants and gaining importance in agroecosystems (Jha and Saraf 2015). This tripartite association was not only effective in nodule formation, AMF colonization, nitrogen fixation but also supported the faba bean growth under alkalinity stress (Abd-Alla et al. 2014).

**Table 1.** PGPR based biofertilizers used in agriculture

Types of biofertilizers	Name of microbe used	Description
Nitrogen fixers	<i>Rhizobium</i> spp.	Ability to fix atmospheric nitrogen in symbiotic association with plants forming nodules in roots.
	<i>Azospirillum</i> spp.	It is free living and non-symbiotic nitrogen fixer, beneficial to a wide array of crops covering cereals, millets, vegetables, cotton, and sugarcane.
	<i>Azotobacter</i> spp.	Rapidly grow in various agricultural soils fixes high level of nitrogen.
	Cyanobacteria	Some cyanobacteria have terminally differentiated, specialized structures called heterocyst and considered as photodiazotrophs. Their presence in rice fields provided aid in nitrogen uptake.
	<i>Azolla</i> spp.	It helps symbiotic relation with blue green algae in the roots of several crops especially rice.
Phosphate solubilizers	<i>Pseudomonas</i> spp., <i>Bacillus</i> spp., <i>Rhizobium</i> spp., <i>Burkholderia</i> spp. and Mycorrhiza	PSB and Mycorrhiza help in uptake of phosphorous in plants by solubilization of phosphate via different mechanisms.
Zinc solubilizers	<i>Bacillus</i> spp., <i>Pseudomonas</i> spp.	Solubilization of zinc compounds like zinc oxide (ZnO), zinc carbonate (ZnCO <sub>3</sub> ) and zinc sulphide (ZnS) is performed by zinc solubilizers and also helps in plant growth and reduces load of chemical and cost of fertilizer.
Potassium solubilizers	<i>Bacillus</i> spp., <i>Burkholderia</i> spp., <i>Pseudomonas</i> spp.	Bacteria can solubilize K, present in the insoluble form of rocks and silicate minerals and enhanced yield of crops.
Phytohormones producers	<i>Pseudomonas</i> <i>Bacillus</i> , <i>Rhizobium</i> , <i>Burkholderia</i> , and Mycorrhiza	Phytohormones such as auxin, cytokinin, gibberellin produced by microorganisms also help in cell proliferation, growth of lateral roots and root hairs.

In plants, Zn is regarded as an essential micronutrient (Tripathi et al. 2015) but due to its transformation to different chemical forms, only very little amount remains available to plant. Various studies now indicate that the release of insoluble and fixed forms of Zn by solubilizing bacteria is an important aspect of increasing soil Zn availability through the production and excretion of organic acids (Sultana et al. 2019). Plant growth promoting fungi (PGPF) have been extensively studied for solubilization of insoluble zinc compounds both *in-vitro* and *in-vivo*. However, some

PGPR of the genera *Acinetobacter*, *Bacillus*, *Gluconacetobacter*, and *Pseudomonas* have also been reported to have ability to solubilize insoluble zinc (Kamran et al. 2017; Mehnaz et al. 2017).

About 40% of the potential global crop yield is destroyed by pests (invertebrates, plant pathogens and weeds) before it is harvested and another 20% is destroyed postharvest (Chandler et al. 2010). However, for sustainable agriculture, use of chemical-based pesticides should be relinquished because of their negative impact on the environment. Workers around the world are now using biopesticides (Table 2) and trying to minimize the use of conventional chemical pesticides. Biopesticides are derived from natural materials such as animals, plants, bacteria, and minerals. Worldwide, approximately 1400 biopesticide products are being sold and their number is increasing day by day. For global status of biopesticide use and regulation as well as availability one can see review by Arora et al. (2016). Microbial pesticides are also known as biocontrol agents (BCAs). Microbes as biopesticides offer the advantage of higher selectivity and lower or no toxicity in comparison to conventional chemical pesticides (Hell et al. 2008). BCAs can be grouped into three broad categories viz. bacterial, fungal and viral. A widely used bacterial pesticide is *Bacillus thuringiensis* (Bt), accounting for approximately 90 % of the biopesticide market in the USA (Kachhawa 2017). Several species of *Pseudomonas* show biocontrol potential against phytopathogens (Saraf et al. 2008). For example, biopesticides containing *P. fluorescens*, *P. aeruginosa*, and *P. syringae* have been used at large scale (Stockwell and Stack 2007). Fungal biopesticides used against plant pathogens include *Trichoderma harzianum*, which is an antagonist of several soil-borne fungi such as *Rhizoctonia*, *Pythium*, *Fusarium*, and other phytopathogens (Siameto et al. 2010). *Baevuria bassiana* and *Metarhizium anisopliae* are naturally occurring entomopathogenic fungi considered good BCA and infect-sucking pests including *Nezara viridula* L. (green vegetable bug) and *Creontiades* sp. (green and brown mirids) (Mishra et al. 2016).

**Table 2.** Some examples of PGPR based biopesticides used in agriculture

	General or commercial name	Target organism(s)
<i>B. thuringiensis</i> subsp. <i>Aizawai</i>	Turex	Lepidoptera pests
<i>giensis</i> subsp. <i>Israelensis</i>	VectoBac	Lepidopteran pests
<i>B. thuringiensis</i> subsp. <i>Kurstaki</i>	Dipel WP, Batik,	Lepidoptera pests
<i>ubtilis</i>	Kodiak	<i>Fusarium</i> , <i>Pythium</i> , <i>Alternaria</i> and <i>Rhizoctonia</i>
<i>Bacillus amyloliquefaciens</i>	Taegro	<i>Fusarium</i> , <i>Alternaria</i> and <i>Rhizoctonia</i> wilt diseases
<i>icheniformis</i>	EcoGuard	Downy mildew, <i>Fusarium</i> wilt
<i>umilus</i>	Sonata	<i>Alternaria</i>
<i>popilliae</i>	Milky Spore Powder	Japanese beetle grubs
<i>onas chlororaphis</i>	At-Eze	Soil and seed-borne fungi
<i>onas syringae</i>	Bio-Save 10LP	Postharvest diseases
<i>onas. Aureofaciens</i>	Spot-Less	Turf fungal diseases
<i>onas fluorescens</i>	Biomonas, Esvin	Bacterial wilt, root rot
<i>onas alcaligenes</i>	Not Known	Locusts, grasshoppers
<i>onas aureofaciens</i>	Spot-Less	Turf fungal diseases
<i>onas resinovorans</i>	Agriphage	Insect pest control
<i>onas syringae</i>	AgriPhage	Bacterial speck
<i>erium radiobacter</i>	NoGall	Crown gall disease
<i>agglomerans</i>	BlightBan C9-1	Fire blight
<i>a bassiana</i>	Naturalis L, Botanigard	Thrips, whitefly and mites
<i>um anisopliae</i>	Biomagic, Biomet	Coleoptera and lepidopteran insect
<i>rma harzianum</i>	Biozim, Phalada 105	Soil-borne pathogens
<i>rma viride</i>	Monitor, Trichoguard,	Soil-borne pathogens
<i>um lecanii</i>	ABTEC, Ecocil	Whitefly, coffee green bug,
<i>oligandrum</i>	Polyversum	Root rots
<i>rma gamsii</i>	Remedier, Bioten	Soil-borne pathogens
<i>rma polysporum</i>	Binab T	Soil and foliar diseases
<i>yces fumosoroseus</i>	Preferal WG	Greenhouse whiteflies
<i>a destruens</i>	Smolder	Herbicide – dodder
<i>oleophila</i>	NEXY	Postharvest disease
<i>us flavus</i>	Afla-guard	<i>Aspergillus flavus</i> producing aflatoxin

Baculovirus is the main virus that is commercially used for designing phage pesticides. As of 2010, over 24 baculovirus species have been reported to be registered for use in insect pest management throughout the world (Arora et al. 2016). Currently, most commonly used microbial biopesticides are biofungicides (*Trichoderma*, *Pseudomonas*, and *Bacillus*), bioherbicides (*Phytophthora*) and bioinsecticides (Bt) (Kachhawa 2017). Data indicates that among the biopesticide market for all crop types, bacterial biopesticides claim about 74%, fungal

biopesticides, 10%, viral biopesticides, 5%, predator biopesticides, 8%, and “other” biopesticides, 3% (Chandler et al. 2011; Siegart et al. 2015).

Bioformulation contains both properties of biofertilizer and biopesticides (Nagachandrabose 2018). Moreover, preparation of the formulation of effective inoculants is a multistep process in which microorganisms contained in a particular carrier with adhesives and additives help in protection, storage, and transport. A good formulation provides an optimal condition to persist microorganisms in soil and maximize its efficacy in order to maximal benefits after inoculation to the host plant (Nagachandrabose 2018). The inoculants should be cost-effective and easy to handle and use, to ensure that the microorganism delivered to the plant in most appropriate form and manner (Arora et al. 2016). There are different ways to deliver PGPR in the field or in contaminated sites. Bioformulation is a biologically active product containing cheap ingredient such as inorganic or organic material or both which maintain shelf-life of microorganisms including PGPR or plant growth promoting fungus (PGPF). Development of PGPR based bioformulations is easy to handle and simple method for delivery in the field. PGPR based bioformulations are generally applied as treatment of seeds, root dip suspension before transplanting, soil amendment, drip irrigation, as well as foliar spray. Two major types of bioformulations have been generally developed; 1) solid and 2) liquid. PGPR based-solid bioformulation contain cheap materials such as talc powder, peat, saw dust, sugarcane bagasse, rice husk, wheat bran, tea waste and, calcium carbonate for use as solid bioformulation to maintain pH. Whereas, in PGPR based-liquid bioformulation, potato broth, liquid waste of rice, paneer whey, minimal media known as peptone water, vegetable oils (rapeseed, palm oil, and castor oil) are used as carrier material. The detailed knowledge of bioformulation is given in the book “Bioformulation: for Sustainable Agriculture” edited by Arora et al. (2016). Development of novel PGPR based-bioformulations is a challenging task because, in all bioformulation, ingredients or carrier materials is a major component to maintain the shelf life of bacteria.

Usually the term solid bioformulation refers to preparation of product using a solid carrier. The carrier is the delivery vehicle of live microorganisms from the laboratory/factory to the field; however no universal carrier or formulation is presently available for the release of microorganisms into soil (Malusá et al. 2012). Moreover, a good carrier should have the capacity to deliver the standard number of viable cells in good physiological condition at the right time (Bashan and De-Bashan 2005). Solid carriers can be divided into three basic categories: (i) soils (peat, coal, clays and inorganic soil) (ii) plant waste materials (iii) inert materials *viz.* vermiculite,

ground rock phosphate, polyacrylamide gels and alginate beads (Bashan 1998; Arora et al. 2016). Inoculants come in four basic dispersal forms (powders, slurries, granules and liquids). The use of each type of inoculant depends upon the market availability, choice of farmers, cost and the need of a particular crop under specific environmental conditions. Thus, bioformulation is a type of living product. Utmost care is needed from preparation to production and finally end use to maintain optimum microbial population delivery to the plant.

Liquid bioformulation contain liquid carrier with beneficial plant growth promoting microorganism(s). Application method (foliar, soil and roots) of liquid bioformulation on the plant is easy as compared to solid bioformulations (Goljanian-Tabrizi et al. 2016). There are different carriers used for the development of liquid bioformulation such as dairy wastes, rice broth waste, potato broth waste, molasses etc. Liquid bioformulations are considered better in comparison to solid, as they have longer shelf-life, can easily colonize on the root or plant surface, can perform well in pots or field conditions, and have low cost (Goljanian-Tabrizi et. al. 2016; Nagachandrabose 2018). Kalita et al. (2015) developed a novel bacterial (*Bacillus cereus* MTCC 8297, *Pseudomonas rhodesiae* MTCC 8299 and *Pseudomonas rhodesiae* MTCC 8300) based liquid bioformulation enhancing the growth and total biomass content of the plants for foliar application. The bioformulation of *Pseudomonas fluorescens* (Pf1 and TDK1) and *Beauveria bassiana* (B2) strains showed biocontrol against leafminer insect and reduced the collar rot disease of groundnut (Senthilraja et al. 2010). Oil based bioformulation developed by using *Bacillus velezensis* FZB42 showed biocontrol potential against *Fusarium solani* in-vitro condition and simultaneously reduced the severity of damping off in *Solanum lycopersicum* seedlings (Raj et al. 2019).

Overall it can be said with conformity that PGPR can be excellent options for enhancement of crop yield by eco-friendly and sustainable manner. *Bacillus* sp. and *Pseudomonas* sp. are eco-friendly tools for promoting plant growth and productivity. Hence, novel strains from these genera can be used for development of bioformulations. These tools may enhance the livelihood of farmers through organic farming of agricultural important crops and especially medicinal plants. The exploitation of PGP strains (*Bacillus* sp. and *Pseudomonas* sp.) as cheap and effective bioformulation 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 industries.

*Chapter-04*  
*Isolation of Rhizobacteria from*  
*Various Medicinal Plants Including*  
*Stevia rebaudiana from Diverse Areas*



## **1. Introduction**

Soil microbes play a predominant role in agro-ecosystems. They can affect soil nutrient cycling, organic matter formation, decomposition and soil aggregation, thus influencing ecosystem stability. A lot of microbes inhabit in the rhizosphere, phyllosphere and endophytic region of the plants (Mishra et al. 2016). Among all, the eco-system of the rhizospheric soil is very complex due to the activities and nature of physical, chemical, and biological components (Tewari and Arora 2018). There are different types of interactions occurring in the soil system such as microbes to other microbes, microbes to host plant and microbes to environment, and overall these interactions create conditions that promote plant growth (Bever et al. 2010). Microbes play an essential role in making of minerals available to the plants and they also retain large quantities of nutrients (such as nitrogen, phosphorus, potassium, sulfur, etc.) which helps to prevent these nutrients from being leached or washed away (Kumar and Gopal 2015). Microbes are vital to the agro-ecosystems, being integral part of the carbon and nitrogen cycle and affecting plant growth and productivity. There are many nutrients present in the soil, but without microbial action, plants are generally unable to utilize them. The rhizospheric microbes are important for growth and nutrients uptake of plants and their health in the field and either directly or indirectly affect the composition, biomass and functioning of plant communities in the natural ecosystems (Jacoby et al. 2017). The rhizosphere is a complex structure between plant roots and soil microbes where interactions among a innumerable microorganisms, soil and plants takes place affecting plant growth, soil fertility and tolerance (of plant) to biotic and abiotic stress (Bakker and Schippers 1987). This hotspot area (rhizosphere) for microbial interactions and activities has received ample attention from scientists in different disciplines.

*Stevia rebaudiana* is an important medicinal crop being substitute for sugar, superior flavor outline, extensive medicinal properties, and also of agronomic interest (Modi et al. 2014). In the rhizosphere of plants, various microorganisms exist and play several beneficial roles for both the plant and environment (Ahemad and Kibret 2014). In the first objective of the present study, bacteria were isolated from the rhizospheric region of different medicinal plants including *S. rebaudiana*. Afterward, selected bacterial isolates were identified on the basis of morphological and biochemical characteristics.

## 2. Materials and Methods

### 2.1 Geographical location and climatic conditions

Lucknow is the capital city of Uttar Pradesh, India. It is in subtropical, semiarid zone falling in between latitude 26.8467° N and longitude 80.9462° E, with an elevation of 123 meters from the sea level. The average annual rainfall is 896.2 millimeters mainly from the south-west monsoon winds, and occasionally frontal rainfall occurs in winters. The minimum and maximum temperature is recorded in between 4°C to 45°C in Lucknow city.

### 2.2 Sample collection

The soil samples were collected from the rhizospheric region of six different medicinal plants i.e. *S. rebaudiana*, *Curcuma longa*, *Withania somnifera*, *Trigonella foenum-graecum*, *Aloe barbadensis* and *Mentha arvensis*, in sterile polybags and further kept at 4°C (icebox) before isolation of rhizobacteria. The rhizospheric soil samples were collected from different locations of Lucknow, India.

### 2.3 Isolation, purification, and maintenance of rhizobacteria

For isolation, 1 gm of soil sample was dissolved in 9 ml of sterile water (pH 7.1) from the ninth ( $10^{-9}$ ) dilution, 0.1 ml of soil sample was spread on nutrient and King's B agar (Aneja 2005) and incubated at 28°C for 48h for isolation of rhizobacteria. After the incubation period, mixed colonies were observed on the Petri plates. The fluorescent, white and yellowish colonies were picked up and further purified by streak plate method.

### 2.4 Morphological characterization

#### 2.4.1 Gram's staining

Fresh culture of all isolates were taken and smeared on the slide as per Aneja (2005). Further, crystal violet solution (crystal violet: 10 gm; ammonium oxalate: 4 gm; ethyl alcohol: 70%, distilled water: 100 ml) was flooded on the prepared smear for 45 seconds and then washed by deionized water. Afterward, few drops of Gram's iodine (iodine: 1 gm; potassium iodide: 2 gm; ethyl alcohol: 25 ml; distilled water: 100 ml) were put on the smear and washed with distilled water followed by 95% ethyl alcohol for 30 seconds

followed by washing with sterile water. In the last step, smear was stained with safranin (counterstain) (2.5% safranin in ethyl alcohol: 10 ml with distilled water: 100 ml) for 1 min (Gram 1884). Afterwards, the safranin was washed with distilled water and the smear was then air dried and examined under microscope.

#### **2.4.2 Observation of cell through compound and phase contrast microscopy**

Cell morphology (of the isolates) was checked by compound and phase contrast microscopy (Krieg and Holt 1984). Briefly, pure cultures were placed on a slide under microscope. The phase contrast microscope was focused at lowest magnification phase objective (10x Ph1 objective with a Ph1 phase annulus) and corresponding phase annulus in place and look at the phase plate and phase ring through the phase microscope to determine shape, size, color, and appearance (Krieg and Holt 1984).

### **2.5 Biochemical characterization**

#### **2.5.1 Catalase test**

A loopful of bacterial culture was taken on a glass slide and one or two drops of hydrogen peroxide (3%) were dropped on it (Aneja 2005). The presence of gas bubbles after few seconds is the indication for positive result.

#### **2.5.2 Lipid hydrolase test**

Overnight grown culture of isolates were spotted on lipid agar plates containing (gm/l); peptone 10, sodium chloride 5, calcium chloride 0.1, Tween 10, agar 20 and distilled water 1 liter. The plates were incubated at 28°C for 48 h (Aneja 2005). The clear zone around the colony is a indication for positive result and absence of this indicates negative result.

#### **2.5.3 Urease test**

For testing urease, 24 h old culture of bacteria were inoculated in sterilized urea agar medium containing (gm/l); yeast extract: 0.1,  $\text{KH}_2\text{PO}_4$ : 9.1,  $\text{Na}_2\text{HPO}_4$ : 9.5, urea: 20, phenol red: 0.01, agar: 16 and distilled water: 1 litre. The test tubes were incubated for 48 h at 28°C (Frazier et al. 1967). After incubation, result was observed.

#### **2.5.4 Citrate utilization test**

Citrate utilization test was done on Simmons citrate agar containing (gm/l); ammonium dihydrogen phosphate, 1; dipotassium hydrogen phosphate, 1; sodium chloride, 5; sodium citrate, 2; magnesium sulfate, 0.2; bromothymol blue, 0.08; agar, 20; in one

liter of distilled water (Simmons 1926). Streaking was done on the prepared slants with overnight grown culture. After incubation, result was observed as appearance of blue color for positive result and no color change indicates negative for citrate utilization.

#### **2.5.5 Protease test**

Skim milk agar (skim milk powder 100 gm/l, agar 20 gm/l and distilled water 1000 ml) was prepared for checking protease activity. Overnight grown cultures were taken and spotted on freshly prepared skim milk agar plates and then incubated at 28°C for 24 h (Narendra et al. 2012). The presence of zone was observed around the spotted colony which shows a positive indication for protease test.

#### **2.5.6 Sulphide, Indole, Motility (SIM) test**

The SIM agar medium was prepared in the test tubes and the cultures were stabbed into them (Aneja 2005). Afterward, culture tubes were incubated for 48 h at 28°C. After incubation, the development of black color along with line of stabbing is considered to be a positive test but no such black coloration shows negative result. The 24 h old bacterial culture were inoculated on SIM agar test tubes and further incubated for 72 h at 28°C. Subsequently, 1 ml of Kovac's reagent was added into it. Development of cherry red color at the time of addition of Kovac's reagent displays as a positive test for indole production (Aneja 2005). For motility, the overnight grown bacterial culture was stabbed straight into the test tubes of SIM agar medium and incubated at 28°C for 24 h. Growth along the line of stab was observed for a positive test.

#### **2.5.7 Endospore staining**

The smear was prepared on the clean slide. After heat fixing smear was flooded with malachite green. Then the slide was heated to steaming for 5 min. Time to time more stain was added to the smear (Aneja 2005). The slide was further washed through slow running distilled water. Counterstain was done with safranin for 30 seconds. The slide was washed with distilled water and further left to dry and finally observed the cell under a microscope (Aneja 2005).

#### **2.5.8 Gelatinase production**

Prepared gelatin media (Hi-Media, Mumbai) was poured in test tube and isolated bacteria were inoculated into it and subsequently incubated at 28°C for 48 h. After incubation, culture was placed in the refrigerator at 4°C for 15 min for checking gelatinase production (Aneja 2005). After incubation, the liquid medium in the test tube

indicates positive results, and no liquefaction means negative result for gelatinase production

### **2.5.9 Intrinsic antibiotic resistance**

Antibiotic resistance test was observed by using the antibiotic disc on Mueller Hinton agar plate. Briefly, the log phase culture of isolates were spread on the medium. The antibiotic disc was placed over the surface of the medium. The antibiotic resistance was detected by the zone of inhibition formed around the disc after 48 h incubation at 28°C. Following antibiotics were procured (from HiMedia) and used: Gentamycin (10mg), ampicillin (10mg), streptomycin (10mg), erythromycin (10mg), amikacin (10mg) and chloramphenicol (30 mg). This experiment was carried as per Aneja (2005).

### **2.5.10 Sugar fermentation**

Triple sugar iron agar (lactose, glucose, and sucrose) were prepared individually in the test tube slant and isolated bacteria were streaked and further incubated at 28°C for 48 h (Aneja 2005). After incubation, color change of the medium was observed as an indication of sugar fermentation.

### **2.5.11 Starch hydrolysis**

Isolates were streaked on starch agar plates and incubated for 24 h. After the incubation period, iodine solution was flooded on the plates for 5-30 seconds and result was observed (Aneja 2005). Absence of blue coloration around the colonies indicates positive result.

## **2.6 Specific test for identification of fluorescent pseudomonads**

### **2.6.1 Fluorescein production**

Specific media for fluorescence production was prepared. *Pseudomonas* minimal media containing casein enzyme hydrolysate (g/l): 10, protease peptone 10, dipotassium hydrogen phosphate 1.5, magnesium sulfate 1.5, agar 20, and distilled water 1 liter (Gill et al. 1986). The prepared medium was sterilized and poured in Petri plates. Afterward, it was streaked with an overnight grown pure culture and plates were incubated at 28°C for 24 h. The plates were then kept under ultraviolet light at 280 nm, and further the colonies with yellowish green fluorescence were selected.

### **2.6.2 Pyocyanin production**

Pyocyanin production media containing peptone 20g/l, magnesium chloride 1.4g/l, potassium sulfate 10g/l, and agar 20g/l was prepared and poured in Petri plates and

streaking was done on the poured plates with 24 h old bacterial culture and incubated at 28°C for 24 h (Gill et al. 1986). After incubation, Petri plate was observed under U.V. light for detection of pyocyanin.

### **2.6.3 Levan production**

Overnight growth culture was spotted on nutrient agar plates containing 5% sucrose for levan production and the control plate was left without sucrose and incubated (time: 48 h, at 28°C) (Yasuda and Katoh 1987). The presence of mucoid, gummy or large colonies was observed for an indication of Levan production.

### **2.6.4 Oxidase production**

Oxidase test was done by using oxidase disc. The overnight cultures of isolates were grown in nutrient broth. Oxidase disc was placed on a glass slide and flooded with overnight grown bacterial cultures. The result was observed within 10 sec at 28°C (Aneja 2005). The appearance of the blue color was observed for positive test.

### **2.6.5 Pectinolytic activity**

Peeled potato was washed and approximately 1 cm thick slice was cut off. Then slice was dipped into alcohol and dried in the presence of flame. The slice was placed in Petri plate and after sterilization distilled water was filled (70%) in it up to depth of 3-4 mm. A nick in the center of the potato was made with the help of sterilized forceps and inoculated with a loopful of fresh bacterial culture (24 h old). The plates were then incubated at 28°C for 24 h (Beg et al. 2000). After incubation, the plates were observed for rot formation as an indication of pectinolytic test.

### **2.6.6 Arginine dihydrolase test**

The overnight grown bacterial culture was spotted on the nutrient agar medium supplemented with arginine and phenol red and incubated for 24 h at 28°C (Møller 1955). The plate was observed for zone of clearance after incubation and cultures showing clear zone around the colony were scored as positive for the test.

### **2.6.7 Growth at 4°C**

The nutrient broth was prepared in the test tubes and autoclaved at 121°C for 15 lb pressure. The test tubes were inoculated with 24 h old bacterial culture and incubated for 24-48 h at 4°C (Frazier et al. 1967). After the incubation period, optical density was taken at 610 nm by spectrophotometer (model: Evolution 201, Thermo Scientific USA).

### 2.6.8 Growth at 41°C

The nutrient broth was prepared in test tube and autoclaved. The 24 h old bacterial culture was inoculated into each test tube and incubated for 24-48 h at 41°C in an incubator (Model: REMI CI- 12 Plus, India) (Yasuda and Katoh 1987). After the incubation period, the bacterial growth was checked by measuring optical density at 610 nm through spectrometric analysis (Thermoscientific Evolution 201).

## 3. Results

### 3.1 Sample collection

The soil samples were collected from three locations of Lucknow, Uttar Pradesh, India including: BBA University Campus, CSIR-CIMAP and South City. On the basis of the previous study of Arora et al. (2001), rhizosphere region was selected for isolation of beneficial rhizobacteria. Details of the isolated bacteria from the rhizosphere of host plants and their locations are mentioned in Table 3.

### 3.2 Isolation of rhizobacteria

A total of 11 rhizobacteria (STJP, STJP12, STJP18, STJP20, STJP22, STJP30, JPN1, JPN2, JPW, NPSP, and NPSP12) were isolated (Figure 8) from different medicinal plants (Table 3). Pure cultures (Figure 9) of the isolates were maintained on the same medium (as that used for isolation) at 4°C (Model: Labocon -86 °C ULT Freezer Upright LUF-86-301) for using in further studies.

### 3.3 Morphological characterization

Out of eleven, five isolates showed fluorescence on the KB medium plate which was further examined for morphology by microscopy. Morphologically, five isolates (JPN1, JPN2, JPW, NPSP and NPSP12) were found to form smooth, circular, non-mucoid colony and were Gram-negative, motile and rod-shaped, while six isolates STJP, STJP12, STJP18, STJP20, STJP22, and STJ30 showed circular gummy and off white colonies and were Gram positive.

### 3.4 Biochemical characterization

The result of the biochemical tests of the isolates are mentioned in Table 4. After addition of H<sub>2</sub>O<sub>2</sub> on a glass slide, it showed the liberation of effervescence by isolates (Figure 10). It is an indication of a positive result for catalase. All isolates produced catalase except STJP12 (Table 4). The ability of bacteria to hydrolyze the fats/lipid into glycerol and fatty acid by lipase enzyme was checked. In this regard, five isolates

(JPN1, JPN2, JPW, NPSP, and NPSP12) showed positive (Figure 11) and remaining (six) isolates were negative. Urea is an organic compound formed by decarboxylation of amino acids. Urease is checked for the ability of an organism to breakdown urea into carbon dioxide and ammonia by urease enzyme. In the present study, only four isolates (JPN1, JPN2, JPW, and NPSP12) showed urease activity (Figure 12). Citrate can be a sole source of energy for some microorganisms to carry out metabolic activities. In this regard, except three (JPN1, JPN2, and NPSP12), all isolates were found to be positive for citrate utilization (Figure 13). This result was confirmed by change of color from green to blue after 24 h of incubation.

The protease cleaves the peptide bond present in the protein or enzyme. Isolate STJP, JPN1, JPN2, and NPSP were found to be positive for protease enzyme (Figure 14), while remaining isolates (JPW, NPSP12, STJP12, STJP18, STJP20, STJP22, and STJP30) did not show protease activity as confirmed by test. SIM is a specific semi-solid media which is used for the differentiation of Enterobacteriaceae based on their ability to produce hydrogen sulfide, to breakdown indole from tryptophan which shows motility. In the sulfide test, none of the isolates produced hydrogen sulfide. The negative result was confirmed by presence of no black color on SIM medium. In the indole production, isolated bacteria did not show color change after the addition of Kovac's reagent. Hence, all isolates were negative for indole (Figure 15). Motility is the ability of a microorganism which can move from one place (by flagella mainly) to another by itself. Isolate STJP and NPSP showed a positive test for motility (Figure 16) and their result was confirmed by diffused and foggy growth appearance throughout the SIM medium. Isolates STJP, STJP12, and STJP18 showed endospore formation as checked after staining of stationary phase cultures. Gelatinase is an enzyme, proteolytic in nature and used as a solidifying agent. Only five isolates (JPN1, JPN2, JPW, NPSP, and NPSP12) produced gelatinase. All of the isolates showed resistance against gentamicin, chloramphenicol, amikacin, and streptomycin. Only two isolates (NPSP and STJP) did not show resistance to erythromycin, while other isolates were found to be resistant (against erythromycin) (Figure 17). All the rhizobacterial isolates were able to utilize different carbon sources (lactose, glucose, and sucrose) (Figure 18). Further, isolates were observed for hydrolysis of starch (Figure 19). Isolates JPN1, JPW, NPSP, NPSP12, STJP, STJP12, STJP18, STJP20, STJP22, and STJP30 showed hydrolysis of starch, while JPN2 did not show starch hydrolysis. Selected isolates were grown in NB medium containing NaCl and showed salt tolerance with different concentrations (2%

to 20% NaCl). Among all isolates, bacterium STJP showed highest salt tolerance (upto 16% NaCl) concentration, but further increase in salt concentration, was inhibited the growth of isolate (Figure 20).

### 3.5 Specific test for pseudomonads

Only five isolates (JPN1, JPN2, JPW, NPSP, and NPSP12) showed fluorescence on the plate contained minimal medium (Figure 21, Table 4). Of these only two isolates JPW and NPSP produced pyocyanin on the medium as confirmed by observation under UV light (Figure 22). However, some compounds (magnesium chloride and potassium sulfate) augment the production of pyocyanin and inhibit the effect of fluorescein pigment in the medium. Levan is a carbohydrate molecule, belonging to a group of fructans. Only one isolate (NPSP) produced levan (Figure 23) and the result was confirmed by the presence of large mucoid colony on the medium. Oxidase is an enzyme which helps in the electron transport chain. When overnight grown culture was flooded in the medium, the dark blue/purple color was observed within 10 seconds in the presence of oxidase disc (Figure 24). Isolates JPN2, JPW, NPSP, NPSP12, and STJP showed oxidase activity and remaining isolates were negative for this activity. Only two isolate (NPSP and JPW) showed pectinolytic activity (Figure 25). The result was confirmed by rot formation on the potato after 24 h of incubation. Arginine dihydrolase is an enzyme which produces ammonium compound from arginine (Figure 26). Isolates JPN1, JPN2, and NPSP12 showed yellow-green, diffusible fluorescence color on the King's B agar medium after 24 h of incubation, while isolates JPW and NPSP produced blue-green diffusible colonies. Remaining isolates did not show fluorescence. Isolates NPSP, JPW, and JPN1, JPN2, NPSP12 showed growth at 41 °C and 4 °C (Figure 27) on King's B broth.

On the basis of morphological and biochemical characters, five isolates JPN1, JPN2, JPW, NPSP, and NPSP12 were found to be members of genus *Pseudomonas*, while six isolates STJP, STJP12, STJP18, STJP20, STJP22, and STJ30 were that of genus *Bacillus* (Bergey's Manual of Systematic Bacteriology; Garriety 2005).

## 4. Discussion

Soil contains a diverse group of microorganisms and divided into two zones on the basis of habitat; 1) rhizosphere; (made up of two words; rhizo means roots and sphere-surrounding zone). The term rhizosphere was first coined by Hiltner (1904), a German soil microbiologist. According to Sturz et al. (2000), the rhizosphere boundary is further

divided into two areas; a) edaphosphere (soil occurs on lower side of the roots) and b) histosphere (soil is present on the upper side of plant roots), 2) rhizoplane; to denote the soil particles closely adhering to root surface.

In the present study, eleven rhizobacteria were isolated from rhizospheric soil of the different medicinal plants by serial dilution method. Serial dilution is a good and effective method for isolation of microbes from soil sample (Ben-David and Davidson 2014). Isolate NPSP, JPN1, JPN2, JPW, and NPSP12 were characterized as fluorescent Pseudomonads on the basis of phenotypic and biochemical tests as per Bergey's Manual of Systematic Bacteriology (Garrity 2005). Some tests such as oxidase, arginine dihydrolase, pyocyanin, levan production, grown at 4°C or 41°C and color diffusible by the colony are very useful for differentiation of *Pseudomonas aeruginosa* and *P. fluorescens* from other pseudomonads (Haynes 1991; Tang et al. 2017; Tewari and Arora 2018). The genus *Pseudomonas* is one of the most important genera and plays a significant role in many sectors such as agriculture, medical industries, food industries, and bioremediation. In this genera, different species and strains are being reported, due to the increased agronomic interest (Ben-David and Davidson 2014). According to Mulet et al. (2010), approximately 118 species have been discovered in the genus *Pseudomonas* and their major roles are recorded in growth and productivity of plants. Radhapriya et al. (2015) reported that *Pseudomonas aeruginosa* RRALC3 showed plant growth promoting attributes and increased the biomass, nutrients and carbon contents of *Pongamia pinnata* in degraded forest soil. Kumar et al. (2015) observed that *Pseudomonas* sp. P17 showed significant growth promotion in terms of root length, dry mass, chlorophyll, carbohydrates, nitrogen, calcium, iron, and manganese in pigeon pea. Tewari and Arora (2018) reported that the fluorescent Pseudomonads produced siderophore, HCN, IAA and enhance the growth of sunflower. In addition this bacteria inhibited the growth of *Macrophomina phaseolina*, a fungal pathogen.

In present study, isolates STJP, STJP12, STJP18, STJP20, STJP22, and STJP30 were found to belong to the genus *Bacillus* (Bergey's Manual of Systematic Bacteriology; Garrity 2005). *Bacillus* are Gram-positive, rod-shaped, obligate aerobe as well as facultative anaerobe, spore-forming bacteria, member of the Firmicutes phylum and omnipresent in nature (Radhakrishnan et al. 2017). Bacilli play several roles in the environment and industries and one of these is as PGPR, both direct and indirect (Radhakrishnan et al. 2017; Rees et al. 2019). *Bacillus* spp. can survive under stress conditions such as high saline, and high or low temperature or pH conditions due to the endospore forming property (Mishra et al. 2016). Many *Bacillus* and

*Pseudomonas* spp. were isolated and identified on the basis of phenotypic and biochemical characters from different medicinal plants by the researchers (Reyes et al. 1981; Fangio et al. 2010; Sandman and Ecker 2014; Celandroni et al. 2019). A lot of literature is available on *Bacillus* spp. and *Pseudomonas* spp. proving their tremendous roles in plant growth promotion and sustainable agriculture (Molina-Santiago et al. 2018). *Bacillus* is recorded as an effective genus belonging to PGPR group and are very well known for enhancement of crop growth and yield under biotic and abiotic stresses (Kumar et al. 2011). A beneficial PGPR *Bacillus pumilus* TUAT1 was used as a biofertilizer, for growth enhancement of rice (Ngo et al. 2019). *P. fluorescens* (Pf1) and *Bacillus subtilis* were found to be good plant growth promoters and recorded an enhancement of seed germination and plant growth (Bharati et al. 2004). The strains of *Bacillus* and *Pseudomonas* solubilized essential minerals like zinc, phosphate and potassium and enhanced growth and productivity of plants (Maheshwar and Sathiyawani 2012). Hence, *Bacillus* and *Pseudomonas* genus could be effective in the development of bioformulation and their applications are essential for food security, healthy foods, prevention of health hazards associated with chemical based fertilizers and pesticides, and for sustainable agriculture.

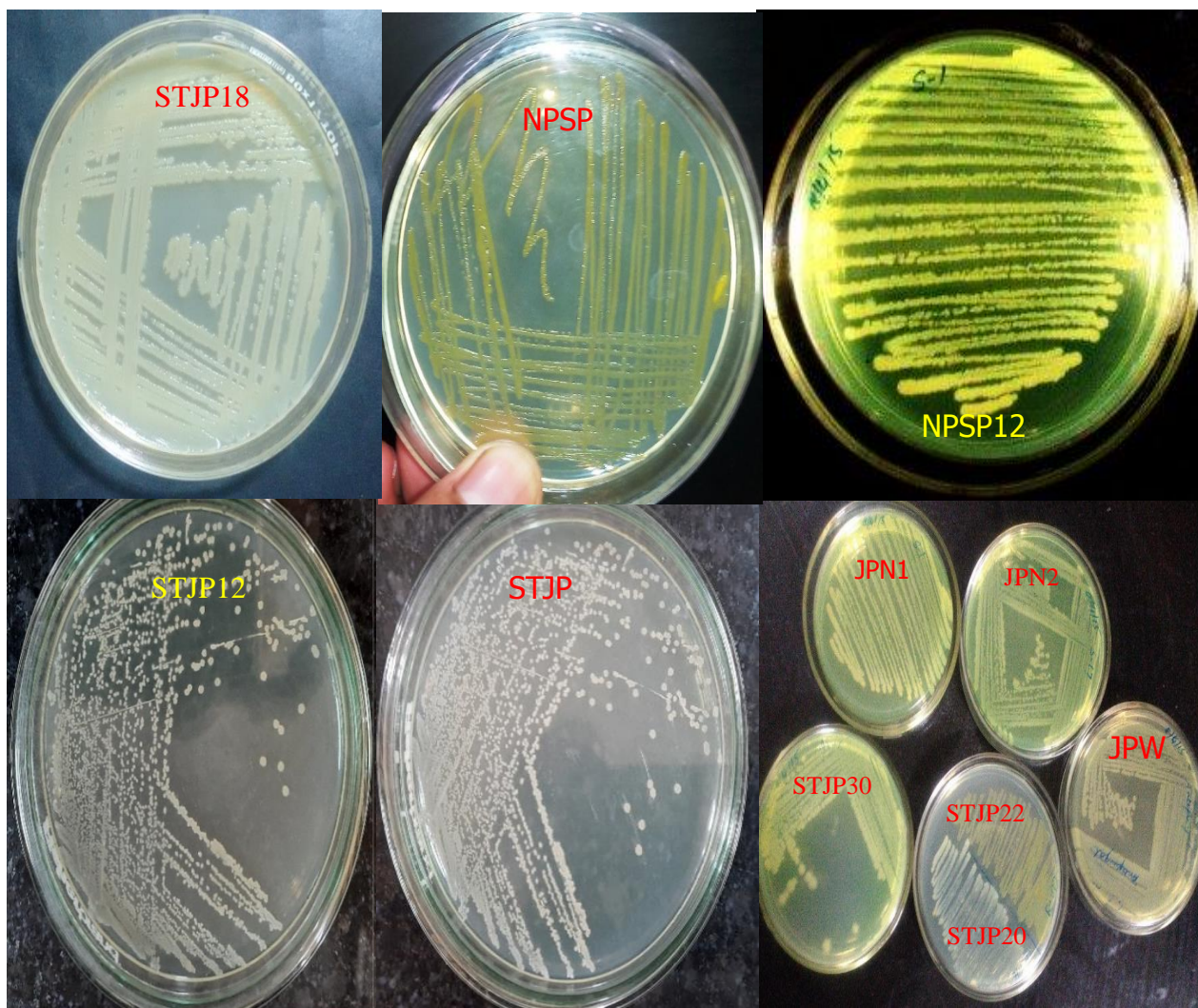
**Table 3.** Bacterial isolates and their hosts and sample collection sites

S. No.	Name of medicinal plant	Bacterial isolates	Naming of the Isolate	Sample collection sites
1.	<i>S. rebaudiana</i>	6	STJP, STJP12, STJP18, STJP20, STJP22, STJP30	CIMAP-Lucknow, U.P., India
2.	<i>Mentha arvensis</i>	1	NPSP	CIMAP-Lucknow, U.P., India
3.	<i>Aloe vera</i>	1	JPN1	BBA University Campus, Lucknow
4.	<i>Ocimum sanctum</i>	1	JPN2	BBA University Campus, Lucknow,
5.	<i>Calotropis gigantea</i>	1	NPSP12	South City, Lucknow
6.	<i>Withania somnifera</i>	1	JPW	South City, Lucknow

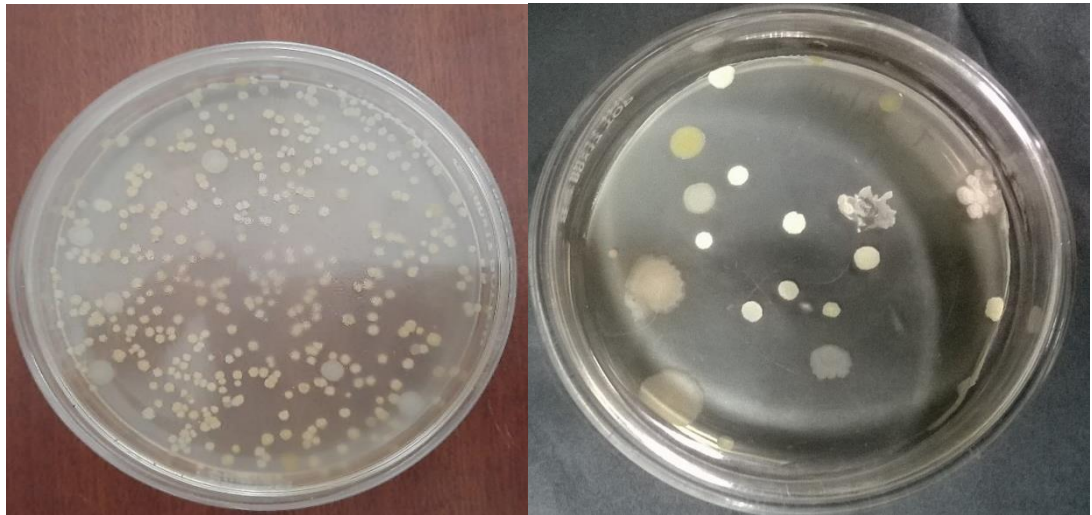
**Table 4.** Biochemical characterization of isolates

Bacterial isolates	JPN 1	JPN 2	JP W	NPS P	NPSP1 2	STJ P	STJP1 2	STJP1 8	STJP2 0	STJP2 2	STJP3 0
Gram staining	-	-	-	-	-	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	-	+	+
Lipase test	+	+	+	+	+	-	+	-	-	-	-
Urease test	+	+	+	-	+	-	-	-	-	-	-
Citrate utilization	-	-	+	+	-	+	+	+	+	+	+
Protease test	+	+	-	+	-	+	-	-	-	-	-
Sulphide test	-	-	-	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	-	-	-	-	-	-	-
Motility test	+	+	+	+	+	+	+	+	+	+	+
Gelatinase production	+	+	+	+	+	-	-	-	-	-	-
Glucose utilization	+	+	+	+	+	+	+	+	+	+	+
Fructose utilization	+	+	+	+	+	+	+	+	+	+	+
Sucrose utilization	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	-	+	+	+	+	+	+	+	+	+
Pigment production	<b>YGD</b>	<b>YGD</b>	<b>BGD</b>	<b>BGD</b>	<b>YGD</b>	<b>No</b>	<b>No</b>	<b>No</b>	<b>No</b>	<b>No</b>	<b>No</b>
Pyocyanin production	-	+	+	-	-	-	-	-	-	-	-
Pectinolytic activity	-	-	+	+	-	-	-	-	-	-	-
Levan production	+	+	-	-	+	-	-	-	-	-	-
Oxidase test	-	+	+	+	+	+	-	-	-	-	-
Arginine dihydrolase test	+	+	+	+	+	-	-	-	-	-	-
Growth at temperature 4°C	+	+	-	-	+	-	-	-	-	-	-
Growth at temperature 41°C	-	-	+	+	-	-	-	-	-	-	-
Endospore staining	-	-	-	-	-	+	+	+	-	-	-
Fluorescence test	+	+	+	+	+	-	-	-	-	-	-

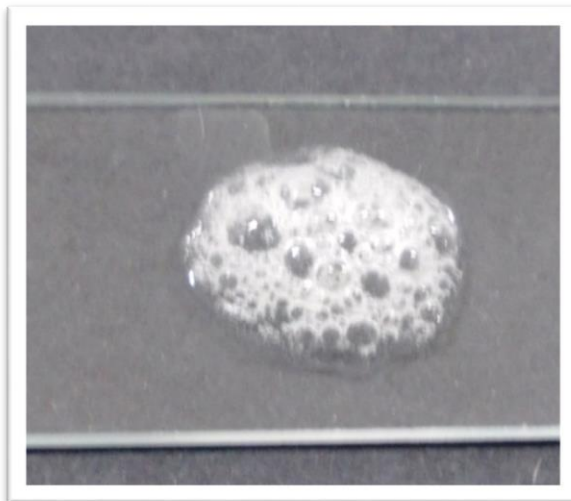
(Where +; positive result; -; negative result; BGD = Blue-green diffusible, YGD= Yellowish-green diffusible)



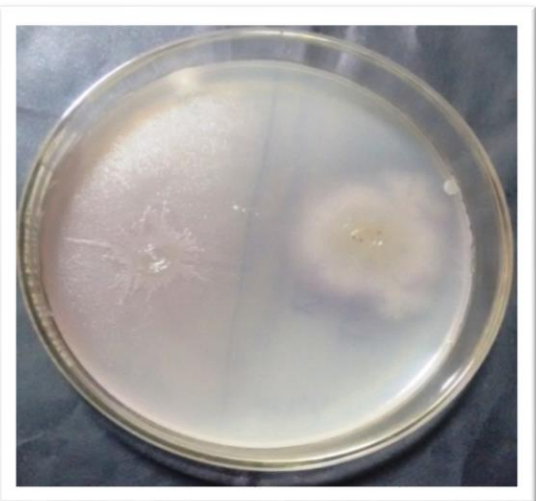
**Fig. 9** Pure culture of isolates



**Fig. 8.** Spreading plate showing bacterial colonies



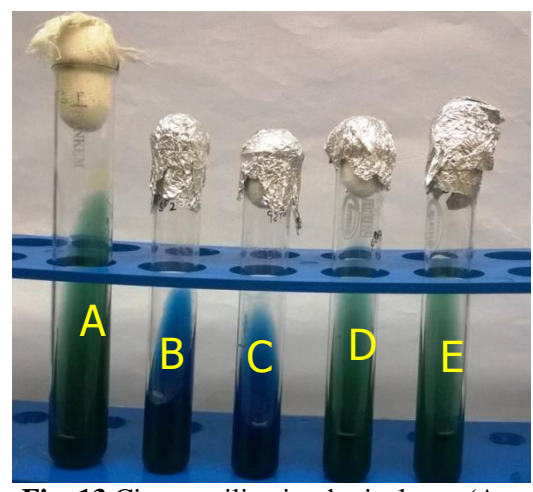
**Fig. 10** Catalase produced by NPSP



**Fig. 11** Lipase produced by NPSP



**Fig. 12** Urease test by isolates (A- control, B-JPN1, C-JPN2, D-JPW, E-NPSP12)



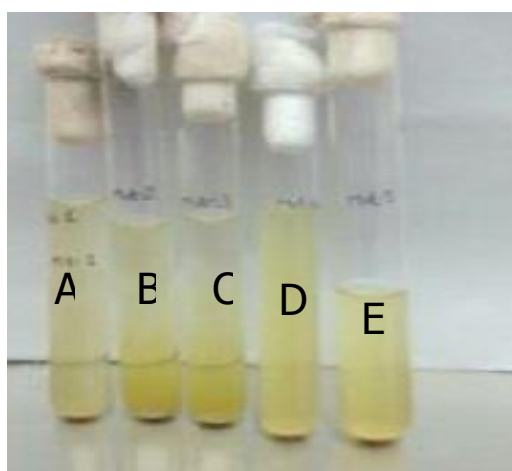
**Fig. 13** Citrate utilization by isolates (A- control, B-NPSP, C-STJP, D-JPN1, E-JPN2)



**Fig. 14** Protease test by STJP



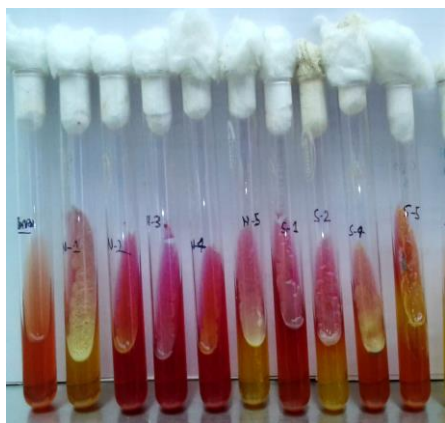
**Fig. 15** Motility test by isolates (A-control, B-STJP, C-NPSP, D-JPN1, E-JPN2)



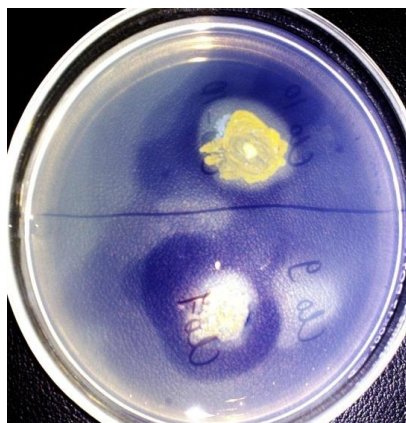
**Fig. 16** Hydrogen sulphide test by isolates (A-control, B-STJP, C-STJP12, D-JPW, E-STJP12)



**Fig. 17** Antibiotic sensitivity test by isolates



**Fig. 18** Sugar utilization by various isolates



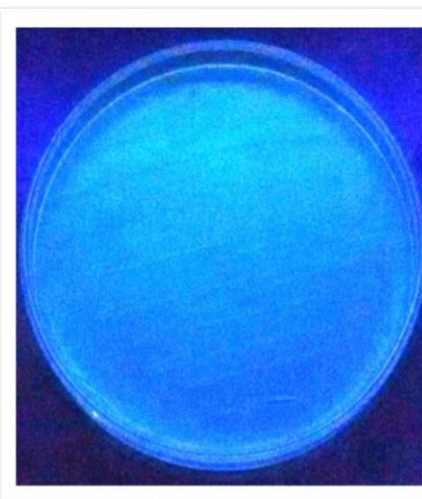
**Fig. 19** Starch hydrolysis by STJP and NPSP



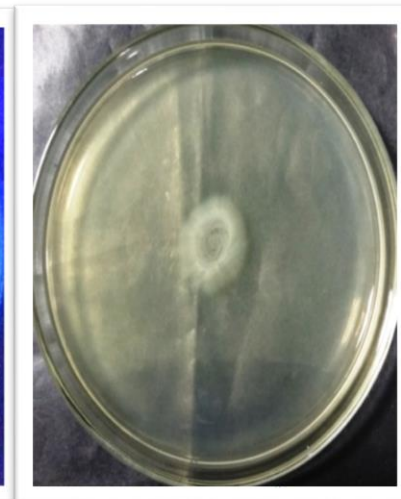
**Fig. 20** Salt tolerance (10%) activity by STJP



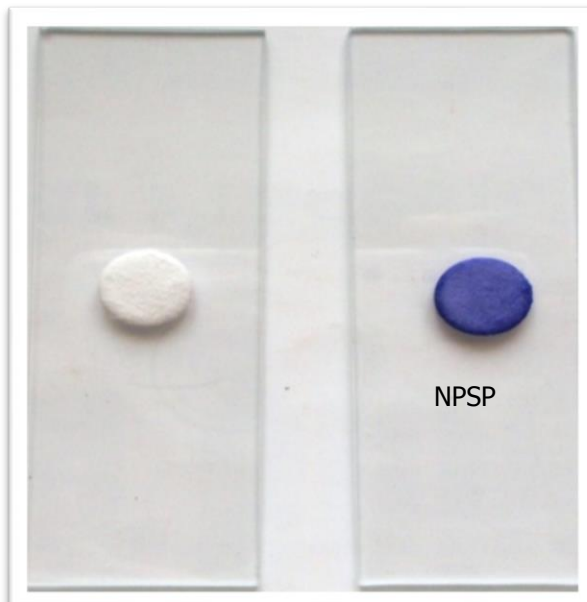
**Fig. 21** Fluorescein production by NPSP under U.V. rays



**Fig. 22** Pyocyanin production by JPW



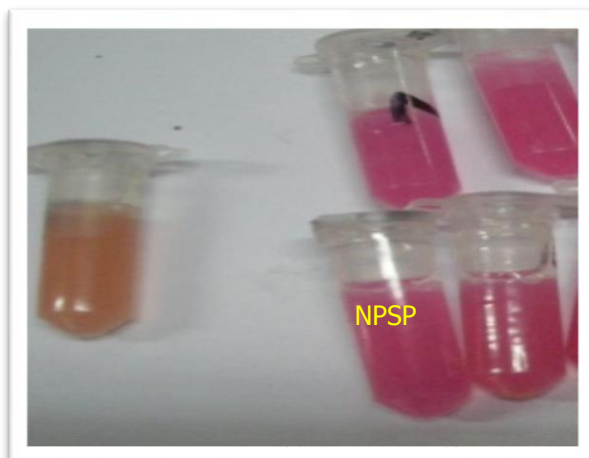
**Fig. 23** Levan production by NPSP



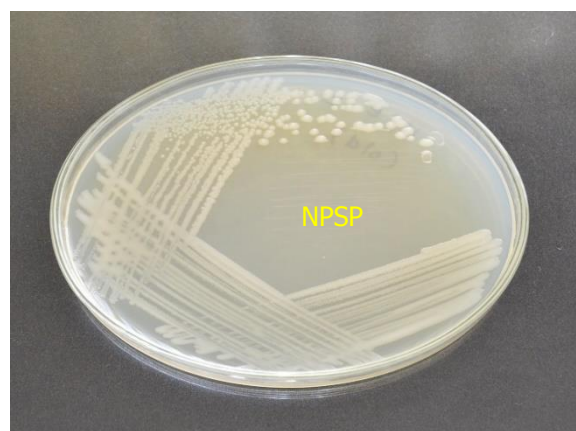
**Fig. 24** Oxidase production by NPSP



**Fig. 25** Pectinolytic activity shown by NPSP



**Fig. 26** Arginine dihydrolase shown by NPSP



**Fig. 27** Growth of bacterium NPSP at 41°C

*Chapter-05*  
*Screening of Isolated Rhizobacteria for  
their PGP Traits as Well as Biocontrol  
Activity*



## **1. Introduction**

Chemical based fertilizers and pesticides are used to enhance the growth and productivity of agricultural crops from several years. Although, these chemicals increase the crop yield, but also affect the soil microflora, deteriorating the quality of agro-ecosystems and aquatic resources and cause several health hazards (Oves et al. 2012). Most of the chemicals persist in the soil in insoluble form and cause biodiversity loss (Sharma et al. 2013). Apart from this, the accumulated chemical based inoculants in the soil may also reach in the water bodies, causing eutrophication (Ayangbenro and Babalola 2017). In addition, loss of soil fertility and soil salinity increase due to use of a large amount of fertilizers and pesticides in the agricultural soils (Saraf et al. 2014).

To overcome this problem, an economically attractive and eco-friendly approach of using PGPR as biofertilizers and biopesticides is increasing globally. PGPR can be important biofertilizers due to their abilities to fix nitrogen, solubilize phosphates from the soil, production of phytohormones such as indole-3 acetic acid (IAA), gibberellic acid, and secretion of iron chelators such as siderophores, apart from solubilization of several other minerals including zinc (Zn) and potassium (K) and are foremost to eco-friendly and sustainable agriculture (Alori et al. 2017; Prakash and Arora 2019). PGPR can also protect host plants indirectly and can be utilized as biopesticides by inhibition of fungal phytopathogens. This is achieved by secretion of antibiotics, hydrogen cyanide, lytic enzymes such as chitinases,  $\beta$ -1, 3 glucanase and salicylic acid (Nagarajkumar et al. 2004). Besides these, PGPR can also tolerate or work at high temperature, variable pH, and high saline conditions as reported by several researchers (Kumar et al. 2011; Kumar et al. 2018; Tewari and Arora 2018). In this way PGPR can also protect the plant from abiotic stresses.

PGPR based inoculants can be cheaper and are of particular interest in the nutrient deficient soils, and in presence of fungal disease(s) (Shen et al. 2016). Among PGPR, members of the genus *Pseudomonas* and *Bacillus* are widely used on the agricultural crops for enhancement of yield and protection against fungal pathogens (Kumar et al. 2001; Bais et al. 2004). Both genera are reported to facilitate the plant growth by

solubilization of minerals, production of metabolites and phytohormones with excellent colonizing property of the roots (Ahmad et al. 2018). In addition, *Bacillus* and *Pseudomonas* have added advantage as their communities, found in the rhizosphere of crops can survive stress conditions very well (Abd\_Allah et al. 2018). Use of *Bacillus* and *Pseudomonas* spp. to increase crop production can be a very important practice for environment friendly, durable agriculture and control of phytopathogens (Alori et al. 2017). *Pseudomonas* and *Bacillus* are excellent PGPR due to their ability to several important PGP metabolites such as siderophores, phytohormones, growth hormones and mineral solubilisation abilities (Souza et al. 2015; Mukhtar et al. 2017). Members of these genera are also very good biocontrol agents because they are known to produce different types of antibiotics, hydrogen cyanide, antifungal toxins, antifungal enzymes etc. (Rijavec and Lapanje 2016; Gómez et al. 2017). In the present study rhizobacteria isolated from different medicinal plants were checked for the production of PGP properties and metabolites for further selection of suitable strains so as to use them in fields.

## **2. Materials and Methods**

### **2.1 Plant growth promoting characteristics**

#### **2.1.1 Indole-3 acetic acid production**

Log phase cultures of all the eleven bacterial isolates were inoculated in the nutrient broth containing glucose (5.0 g/l), yeast extract (0.025 g/l), L-tryptophan (0.204 g/l) (Brick et al. 1991). Inoculated broth was incubated at 28°C for 72 h. After incubation, the broth was centrifuged and supernatant taken. Further, 2 ml Salkowski reagent (2% of 0.5 M FeCl<sub>3</sub> in 35% H<sub>2</sub>SO<sub>4</sub> solution) was added into culture supernatant and incubated for 30 minutes. The color change of the sample, after incubation, was observed for IAA production (Brick et al. 1991). The changed color of the sample was analyzed by spectrophotometer (Thermo scientific-Evolution 201) at 530 nm for quantitative estimation of IAA by using standard curve.

#### **2.1.2 Gibberellic acid production**

The overnight grown culture of bacterial isolates were inoculated in nutrient broth. Inoculated broth was incubated for 48 h at 28°C. After incubation, the culture broth was centrifuged at 10,000 rpm (15 min at 4°C). The pH of the obtained supernatant was maintained at 2.5. Subsequently, extraction of the supernatant was done through ethyl acetate and sodium hydro-carbonate (NaHCO<sub>3</sub>) as per liquid-liquid extraction method

(Berríos et al. 2004). The amount of gibberellic acid was examined from the obtained supernatant at 254 nm by UV spectrophotometer (Berríos et al. 2004). For standard, gibberellic acid was procured from the Sigma-Aldrich.

### **2.1.3 Phosphate solubilization**

The solubilization of phosphate activity was checked by using Pikovskaya's agar (Pikovskaya 1948). Briefly, the log phase culture was spotted on the Pikovskaya's agar plates containing 0.5% tri-calcium phosphate (TCP) and incubated for 24-72 h at 28°C for checking phosphate solubilization. Afterward, isolates were also grown for 24-72 h in National Botanical Research Institute Phosphate (NBRIP) broth medium at 30°C to check the amount of phosphate solubilization. The soluble P content ( $\mu\text{g ml}^{-1}$ ) was determined by spectrophotometer at 600 nm (Nautiyal 1999).

### **2.1.4 Phosphatase enzyme assay**

The phosphatase enzyme assay was done as per the method of Tabatabai and Bremner (1969). Briefly, 100  $\mu\text{l}$  bacterial culture was inoculated in NBRIP broth (50 ml) and incubated at 30°C for 7 days. After incubation (24 h), the broth culture was centrifuged (10,000 rpm for 10 min at 4°C) and the supernatant collected. One ml of supernatant was mixed with modified universal buffer (4 ml; pH: 11) and subsequently, disodium p-nitrophenol phosphate (1 ml; 0.025 mM) was added in the supernatant and incubated at 28°C for 60 min. After incubation, 1 drop of toluene was added and further incubated (for 1 h at 37°C) and finally 0.5 M of  $\text{CaCl}_2$  and NaOH (ratio: 1:4) was mixed for inhibition of the reaction. The obtained sample was filtered via Whatman filter paper no 42 and quantity of enzyme was examined in 1  $\mu\text{mol}$  of p-nitrophenol/ml/min by spectrophotometer (at 420 nm). For standard, p-nitrophenol was used for the quantification of alkaline phosphatase.

### **2.1.5 Zinc solubilization**

To check Zn solubilization, modified Pikovskaya's medium (containing (gm/l) glucose 1, ammonium sulphate 1, dipotassium hydrogen phosphate 0.2, magnesium sulphate 0.1, yeast extract 0.2) was prepared and autoclaved. Afterward, 0.1% of zinc oxide, zinc sulfate, and zinc carbonate were added (separately) and plates were allowed to cool. Later a loopful bacterial culture was spotted on the modified Pikovskaya's medium plates and incubated at 30°C for 48-72 h (Pikovskaya 1948). The clear zone appearance around the colony was observed for zinc solubilization by the isolates.

For quantitative estimation of Zn, the fresh culture of isolates were inoculated in minimum salt broth medium (MSM) containing [(g<sup>-L</sup>); KCl: 0.2, K<sub>2</sub>HPO<sub>4</sub>: 0.1, MgSO<sub>4</sub>: 0.2, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>: 1, dextrose: 10], and inorganic Zn compound (ZnO: 0.1g/L; Zn SO<sub>4</sub>: 0.1g/L; ZnCO<sub>3</sub>: 0.1g/L) (Sigma-Aldrich) added into separate flask and pH was maintained at 7.0 (Saravanan et al. 2004). The inoculated broth was incubated at 120 rpm and 28°C for 5 days. Afterward (24 h incubation), the culture broth was centrifuged and supernatant was taken and checked through atomic absorption spectrometry (AAS) (AA240FS Fast Sequential AAS, USA) for solubilisation of available Zn content (Fuwa et al. 1964).

### 2.1.6 Potassium solubilisation

In this experiment, Aleksandrov medium (g<sup>-L</sup>; K<sub>2</sub>HPO<sub>4</sub>.3H<sub>2</sub>O: 0.2, CaPO<sub>4</sub>: 0.2, CaCO<sub>3</sub>: 0.01, MgSO<sub>4</sub>.7H<sub>2</sub>O: 0.05, C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>: 0.5, FeCl<sub>3</sub>: 0.0005, pH: 7.2) supplemented with mica powder (0.1 g/L) (Sigma, USA) as the sole source of insoluble potassium (K) was taken to check the ability of K solubilization by the isolates (Hu et al. 2006). The fresh culture of isolates was spotted on the sterilized Aleksandrov agar and incubated at 28°C for 72h. After incubation, solubilisation of K was observed qualitatively as a clear zone around the spotted bacterial colony.

For quantitative estimation, log phase of isolates were inoculated in Erlenmeyer flask (EF) containing 100 ml Aleksandrov broth (Ab) containing mica powder and incubated (120 rpm, 30°C, 120 h); for control, uninoculated broth was taken. After incubation, Aleksandrov broth was centrifuged (REMI CM-12 PLUS) for 10 min at 10,000 rpm and collected supernatant was used for examination of soluble K by AAS (AA240FS Fast Sequential AAS, USA) at 766.5 nm (Parmar and Sindhu 2013).

### 2.1.7 Nitrogen fixation

Nitrogen fixation was checked by all the selected isolates on the nitrogen free Jensen's media (Jensen 1942). Briefly, a loopful culture of isolates were streaked on the nitrogen-free Jensen's media containing bromothymol blue (an indicator dye) and incubated (48h, 28°C). After incubation, result was checked, the coloured zone around the streaked colony is indicative of positive result.

### 2.1.8 Ammonia production

For examination of ammonia production, peptone water medium was poured into test tubes (5 ml) and autoclaved. The log phase culture of the isolates was inoculated in the test tubes containing peptone medium and incubated for 72 h at 28°C. After incubation,

one ml of Nessler's reagent was added into each culture tubes. The intensity of the color change in the test tubes indicates the level of ammonia production (Marques et al. 2010).

### **2.1.9 Siderophore production**

#### **2.1.9.1 Chrome-azurol S (CAS) assay**

Selected isolates were examined for siderophore production as per Schwyn and Neilands (1987). For the preparation of siderophore media/blue agar media, CAS dye (60.5 mg) was dissolved in distilled water (50 ml) and properly mixed with  $\text{Fe}^{3+}$  solution (1 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 10 mM HCl). This solution was slowly added with 72.9 mg HDTMA dissolved in 40 ml water. After the development of a blue color, the media was autoclaved. In addition, another solution containing distilled water: 750 ml, pipes buffer: 30.24 gm, agar: 30.24 gm, NaOH solution: 12 gm of 50 % (w/v) to make the final pH 6.8, was separately autoclaved. Subsequently, 30 ml casamino acid (10 %) was added into the sterilized solution at the temperature of 50°C. The developed dye solution was further added in blue color media with sufficient agitation to attain mixing without generation of foam. The media was then poured in Petri plates. Isolates were spotted on the prepared media and result was measured for color change of the medium from blue to orange after incubation.

#### **2.1.9.2 Carboxylate type of siderophore**

For quantifying carboxylate type of siderophore, isolates were inoculated in nutrient broth (NB) and incubated for 72 h at 28°C (130 rpm). After incubation, the culture was centrifuged to obtain supernatant (5000 rpm, 15 min at 4°C) and the collected sample was taken to examine the siderophore production (Reeves et al. 1983). The siderophore quantity was expressed in  $\mu\text{mol}$  benzoic acid/ml.

#### **2.1.9.3 Hydroxamate type of siderophore**

The fresh culture of isolates were grown in the iron-deficient Grimm-Allen broth medium containing (gm/l) citric acid: 1; sucrose: 20; ammonium acetate: 3,  $\text{K}_2\text{HPO}_4$ : 3,  $\text{K}_2\text{SO}_4$ : 1, and pH was maintained at 6.8. Further, thiamine and  $\text{ZnCl}_2$  were added in the broth (Grimm and Allen 1954). Afterward, the broth was centrifuged and supernatant collected. In the supernatant, freshly prepared 2% of aqueous  $\text{FeCl}_3$  solution was added in the ratio of 1:1 and the absorbance was read between 420-450 nm by spectrophotometer (Neilands 1981)

## **2.2 Biocontrol activities of isolates**

### **2.2.1 HCN production**

For hydrogen cyanide (HCN) production, the overnight grown culture was taken and streaked on the nutrient and King's B plates containing glycine 4.5 gm/l (Bakker and Schipper 1987). The lid of the Petri plate was covered with filter paper (Whatmann no. 42) which was dipped in 0.5% picric acid in 2% sodium carbonate solution. Then the plates were sealed with parafilm and incubated at 28°C for 72 h. The color change was observed on the filter paper for an indication of HCN production (Bakker and Schipper 1987). For the quantitative estimation of HCN, the filter paper was removed from the lid of the Petri plate and dissolved in 10 ml sterile water and absorbance was measured at 625 nm (Meena et al. 2001).

### **2.2.2 Salicylic acid production**

Overnight grown culture was inoculated in 50 ml of succinate medium containing (gm/l) succinic acid: 4, K<sub>2</sub>HPO<sub>4</sub>: 6, KH<sub>2</sub>PO<sub>4</sub>: 3, NH<sub>2</sub>SO<sub>4</sub>: 1, MgSO<sub>4</sub>: 0.2 and distilled water: 1000 ml in the conical flask (250 ml) and left for incubation in a rotary shaker at 28°C for 48 h (Meyer and Abdallah 1978). The medium was then centrifuged at 6000 rpm for 5 min. After centrifugation, 4 ml of cell-free culture supernatant was taken in the test tubes and acidified with 1N HCl to maintain at pH at 2, and the acidic supernatant was extracted using chloroform (CHCl<sub>3</sub>) solvent. Afterward, 5 µl of 2M FeCl<sub>3</sub> was added in the sample and the changed color of the sample was observed for the production of salicylate. The developed color was further read at 527 nm for quantitative estimation of salicylic acid by spectrophotometer (Evolution 201, Thermo Scientific, USA).

### **2.2.3 Lytic enzymes production**

#### **2.2.3.1 Chitinase activity**

Isolates were grown in chitin-peptone medium (peptone: 0.2%, glucose: 0.5%, colloidal chitin: 0.2% (prepared in the lab), K<sub>2</sub>HPO<sub>4</sub>: 0.1%, NaCl: 0.05%, MgSO<sub>4</sub>.7H<sub>2</sub>O: 0.05% and pH: 6.9) and incubated for 96 h at 28°C (Lim et al. 1991). After incubation, culture medium was centrifuged (12,000 rpm for 20 min at 4°C) and supernatant taken as enzyme source. Further, 0.25 ml of the obtained supernatant, 0.5 ml colloidal chitin and 0.3 ml of 1M sodium acetate buffer (pH 5.3) was mixed for reaction and incubated in the water bath (4 h at 50°C). The chitinase activity was examined by calculating the release of reducing sugar (Nelson 1944). One unit of chitinase was reported as 1 nmol

of glucanase per minute per mg of protein. For the standard of protein, bovine serum albumin was used (Bradford 1976).

### 2.2.3.2 $\beta$ -1, 3 glucanase activity

Isolates were grown in peptone medium amended with laminarin [(0.2%) (Sigma)] at 28°C for 96 h as per Lim et al. (1991). After incubation, growth media was centrifuged (12,000 rpm for 20 min at 41°C) and supernatant was taken as enzyme source. Further, 0.3 ml of phosphate buffer (0.1 M) and 0.5 ml of laminarin (0.2%) were added in obtained enzyme source (0.3 ml) for reaction mixture (Lim et al. 1991). This mixture was further incubated for 2 h at 40°C in the water bath. For determination of  $\beta$ -1, 3 glucanase, a prepared sample was measured as 1 nmol of glucose released per minute per mg of proteins. For the standard of protein, bovine serum albumin was used (Bradford 1976).

### 2.2.3.3 Cellulase activity

Pure culture of isolates was spotted on carboxymethyl cellulose (CMC) medium containing ( $\text{gm/L}^{-1}$ )  $\text{KH}_2\text{PO}_4$ : 0.5,  $\text{MgSO}_4$ : 0.25, cellulose: 0.25, gelatin: 2 and agar: 20, respectively. Afterward, bacterial culture was incubated at 28°C. After incubation, iodine solution was added for the confirmation of enzyme activity (Aneja 2005). The presence of a clear zone around the spotted colony was observed for cellulase activity.

## 2.3 Statistical analysis

All experiments were done taking five replica and data were represented as the mean  $\pm$  standard deviation (SD). To check the result validity, an analysis of variance (ANOVA) (Gomez and Gomez 1984) was done and the finally Duncan's multiple range test ( $p < 0.05$ ) was implemented to check the significant differences of different sets.

## 3. Results

### 3.1 Plant growth promoting characteristics

#### 3.1.1 IAA production

All isolates produced IAA (Figure 28). Among them, isolate STJP produced highest amount of IAA (30.59  $\mu\text{g/ml}$ ) followed by NPSP (28.59  $\mu\text{g/ml}$ ). Isolates STJP20, JPN2, STJP22, JPW, NPSP12, JPN1, STJP30, STJP18, and STJP12 also produced IAA at the level of 19.32  $\mu\text{g/ml}$ , 18.23  $\mu\text{g/ml}$ , 18.23  $\mu\text{g/ml}$ , 15.28  $\mu\text{g/ml}$ , 15.36  $\mu\text{g/ml}$ , 8.39  $\mu\text{g/ml}$ , 8.36  $\mu\text{g/ml}$ , and 5.36  $\mu\text{g/ml}$ , respectively (Table 5).

### 3.1.2 Gibberellic acid production

Among all, six isolates JPN1, JPN2, STJP12, STJP18, STJP20, and STJP22 produced less than 5 µg/ml of gibberellic acid. Among all isolates, STJP produced higher gibberellic acid (9.60 µg/ml) followed by NPSP (8.22 µg/ml). Isolate JPN1 produced minimum quantity of gibberellic acid as compared to other isolates (Table 5).

### 3.1.3 Phosphate solubilization

All isolates solubilized phosphate (Figure 29). Among them, six isolates showed greater than 6 mm zone of phosphate solubilization on the PKV agar plates. Further, isolates were screened for the quantity of phosphate solubilization on NBRIP. All isolates were able to carry out phosphate solubilization ranging from 21 to 610.33 µg ml<sup>-1</sup> (Table 5). Among them, isolate STJP showed highest phosphate solubilizing activity (610.33 µg ml<sup>-1</sup>) followed by NPSP (325 µg ml<sup>-1</sup>).

### 3.1.4 Phosphatase enzyme assay

The production of alkaline phosphatase by isolates were checked in NBRIP medium (on the basis of phosphate solubilization ability). Isolate STJP produced maximum phosphatase enzyme (81.45 U/ml) after 96 h incubation period which was further reduced with increasing of the incubation period. Other isolates did not show significant amount of enzyme production as compared to STJP.

### 3.1.5 Zinc solubilization

Isolate STJP solubilized maximum amount of ZnO (5.40 mg/l), ZnSO<sub>4</sub> (3.20 mg/l), and ZnCO<sub>3</sub> (3.80 mg/l), after 4 days of incubation (Table 6; Figure 30 and Figure 31), but further increment of incubation time brought a reduction in the solubilization of Zn. NPSP also solubilized ZnO, ZnSO<sub>4</sub>, and ZnCO<sub>3</sub>, however, it was significantly lower as compared to isolate STJP. Remaining isolates did not show a significant quantity of Zn solubilization.

### 3.1.6 Potassium solubilization

Isolate STJP solubilized maximum K (4.41 mg/l) at 5 days of incubation (Figure 32) as compared to other isolates, but further increase in the incubation time did not significantly affect the solubilization of K. After STJP, NPSP solubilized good quantity of K (2.24 mg/l). Four isolates (JPN2, STJP18, STJP20, and STJP30) did not solubilize K in Aleksandrov broth.

### 3.1.7 Nitrogen fixation ability

Three isolates JPN1, JPN2, and STJP12 showed a positive result for nitrogen fixation ability, while remaining isolates including STJP did not fix nitrogen (Figure 33).

### 3.1.8 Ammonia production

The result was observed as the intensity of the color (yellow) as a positive indication for ammonia production (Figure 38). STJP produced ammonia but result was not better than STJP20. In this study, remaining isolates were given very limited amount of ammonia (Table 5).

### 3.1.9 Siderophore production

The positive result of siderophore was confirmed by a change in color of the media from blue to orange due to iron chelation (Figure 37). Among all isolates, STJP produced carboxylate (16.06  $\mu\text{g/ml}$ ) and hydroxamate types (3.21  $\mu\text{g/ml}$ ) of siderophore and this result was recorded highest as compared to other isolates. The minimum carboxylate (1.12  $\mu\text{g/ml}$ ) and hydroxamate (0.12  $\mu\text{g/ml}$ ) types of siderophore production were recorded by isolate STJP22 (Figure 38; Table 6). A total of five isolates (JPN1, JPN2, JPW, NPSP12, and STJP12) did not produce carboxylate type of siderophore. Three isolates (NPSP12, STJP12, and STJP22) were unable to produce hydroxamate type of siderophore (Table 6).

## 3.2. Biocontrol activities

### 3.2.1 HCN production

Bacterial isolates NPSP and STJP did not show HCN production while other isolates (JPN1, JPN2, JPW, NPSP12, STJP12, STJP18, STJP22, and STJP30) produced HCN (Figure 34). Among all isolates, JPN1 showed maximum HCN production followed by STJP22 as confirmed by a color change of filter paper from yellow to brown (Table 6).

### 3.2.2 Salicylic acid production

Isolate NPSP (16.80  $\mu\text{g/ml}$ ) produced highest amount of salicylic acid followed by STJP (12.80  $\mu\text{g/ml}$ ) (Figure 35, Table 7). Isolate STJP22 recorded lowest production of salicylic acid. However, four isolates (NPSP2, STJP12, STJP18, and STJP20) were unable to produce salicylic acid (Table 7)

### 3.2.3 Lytic enzymes production

#### 3.2.3.1 Chitinase activity

All (but one) isolates showed chitinase activity (in between 6.38 nmol/min/mg to 75.58 nmol/min/mg). Isolate STJP (75.58 nmol/min/mg) recorded highest chitinase activity followed by NPSP (02.36 nmol/min/mg) (Table 7). Only one isolate NPPsP2 was unable to show chitinase activity.

#### 3.2.3.2 $\beta$ 1, 3 glucanase

All the isolates were tested for  $\beta$ -1, 3 glucanase activity. Among all, isolate STJP was found to be maximum producer of  $\beta$ -1, 3 glucanase (220.36 nmol/min/mg) followed by NPSP (48.50 nmol/min/mg) (Table 7). Minimum amount of  $\beta$ -1, 3 glucanase was recorded by STJP30 (2.35 nmol/min/mg) and two isolates (NPPsP2 and STJP20) were unable to produce  $\beta$ -1, 3 glucanase.

#### 3.2.3.3 Cellulase activity

Among all, isolate NPSP and STJP showed a positive result for cellulase production and their result was confirmed by the presence of a clear zone around the spotted colony (Figure 39).

## 4. Discussion

Rhizobacteria have been extensively used for enhancement of plant growth and control of disease against the fungal pathogens (Beneduzi et al. 2012). On the basis of plant growth promoting characters isolates NPSP and STJP were found to be the best PGPR (amongst the 11 isolates). As mentioned in the previous chapter, on the basis of morphology and biochemical tests, isolates NPSP and STJP maybe *Pseudomonas* sp. and *Bacillus* sp (Bergey's Manual of Systematic Bacteriology; Garrity 2005). In the present study, two of the isolates NPSP and STJP produced a significant amount of IAA at the level of 28.59  $\mu$ l, and 30.59  $\mu$ l, respectively. IAA is a major plant growth hormone which helps in several stages of plant growth such as cell division and enlargement, pigment formation, responses to light and gravity, resistance to stressful conditions and tissue differentiation (Myo et al. 2019; Kang et al. 2019). Many rhizobacteria belonging to the genera *Bacillus* and *Pseudomonas* are able to synthesize IAA and influence the plant growth by increasing plant growth and crop yields (Hazzoumi et al. 2014; Lyu et al. 2019).

Isolates NPSP and STJP produced a significant quantity of gibberellic acid (9.60 $\mu$ l, and 8.22 $\mu$ l) and solubilized phosphate (610.33 $\mu$ l, 325 $\mu$ l). Gibberellic acid is

another phytohormone, which plays a key role in the enhancement of plant growth by upregulation of cell elongation, cell division, growth of hypocotyl and stem, size of leaf and root meristematic tissue (Gupta and Chakrabarty 2013). These two genera (*Pseudomonas* and *Bacillus*) of PGPR are often associated with higher plants, producing gibberellic acid and improving the growth and yield of crops (Lyu et al. 2019). Integrated phytohormones production by the plant growth promoting bacterium (*Bacillus tequilensis*) was found to increase growth of soybean crop under biotic and abiotic stress conditions (Kang et al. 2019). Phosphorus (P) is the second most important macronutrient in the soil (after nitrogen), which is required to be added to soil so as to maintain growth and development of the plant. In agricultural soil, most of the P occurs in insoluble form such as ferric phosphate ( $\text{FePO}_4$ ) and aluminium phosphate ( $\text{AlPO}_4$ ) and this insoluble form cannot be utilized by the plants (Wang et al. 2017). It has been reported that *Bacillus* spp. are reported to be good producers of IAA along with being phosphate solubilizer (Walpola and Yoon 2013; Prakash and Arora 2019). Scientists are trying day by day to investigate an effective IAA producing rhizobacterial strain (Mukhtar et al. 2017) and also having other PGP traits like phosphate solubilizer (Prakash and Arora 2019). PGPR have ability to solubilize inorganic phosphate by one of the several mechanisms such as producing phosphatase enzyme, releasing organic acids (gluconic, oxalic and succinic acids), ion exchange and chelation method (Sharma et al. 2013; Behera et al. 2017; Wei et al. 2018). Our report displayed that isolate STJP was found to be most effective in solubilization of phosphate and simultaneously showed several other PGP characters (better than other isolates). Simultaneous phytohormone production and phosphate solubilization activities that too in significant amounts makes STJP a very important and effective PGPR.

In the present study, isolate STJP produced highest phosphatase enzyme (81.45 U/ml) at 96 h of incubation as compared to other isolates. There are several studies on the production of phosphatase by *Bacillus* spp. (Behera et al. 2017; Prakash and Arora 2019). The phosphatase enzyme breaks insoluble phosphate especially tricalcium phosphate (TCP) into phosphate ions and thus are easily taken up by plant's roots (Sharma et al. 2013).

Siderophores play an important role in chelation of micronutrients such as iron even under limiting conditions (Arora and Verma 2017). On the basis of the functional group, siderophore can be divided into carboxylate, catecholate and hydroxamate types.

In the current study, isolate STJP and NPSP produced carboxylate (16.06  $\mu\text{l}$ , 4.23  $\mu\text{l}$ ) and hydroxamate type (3.21  $\mu\text{l}$ , 2.25  $\mu\text{l}$ ) of siderophores. *Pseudomonas* and *Bacillus* are very well known to produce different types of siderophores (Santoyo et al. 2012). In fact production of siderophores provides a great advantage to these very important PGPR in colonizing the rhizosphere region of diverse crops (Bhattacharyya and Jha 2012). Walpola and Yoon (2013), reported that *Pseudomonas agglomerans* and *Bacillus anthina* as good siderophore producers. Siderophores also help in chelation of several other micronutrients (apart from iron)  $\text{Al}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Zn}^{2+}$  (Ahmed and Holmström 2014). These micronutrients are very essential for plant growth and productivity (Arora and Verma 2017). Siderophores also play very important role in biocontrol of fungal phytopathogens by starving them off iron (Li et al. 2014).

Apart from the above mentioned PGP characters, isolates solubilized K and Zn in *in-vitro* conditions. Previous studies also supported that *Bacillus* spp. and *Pseudomonas* spp. are involved in the solubilization of K and Zn minerals in *in-vitro* as well as in the soil system (Shakeel et al. 2015; Etesami et al. 2017). K is the third most essential nutrient in the soil (macro), the eighth most abundant element on earth's crust, most abundant cation in plant cells and most ample nutrient in leaves after nitrogen (N) (Wang et al. 2013; Hasanuzzaman et al. 2018). K 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, increase root, and growth regulation of crops (Wang et al. 2013). In addition, K plays a positive role in the physiological status and enzymatic activities of the plant and the carbohydrate metabolism (Bräsen et al. 2014). Zn is another essential micronutrient, mandatory for normal growth and yield of plant by playing an imperative role in physiological processes such as regulation of IAA, role in plant immune system, repair of cellular membranes, carbohydrate metabolism, regulation of auxin synthesis, components of protein and enzymatic reactions (Dinesh et al. 2015). *Bacillus* spp. and *Pseudomonas* spp. are well known to solubilize Zn and its uptake in plants resulting in the improved growth, productivity and nutrients quality (Kamran et al. 2017).

In the present study, some of the isolates were able to produce HCN. It is a volatile compound produced by many PGPR and plays a significant role in the inhibition of fungal pathogens. HCN producing PGPR mainly belong to the genus of *Pseudomonas* and *Bacillus*. Some species of *Bacillus* and *Pseudomonas* are well known

to produce HCN and their roles as antagonistic biocontrol agents against fungal phytopathogens are very well known (Lateef et al. 2015).

In the current study, isolate NPSP produced high amount (16.80  $\mu$ l) of salicylic acid. Salicylic acid is a natural phenolic compound present in many plants and its role is recorded in the growth promotion of plant by inhibition of pathogens (Meyer et al. 1992). It is a chief plant growth hormone, prevents the entry of fungal phytopathogens inside the plants (Dieryckx et al. 2015) and induces systemic resistance (ISR) against phytopathogens (Beneduzi et al. 2012). Salicylic acid produced by *P. fluorescens* and *Bacillus amyloliquefaciens* CNU114001 plays an important role in the growth inhibition of *Rhizoctonia solani*, and *Alternaria* sp. phytopathogens (Nagarajkumar et al. 2004; Kang et al. 2015). Mandal and Mallick (2009) demonstrated that exogenous application of salicylic acid on tomato crops enhanced ISR against *Fusarium oxysporum* sp. *lycopersici*.

Isolate STJP showed the highest chitinase activity (75.58 nmol/min/mg) as compared to other isolates. Chitinase is a cell wall (fungal) degrading enzyme, belonging to glycosyl hydrolase family, breakdowns the glycosidic bond present in the chitin, and mainly secreted by rhizobacteria (Kumar et al. 2018). This enzyme can augment the plant's defence system by inhibition of fungal phytopathogens due to degradation of chitin present in their cell wall and their role is further reported in the enhancement of plant growth and productivity (Kumar et al. 2018). Many rhizobacteria including pseudomonads use chitin as an energy source by degrading it with the help of chitinase. Isolate STJP and NPSP produced  $\beta$ -1, 3 glucanases as well, at 220.36 nmol/min/mg, 148.50 nmol/min/mg, respectively. This enzyme has the ability to breakdown glycosidic bond in the cell wall of fungal pathogens and also helps in cell division, flower formation, seed maturation and transferring of materials through plasmodesmata (Arora et al. 2008). *Bacillus halodurans* strain C-125 produced  $\beta$ -1, 3 glucanase enzyme and played an important role in the fungal growth inhibition (Chen et al. 2015).

Overall isolates STJP and NPSP were found to be very good PGPR because of their ability to produce different PGP metabolites and activities. While STJP showed production of IAA, gibberellic acid, siderophore, salicylic acid, chitinase and  $\beta$ , 1,3 glucanase activity it also showed solubilisation of potassium, phosphate, and zinc. Isolate NPSP was only given PGP test including nitrogen fixation, but their result was not good when compared with isolate STJP. However, isolate STJP was more effective

in showing PGP activities both qualitatively and quantitatively. Such isolates can be very important for use as bioinoculants. PGPR showing multiple growth promoting traits are in demand as they can perform several activities simultaneously and can help the plants in numerous ways.

**Table 6.** Siderophore production and zinc solubilization by isolates

Isolates	Siderophore production		Zinc solubilization		
	Carboxylate type ( µg/ml)	Hydroxamate type (µg/ml)	Zinc oxide (µg/ml)	Zinc sulphate (µg/ml)	Zinc carbonate (µg/ml)
JPN1	-	2.01±1.12	1.24±0.45	2.30±0.42	3.46±0.76
JPN2	-	1.12±1.12	3.56±1.12	5.56±0.45	3.46±1.12
JPW	-	2.50±0.46	1.10±0.12	0.42±0.45	0.98±0.42
<b>NPSP</b>	4.23±0.48	2.25±1.12	2.50±1.14	1.20±1.12	3.9±1.12
NPSP12	-	-	1.45±0.12	2.42±1.12	-
<b>STJP</b>	<b>16.06±1.23</b>	<b>3.21±0.21</b>	<b>5.40±0.98</b>	<b>3.20±0.12</b>	<b>3.80±1.11</b>
STJP12	-	-	0.14±0.14	0.74±0.16	0.86±0.46
STJP18	5.48±1.18	1.12±0.14	0.78±0.24	0.15±0.12	1.18±0.12
STJP20	5.24±1.22	2.12±0.46	2.45±0.46	-	2.45±0.36
STJP22	5.60±0.48	-	0.78±0.02	0.98±0.4	-
STJP30	1.12±0.02	0.12±0.01	2.56±1.44	1.47±0.74	1.89±0.78

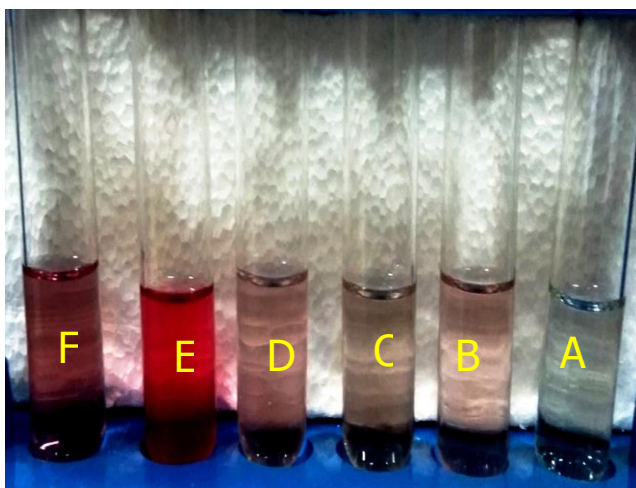
Where, O.D= Optical density, µg= microgram, + = positive; - = negative

[Data are mean of three replicates ± standard error of means. Means, followed by the same letter in a column are not significantly different (P = 0.05) by Duncan's multivariate test (DMRT)]

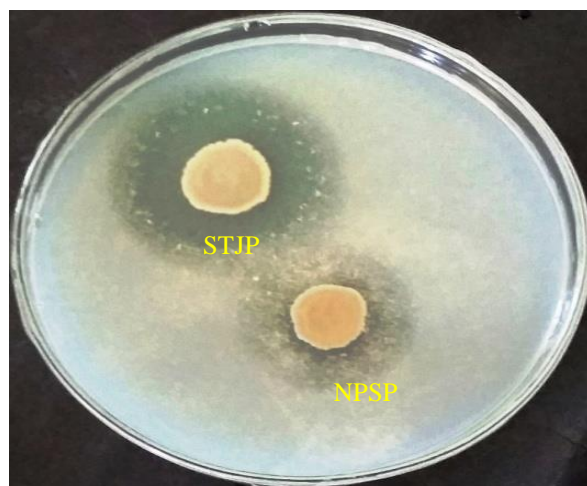
**Table 7.** Biocontrol activities shown by isolates

Isolates	HCN production (O.D)	Salicylic acid production ( $\mu\text{g/ml}$ )	Chitinase activity (nmol/min/mg)	$\beta$ , 1,3 glucanas) (nmol/min/mg)
JPN1	$0.925 \pm 0.01$	$6.40 \pm 1.12$	$5.56 \pm 0.48$	$12.56 \pm 5.64$
JPN2	$0.712 \pm 0.01$	$10.80 \pm 2.86$	$24.60 \pm 1.40$	$98.56 \pm 4.36$
JPW	$0.211 \pm 0.02$	$8.86 \pm 2.48$	$45.64 \pm 4.56$	$120.36 \pm 2.48$
<b>NPSP</b>	-	$16.80 \pm 2.46$	$02.36 \pm 1.24$	$048.50 \pm 4.25$
NPSP12	$0.123 \pm 0.02$	-	-	-
<b>STJP</b>	-	$12.80 \pm 1.46$	$75.58 \pm 4.54$	$220.36 \pm 8.25$
STJP12	$0.453 \pm 0.02$	-	$23.25 \pm 2.24$	$123.60 \pm 4.20$
STJP18	$1.123 \pm 0.02$	-	$36.40 \pm 1.14$	$45.68 \pm 2.42$
STJP20	-	-	$12.36 \pm 0.46$	-
STJP22	$0.864 \pm 0.03$	$2.36 \pm 0.86$	$8.42 \pm 0.86$	$46.36 \pm 2.25$
STJP30	$0.115 \pm 0.01$	$8.64 \pm 0.98$	$6.38 \pm 0.78$	$2.35 \pm 0.40$

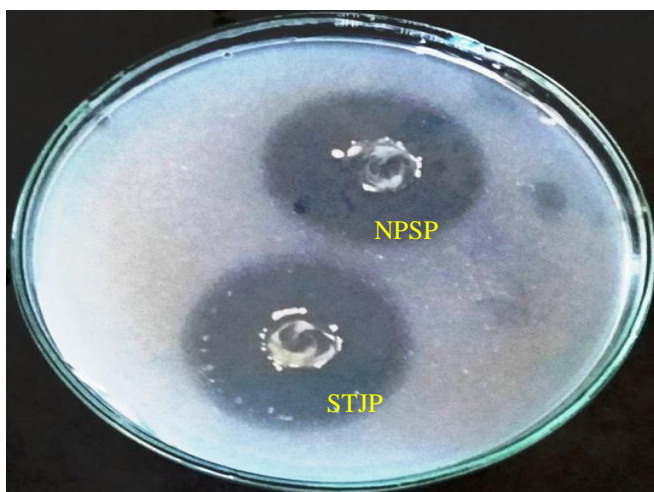
(Data are mean of three replicates  $\pm$  standard error of mean); - = negative



**Fig 28.** IAA produced by isolates (A-control, B-JPN1, C-JPN2, D-STJP12, E-STJP, F-NPSP)



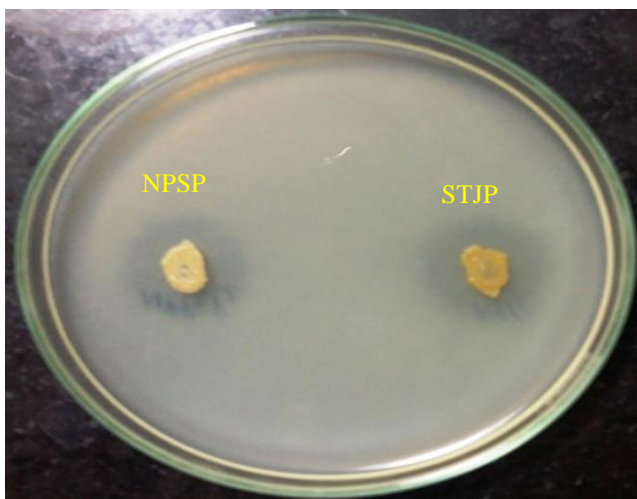
**Fig. 29** Phosphate solubilised by NPSP and STJP



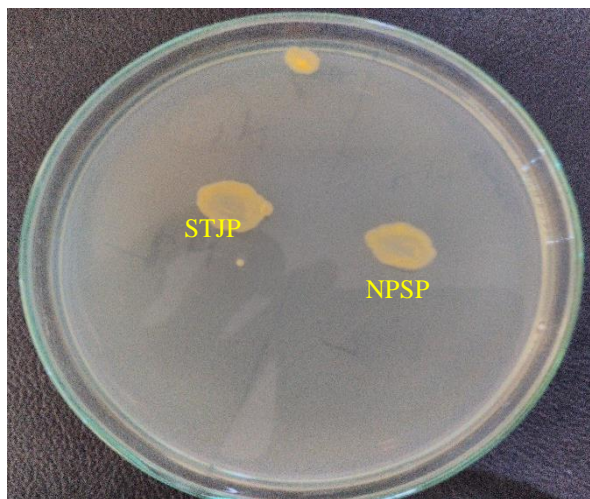
**Fig. 30** Zinc oxide solubilised by STJP and STJP30



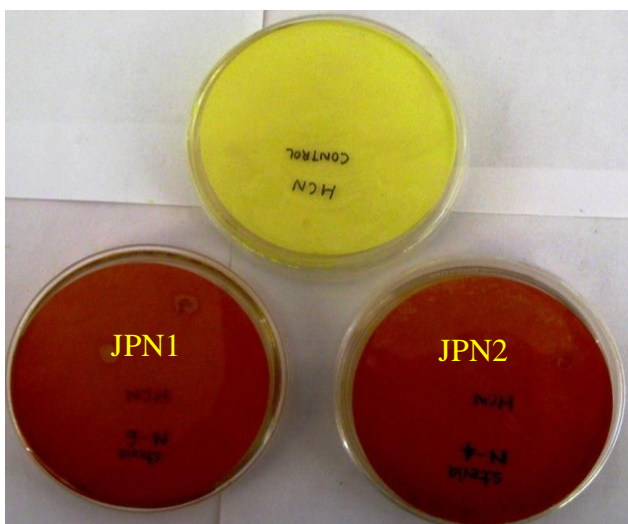
**Fig. 31** Zinc sulphate solubilisation by NPSP and STJP



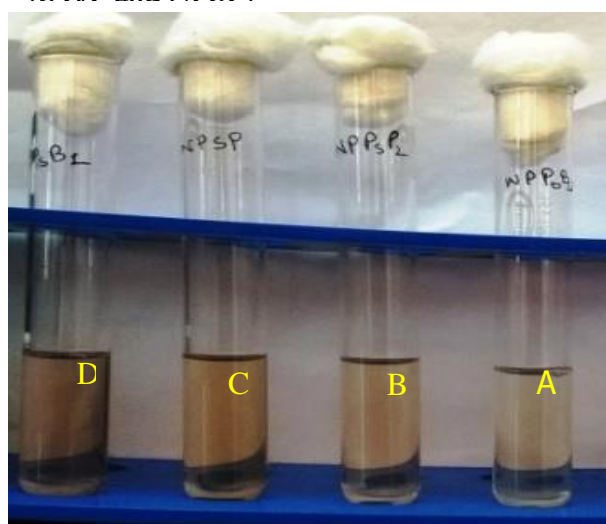
**Fig. 32** Potassium solubilization by STJP and NPSP



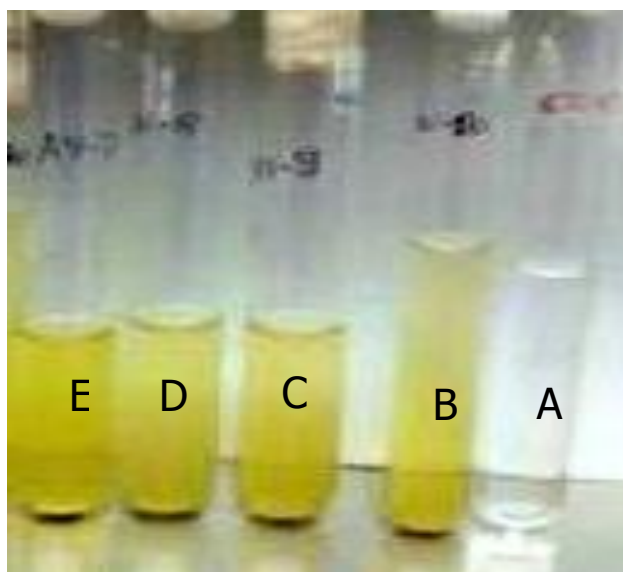
**Fig. 33** Growth on nitrogen free media (STJP and NPSP)



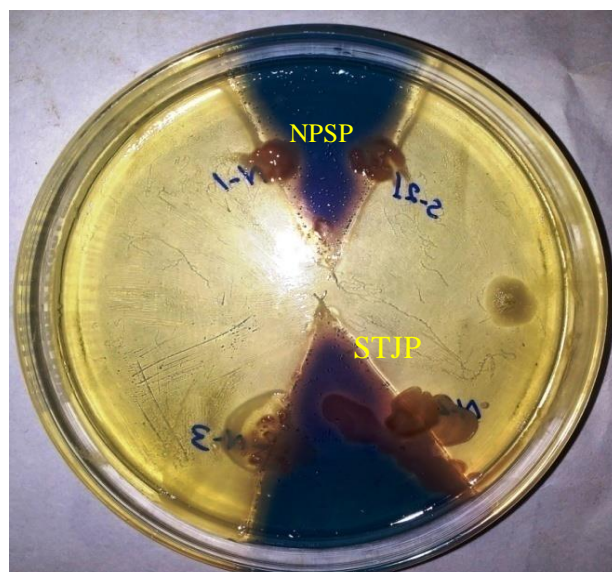
**Fig. 34** HCN produced by JPN1, and JPN2



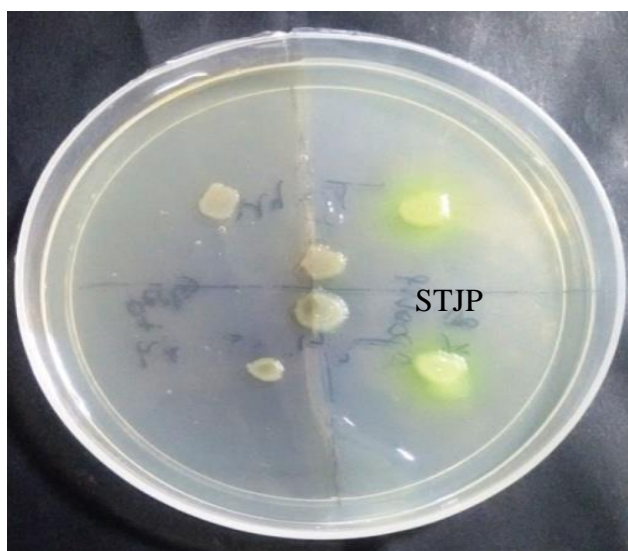
**Fig. 35** Salicylic acid produced by isolates (A-control, B-JPN1, C-STJP, D-NPSP)



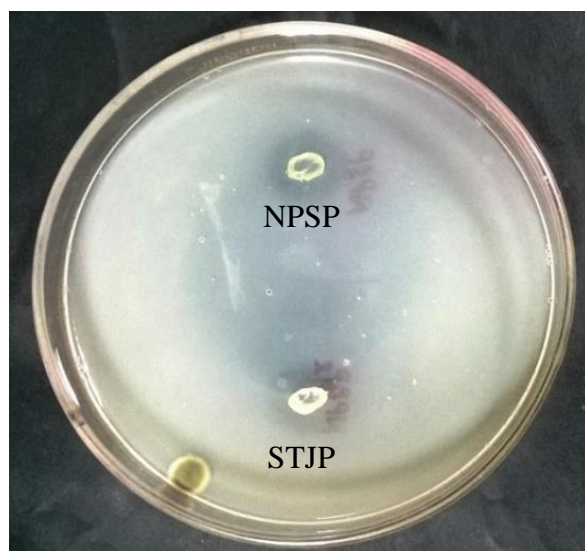
**Fig. 36** Ammonia production by isolates (A-control, B-JPN-1, C-JPN2, D-JPW, E-NPSP)



**Fig. 37** Siderophore (CAS) production by STJP



**Fig. 38** Siderophore (hydroxamate type) produced by STJP



**Fig. 39** Cellulase production by NPSP and STJP

*Chapter-06*  
*Study of Biocontrol Potential of*  
*Isolated Rhizobacteria Against*  
*Alternaria alternata A Fungal Leaf*  
*Pathogen of S. rebaudiana*



## **1. Introduction**

According to estimates, the world population is expected to reach 9 billion by 2050. Hence the demand for food is continuously rising to so as to feed the ever growing population. Currently, the total cost of annual food production is 1.3 trillion US dollars (USD) worldwide, out of which 500 billion USD (31–42% foods) are lost every year due to biotic stresses such as weeds, insects, fungus, virus and bacterial diseases (Watts et al. 2018). Among all pathogens, yearly 120 billion USD (about 20%) are wasted mainly due to fungal and bacterial diseases during the crop production and after harvesting (Shuping and Eloff 2017). However, a drastic decrease in the crop yield and economy due to phytopathogens are often more severe in developing countries like India (Dhlamini et al. 2005). Deadly fungal phytopathogens such as *Alternaria alternata*, *Fusarium* spp. and *Macrophomina phaseolina* are commonly responsible for the reduction of crop productivity all around the globe (Singh and Pandey 2019). *A. alternata* is known to cause leaf spot disease in several agricultural crops including *Stevia rebaudiana*. The leaf spot disease is identified by the presence of dark brown color or necrotic lesions on the leaf (Shakeel et al. 2015). The symptoms of this disease is appeared during pathogenesis, produced by toxicity of fungal metabolites during active phase of plant growth Dhlamini et al. 2005). Fungal phytopathogen *Alternaria* is one of the most common genera, occurs everywhere with endophytic, saprophytic and pathogenic properties and it is responsible for the reduction of crop productivity (Ghosh et al. 2018; Singh and Pandey 2019). The spores of the *Alternaria* species (spp.) are dispersed and can spoil cereals, grains, fruits, foods and damage vegetative parts of the plants and ultimately decrease their nutritive value by secretion of several toxic metabolites such as alternariol, monomethyl ether, altertoxins, altenuene, alternariol, porrotoxin, tenuazonic acid, tentoxin, zinniol, curvularin, and brefeldin A (dehydro-) (Meena et al. 2017). *Alternaria* spp. recorded 20% spoilage of some agricultural crops, and up to 80% reduction of yield (Nowicki et al. 2012). *M. phaseolina* causes charcoal rot, dry rot, wilt, leaf blight, stem blight and damping off disease in a wide range of host plants and ultimately reduce the crop's productivity (Tewari and Arora 2018), while *Fusarium* spp., a fungal pathogen causes several diseases in agricultural crops such as seedling blight, and white rot disease (Singh and Pandey 2019).

Around the globe about 890 synthetic chemicals are approved as pesticides, whereas marketed products are estimated to be approximately 20,700 and among them organophosphorus insecticides are the biggest group (Stenersen 2004). The use of pesticides is not evenly distributed among various crops, and data indicates that 93 % of all row crops such as corn, cotton, and soybeans are treated with some type of pesticide, whereas percentage of forage crop is less than 10 (Pimentel 1993). Albeit synthetic chemicals enjoy a great reputation in enhancing crop productivity and checking several plant diseases but are also proving as environmental havoc (Fenske and Day 2005; Colt et al. 2007). In recent years their role in damaging agro-ecosystems is very well known. The scenario in developing countries is worse and studies show that although these regions use only 20 % of the world's agrochemicals, they suffer 99 % of deaths from pesticide poisoning (Kesavachandran et al. 2009). Among them farmers are the main victims and occupational exposure is very high with lack of technical education (Konradsen et al. 2003; Coronado et al. 2004; Khan et al. 2009a). According to the World Health Organization (WHO), approximately 20,000 workers die from pesticide exposure every year (Pimentel et al. 1992)

According to Food and Agriculture Organization (FAO) estimates developing countries are more vulnerable to loss in the productivity because agriculture plays a lead role in economic growth of these nations. In the last few years, chemical pesticides are commonly applied to the crops for protection against fungal diseases and enhancement of growth and productivity of agricultural crops (Mishra and Arora 2016). Although, pesticides control fungal pathogens, but they have given birth to many health hazards in animals and human, leading to loss of soil fertility, causing deleterious effects on environmental micro and macroflora and agro-ecosystems as whole (Flandroy et al. 2018). The situation thus calls for new management strategies to control plant diseases along with increasing the crop productivity. The new viable solution is available in the form of plant-associated microorganism(s) which are now being applied in agricultural fields in the form of biopesticides (Ruiu 2018). One of the objective of the present study was to isolate and select biocontrol agents against a broad range of fungal pathogens such as *A. alternata*, *Fusarium sp.* and *M. phaseolina*. The selected isolate will be used for production of bioformulation to protect the important medicinal plants (including *S. rebaudiana*) from these phytopathogens.

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## 2. Materials and Methods

### 2.1 Evaluation of antagonistic activity

All isolates were evaluated for antagonistic activity against fungal pathogens so as to determine their biocontrol potential. The fungal phytopathogens selected for biocontrol study were *A. alternata* strain P1 (Accession number - KX494864), *Fusarium* spp (Accession number- ITCC No. 2193), and *M. phaseolina* (Accession number: ARIFCC257). All phytopathogens were procured from Laboratory for Rhizospheric Microbiology and Sustainable Agriculture, School for Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow and grown (at 26°C) and maintained on potato dextrose agar (PDA) and Czapek dox agar (CDA) at 4°C for further use and experiments.

### 2.2 Rapid screening technique

A rapid screening test is a simple process, done in the laboratory on the Petri plate for screening of antagonistic microorganisms. For rapid screening test, mycelia and hyphae were cut with the help of cork borer and kept in the center of prepared PDA plate and four different isolates were spotted or streaked around the fungal pathogens (*A. alternata*, *Fusarium* spp. and *M. phaseolina*) (Schoeman et al. 1999). Afterward, the Petri plate was incubated for 120 h at 28°C and the result was observed after incubation to measure the fungal growth inhibition rate.

### 2.3 Dual culture method

Dual culture method was performed on PDA (HiMedia, Mumbai). Fungal mycelium was cut by cork borer (from a PDA plate grown with the phytopathogens after 7 day incubation period) and placed on the centre of Petri plate. Afterward, the fresh culture of selected isolates was streaked on two edges of Petri plates (Arora et al. 2001). Further, Petri plates were sealed with parafilm and then incubated at 28°C for 7 days. After incubation period, the inhibition of fungal growth was observed. The inhibition zone was calculated by using formula  $100 \times (C-B)/C$ ; where; C was the diameter of control plate of fungus and B was measured off the area of grown fungus in the dual culture plate.

## 2.4 Analysis through scanning electron microscopy

For sample preparation of scanning electron microscopy (SEM), the inhibited fungal growth of *A. alternata* (because this pathogen is the very important phytopathogen of *S. rebaudiana*, hence it was selected for study) on the Petri plate was selected for SEM analysis as per Karnovsky (1965). The fungal mycelia were picked up and fixed with modified Karnovsky's fixative (2% glutaraldehyde and 2% paraformaldehyde in sodium cacodylate buffer (0.05 M; pH 7.2) and incubated at 4°C for 12 h (Karnovsky 1965). Further, the fixed specimen sample was washed three times with sodium cacodylate buffer (0.05 M) for 12 min followed by washing with distilled water. Subsequently, dehydration was done of the specimen with ethanol solution as sequences of 30%, 50%, 70%, 80%, and 90% (time: 5-10 min), and lastly, 100% (three times) for dehydration of cell for 10 min (Karnovsky 1965). Afterward, the specimen was dried by using hexamethyldisilazane and with the help of a sputter coater, the specimen was coated by gold (MSC-101, JOEL). The observation of the specimen was done under SEM (Model No. JSM-7610F, JEOL, Japan).

## 2.5 Pathogenicity/hemolysis test

For the pathogenicity test, the selected isolates NPSP and STJP were spotted on the center of the plate containing blood agar medium (Aneja 2005). Subsequently, plate was incubated for 48 h at 36°C. After incubation, the result was observed through a clear zone present around the bacterial colony, which indicates a positive result for the hemolysis test and the absence of zone shows a negative result for hemolysis.

## 2.6 SEM and molecular characterization

On the basis of biocontrol and PGP activities, two of the selected isolates NPSP and STJP were morphologically identified through SEM and molecular gene sequencing. The procedure of SEM was as described as above. Further, 16S rRNA characterization of isolates STJP and NPSP was carried out using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (3'-CGGTTACCTTGTTACGACTT-5') for amplification (Weisburg et al. 1991). The DNA sequence purified from the gel and the sequences were analyzed using the gapped BLASTn (<http://www.ncbi.nlm.nih.gov>) search algorithm and aligned to their nearest

neighbor. The evolutionary neighbor distances of isolate NPSP and STJP and their related taxa were calculated using mega 7.00 software and the sequences were submitted to National Centre for Biological Information (NCBI) to obtain the accession number. Afterward, selected isolate (on the basis of PGP traits) was submitted in the National Bureau of Agriculturally Important Microorganisms Culture Collection (NAIMCC), Uttar Pradesh, India (a national culture collection center) to obtain the accession number.

### Experimental details

#### DNA isolation

Details of DNA isolation protocol and conditions were as mentioned below-

**Table 8. a)** PCR conditions

Reaction Mixture (50 $\mu$ l)		Cycling Conditions		
Template DNA	100 ng	Initial Denaturation	minutes at 95°C	
Forward Primer	0.3 mM	Denaturation	30 seconds at 95°C	35 Cycles
Reverse Primer	0.3 mM	Annealing	30 seconds at 52°C	
Master Mix	25 $\mu$ l	Extension	2 minutes at 72°C	
Nuclease-Free Water	Volume makeup 50 $\mu$ l	Final Extension	15 minutes at 72°C	

#### b) Primer Details

S.No	Oligo Name	Sequence (5' $\rightarrow$ 3')	Tm (°C)	GC-Content
1	27F	AGAGTTTGATCMTGGCTCAG	56.3	47.5%
2	1492R	CGGTTACCTTGTTACGACTT	55.3	45%

### 2.7 Statistical analysis

All data were calculated by one-way analysis of variance (ANOVA) and means were compared by Duncan's multiple range test (DMRT) (Gomez and Gomez 1984) using software of statistically package social science (SPSS) version 15.

## 3. Results

### 3.1 Rapid screening technique

In the rapid screening test, all the eleven bacterial isolates were checked on the basis of maximum growth inhibition of *A. alternata*, *Fusarium spp.* and *M. phaseolina*. The bacterial isolates were further selected for the study by dual culture test (Figure 40).

### 3.2 Dual plate method

All isolates (eleven) showed growth inhibition of *A. alternata* (shown in figure 41) *in-vitro* conditions as confirmed by dual culture method. Among all isolates, isolate STJP was found to be the most effective in terms of mycelial growth inhibition of *A. alternata* followed by isolate NPSP (Figure 42). Zone of inhibition of 14.8 mm (of *A. alternata*) by isolate STJP was observed. In the current study, isolates STJP30 and JPW showed minimum inhibition of growth of *A. alternata* (Table 9).

Selected isolates were further checked for biocontrol activity against *Fusarium* sp. Among all the isolates, STJP (6.8 mm) was found to be the most effective one in disrupting mycelial growth of *Fusarium* sp. followed by NPSP (6.5 mm) (Figure 43). However, isolates NPSP12 and STJP12 were unable to inhibit the growth of *Fusarium* sp., whereas other isolates JPN1, JPN2, JPW, STJP18, STJP20, STJP22, and STJP30 recorded growth inhibition (of *Fusarium* sp.) by 2.5, 6.5, 1.2, 3.5, 2.4, 3.8 and 3.1 mm, respectively.

Isolates were further checked against *M. phaseolina* for biocontrol activity. Among all isolates, NPSP showed maximum disruption (8.6 mm) of fungal growth followed by STJP (6.8 mm) (Figure 44). Isolates JPN1, JPW, STJP12, STJP18, STJP22, and STJP30 also recorded growth inhibition of *M. phaseolina* with the inhibition rate of 3.6, 3.6, 2.5, 2.4, 2.4, 6.4, and 4.6 mm, respectively. However, in this study, isolate JPN2, NPSP12, and STJP20 did not show any biocontrol activity against *M. phaseolina*.

From the dual culture (Figure 45) and control plates (Figure 46), *A. alternata* sample was taken to image by phase contrast microscope. Results clearly indicate that bacterium STJP showed antagonistic activity.

### 3.3 Scanning electron microscope (SEM) analysis

For this, dual culture plate showing the antagonistic activity of isolate STJP against *A. alternata* was taken. After the successful result of biocontrol activity, it was selected for SEM analysis. For this, dual culture plate was chosen in which STJP inhibited the growth of *A. alternata* and the control plate (*A. alternata*) was also taken for result interpretation. From the control plate, straight large catenate conidia chains with transverse septa of *A. alternata* were seen clearly (Figure 47), whereas from bacterial (STJP) treated (dual) plate, the destruction, and ruptured hyphae of fungus were observed (Figure 48). Further, more magnified and clear image indicates that no conidia

were observed in the bacteria treated plate (Figure 48). Through SEM image, it was confirmed that isolate STJP showed severe antagonistic activity against *A. alternata*.

### 3.4 Pathogenicity/hemolysis test

Isolates including STJP did not give hemolysis test as confirmed by absence of zone around the colony on the blood agar media (figure not shown). The absence of zone around the colony indicated non-pathogenic character of the bacteria.

### 3.5 SEM and molecular identification of bacterial isolates

On the basis of phenotypic and biochemical traits, NPSP was found to be a member of genus *Pseudomonas* (Bergey's Manual of Systematic Bacteriology; Garriety 2005). For confirmation of shape and size of selected isolate NPSP, SEM was used and found to be a mono-flagellated bacterium with cell size of 1-5 x 0.1-0.5  $\mu\text{m}$  (Figure 49), while isolate STJP (member of genus *Bacillus*) was found to be flagellated bacterium with the peritrichous arrangement and cell size of 1.0-1.28 x 0.5-0.7  $\mu\text{m}$  (Figure 50). Further, through 16S rRNA gene sequencing NPSP showed 99 % similarities with *Pseudomonas aeruginosa* PAO1 (Figure 51). After submission of gene sequence data in the NCBI database, the accession number was found to be KX372539.1. Similarly, STJP was found to be a member of genus *Bacillus* sp. The comparison of the whole 16S rRNA gene sequence of STJP showed 99% similarities with *Bacillus safensis* strain NBRC 100820. The gene sequence was submitted to the NCBI database with accession number KX372540.1 (Figure 52). Based on PGP characters and Biocontrol potential isolate STJP was selected for further experiments. Isolate STJP was also submitted to National Bureau of Agriculturally Important Microorganisms Culture Collection (NAIMCC), Kushmaur, Maunath Bhanjan, Uttar Pradesh, India and the accession number of the strain is TB-2833.

## 4. Discussion

Rhizospheric microorganisms especially *Pseudomonas* spp. and *Bacillus* spp. produce a number of secondary metabolites from *Pseudomonas* spp. and from *Bacillus* spp. Wu et al. 2013; Chalasani et al. 2015; Tewari and Arora 2018). Secondary metabolites of bacteria are well known as anti-fungal compounds and play an important role in biocontrol mechanism or growth inhibition of fungal pathogen(s) (Sansinenea and Ortiz 2011; Boottanun et al. 2017). Biocontrol is a reliable or ideal method for the screening

of potential biocontrol agent (BCA) and its application on the plants for disease management (Benítez et al. 2004; Arora and Mishra 2016). BCA are an alternative option of chemical pesticide because of being safe, eco-friendly, economical, and may provide long term defense against diseases ((Fravel, 2005). Thus, low-cost technology as use of BCA for suppression of diseases and increase plant growth has now become a demanding sector for research and development in many countries like India (Bandopadhyay and Das 2017).

Soil-borne fluorescent pseudomonads and *Bacillus* spp. have been widely used for the control of different fungal pathogens such as *A. alternata*, *M. phaseolina* and *Fusarium* sp. and they increase the growth and productivity of agricultural crops (Saraf et al. 2014; Lastochkina et al. 2019). In the current study, isolates from the rhizosphere of various medicinal plants were checked for their biocontrol activity against three fungal phytopathogens, *A. alternata*, *M. phaseolina*, and *Fusarium* sp. *in-vitro* condition. In the current study, plant growth promoting isolates STJP and NPSP from the rhizosphere of medicinal plant and identified to be *Bacillus safensis* and *Pseudomonas* sp. was tested as potent PGPR and BCAs. However, *Bacillus* sp. STJP was found to be maximum growth inhibitor of *A. alternata* and *Fusarium* sp. followed by *Pseudomonas* sp. NPSP. This study has already shown (Objective 2) that *B. safensis* STJP produced several antifungal traits such as siderophore, salicylic acid, chitinase and  $\beta$ -1, 3 glucanase activities which can be responsible for inhibition of fungal growth. Cell wall lytic enzymes such as chitinase,  $\beta$ -1, 3 glucanase and production of salicylic acid are known to be involved in the biocontrol activity and induced immunity against phytopathogens and are also well known to be produced by the members of the genera *Bacillus* and *Pseudomonas* (Saraf et al. 2014; Shafi et al. 2017; Carmona-Hernandez et al. 2019). Lytic enzymes viz.  $\beta$ -1, 3 glucanase and chitinase are capable of degrading  $\beta$ -1, 3 glucans, and chitin which are the major components of the fungal cell wall (Kumar et al. 2018). Salicylic acid is a chief plant hormone, which prevents the phytopathogens and induced systemic resistance (ISR) against fungal pathogens of plant (Beneduzi et al. 2012; Zhang et al. 2013; Dieryckx et al. 2015; Meena et al. 2017). Research has also shown that salicylic acid is involved in the growth enhancement of plants through the local and systemic response against fungal pathogens including *A. alternata* (Jayapradha and Raja 2016; Hasanuzzaman et al. 2018).

*Alternaria* is a major fungal pathogen not only of *S. rebaudiana*, but it causes disease on cereals (rice, wheat, maize, pea, and gram), vegetables (potato, and tomato)

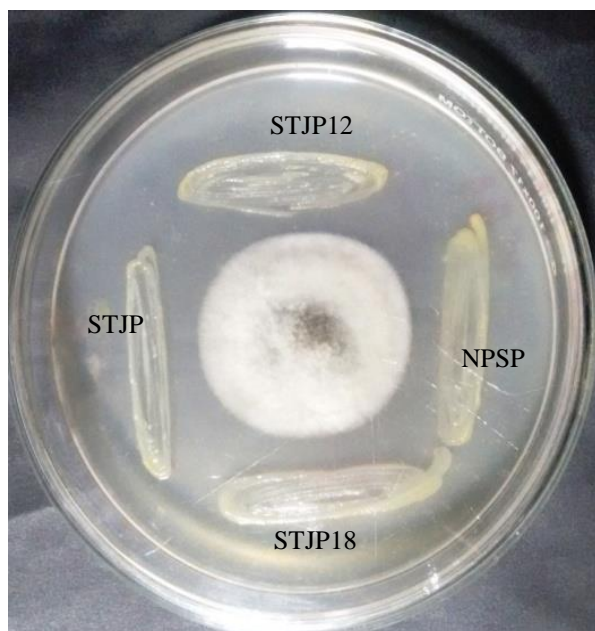
and other medicinal crops (pepper, canola, *Mentha* and etc.) as well (Jayapradha and Raja 2016; Schiro et al. 2018). Abdelmoteleb et al. (2017) observed that *Bacillus* sp. reduced 54% growth of *A. alternata*. Pane and Zaccardelli (2015) reported *Bacillus* strain 15F-III showed biocontrol activity against *A. alternata* by the production of volatile compounds. However, the ability of biocontrol potential by isolate STJP was found to be better than the earlier reported isolates of *Bacillus* spp. (Pane and Zaccardelli 2015; Torres et al. 2017; Abdelmoteleb et al. 2017). The result of antagonistic activity of *B. safensis* STJP showed narrow-spectrum antimicrobial property because it significantly inhibited growth of *A. alternata* only, however, this beneficial property results in inhibition of selective phytopathogen (*A. alternata*) not harming other fungi in soil. Thus such BCA will not disturb beneficial microflora in rhizosphere. Several *Bacillus* spp. show narrow-spectrum antimicrobial properties under laboratory conditions and their metabolites are extensively used to control soil-borne diseases in pot and field conditions (Harbut et al. 2015; Fira et al. 2018). *Bacillus* isolate W19 showed antagonistic activity against fungal pathogen and reduced wilt disease of banana plants and increased plant productivity (Bubici et al. 2019). Pane and Zaccardelli (2015) reported *Bacillus* strain 15F-III showed biocontrol activity against *A. alternata* by the production of volatile compounds and siderophore. In the current study, STJP also showed growth inhibition of *M. phaseolina*. (Torres et al. 2017) observed that *B. subtilis* strain PGPMori7 and *B. amyloliquefaciens* isolate PGPBacCA1 as biocontrol agents against wide type of phytopathogens. The ability of biocontrol potential by STJP was found to be better than reported earlier for *Bacillus* spp. (Torres et al. 2017). Fluorescent Pseudomonads were evaluated for their potential to protect the plants from phytopathogenic *Alternaria* and *Fusarium* fungi and was found to control growth and distribution of these pathogenic fungi in field and also enhanced the growth of wheat crop (Gdanetz and Trail 2017). Beside these, it is conceivable that fluorescent pseudomonads could suppress the formation of *Alternaria* and *Fusarium* toxins and thus enhance the quality and quantity of crop under field conditions (Müller et al. 2018). Uzair et al. (2018) reported *Pseudomonas* strain PS24 which exhibited biocontrol activities against several phytopathogenic fungi (*Rhizopus microsporus*, *F. oxysporum*, *Aspergillus niger*, *A. alternata*, and *Penicillium digitatum*) by mechanisms of multiple PGP attributes such as phosphate solubilization, indole acetic acid (IAA), siderophore, and HCN production.

Overall, in the present study, *B. safensis* STJP was found to be better PGPR on the basis of PGP traits, hence, it was selected for further study. Isolate STJP was also submitted to culture collection centre (NAIMCC) so that this important strain is secured and can be used in future for developing bioinoculants for sustainable agricultural practices.

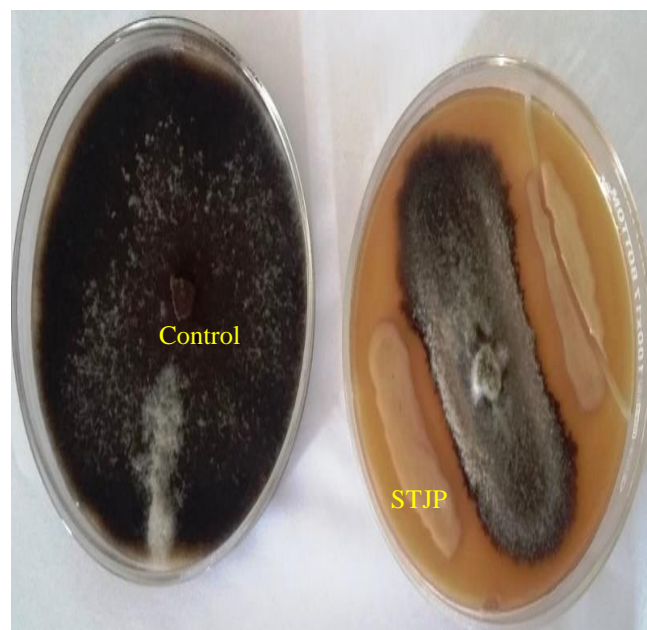
**Table 9.** Antagonistic activities of isolated rhizobacteria against fungal pathogens

Isolates	<i>alternata</i> (mm)	<i>M. phaseolina</i> (mm)	<i>Fusarium sp.</i> (mm)
JPN1	5.5 ± 0.24	2.5 ± 0.42	3.6 ± 0.12
JPN2	2.2 ± 0.24	6.5 ± 0.35	-
JPW	1.2 ± 0.01	1.2 ± 0.86	-
<b>NPSP</b>	5.2 ± 0.46	1.5 ± 0.48	3.6 ± 0.14
NPPSP2	5.5 ± 0.14	-	-
<b>STJP</b>	<b>14.8 ± 0.98</b>	<b>6.5 ± 0.26</b>	<b>6.8 ± 0.12</b>
STJP12	2.1 ± 0.08	-	2.5 ± 0.12
STJP18	3.5 ± 0.06	3.5 ± 0.46	2.4 ± 0.12
STJP20	3.6 ± 0.06	2.4 ± 0.12	8.6 ± 0.12
STJP22	2.4 ± 0.10	3.8 ± 0.24	6.4 ± 0.12
STJP30	1.2 ± 0.02	3.1 ± 0.24	4.6 ± 0.12

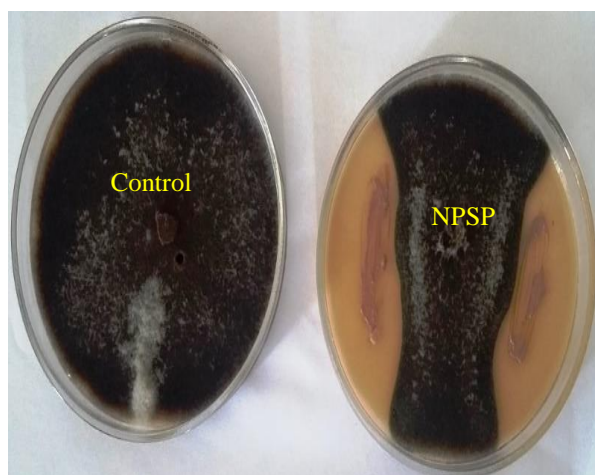
(Data are means of five replicates ± standard deviation); - = negative



**Fig. 40** Antagonistic activities shown by STJP, NPSP, STJP12, and STJP18 by rapid screening



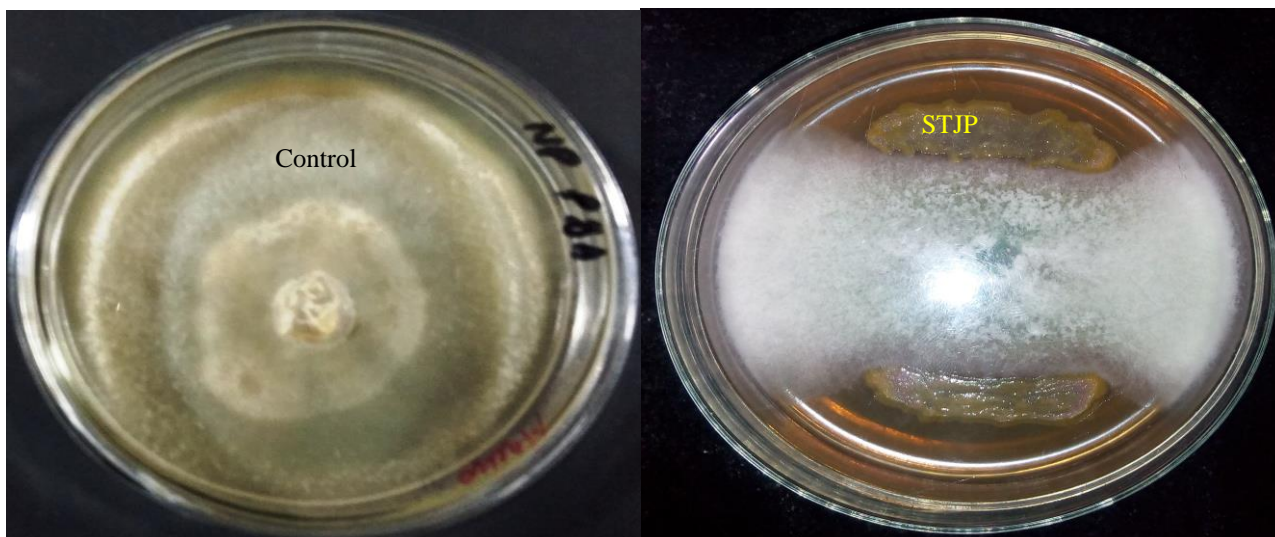
**Fig. 41** Growth inhibition of *A. alternata* by isolate STJP



**Fig. 42** Growth inhibition of *A. alternata* by isolate NPSP



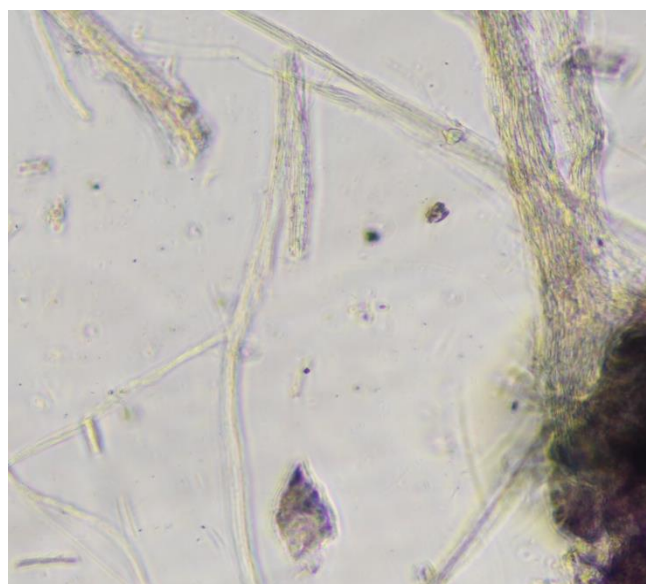
**Fig. 43** Growth inhibition of *Fusarium* sp. by isolate STJP



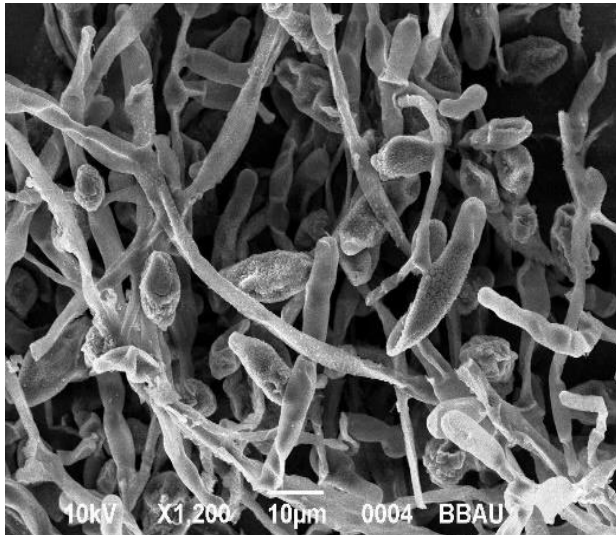
**Fig. 44** Growth inhibition of *M. phaseolina* by isolate STJP



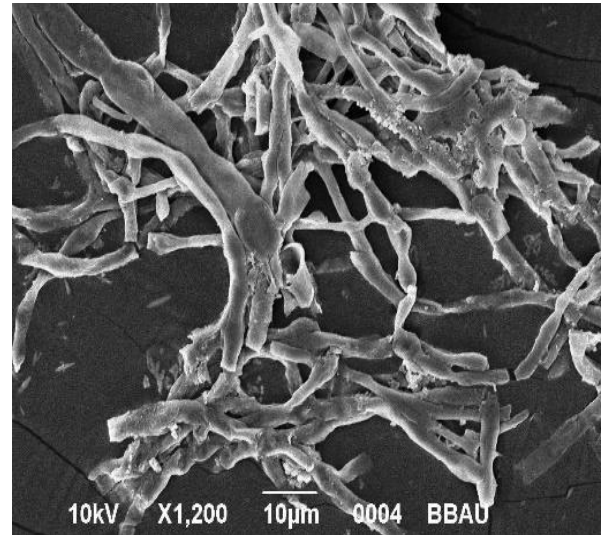
**Fig. 45** Phase contrast image of *A. alternata* (from control plate)



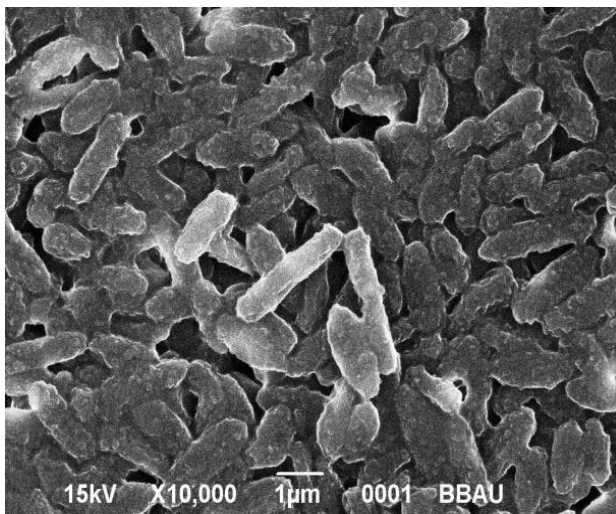
**Fig. 46** Phase contrast image of *A. alternata* (from dual plate)



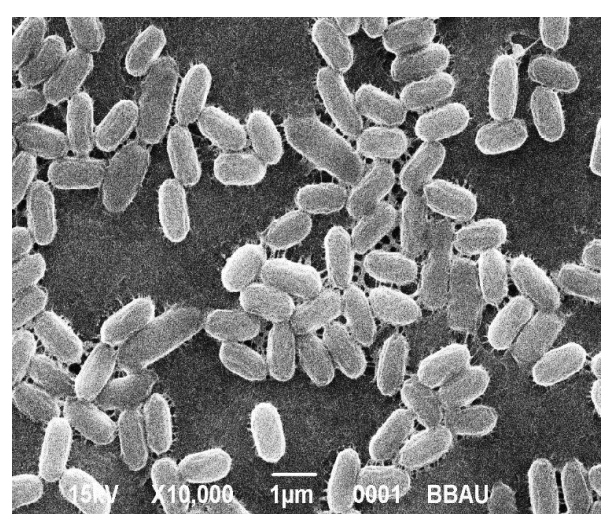
**Fig. 47** SEM image of *A. alternata* (from control plate)



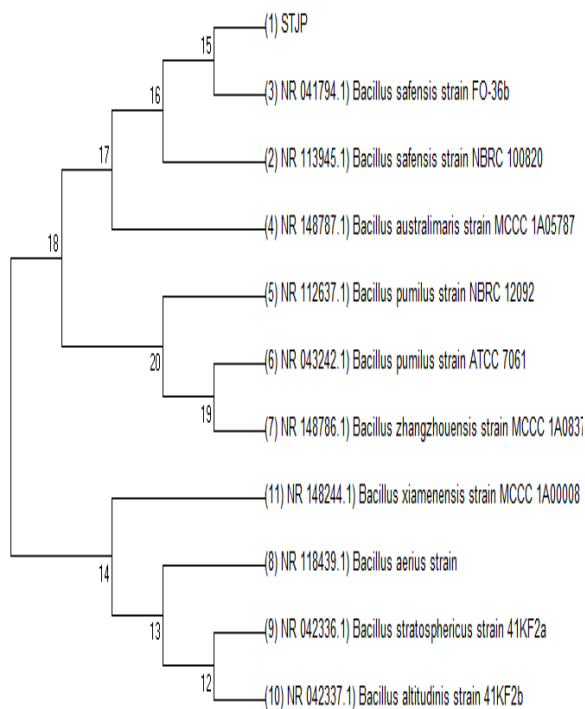
**Fig. 48** SEM image of *A. alternata* (from dual plate)



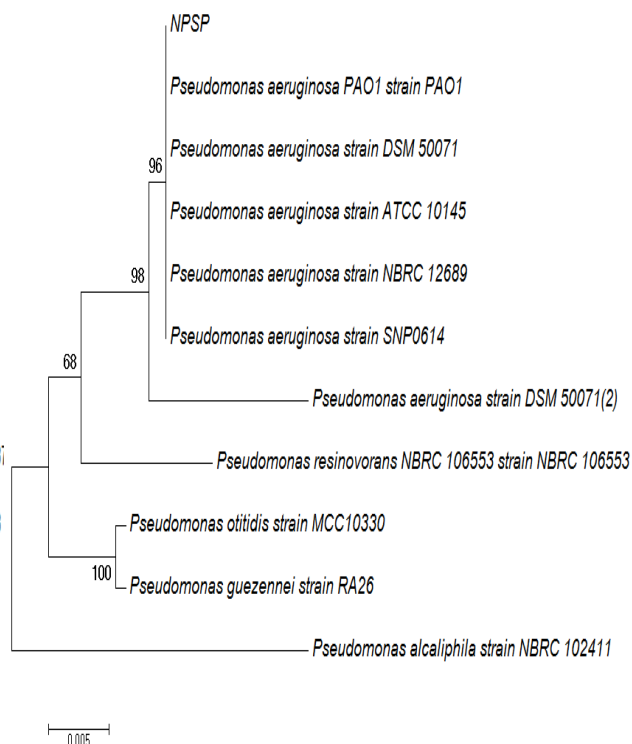
**Fig. 49** SEM image of bacterium NPSP



**Fig. 50** SEM image of bacterium STJP



**Fig 51.** Phylogenetic tree of isolate STJP by neighbour joining method



**Fig 52.** Phylogenetic tree of isolate STJP by neighbour joining method

*Chapter-07*  
*Study of Mechanisms Involved in  
Biocontrol and Extraction and  
Identification of Bioactive Compounds*



## **1. Introduction**

Soil is the best place for the flourishing of microbial population on Earth and most of the beneficial microorganisms occur in the rhizosphere region of plants (Khan 2005). PGPR produce a number of secondary metabolites such as phenazine, pyrrolnitrin, oomycin A, geldanamycin, pyoluteorin, pyocyanin, and 2-4 diacetylphloroglucinol reported from *Pseudomonas* (Tewari and Arora 2018) and bacitracin, polymyxins, lipopeptide surfactin, subtilosin, lantibiotic, iturin and gramicidin from *Bacillus* sp. (Chalasanani et al. 2015) which are involved in biocontrol. Several of the secondary metabolites produced by rhizospheric bacteria are known as anti-fungal compounds and play an important role in biocontrol mechanisms against fungal pathogens to enhance plant health (Sansinenea and Ortiz 2011; Boottanun et al. 2017). Antagonistic metabolites are a reliable method for the identification of potential biocontrol agents and for disease management (Arora et al. 2001; Saraf et al. 2014; Lastochkina et al. 2019). Biocontrol agent is a very specific strategy to crop productivity and sustainable agriculture (Saraf et al. 2014).

*B. safensis* STJP is able to produce a number of secondary metabolites with very different structures and nature. *B. safensis* strains are previously known to play different roles in the environment, and industries (Baruzzi et al. 2011). Among all functions, the biocontrol activity of *Bacillus* spp. are considered to be good by researchers (Sansinenea and Ortiz 2011; Harwood et al. 2018), but there are limited reports available on *B. safensis* for biocontrol activity against phytopathogens. However, *B. safensis* produce several metabolites such as antibiotics, toxin, pigment, growth regulators, pheromone, hormones, organic metabolites such as lipopeptides, bacilomycins, fengycins, and surfactins and other bioactive compounds, but their major roles are considered in enhancement of plant growth rather than protection against phytopathogens (Lateef et al. 2015; Shafi et al. 2017; Lastochkina et al. 2019). Secondary metabolites from microbial origin are now being used in several industries including pharmaceuticals (Prakash and Arora 2019). However, there is an urgent need for extraction and identification of metabolites from *B. safensis* at large scale, for fruitful utilization by agricultural industry, in particular as biocontrol agents. In the present study, the liquid-liquid extraction process has been used for the extraction of

metabolites from broth culture of *B. safensis* STJP, which were further characterized through thin layer chromatography, Fourier transform-infrared (FT-IR) spectroscopy and gas chromatography-mass spectrometry (GC-MS).

## **2. Materials and Methods**

### **2.1 Extracellular or intracellular metabolites production**

The nutrient broth was prepared and inoculated with overnight grown bacterial culture and incubated at 30°C for 24 h. After incubation, the medium was centrifuged at 5,000 rpm for 15 min to get the supernatant. Subsequently, two wells were made on the prepared PDA plate with the help of sterilized cork borer and obtained supernatant and bacterial pellet were added. Supernatant and bacteria were further transferred into both wells of Petri plates (separate) containing *A. alternata*, to check whether the biocontrol activity is extra- or intra-cellular (Kersten and Kirk 1987). *A. alternata* (disc) was kept on the center of the plate without any well or supernatant to act as control. The plates were incubated at 28°C for 120 h. After incubation, the result was observed.

### **2.2 Production of volatile or non-volatile compounds by seal plate method**

Bacterial isolates were used for the production of antifungal volatile compound as per Fernando et al. (2005). Briefly, PDA and nutrient agar (NA) was prepared and poured in the sterilized Petri plate. Mycelia of *A. alternata* (5 mm) was cut with the help of cork borer from the margin of an actively growing culture and kept at the center of Petri plate containing PDA. The overnight grown bacterial culture was spread on the bottom of second Petri plate containing NA. Petri dishes containing fungal mycelia were inverted over the NA and both plates were immediately wrapped with parafilm. The plates were further incubated for 120 h at 26°C. Every 24 h incubation, growth inhibition of *A. alternata* by bacterial isolates were observed as compared to control plate (contained only mycelia). After 120 h, fungal mycelia were detached from the plate and further confirmed for viability of the fungus in a fresh Petri plate containing PDA. The experiments were repeated three times with five replicas.

### **2.3 Extraction and purification of metabolites**

For extraction of antifungal metabolites, 200 ml nutrient broth was prepared in a 500 ml conical flask and fresh culture of *B. safensis* STJP was inoculated into it. The inoculated medium was incubated in a rotatory shaker (120 rpm; 30°C; incubation for

7 days) (Labtech Model No: LS1-3016R). After the incubation period, the broth was centrifuged (10,000 rpm for 15 min at 4°C) (Model no: Sigma 3-18KS) and obtained supernatant was dissolved in the solvents; chloroform + ethyl acetate + dichloromethane (DCM) in a ratio of 1:2:1 as per Hennequin et al. (2006). Further, 50 ml sample (reaction mixture) was filled in the separating funnel and mixed vigorously for 15 min. The funnel was then hanged on the stand and this process was continuously done for 2 h. The transparent layer of the solvent containing metabolites was removed from the separating funnel and the sample was filtered via Whatman number 42 containing 5 gm of sodium sulphate (anhydrous compound). The obtained filtered sample containing antifungal metabolites was kept in a sterilized beaker, and finally, solvent was evaporated by using a rotatory vacuum evaporator (Model No: UTS: 1.53, Thermo-scientific).

#### **2.4 Metabolite identification by thin layer chromatography**

Thin layer chromatography (TLC) was used for separation of different compounds present in the crude metabolite as per Reddy et al. (2009). Briefly, TLC was done on freshly prepared silica plate (20cm long x 10 cm width) by using mobile phase [chloroform: methanol (80:20)]. The crude metabolite (10 µg) was spotted on the silica plate and the solvent was allowed to run from bottom to top. The retardation factor (RF) value was calculated on the basis of solvent and metabolite fraction height (Kagan and Flythe 2014). The running lane was then dried thoroughly and the fraction of the compound was analyzed under UV light. Different bands of metabolites were scraped separately into microcentrifuge tubes and further extracted with 15 ml of ethyl acetate as per Gocan (2002). After centrifuged, supernatant was taken after removal of silica residue and further identified.

#### **2.5 Metabolite identification by Fourier transform infrared spectroscopy (FT-IR)**

To analyze the functional groups of purified metabolites (by *B. safensis* STJP), the sample was dried in a hot air oven at 60°C and mixed with KBr to analyze the absorption spectra of the metabolites as per Reddy et al. (2009). Briefly, the dried sample of metabolites was mixed with KBr (IR Grade; purity ≥ 99%) (1:30 ratio) and the reaction mixture was thinly ground and further fused into a thin pellet (13mm X 1mm). This process was prepared manually by PCI hydraulic Press with a 15 tons capacity under vacuum pressure. Afterward, spectra absorbance was observed by FTIR (Model:

Nicolet™ 6700, Thermo Fisher Scientific, USA). Further, the result was analyzed in the mid-infrared region from 4000 to 450  $\text{cm}^{-1}$  with a resolution of 4  $\text{cm}^{-1}$ . For spectrum peak, scanning was done and the pure KBr as a background spectrum was kept to measure in the ambient air. The processing of data was done using a software OMNIC™ (v7.4).

## 2.6 Gas chromatography-mass spectrophotometry

Gas chromatography (GC) was performed using a Varian Star 3400 CX series GC with a flame ionization detector. Further, 15 m DB-1 megabore column of 100% dimethylpolysiloxane was used for the separation of the bacterial sample (Shojania et al. 1999). The sample was injected into a fused silica column [(25 m X 0.22 mm X 0.25  $\mu\text{m}$  (length X width X thickness)] at 22°C which was focused cryogenically with chilled acetone (Time: 2 min). This column was previously joined to a Hewlett-Packard 5890 gas chromatograph linked to a selective Hewlett-Packard mass detector. For the carrier gas, helium was used and the flow rate was adjusted at 1 ml/min. The temperature of GC-column was designed from 35-200°C at 4°C/min ramp rate. The result obtained in the form of unknown compounds were compared with mass spectra using NIST/EPA/NIH mass spectroscopy library (Version 17) as per Shojania et al. (1999).

## 2.7 Inhibition of fungal growth by identified metabolites

The antifungal compound phenol, 2, 4-bis(1,1-dimethylethyl) was selected on the basis of major/concentration in GC-MS sample and was also purchased from Sigma Aldrich, USA (synthetic chemicals) for checking antifungal activity against phytopathogen *A. alternata* by different concentrations of the metabolite (Schmitz 1930). The different concentrations of extracted crude metabolite (10ppm, 20ppm, 30ppm, 40ppm, 50ppm, 60ppm, 70ppm, 80ppm, 90ppm, 100ppm) were prepared and poured in PDA medium on separate Petri plates. Wells were made on the poured PDA plate with the help of sterilized cork borer. Fungal mycelia were kept at the center of the plate and prepared concentration of metabolites was inoculated in the wells and plates were further incubated for 5 days at 28°C. After incubation, the result was observed.

## 2.8 Statistical analysis

All experiments were performed in triplicates. Data were analyzed using ANOVA and the means was statistically compared using Duncan's multiple range test (DMRT) using software SPSS version 15.

### 3. Results

#### 3.1 Extracellular or intracellular metabolite production

The supernatant extracted from *B. safensis* STJP showed the inhibition zone around the well (where supernatant was poured), but no such inhibition zone was observed around the wells in which pellet was poured. This result confirmed that bacterial strain STJP is producing extracellular fungicidal metabolites in the medium (Figure 53).

#### 3.2 Volatile or non-volatile compounds production by seal plate method

In the seal/dual/divided plate method, isolate STJP inhibited the growth of *A. alternata* present in a second Petri dish. This result confirmed that *B. safensis* STJP produced volatile compound as biocontrol metabolite (Figure 54). For the confirmation of result, mycelia and conidia of the fungus failed to germinate, when plated on fresh PDA plates by seal plate method. The normal mycelial growth and conidia germination was observed in the control plate. Overall, bacterium STJP was considered an effective strain for volatile compounds production.

#### 3.3 Metabolite identification by thin layer chromatography

After extraction (Figure 55) and evaporation of the solvent, a crude antifungal metabolite was obtained. The sample containing metabolite reached at 7.5 cm with retention time 45 min. On the TLC plate (Figure 56), three bands were detected under ultraviolet (UV) light (254 nm) in order of decreasing R<sub>f</sub> values, as shown in Figure 56. Compounds with R<sub>f</sub> value of 0.86, 0.72, 0.14 were seen on the TLC plate.

#### 3.4 Metabolite identification by fourier transform infrared spectroscopy

FTIR spectrum image was taken of the bacterial metabolite sample (Figure 57). The absorption peaks of the FTIR spectrum were analyzed as described by Lambert (1987) to detect the relevant functional groups and chemical bonds of organic compounds.

The absorption peak at 3610.0–3355.4 cm<sup>-1</sup> indicated the –OH group (-OH stretching) with strong intensity corresponded to the existence of alcohol, phenol, and carboxylic acid present in the metabolite sample (Table 10). Absorbance peak at 3406.5 cm<sup>-1</sup> is attributed to O-H stretching corresponded to the presence of carboxylic and phenolic compounds. The absorbance peak 1620.8-1434 cm<sup>-1</sup> indicated the C-C stretching resembled the presence of aromatic compounds such as benzene, phenol, and

toluene, and absorbance peak  $1115.6\text{ cm}^{-1}$  indicated the alkyl halide and peak  $800.7\text{-}662.2\text{ cm}^{-1}$  showed the presence of aliphatic compounds such as alkane, alkyne or alkene and the absorbance peak at  $601.1\text{ cm}^{-1}$  attributed to alkyl halide and peak  $465.2\text{ cm}^{-1}$  showed the alkane group (Table 10). Overall, the absorbance spectrum of bacterial metabolite (STJP) showed the presence of alcohol, alkane, phenol, alkyl halide, and aromatic rings.

### 3.5 Gas chromatography-mass spectrophotometry

The GC-MS analysis of chloroform + ethyl acetate + DCM extract has revealed the presence of various organic compounds, including fatty acid, alcohol, phenol, and other aliphatic compounds at different RT identified using NIST mass spectral library (Figure 58). The major peaks were detected at retention time (RT) 21.439, 25.191, 27.783, 29.374, 29.765, 31.536, 27.273, 32.263, 37.481 and 44.258 which corresponded to the presence of phenol, 2,4-bis(1,1-dimethylethyl), docosane, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, 5,10-diethoxy-2,3,7,8-tetrahydro-1h,6h-dipyrrolo[1,2-a:1',2'-d]pyrazine, hexadecanoic acid, cyclohexanol, 1-butyl-, 1-proline, n-valeryl-, heptadecyl ester, dotriacontane, d-Ribose, 2-deoxy-bis(thioheptyl)-dithioacetal, 2-tert-Butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol, respectively, based on matching with NIST database (Table 11).

Different minor peaks were also examined at RT 21.038, 27.006, 27.085, 27.396, 28.725, 28.817, 28.906, 29.557, 29.614, 32.895, 33.549, 35.898, 37.239, 37.743, and 39.581 which resembled to the presence of nonadecane, octadecane, 3-hexadecanol, isopropyl tetradecanoate, heptadecane, 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, eicosane, 3-ethyl-3-undecanol, 1,2-benzenedicarboxylic acid, ditridecyl ester, tetratetracontane, hexacosyl acetate, hexatriacontane, 1,3,5-trisilacyclohexane, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, and 4,4'-((p-phenylene)diisopropylidene)diphenol, respectively (Table 11 and Figure 58).

### 3.6 Fungal growth inhibition by metabolite

The different concentrations of purified metabolite were tested for antagonistic activity against *A. alternata*. It was noted that 40 ppm metabolite concentration was capable for 50% growth inhibition of fungal pathogen. With further increase in the concentration of metabolite the rate of inhibition also increased.

#### 4. Discussion

The results showed that *B. safensis* STJP showed broad-spectrum antimicrobial property against several phytopathogens such as *A. alternata*, *M. phaseolina*, and *Fusarium* sp. Harbut et al. (2015) reported that *Bacillus* sp. displayed broad-spectrum antimicrobial activity and was effectively used to control soil-borne diseases in field conditions. *Bacillus* strain W19 showed antagonistic activity against *Fusarium* sp. and reduced the wilt disease of banana plants and other soil-borne pathogens (Bubici et al. 2019). A number of species of *Bacillus* genus are capable of producing a wide collection of antimicrobial substances to inhibit phytopathogens including volatile compounds, lipopeptides, hydrolytic enzymes and macrolactins (Fira et al. 2018).

*B. safensis* STJP produced volatile compounds and inhibitory to survival (conidia), infection (mycelia), reproductive structures (conidiospore) of *A. alternata*. The present study confirmed the presence of antifungal volatile compounds. This is an interesting phenomenon; volatile antibiotics can be very effective in controlling the fungal pathogens in the soil, with far reaching impact and inhibiting the fungal mycelia from rhizosphere region. Several volatile compounds producing bacteria were isolated from rhizospheric region (Griffin et al. 2010; Lim et al. 2017). In the present study, *B. safensis* STJP produced extracellular enzymes or metabolites as confirmed by different methods. Further, these metabolites were checked and found to be volatile in nature as confirmed by dual plate method. After the confirmation of the nature of compounds (volatiles), the extracted metabolites were analyzed through FTIR. Most of the compounds detected in the metabolites sample by FTIR were aliphatic and aromatic phenolic compounds.

However, FTIR is not a reliable method for the detection of antifungal compounds or any other bacterial metabolites (Wang et al. 2013). These methods only predict functional group. Hence the samples containing metabolites were further identified by GC-MS.

The key mode of action of antifungal compounds show fungicidal effect on the mycelia or conidia of *A. alternata* as studied previously and confirmed by SEM, which showed no germination of *Alternaria* spore and destruction of conidia and hyphae. In the presence of antifungal compounds, *Alternaria* spore and candida would reduce the apothecial formation which would decimate ascospore production, it is a key factor for the pathogenesis of crops, and would consequently reduce diseases (Schiro et al. 2018).

*B. safensis* STJP produced the volatile compounds *in vitro* and this result can be good for management of soil borne fungal pathogen such as *A. alternata*. This might indicate the use of these bacteria as soil amendments for the controlling of soil-borne fungal diseases. Destruction of the fungal mycelia can greatly limit primary inoculum production and establishment of the disease in the agricultural crops (Raaijmakers et al. 2009).

The major peak of GC-MS was detected as phenol, 2, 4-bis(1,1-dimethylethyl)/2,4-di-tert-butylphenol. This is a member of the class of phenol carrying two tert-butyl substituents at positions 2 and 4 and was reported a bacterial metabolite that is an important biocontrol metabolite along with antioxidant property (Dharni et al. 2014). Pyrrolo[1,2-a]pyrazine-1,4-dione was recorded as strong antioxidant agent produced by bacteria and it is useful in prevention against free radical associated diseases (Ser et al. 2015). Hexahydro-3-(2-methylpropyl) showed antagonistic activity against bacterial pathogens along with anticancer properties (Lalitha et al. 2016). Further, 5,10-diethoxy-2,3,7,8-tetrahydro-1h,6h-dipyrrolo[1,2-a:1',2'-d]pyrazine and hexadecanoic acid detected in metabolite sample showed antifungal activity against fungal pathogens (Li et al. 2014). However, other compounds detected in bacterial metabolites such as cyclohexanol, 1-butyl-, 1-proline, n-valeryl-, heptadecyl ester, dotriacontane, d-Ribose, 2-deoxy-bis(thioheptyl)-dithioacetal, and 2-tert-Butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol are also reported to show antimicrobial, antioxidant and medicinal property (Nicolaou et al. 2011; Shakir et al. 2014; Shpakovsky et al. 2018).

The compounds nonadecane and octadecane were observed in metabolite sample of GC-MS, but their function is not reported in antagonism against phytopathogens. Another compound, 3-hexadecanol, is widely used in several industrial applications such as pharmaceuticals, cosmetics, perfumery, detergents and emulsifier (Barbizan et al. 2009). It is also reported as a type of pesticide but not yet approved (Ciancio et al. 2016). The compound isopropyl tetradecanoate does not show antimicrobial activity but it is well known in industrial applications such as food, pharmaceutical (as lubricant), and cosmetics (Barbizan et al. 2009). The volatile compound heptadecane is also considered a plant metabolite and is reported to be produced by *Bacillus* sp. (Moses et al. 2017). The compound 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione is an oxaspiro compound and its role have not yet been detected for antimicrobial activity. However, eicosane is a volatile compound

isolated from the microorganisms but its role has not been reported for biological control (Zhang et al. 2013). However, the function of some compounds such as 3-ethyl-3-undecanol, tetratetracontane, hexacosyl acetate, 1,3,5-trisilacyclohexane, 4,4'-((p-phenylene)diisopropylidene)diphenol and hexatriacontane is not clear and 1,2-benzenedicarboxylic acid show toxicity when concentration is high, but toxicity is recorded for fungus and bacteria and not for humans (Clark et al. 2004). Ditridecyl ester and hexadecanoic acid are used in several industries rather than as agrochemicals (Zhang et al. 2009), while 2-hydroxy-1-(hydroxymethyl)ethyl ester is used as a hypocholesterolemic, nematocide, antiarthritic, hepatoprotective, antiandrogenic, hypocholesterolemic 5-Alpha reductaseinhibitor, antihistaminic, anticoronary, insectifuge, antieczemic and antiacne (Ma et al. 2018).

On the basis of major peak of GC-MS, selected phenol, 2, 4-bis(1,1-dimethylethyl)/2,4-di-tert-butylphenol was tested further and recorded 50 % reduction in mycelial growth of *A. alternata* at 40 ppm concentration. Rate of inhibition further increased with increasing concentration of 2,4-di-tert-butylphenol. This is a volatile compound reported by other bacterial strains of *Streptomyces mutabilis* G61 and showed biocontrol activity against fungal pathogens (Belghit et al. 2016). Gao et al. (2018) reported that the volatile organic compounds (VOCs) produced by *Bacillus subtilis* CF-3 inhibited the mycelial growth of common fungal pathogens including *A. alternata*, with a mean inhibition rate of 59.97% and clarified that 2,4-di-tert-butylthiophenol is a key inhibitory VOC produced by *Bacillus* sp. CF-3 (Gao et al. 2018). This organic compound significantly inhibited the germination of fungal spores, disrupt hyphal and cell morphology, and decrease cell membrane fluidity and integrity, resulting in the changes of indexes of pathogen (Zhao et al. 2019).

Overall, result concluded that *B. safensis* is an eco-friendly tool for promoting plant growth by using as a biopesticide. It produces effective antifungal volatile metabolites and proved to be an alternative option for chemical pesticides. Among all volatile metabolite, 2, 4-di-tert-butylphenol was recorded for biocontrol activity against *A. alternata*. The result of present study proposes the application of *B. safensis* in the agricultural soils could be a promising tool of enhancing productivity of crops, controlling phytopathogens and promoting organic farming.

**Table 10.** FTIR spectral data of bacterial metabolites with their corresponding peak assignments

Frequencies observed (cm-1)	Tentative peak assignments	
3610.0	O-H stretching	Alcohol, phenol, carboxylic acid
3555.4	O-H stretching	Alcoholic group
3406.5	O-H stretching	Alcoholic and phenolic group
1620.8	C-C stretching ring	Benzene
1434.4	C-C stretching ring	Aromatic
1115.6	C-H stretching	Alkyl halide
800.7	C-H stretching	Alkane
662.2	-C-H stretching	Alkenes
601.1	-C-Cl stretching	Alkyl halide
465.2	C-H stretching	Alkanes
Frequencies observed (cm-1)	Tentative peak assignments	
3610.0	O-H stretching	Alcohol, phenol, carboxylic acid
3555.4	O-H stretching	
3406.5	O-H stretching	primary, secondary amines, amides
1620.8	C-C stretching ring	Benzene
1434.4	C-C stretching ring	Aromatic
1115.6	C-H stretching	Alkyl halide
800.7	C-H stretching	Alkane
662.2	-C-H stretching	Alkenes
601.1	-C-Cl stretching	Alkyl halide
465.2	C-H stretching	Alkanes

**Table 11. Metabolites identified by GC-MS in extracted sample/ supernatant of *B. safensis* STJP**

Number of Peak	Retention Time	Area	Area %	Height	Height %	Name of the compounds	Base m/z
1	21.038	514207	0.94	258692	1.35	NONADECANE	57.05
2	21.439	15586361	28.42	6568718	34.28	PHENOL, 2,4-BIS (1,1-DIMETHYLETHYL)-	191.10
3	25.191	982050	1.79	490797	2.56	DOCOSANE	57.05
4	27.006	222990	0.41	115581	0.60	OCTADECANE	57.05
5	27.085	1500472	2.74	587269	3.06	3-HEXADECANOL	59.05
6	27.273	3044960	5.55	846893	4.42	1-PROLINE, N-VALERYL, HEPTADECYL ESTER	70.05
7	27.396	862505	1.57	363396	1.90	ISOPROPYL TETRADECONOATE	60.00
8	27.783	3217193	6.78	894942	4.67	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	70.05
9	28.725	241354	0.44	102276	0.53	HEPTADECANCE	57.05
10	28.817	1742938	3.18	728708	3.80	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	57.05
11	28.906	2591555	4.72	1040957	5.43	Eicosane	154.05
12	29.374	7104699	12.95	1768885	9.23	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine	70.05
13	29.557	912367	1.66	401794	2.10	3-ETHYL-3-UNDECANOL #	87.10
14	29.614	951300	1.73	452962	2.36	1,2-BENZENEDICARBOXYLIC ACID, DITRIDECYL ESTER	149.00
15	29.765	3032489	5.53	992583	5.18	HEXADECANOIC ACID	73.05
16	31.536	692718	1.26	217218	1.13	Cyclohexanol, 1-butyl-	81.10
17	32.263	996868	1.82	442820	2.31	DOTRIACONTANE	57.10
18	32.895	869675	1.59	285174	1.49	Tetratetracontane	57.05
19	33.549	754745	1.38	313102	1.63	Hexacosyl acetate	57.05
20	35.898	270808	0.49	128222	0.67	HEXATRIACONTANE	57.05
21	37.239	850950	1.55	382659	2.00	1,3,5-Trisilacyclohexane	130.05
22	37.481	1970794	3.59	757365	3.95	d-Ribose, 2-deoxy-bis(thioheptyl)-dithioacetal	117.05
23	37.743	1089259	1.99	387242	2.02	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	57.05
24	39.581	488780	0.89	215690	1.13	4,4'-(p-Phenylene)diisopropylidene)diphenol	331.25
25	44.258	3857205	7.03	417042	2.18	2-tert-Butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol	57.05
		54849242	100.00	19160987	100.00		



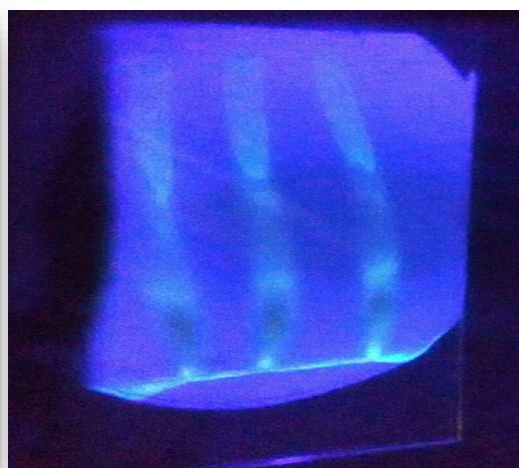
**Fig. 53** Production of extracellular metabolites by isolate STJP



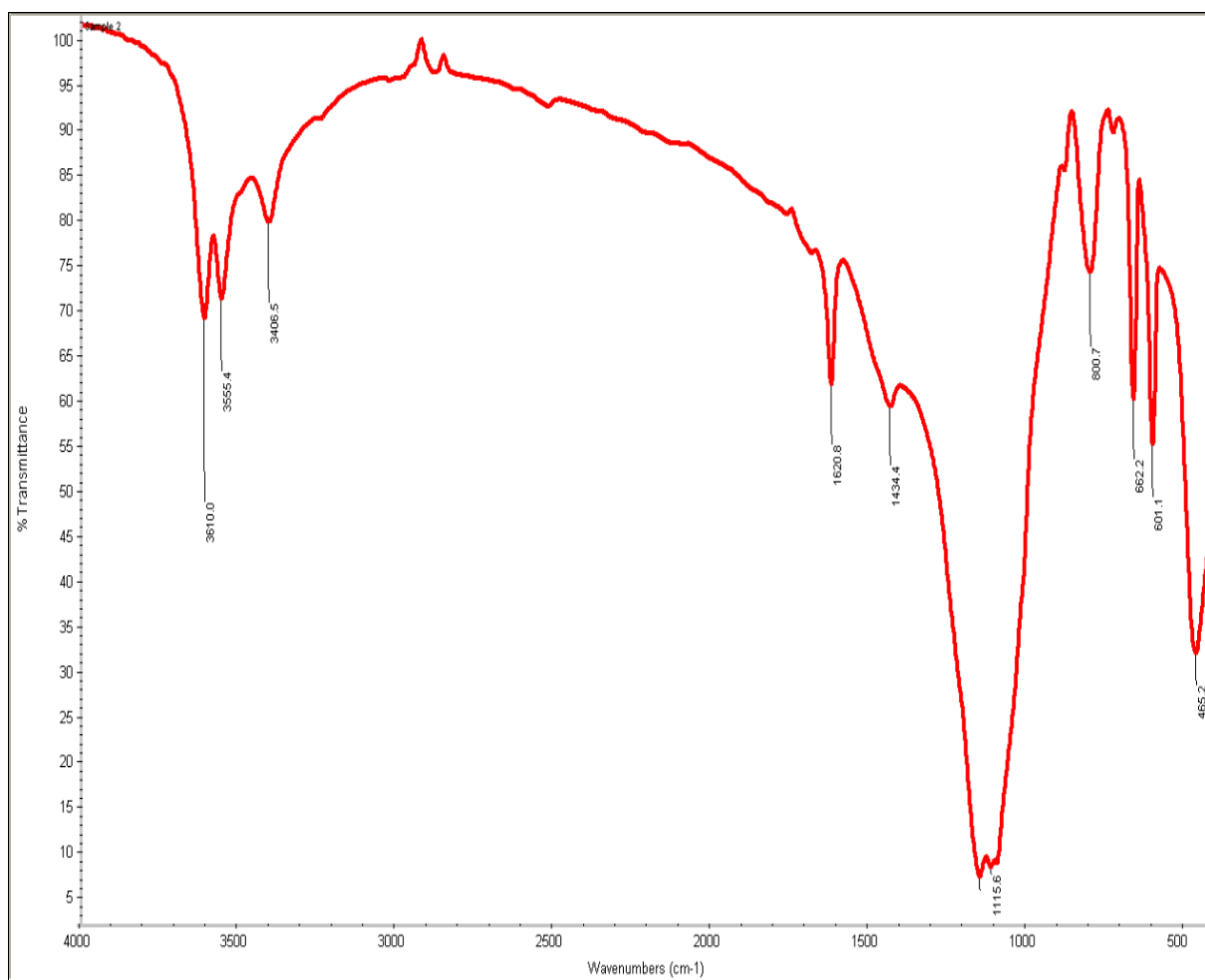
**Fig. 54** Production of volatile compounds by isolate STJP through seal plate method



**Fig. 55** Extraction of metabolites from bacterium STJP by separating funnel



**Fig. 56** Band showing metabolites, extracted from bacterium STJP on TLC plate



**Fig. 57** FT-IR spectra of bacterial (STJP) metabolite

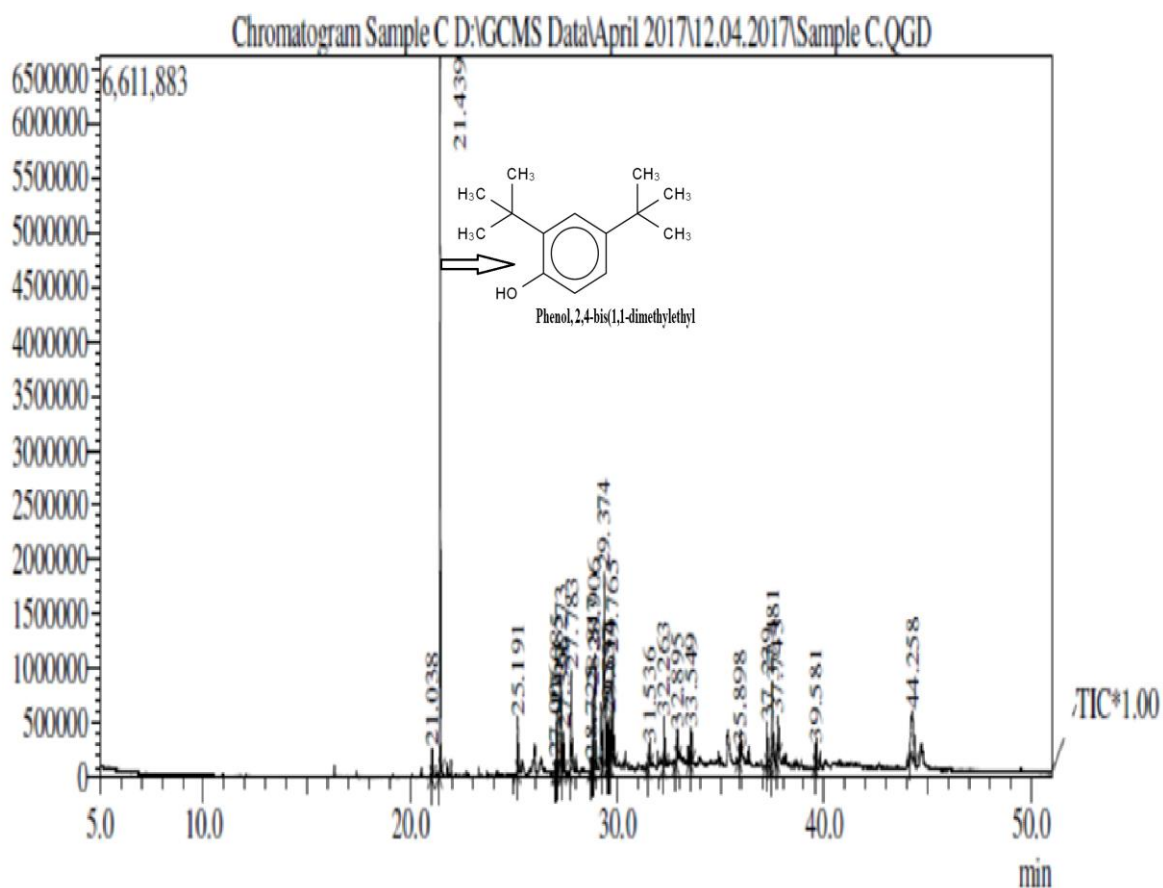


Fig. 58 Identification of bacterium (STJP) metabolites by GC-MS

*Chapter-08*  
*In Vitro and In Vivo Study of  
Selected Rhizobacteria on Plant  
Growth, Disease Management and  
Seed Germination of S. rebaudiana*



## 1. Introduction

Microbes play beneficial roles in plants and ecosystems. The rhizobacteria which show plant growth promoting characteristics and known as PGPR (e.g., *Pseudomonas* spp. and *Bacillus* spp.), can be used as bioformulations (Khan 2005). These rhizobacteria have proven to enhance germination and improve growth of agricultural crops (Backer et al. 2018). PGPR colonize the rhizosphere resulting in acceleration of growth and protection of plant against phytopathogens (Beneduzi et al. 2012). Rhizobacteria play direct role as a biological fertilizer by several PGP traits such as production of phytohormones, siderophores and solubilization of minerals (zinc, potassium, and phosphate) (Zhou et al. 2017). These bacteria can also indirectly help plants by inhibiting pathogenic microorganisms through production of siderophores and antibiotics or secretion of lytic enzymes such as chitinases,  $\beta$ , 1, 3 glucanase, and cellulase (Saraf et al. 2008).

Among PGPR, *Bacillus* is one of the most effective microbe for enhancing growth and yield of crops under biotic and abiotic conditions (Prakash and Arora 2019). The multifaceted properties of *Bacillus* spp. can be an alternative substitute of chemical fertilizers/pesticides (Kang et al. 2019). Zhou et al. (2017) reported that *Bacillus* sp. SA03 when applied on *Chrysanthemum* resulted in increased rate of photosynthesis, biomass and yield of plant. The seeds of radish plant (*Raphanus sativus*) treated with *Bacillus* spp. enhanced fresh and dry weight, photosynthetic pigments, proline content, amino acid and protein contents in plant as observed by Mohamed and Gomaa (2012). Although, many bacterial biofertilizers are available for different crops, but *Bacillus* based fertilizers are known to be better due to their spore forming property and production of several metabolites (Malusa et al. 2012). Application of *Bacillus* sp. is an eco-friendly and sustainable tool for agriculture.

Arbuscular mycorrhizal fungi (AMF) are also commonly used for enhancing the growth and crop productivity and are being used as bio-fertilizers (Sadhana 2014). Beside the plant growth, AMF provide tolerance to host plants against several pathogens and help in survival under stressful conditions like drought, heavy metals, heat, salinity and extreme temperatures (Mosa et al. 2015). AMF form vesicles, arbuscules, and hyphae in plant roots, and also spores and hyphae in the rhizosphere

region (Wu et al. 2013). Formation of hyphal web by the AMF with plant roots significantly improves the root surface area in the soil, causing development and increment in plant growth (Burrows and Pflieger 2002). AMF improve plant nutrient content by increasing the availability as well as translocation of various nutrients inside the plant (Rouphael et al. 2015). They also improve soil fertility by influencing its structure and texture, and hence plant fitness (Vázquez-Hernández et al. 2011). In addition, fungal hyphae can accelerate the decomposition process of soil organic matter (Hildebrandt et al. 2006).

Physical and chemical (1% tetrazolium chloride, hormones chemical based) methods are available for enhancement of seed germination of Stevia crop, but these methods have some limitations (Tandon et al. 2019). Biological approaches are considered to be better as compared to physical and chemical methods (Van Klinken et al. 2008). PGPR are the biological tools which can enhance the germination of seed, growth and yield of Stevia plant. There is very limited research available in relation with PGPR and growth promotion of Stevia. In the current study, isolate *B. safensis* STJP (alone) and in combination with AMF were applied on Stevia and checked for enhancement of plant growth parameters, plant metabolites and soil health.

## 2. Materials and methods

### 2.1 Organisms and seeds collection

*Glomus fasciculatum* (ABTEC) Vesicular-Arbuscular Mycorrhiza (VAM) and Stevia seeds (Variety: ste002) were purchased online from www.amazon.com. *B. safensis* STJP (TB-2833) (isolated and identified as reported in previous chapters) was taken for pot trials. Isolate STJP was selected based on its PGP characteristics as already mentioned (in previous chapters). *A. alternata* (Accession number - KX494864, NCBI), a phytopathogen of *S. rebaudiana* was procured from Laboratory for Rhizospheric Microbiology and Sustainable Agriculture, School for Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow. Seedlings (variety: CIM-Mithi) of *S. rebaudiana* (for pot study) were procured from Central Institute of Medicinal and Aromatic Plants, (CIMAP), Lucknow, Uttar Pradesh, India.

### 2.2 *In vitro* study

For seed germination (of Stevia), culture tubes (size: 100 mm long x 13 mm width) containing water agar were taken. Before experiments, seeds were surface sterilized

with 70% ethanol for 2 min followed by 2 % sodium hypochlorite (5 min) and finally rinsed with distilled water (thrice) (Arora et al. 2001). The surface sterilized seeds were dipped into bacterial broth culture (log phase with  $10^8$  cfu/ml) for 20 min and then allowed to dry for 30 min (Arora et al. 2001). Bacterial coated seeds (of Stevia) were sown on culture tubes containing water agar. These sets included; T-1) Control (Stevia seeds without coating); T-2) Stevia seeds with *A. alternata* (negative control); T-3) Seeds coated with *B. safensis* STJP; T-4) Seeds coated with STJP + *A. alternata*. Three replicates were taken for each set. The tubes (diameter: 20 mm x 70 mm) were kept for 30 days at room temperature. Seed germination percentage (SGP), plant height, biomass, root shoot ratio and germination index (GI) were noted 30 day after sowing (DAS).

### 2.3 *In vivo* (pot) study

The pot experiment was performed in Horticulture Research Farm (HRF), BBA University, Lucknow (latitude  $26.8467^\circ$  N, and longitude of  $80.9462^\circ$  E), India, for 90 days. Selected isolate STJP was grown in NB. Afterward 48 h, growth culture was centrifuged for collecting the bacterial pellet (5000 rpm for 10 min at  $4^\circ\text{C}$ ). Sterilized deionized water was mixed in the pellet to obtain population density of  $10^8$  cfu/ml as per Arora et al. (2010).

Sandy-loamy soil (pH- 7.69; EC - 0.48 dS  $\text{m}^{-1}$ ; N - 108 kg  $\text{ha}^{-1}$ ; K - 89 kg  $\text{ha}^{-1}$ ; P - 4.26 g kg  $\text{ha}^{-1}$ ) was collected from HRF and 6 kg was filled in each clay pot (20 cm x 20 cm x 20 cm). Rootlets of the seedlings of *S. rebaudiana* were disinfected with ethanol (70% for 2 min) followed by sodium hypochlorite (2% for 5 min) and subsequently washed with deionized water for 10 min (Arora et al. 2001). The seedling was then dipped in the bacterial inoculum suspension ( $10^8$  cfu/ml for 20 min). Fungal propagules ( $10^4$  cfu/ml/gm of soil) were added in the pots as per Khare et al. (2011). Subsequently, the seedlings (five per pot) were sowed as per following sets: 1) Control (*S. rebaudiana*), 2) *A. alternata* (negative control), 3) *B. safensis* STJP, 4) STJP + *A. alternata*, 5) Mycorrhiza (*G. fasciculatum*), 6) Mycorrhiza + *A. alternata*, 7) Mycorrhiza + STJP + *A. alternata*, 8) Mycorrhiza + STJP. Five replicates were taken for each set. Irrigation was done using tap water (in equal amount). The plants were uprooted 90 DAS and checked for the shoot and root length, fresh and dry weight, and number of leaves.

### 2.3.1 Effect of STJP on carbohydrate content

Examination of carbohydrate (soluble sugar) was done by Anthrone method (Albalasmeh et al. 2013). Briefly, 100 mg of plant sample (leaves and stem) were homogenized with 10.0 mL of 80% (v/v) ethanol and further centrifuged (6000×g for 5 min). Afterward, 0.5 mL alcoholic extract of sample was mixed with deionized water to make the volume 1 mL to which freshly prepared Anthrone reagent (4 mL) was added. After the development of color, the absorbance of the sample was read at 630 nm by spectrophotometer (Evolution 201, Thermoscientific). The soluble sugar (carbohydrate) was calculated ( $\text{g}^{-1}$  FW of leaf by using a standard curve of glucose (Albalasmeh et al. 2013).

### 2.3.2 Effect of STJP on chlorophyll content

To determine the chlorophylls content (chlorophyll a and chlorophyll b) of plants, 0.5 g of fresh leaves were homogenized with 10 mL of acetone (80%) using mortar and pestle. Afterward, mixture was centrifuged (for 10 min at 5000 rpm) and collected the supernatant. The optical density of the obtained supernatant was read at 645 and 663 nm by spectrophotometer. The chlorophyll content was calculated using the formula as per Burnett (1976).

### 2.3.3 Effect of STJP on antioxidant activity

For estimation of antioxidant activity, the fresh plant sample was dissolved in 6 ml of 0.008% methanol solution containing 2,2-diphenyl-2-picrylhydrazyl (DPPH) as radical scavenging agent (Braca et al. 2001) and stored at 20°C. Further, the mixed sample was shaken well and incubated in dark (15 min). After incubation, sample was analyzed using spectrophotometer at 517 nm for examination of antioxidant activity as per the following equation:

$$[\text{Antioxidant assay (\%)} = (\text{control absorbance} - \text{sample absorbance}) / (\text{control absorbance}) \times 100]$$

### 2.3.4 Effect of STJP on flavonoid activity of plant

The plant sample was crushed and dissolved in methanol. Further, 20  $\mu\text{l}$  of the sample extract was added in a solution of 2%  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ . The mixture was continuously shaken and diluted with double distilled water to make the total volume 10 ml. The absorbance of the sample was measured after 10 min incubation at 440 nm. Flavonoid content was expressed as quercetin equivalents in mg per gram dry material as per Quettier-Deleu et al. (2000).

### 2.3.5 Extraction and estimation of stevioside

The harvested shoots of *S. rebaudiana* were dried in the hot air oven (Thermostatic RSTI-101, India) (at 50°C). The extraction of stevioside was done by hot water method (Rai et al. 2012). Briefly, the completely dry shoots were crushed into powder form and dissolved in the water and further boiled (78±2°C; dried shoot powder 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 (R-100 Thermo Fisher Scientific, USA) (45°C; 75 mbar; 55 rev min<sup>-1</sup>) for complete water vaporization (Megeji et al. 2005).

Obtained dry sample 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 sample was analyzed for stevioside content by using HPLC (Waters 2489 UV/Visible Detector, USA) consisting mixed-mode wax-1 column (pore size: 5µm). 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 (20:80v/v; HPLC Grade, Merck) (flow rate; 1.0 mL/min, pressure; 1100 psi, at 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.

### 2.3.6 Effect of STJP on nutrients uptake in *S. rebaudiana*

After tilling of *S. rebaudiana* (90 DAS), all plants were dried in the hot air oven (Thermostatic RSTI-101 Series, India). For analysis of P, 100 mg dried sample was grinded and digested with 6 ml of H<sub>2</sub>SO<sub>4</sub> and HClO<sub>4</sub> (Ratio: 9:1) using hot plate (for 10 minutes). Digested colourless samples were diluted into 100 ml distilled water. Finally, the prepared sample was examined for P as per Vanadomolybdophosphoric yellow color method (Koeing and Johnson 1942). Available Zn uptake in the plants were determined after the digestion process by using atomic absorption spectroscopy (AAS) (AA240FS Fast Sequential AAS, USA) (Horwitz et al. 1970). Subsequently, K uptake in *S. rebaudiana* plants was evaluated with tri-acid digestion method according to Jackson (1967).

### 2.3.7 Effect of STJP on soil health

After tilling of plants, soil pH and electrical conductivity was measured by the standard method as given by Jackson (1973). The total organic carbon in the soil was examined using wet oxidation method described by Walkely and Black (1934). The available P content in soil (saline and non-saline conditions) was determined by using Kjeldahl digestion method (Taylor 2000) and uptake of P through plants (dried stems and leaves) was determined by vanado-molybdo-phosphoric yellow color method (Koeing and Johnson 1942). The amount of nitrogen (N) was examined by alkaline permanganate method as per Subbiah and Asija (1956). Total nitrogen (N) from the soil sample was analyzed with aqueous solution (HCl/HNO<sub>3</sub>) in a ratio of 3:1 as per standard method (Subbiah and Asija 1956).

### 2.3.8 Statistical analysis

Pot experiment was done taking five replicates and data was represented as the mean  $\pm$  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 different sets.

## 3 Results

### 3.1 *In vitro* study

Among all the sets, STJP showed best results in terms of SGP, shoot length, root length, total fresh weight (TFW), GI, and RSR. The SGP (80%) was recorded highest by STJP treatment as compared to control (Figure 60). The plant length was enhanced by 188.81%, and 88.15% by STJP treatment as compared to control and pathogen treatments (*A. alternata*), respectively (**Table 11**). TFW of plants increased by 40.29% by the treatment with STJP as compared with control plants (**Table 11**). Further, this result (TFW) was observed also to be much better by STJP treatment even in the presence of phytopathogen. The GI of seeds recorded enhancement of 42.40%, 90%, and 442% by the treatment with STJP in compared with STJP + *A. alternata*, control and *A. alternata* (negative control), respectively. Further, RSR was also found to be significantly higher by 236.36% and 54.16% by STJP treatment when it was compared with without any treatment and *A. alternata*. STJP treated with *A. alternata* enhanced 71.17% RSR as compared to control.

### 3.2 *In vivo* pot study

#### 3.2.1 Effect on plant growth parameters

Fresh weight of *S. rebaudiana* was improved with STJP treatment as related with plants without any treatment. Fresh weight increased by 72.99% and 31.33% by the treatments of STJP and mycorrhiza respectively, when compared with control (Table 12). The maximum fresh weight (91.63%) was observed with STJP + mycorrhiza as compared to without application (control) (Table 12). Dry weight is an effective parameter of the growth and development of plant. In this regard, the application of STJP + mycorrhiza treatment showed more pronounced effect in terms of dry weight as an increment of 78.94%, was observed as compared to untreated plants (Table 12). When STJP and mycorrhiza were separately applied on the Stevia plants, 53.07% and 26.66% enhancement in dry weight was observed as compared to negative control (*A. alternata*), respectively (Figure 61). The combined (STJP and mycorrhiza) treatment enhanced (105.03%) plant length as compared to control plant. Although STJP and mycorrhiza treatments (separately) resulted in enhancement in growth parameters, but results were better in combined treatment of fungi and bacteria.

Treatments of STJP + mycorrhiza + *A. alternata*, STJP + *A. alternata*, mycorrhiza + *A. alternata* improved plant length by 121.34%, 64.43%, and 82.10% in comparison with negative control (*A. alternata*), respectively (Figure 61). The number of leaves was enhanced (133.48%) by treatment with STJP + mycorrhiza as compared to *A. alternata*. STJP and mycorrhiza increased leaf number by 98.01% and 35.71% (as compared to control), respectively. Number of leaves by STJP + mycorrhiza + *A. alternata*; STJP + *A. alternata* and mycorrhiza + *A. alternata* treated plants recorded higher (178.43%, 125.02% and 77.29%, respectively) as compared to negative control (Table 12). Overall the treatment STJP + mycorrhiza gave best results in the terms of fresh weight, dry weight, plant length and number of leaves of *S. rebaudiana* plants (90 DAS).

#### 3.3 STJP enhances chlorophyll, carbohydrate, flavonoid and antioxidant activity

STJP + mycorrhiza treated Stevia plants showed maximum chlorophyll content (a and b) (78.01%, 127.18%) as compared to control (Table 13). Chlorophyll a and b were also increased with the treatment of mycorrhiza, but result was not as good, when compared with STJP + mycorrhiza (Table 13). STJP + mycorrhiza even in presence of

*A. alternata* treatment enhanced chlorophyll content (a and b) by 94.59%, and 115.33%, respectively as compared to *A. alternata* (negative control).

Flavonoid and antioxidant activity are the two most important growth parameters of crops (especially medicinal plants). In this regard, STJP and mycorrhizal treatment positively affected flavonoid and antioxidant content in comparison with control (Stevia) plant and results showed maximum increment in flavonoid (253.06%) and antioxidant (109.78%) content in plants, as compared to control. STJP + mycorrhiza in presence of *A. alternata* treated plants showed enhancement (in comparison with control plants) in the flavonoid (487.50%) and antioxidant activity (74.48) followed by STJP treatment alone in presence of phytopathogen. However, mycorrhiza treatment showed 275% and 50.86% increment in the flavonoid and antioxidant content of Stevia plant in the presence of fungal pathogen (*A. alternata*) as compared to negative control. Carbohydrate is one of most important parameter of Stevia plant. The highest carbohydrate content in Stevia plant was recorded when inoculated with STJP + mycorrhiza (74.40%) as compared to control. Mycorrhizal treatment also positively affected the carbohydrate content. Combine treatment of PGPR and mycorrhiza in presence of phytopathogen showed increment in carbohydrates (62.73%) as compared to negative control (*A. alternata*).

### 3.4 STJP enhances stevioside content

The treatment with STJP and mycorrhiza showed significantly high stevioside content (201.48%) in *S. rebaudiana* as compared to control (Figure 62). Only STJP treatment (175.76%) resulted in significantly better stevioside content when compared with control. Among all, STJP + mycorrhiza in presence of *A. alternata* treated plants showed greater (214.40%) amount of stevioside followed by STJP + *A. alternata* (149.82%). However, mycorrhiza treatment with *A. alternata* in Stevia plants also showed good results (62.47%) as compared to negative control (*A. alternata*). Overall results suggested that STJP + mycorrhiza treatment was a more effective method in augmentation of stevioside concentration in the *S. rebaudiana*. This result was compared and confirmed by HPLC (Figure 63).

### 3.5 STJP enhances nutrient uptake in *S. rebaudiana*

Application with STJP + mycorrhiza treatment resulted in ~1.3 folds enhancement of P in the plant tissue when compared with control (Table 14). Bacterial treatment (alone)

and in combination with mycorrhiza in presence of *A. alternata* did not show statistical difference in uptake of P, however results were much better than without treated plants and negative control. Zn and K nutrients uptake in *S. rebaudiana* were significantly enhanced when applied with STJP + mycorrhiza. The quantity of Zn and K was enhanced by 50% and 99.75% respectively, by STJP + mycorrhiza treated *S. rebaudiana* than that of without inoculated plants. STJP and mycorrhiza treatments recorded higher Zn and K content (29.24% and 72.46%) as compared to control. STJP + mycorrhiza in presence of *A. alternata* significantly enhanced Zn (151.02%) and K (37.97%) concentration in comparison with negative control. Mycorrhiza (in presence of *A. alternata*) treated plants showed an increment in uptake of K and Zn as confirmed by statistical result, when it was compared with negative control plants. Overall, STJP + mycorrhiza was found to be best method in uptake of nutrients (P, K and Zn) in *S. rebaudiana*.

### 3.6 Effect of treatments on soil health

After harvesting of crops, soil nutrients were checked. In this regard, it was observed that K, P, Zn, C, and N of STJP + mycorrhiza treated soils was 118.69, 113.69, 464.30, 71.72, and 138.21% higher than without any treated soils. Only STJP treatment was able to increase K, P, Zn, C, and N in the soils as compared to control (Table 14). However, STJP + mycorrhiza even in presence of *A. alternata* resulted in 3.31, 13.22, 60.65, and 51.14% increase of K, P, Zn C, and N, respectively, in comparison with control. Mycorrhiza treatment also improved soil fertility, when it was compared with control (Table 14). The pH is also an important parameter of soils. In this regard, pH was recorded in between 6.8-7.6 by all the treatments including control. However, the most appropriate soil pH (nearly neutral) was observed by STJP treated soils. Overall, STJP + mycorrhiza treatment was found to be a reliable method to improve soil fertility.

## 4. Discussion

Stevia and their metabolite (stevioside) are very important commercially, being used as calorie free sweetener for diabetes patients (Modi et al. 2014). Besides being alternative to sugar, it also controls blood sugar, hypertension, skin disorders, and tooth decay (Angelini et al. 2018). One gm of stevia powder consists of 91% stevioside and is equal to 250 gm refined sugar with no calories (Angelini et al. 2018). Beside these, Stevia leaf contains 6-18% stevioside with rich amount of micro and macronutrients (Modi et

al. 2014). Hence, the cultivation of this commercially important crop is very essential by using eco-friendly approach. In this regard, *Bacillus* spp. are widely reported as PGPR and could be eco-friendly and sustainable method for growth and production of Stevia crop (Molina-Santiago et al. 2018; Prakash and Arora 2019).

Bacterium STJP with mycorrhiza applied on *S. rebaudiana*, significantly increased the growth, biomass, and nutrients uptake as well as the soil health. The increased plant growth by rhizobacteria and mycorrhiza is also reported in previous studies (Miransari 2013; Vafadar et al. 2014; Parewa et al. 2014). Present study clearly shows improved growth, biomass yield, stevioside content as well as nutrient uptake (N,P, K) and chlorophyll content of *S. rebaudiana* by combine application of rhizobacteria and mycorrhiza. Bharati et al. (2016) also reported that combined application of mycorrhiza (*G. fasciculatum*) and bacterium (*Exiguobacterium oxidotolerans*) resulted in enhancement of plant length, number of leaves, growth biomass, nutrient uptake and metabolite content in *M. arvensis*. This PGPR produced phytohormones, and solubilized minerals (Zn, P, and K), leading to greater rate of photosynthesis in the plants by which plant growth and productivity was increased (Bharati et al. 2016). In current study, chlorophylls (a and b) content in *S. rebaudiana* was significantly augmented by the treatment of *B. safensis* STJP. This might be due to production of IAA, siderophore, and solubilization of nutrients like zinc, potassium, and phosphorous. According to Tarraf et al. (2017) maximum chlorophyll content in *Salvia officinalis* L was recorded via IAA and mineral solubilization by PGPR.

In the present study, *B. safensis* STJP inhibited the growth of *A. alternata* by production of volatile compounds (proved in the previous chapter) and increased plant growth and productivity. Hazarika et al. (2019) reported that *Bacillus subtilis* SCB-1 produced volatile compounds to inhibit fungal pathogen and their role was recorded in the improvement of plant growth and yield. *Bacillus* spp. are recorded as effective PGPR which can inhibit the phytopathogens and may improve the photosynthesis rates, nutrients uptake, biomass and yield of plants as observed by earlier studies (Modi et al. 2014; Ahmad et al. 2017). However, lots of literature are available on the mechanism of PGPR for plant growth promotion and yield (Bharati et al. 2016; Zheng et al. 2018; Prakash and Arora 2019), but there are limited reports available on the enhancement of secondary metabolite content of plants.

Stevioside is a chief secondary metabolite of Stevia plant and an excellent substitute of sugar for diabetics (Modi et al. 2014). In this study, *Bacillus* sp. STJP

recorded enhancement of stevioside content may be due to solubilization of minerals viz. P, K, and Zn and by production of hormones (IAA and GA). The high stevioside concentration in *S. rebaudiana* plants was obtained by the treatment of *Bacillus gladioli* strain 10216. The enhancement of metabolite content in plant was observed due to mineral solubilization and growth hormone production by bacteria (Mamta et al. 2010). Mahanty et al. (2017) reported that *Bacillus* sp. containing multiple PGP traits showed enhancement in growth and metabolite content in several medicinal, vegetables and fruits crops. Even in the presence of pathogen, *A. alternata*, metabolite content of Stevia, (when inoculated with *Bacillus safensis* STJP) was significantly increased. Vurukonda et al. (2018) reported that PGPR inoculation was found to control various fungal pathogens including *A. alternata* and increment of metabolite content in plants. Bacterium *Pseudomonas fluorescens* strain CL12 when applied on *Curcuma longa*, increased the growth and metabolite curcumin content due to solubilization of inorganic phosphates, production of IAA and siderophore (Kumar et al. 2016). *B. safensis* also showed good PGP characters, and enhanced growth, and metabolite content in Stevia plant. Based on above study, *B. safensis* can be used as an effective bioformulation that will provide several benefits such as solubilization of minerals, production of phytohormones and siderophores and make the availability to plants. Such strain can be very important in improving the growth, biomass, management of disease, nutrient uptake and stevioside content in *S. rebaudiana*. Hence the strain was submitted in a culture collection centre (NAIMCC-B-02323).

The soil fertility mainly depends on the availability of microbial population (Chowdhary et al. 2018). Plants require soluble nutrients for growth and development. *B. safensis* STJP increased the availability of nutrients to the plant. Overall, *B. safensis* STJP in combination with mycorrhiza enhanced the growth, nutrients, metabolite content, soil health and thus can be suitable candidate for sustainable agriculture.

**Table 12.** Impact of different treatments on plant growth parameters of *S. rebaudiana* in the tubes (30 days)

Treatments	SGP (%)	SL (cm)	RL (cm)	TFW (mg)	GI (%)	RSR (%)
STJP	80	5.1 ± 0.16	3.16 ± 0.16	64.66 ± 1.24	45.00	7 0.15
STJP + <i>A. alternata</i>	60	4.33 ± 0.80	2.70 ± 0.08	53.33 ± 0.94	31.60	0.31 ± 0.18
Control	50	2.63 ± 0.60	2.03 ± 0.32	48.00 ± 1.63	23.60	0.24 ± 0.13
<i>alternata</i>	30	1.46 ± 0.36	1.40 ± 0.28	31.33 ± 0.94	08.30	0.11 ± 0.07

(Data are mean of five replicates ± standard deviation, Where Control is only *Stevia* (without any treatment); SGP: seed germination percentage, SL: stem length; RL: root length; TFW: total fresh weight; GI: germination index; RSR: root shoot ratio)

**Table 13.** Effect of different treatments on growth of *S. rebaudiana*

Treatments	Growth parameters of <i>S. rebaudiana</i>						
	Plant length (cm)		Number of leaves	Fresh weight (gm)		Dry weight (gm)	
	Shoot length	Root length		Shoot	Root	Shoot	Root
Control	17.73±1.84g	5.53±0.74fg	84.00±2.82g	19.96±1.80f	4.63±0.98f	8.36±0.85g	3.07±0.75g
<i>A. alternata</i>	12.23±1.63h	5.43±0.57h	58.66±0.94h	14.26±0.83g	2.90±1.34g	4.80±1.28h	2.40±0.94h
STJP	29.73±1.76c	7.66±0.88bc	166.33±6.34b	36.20±2.08c	10.05±1.73b	14.43±1.54bc	5.43±1.21e
STJP + <i>A. alternata</i>	24.93±1.59de	7.23±1.22c	132.00±4.89e	33.06±1.87d	8.43±0.98d	10.63±2.12f	6.10±1.10d
Mycorrhiza	24.46±1.63e	5.86±0.73efg	114.00±4.32d	21.93±1.99e	8.30±2.49cd	11.30±1.83d	6.40±1.01c
Mycorrhiza + <i>A. alternata</i>	23.86±0.92f	5.53±0.92e	104.00±1.41f	18.08±2.64fg	7.33±1.02e	10.10±1.66e	5.20±1.06f
Mycorrhiza + STJP + <i>A. alternate</i>	33.13±0.57b	5.96±0.44d	163.33±2.35c	37.32±2.45bc	8.43±0.98c	14.23±1.40c	7.30±0.77b
Mycorrhiza + STJP	36.46±1.40a	8.56±0.86a	179.33±5.43a	41.94±2.39a	11.46±2.19a	15.80±2.45a	8.66±0.93a

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 14.** Chlorophylls, carbohydrate, flavonoids and antioxidant activity of *S. rebaudiana* under different treatments

Treatments	Chlorophyll a	Chlorophyll b	Carbohydrate content	Flavonoids	Antioxidant activity
Control	63.23±2.46f	42.56±1.25g	3.36±0.10f	1.96±0.12e	53.56±2.45g
<i>A. alternata</i>	32.56±1.14h	30.12±1.14h	2.12±0.24h	0.48±0.01g	42.56±2.46h
STJP	96.42±4.68b	76.36±4.46b	4.86±0.46b	3.64±1.12b	96.86±4.46b
STJP + <i>A. alternata</i>	80.12±1.41c	62.24±5.40ef	3.64±0.96e	2.64±0.89d	78.36±2.46cd
Mycorrhiza	66.36±2.86de	64.40±2.48de	3.64±0.28de	2.82±0.84cd	76.36±2.48d
Mycorrhiza + <i>A. alternata</i>	40.21±4.34g	60.24±2.40f	2.86±0.36g	1.80±0.78f	64.21±2.13f
Mycorrhiza + STJP + <i>A. alternata</i>	63.36±4.50ef	64.86±3.46cde	3.45±1.12c	2.82±0.84d	74.48±1.12e
Mycorrhiza + STJP	112.56±3.46a	96.69±6.42a	5.86±1.10a	4.96±0.86a	112.36±4.12a

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 15.** Nutrients uptake by *S. rebaudiana* on application of different treatments

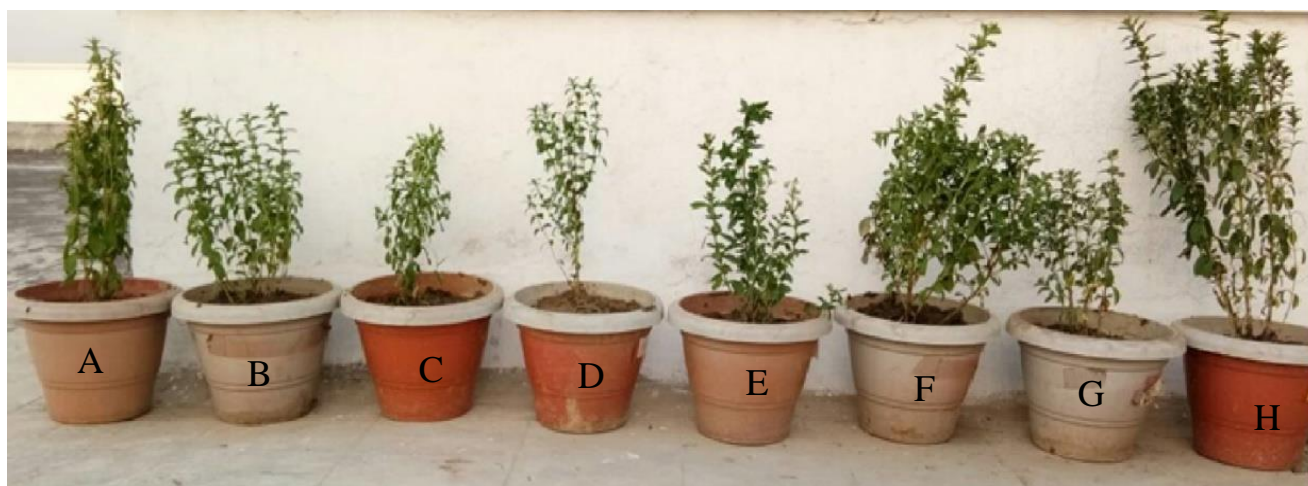
Treatments	Potassium uptake (mg/plant)	Phosphorus uptake (mg/plant)	Zinc uptake (mg/plant)
Control	4.14 ± 0.05 e	140.30 ± 2.77 d	2.12 ± 0.05 c
<i>A. alternata</i>	3.95 ± 0.02 f	123.24 ± 5.58 e	0.98 ± 0.10 d
STJP	7.14 ± 0.11 b	302.00 ± 6.55 b	2.74 ± 0.07 b
STJP + <i>A. alternata</i>	6.14 ± 0.11 c	288.25 ± 6.10 b	2.53 ± 0.05 b
Mycorrhiza	6.04 ± 0.13 c	275.31 ± 6.40 b	2.64 ± 0.08 b
Mycorrhiza + <i>A. alternata</i>	5.37 ± 0.06 d	232.46 ± 5.07 c	2.42 ± 0.06 bc
Mycorrhiza + STJP + <i>A. alternata</i>	5.45 ± 0.10 d	235.65 ± 6.40 c	2.46 ± 0.03 b
Mycorrhiza + STJP	8.27 ± 0.06 a	334.30 ± 2.06 a	3.18 ± 0.06 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).

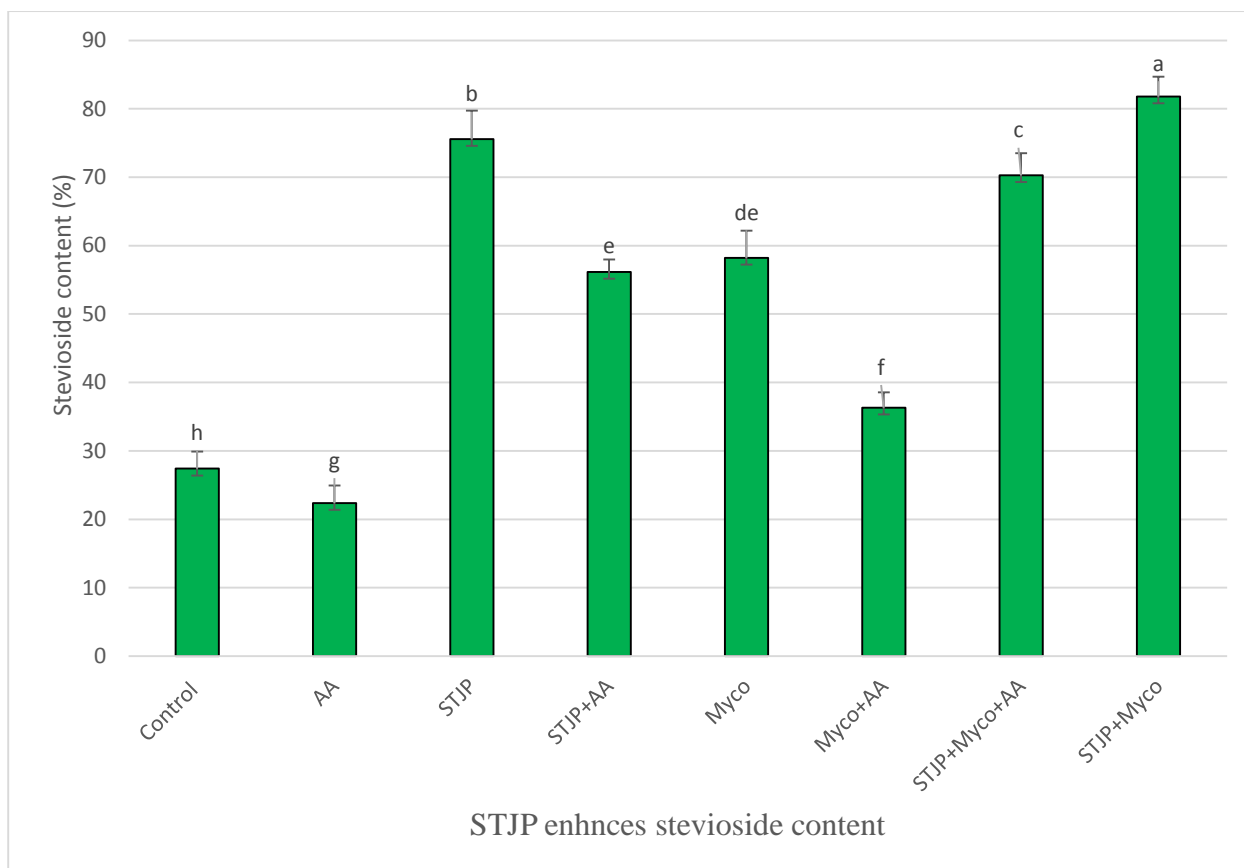
**able 16.** Nutrients content in soils after cultivation of plant (after 90 days)

Treatments	Plant nutrients in soils					
	Potassium uptake (mg/kg)	Phosphorus uptake (mg/kg)	Zinc uptake (mg/kg)	Carbon	Nitrogen	pH
Control ( <i>Stevia rebaudiana</i> )	3.21±0.26e	1.46±0.25h	0.31±0.08h	7.25±0.10f	2.46±0.42d	7.8
<i>Alternaria alternata</i>	2.82±0.28h	1.89±0.14g	0.61±0.04g	5.22±0.42h	2.41±0.98f	7.6
STJP	6.24±1.46b	2.46±0.23b	1.12±0.09c	10.45±2.24b	3.86±1.12b	7.1
STJP + <i>A. alternata</i>	4.14±0.86c	1.98±0.41f	1.24±0.08b	6.45±1.24g	3.46±0.86c	7.8
Mycorrhiza	3.42±0.74d	1.98±0.76de	0.84±0.12e	7.46±1.22e	2.46±0.78d	6.8
Mycorrhiza + <i>A. alternata</i>	2.95±0.86g	1.96±0.24e	0.70±0.06f	7.68±0.89de	1.78±1.12g	7.1
Mycorrhiza + STJP + <i>A. alternata</i>	2.97±0.68fg	2.14±0.78c	0.98±0.20d	7.89±1.24cd	2.96±1.14e	7.2
Mycorrhiza + STJP	7.02±1.45a	3.12±0.12a	1.75±0.31a	12.45±1.48a	5.86±0.74a	6.8

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).

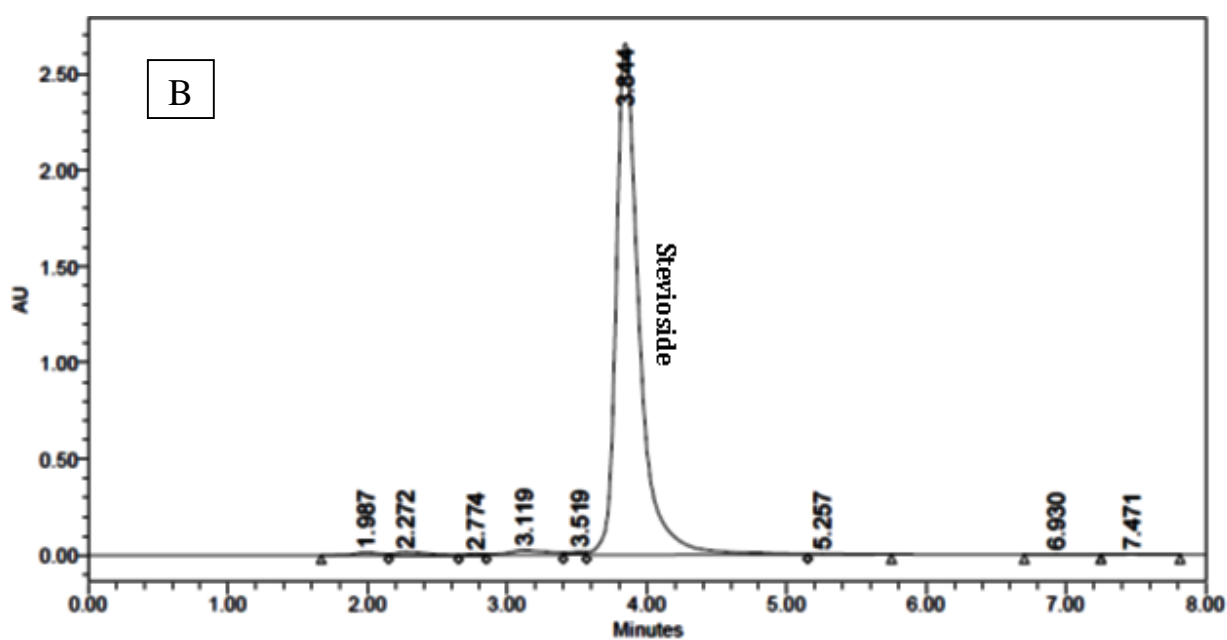
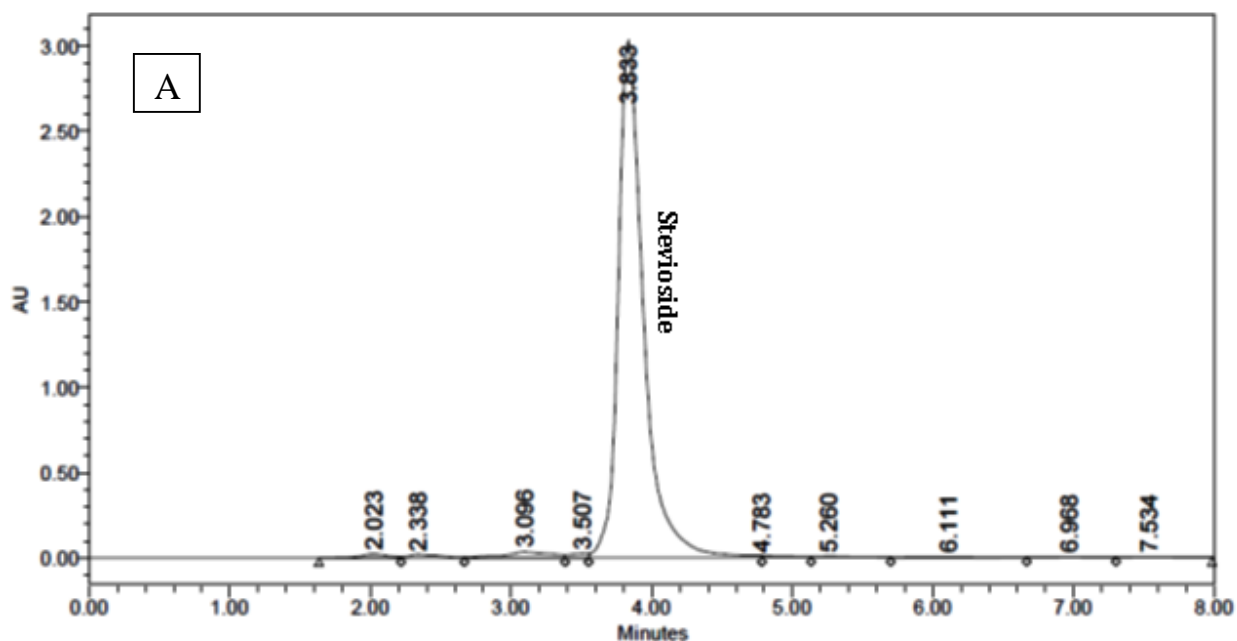


**Fig. 59** Growth promotion of Stevia by different treatments under *in-vivo* condition; Where, **A)** STJP + Mycorrhiza + *A. alternata*, **B)** Mycorrhiza, **C)** control, **D)** *A. alternata*, **E)** STJP + *A. alternata*, **F)** STJP, **G)** Mycorrhiza + *A. alternata*, **H)** STJP + Mycorrhiza



**Fig. 60** Effect of different treatments on stevioside content of *S. rebaudiana*

(Where, STJP: bacterial strain, AA: *A. alternata*, Myco: mycorrhiza (*Glomus fasciculatum*), Data are mean of five replica  $\pm$  standard error of means. Means, followed by the same letter in a column are not significantly different by Duncan's multivariate test (DMRT).



**Fig. 61** HPLC chromatogram (A) showing stevioside content of control *S. rebaudiana* and (B) plants inoculated with STJP

*Chapter-09*  
*Development of Suitable Bioformulation*  
*Using Selected Strain for Stevia Crop*  
*and Further Field Trials To Investigate*  
*The Effect of Prepared Bioformulation*  
*on Plant Growth and Fitness as Well As*  
*on Soil Health*



## **1. Introduction**

*Bacillus* is one of the most important genus amongst PGPR and widely accepted as plant growth promoting and biocontrol agent (Sivasakthi et al. 2014). Due to its ability to increase plant growth and uptake of macro and micronutrients from soil it is mostly used as biofertilizer, while *Bacillus* is also used as a biopesticide because of the biocontrol properties such as production of siderophore and antibiotics, chitinase, and glucanase enzymes (Cawoy et al. 2011; Alori et al. 2017). Multifaceted activities shown by *Bacillus* spp. can be exploited to use them in preparing novel bioformulation which can not only enhance the plant growth but also help in controlling diseases caused due to phytopathogens. Bioformulation or bioinoculants are a good option particularly for the cultivation of medicinal plants (Alori et al. 2017).

However, designing and production of bioformulation which is successful in fields under diverse conditions is not an easy task to achieve and requires effective and potential PGPR strain, a suitable carrier and may be an adhesive or sticker. Protection, storage, and transport of bioformulation is a challenging task for agro-industry, market, and end user (Arora and Mishra 2016). However, a good formulation provides an optimal condition to maintain microorganism population in the carrier and maximize its efficacy in order to obtain maximal benefits after inoculation (Berninger et al. 2018). The inoculants should be cost-effective and easy to handle and use, to ensure that the microorganism is delivered to the plant in the most appropriate form and manner (Singh et al. 2014). Bioformulations may be in liquid or solid forms, but liquid are considered better as they have longer shelf-life, can easily colonize on the root or plant surface, can perform well in field conditions, and are cheaper (Goljanian-Tabrizi et al. 2016; Nagachandrabose 2018).

The most important secondary metabolites steviol glycosides (SGs) are present in *S. rebaudiana* plants having low calorie and high sweetener properties (Casas-Grajales et al. 2019). Stevioside and Rebaudioside A are the two major SGs and these molecules are glucosylated derivatives of diterpenoid steviol (Wang et al. 2013). The biosynthesis of SGs shows plastidial 2-C-methyl-D-erythritol-4-phosphate pathway/MEP pathway, which is a similar pathway with gibberelins (Mathur et al. 2017). It has many steps in MEP pathway, among them, the last step of SGs

biosynthesis is present in cytosol of plant cell, catalysed by 4 UDPglycosyltransferases (UGTs) enzymes (Modi et al. 2014). UGT85C2 joins one molecule of D-glucose to C-13 of steviol to produce steviolmonoside metabolite. The next step of the process (glycosylation) leads to the formation of steviolbioside metabolite, catalysed by a UGT enzyme, but it has not yet been characterized by researchers (Modi et al. 2014). UGT74G1 enzyme is involved in the formation of stevioside metabolite by glycosylation process of C-4 carboxylic acid of steviolbioside. Afterward, rebaudioside A is formed by the addition of a D-glucose molecule of the C-3' of the C-13 glucose of stevioside, catalysed by UGT76G1 enzyme (Ceunen and Geuns 2013). A lots of work has been done on the regulation of SGs genes (Ceunen and Geuns 2013; Modi et al. 2014; Chen et al. 2014). PGPR colonize the plant tissue and stimulate the growth, productivity, and accretion of the secondary metabolite of plants (Arora and Mishra 2016).

The present study was focused on the development of bioformulation to increase the stevioside content in *S. rebaudiana* by utilizing plant growth promoting strain *Bacillus safensis* STJP (NAIMCC-B-02323). In this regard, the impact of bioformulation on various growth parameters like root and shoot length, fresh and dry weight, number of leaves, growth parameters, chlorophyll content, antioxidant content, flavonoid content plus nutrient uptake in plants and soil health has been investigated. In addition, relative highest transcript profiles of candidate genes have also been investigated in the biosynthesis of SGs in *S. rebaudiana* using real time polymerase chain reaction (RT-PCR). The developed bioformulation may prove to be a noble asset for the farmers involved in the cropping of *S. rebaudiana*.

## **2. Material and methods**

### **2.1 Development of solid bioformulation**

Development of bioformulations was done using selected bacterium *B. safensis* STJP NAIMCC-B-02323 (on the basis of PGP characters and biocontrol activity). For the development of solid bioformulations, different carriers such as wheat bran, tea compost, potato peel, mustard cake, and talc were used along with gum arabic (source: *Acacia arabica*, used as an adhesive material). Calcium carbonate was used to maintain the pH of the bioformulations. The carrier was autoclaved and later on the bioformulation was prepared using following compositions; 74% carrier(s), 15 %

inoculum (fresh culture of *B. safensis* STJP), 10 % gum arabic and 1% of calcium carbonate in per Kg of the carrier (Ibrahim et al. 2004). These ingredients were mixed carefully and homogenously. The prepared formulation was packed into sterilized polybags and stored for nine months. The composition of different bioformulations are shown in Tables 15 and 16.

## 2.2 Development of liquid bioformulation

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. Carriers were selected because of ease of availability, being waste products and hence economical. For the preparation of liquid bioformulation, the bacterial isolate STJP was grown in nutrient broth (at 30°C for 48 h). After the growth period, bacterial population was maintained at  $10^8$  cells/ml with the help of deionized water as per the method described by Udekwu et al. (2009). Further, 10% bacterial inoculum and 5% glycerol were mixed in each carrier (85%; potato broth, rice broth, paneer-whey, and molasses) for preparation of bioformulation (Goljanian-Tabrizi et al. 2016). The pH (Model: Manti Lab MT-103M Digital pH meter, India) of all carriers were adjusted at neutral and prepared bioformulations were stored at 30°C for nine months.

## 2.3 Examination of physiochemical properties of bioformulation

After storage (of bioformulations) insurance of quality including pH, electrical conductivity (EC) (Cyberscan PCD 6500, India), shelf life, 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).

## 2.4 Impact of bioformulation on growth of *S. rebaudiana*

On the basis of availability, maintaining good bacterial population, being non-pathogenic, physio-chemical characteristics and being eco-friendly, paneer whey based bioformulation was selected for further study. The field experiment was performed in Horticulture Research Farmhouse (HRF) during February to April 2017 (90 days) and repeated in February to April 2018 for 90 days (Arora et al. 2001). The soil was saline

(pH: 8.1; electrical conductivity: 8.2 dS/m, pH) and naturally infested with *A. alternata* ( $10^2$  CFU/gm of soil). The field experiment was performed using a completely randomized block design (CRD) with five replicates of each treatment in a standard plot size of 200 m<sup>2</sup> (15m × 10m), where each block was 2 m × 2 m, with following treatments: 1) Control (*A. alternata*/infested field), 2) *B. safensis* STJP (bioformulation), 3) Mycorrhiza (*Glomus fasciculatum*), 4) Mycorrhiza + *B. safensis* STJP ( ). Approximately, 0.20 m distance was left in between each block and seedling of *S. rebaudiana* was sown in 35 cm row-width. Equal amount of water was used for irrigation as per requirement (two times in a week). The bacterial based bioformulation was applied on seedling of Stevia plant through root dip method (Mamta et al. 2010). No chemical fertilizers nor any pesticides were used in this experiment. After the harvesting of plants, growth parameters in checking root and shoot length, fresh and dry weight, and number of leaves were observed after harvesting (30, 60 and 90 days).

## **2.5 Impact of bioformulation on chlorophyll, carbohydrate, flavonoid, and antioxidant activity in *S. rebaudiana***

The physiological parameters viz. carbohydrate content, chlorophyll a and chlorophyll b, antioxidant activity, flavonoid content were checked as per the standard protocols described in the previous chapter (Chapter 8).

## **2.6 Extraction and estimation of stevioside**

Dried *S. rebaudiana* plant sample were taken for extraction and estimation of stevioside content as per standard protocol described earlier (chapter 8 and section 2.5).

## **2.7 Effect of bioformulation on nutrients uptake by *S. rebaudiana* and soil health**

After tilling of *S. rebaudiana* (90 DAS), all plants were dried and used to estimate nutrient (K, Zn, and P) uptake in plant. The same process was used for the estimation of soil nutrients as described in previous chapter (8).

## **2.8 Investigation of overexpression genes in synthesis of steviol glycosides**

### **2.8.1 Plant sample and treatments**

For the investigation of overexpression genes, seedlings of plants were grown in the field. *S. rebaudiana* control and inoculated with *B. safensis* STJP. For sample, same stage, uniform axial positioned leaf of control and treated plants were taken for the study of gene expressions.

### 2.8.2 Primer designing and validation

Sequences of candidate genes were taken from model organism and the primers were designed using a Gene Runner software (version 6) (Table 23). Beside, primers for three major genes involved in the biosynthesis of SGs; UGT85C2, UGT74G1, and UGT76G1 was designed by Gene Runner software (version 6). Primers are provided to make a stock solution (100 pmol with 0.3 x TE buffer) and further this solution was diluted 10 pmol for PCR master mix preparation. For the primer validation, 1.0 µl and 0.2 µl samples (in 25 µl and 20 µl systems) were taken each for DNA confirmation and real time experiments, respectively (Kumar et al. 2012). cDNA sample was taken for control and run in real-time PCR using all the selected primers. The step of PCR (cycling conditions) before the melting curve of designed genes and all validation procedure was followed (Figure 69) as per Kumar et al. (2012).

### 2.8.3 RNA extraction and cDNA synthesis

For isolation of RNA, TRIzol reagent was used as per Ghawana et al. (2011). Briefly, 50 mg of leaf and tissue and 1 ml of TRIzol were mixed and the reaction mixture was homogenized. Further, 0.2 ml of chloroform was added to lyse the cells or tissues and centrifuged at 13,000 rpm for 10 min. The aqueous phase (upper part of the sample) was mixed with iso-propanol (volume: 0.6) and further centrifugation was done to obtain a sample as precipitate form. Afterward, the sample was rinsed with 75% chilled ethanol. The obtained RNA was dissolved in treated water of DEPC (diethylpyrocarbonate) (Modi et al. 2014).

According to the manufacturer's instructions, the cDNA was primed from cDNA synthesis kit (Fermentas). Reaction mixtures (20 µl) containing total RNA (5 µg), dNTP mix, reverse transcriptase, RNase inhibitor and oligo dT primers were incubated for 60 min at 37°C followed by 10 min at 65°C to denature enzyme present in the sample and finally obtained samples were diluted 5 times and used as a working solution.

### 2.8.4 Housekeeping/ endogenous genes

Two housekeeping genes viz. act and 18S rRNA were nominated for the study of relative gene expression. Gene Runner software (version 6) was used to design primer of each candidate genes as per Kumar et al. (2012).

### 2.8.5 Quantification of steviol glycosides using real-time PCR

The gene expression was performed by reverse transcriptase-polymerase chain reaction (RT-PCR) using act and 18S rRNA as endogenous control (Radonić et al. 2004). cDNA synthesis was prepared as described above and PCR was carried out using 1 µl of cDNA template in a 25 µl reaction. For each gene, conditions of PCR cycling were optimized to achieve amplification beneath the exponential phase of PCR amplification as per instructions. Signal intensities were examined and image were captured using a gel documentation machine (Model: iBright™ CL1000, Thermo Fisher Scientific, USA). AD-1000 software was used for the calculation of integrated density value (IDV) to examine deviations in the expression of candidate genes (Modi et al. 2014). The experiment of the PCR was replicated three times using first strand cDNA of two independent RNA preparations and for confirmation, one-time gel image was taken.

### 2.8.6 Analysis by bioinformatics

BLAST was carried out at NCBI (<http://www.ncbi.nlm.nih.gov>) for similarity. Gene alignment was checked of all sequences using ClustalW (<http://ebi.ac.uk/>) and multalin program (<http://prodes.toulouse.inra.fr/multalin/multalin.html>) at different settings. Primers were designed and analyzed using Gene Runner software (version 6).

## 2.9 Statistical analysis

Field experiment was planned in a completely randomized block design (CRD) with five replica and data was represented as the mean  $\pm$  standard deviation (SD). To check the result validity, 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 different sets. Real-time PCR of primers (genes) was designed CRD, taken with three replica and statistical analysis of relative quantification of candidate genes was performed using Data Assit v3.01 01 (Woo et al. 1999).

## 3 Results

### 3.1 Solid bioformulation

A total of four solid bioformulations [tea compost based bioformulation (TC-BF), wheat bran and potato based bioformulation (WP-BF), talc based bioformulation (T-BF) and wheat bran and mustard cake based bioformulation (WM-BF)] were prepared

using agri-wastes and talc powder. Among all, talc based bioformulation was found to be good on the basis of pH, shelf-life and moisture content as compared to other bioformulation and it has been described in next section (3.4) (Table 15 and Figure 64).

### 3.2 Liquid bioformulation

A total four liquid bioformulations [paneer whey liquid bioformulation (P-WBF), rice broth liquid bioformulation (R-BF), potato broth based bioformulation (P-BF), and molasses based bioformulation (M-BF)] were developed and found to be good in terms of quality parameters such as pH, shelf-life and nutrient characteristics.

### 3.3 Physiochemical and biological properties of bioformulations

Physiochemical and biological characteristics of solid and liquid bioformulations were checked (Table 17). Among them, liquid bioformulation were found to be better than solid bioformulation on the basis of pH, shelf-life, nutrients characteristics and being free of contamination (Table 17). 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 (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 ninth month 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.

### 3.4 Impact of bioformulations on growth of *S. rebaudiana*

P-WBF positively affected the plant growth parameters as observed after 30, 60 and 90 days after sowing (DAS) (Figure 65). Among all treatments, P-WBF plus mycorrhiza recorded maximum increment in vegetative parameters as compared to control (Table 19, 20 and 21).

*B. safensis* STJP (P-WBF) and mycorrhiza treatment enhanced fresh and dry weight by 91.36% & 78.94%, 73.41% & 104.82% and 136.30% & 184.22% after 30, 60, and 90 days, in comparison with control plant, respectively. P-WBF also enhanced fresh weight (72.99%, 59.10%, 92.64%) and dry weight (53.07%, 81.97%, 131.03%) of plants in comparison with negative control after 30, 60, and 90 DAS. However, the application of mycorrhiza corresponded to 31.33% & 26.66%, 42.10% & 57.86%, and 20.76% & 94.18% rise in fresh and dry weight in the presence of phytopathogen after 30, 60, and 90 days, respectively (Figure 66).

P-WBF and mycorrhiza treatment enhanced plant length and number of leaves by 105.03% & 98.55%, 94.55% & 93.22% and 130.34% & 81.22%, respectively, as compared to negative control after 30, 60, and 90 DAS. In the presence of phytopathogen (*A. alternata*), the plant length and number of leaves of *S. rebaudiana* treated with P-WBF (only) was increased by 67.51% & 86.15%, 71.42% & 66.95%, and 65.22% & 65.51%, respectively after 30, 60, and 90 DAS. The number of leaves and plant length was further increased by the treatment of mycorrhiza alone, when it was compared with negative control (*A. alternata*). Overall results suggested that combine treatment P-WBF and mycorrhiza is a reliable method for enhancement of number of leaves, plant length, fresh and dry weight of *S. rebaudiana*.

### 3.5 Impact of bioformulations on chlorophylls, carbohydrate, flavonoid, and antioxidant activity in *S. rebaudiana*

*S. rebaudiana* grown in the field showed higher chlorophyll a (57.71%) and chlorophyll b (22.31%) content by treatment with P-WBF even in the presence of phytopathogen (Table 21). A significant rise in chlorophylls content (a and b) was observed after combine treatment of P-WBF and mycorrhiza (132.85%, 39.80%) as compared to control. Plant treated with mycorrhiza was also found to be better in terms of chlorophyll a and chlorophyll b in the presence of phytopathogen.

There was 86.99% and 75.11% enhanced flavonoid and antioxidant activity by the treatment with P-WBF and mycorrhiza, in comparison with the control plant and this result showed highest among all the treatments. P-WBF and mycorrhiza also improved the flavonoid (56.91%, 16.26%) and antioxidant activity (50.39%, 12.63%) (in the plant) in the presence of phytopathogen.

In the presence of phytopathogen, mycorrhiza, P-WBF and P-WBF+mycorrhiza increased carbohydrate content by 25.64%, 50.39%, and 103.84% in the plants, respectively as compared with control. The protein content was enhanced by 227.55% as compared to control by the combined treatment of P-WBF and mycorrhiza. Overall results suggested that P-WBF and mycorrhiza was best treatment for enhancement of antioxidant activity, chlorophylls, carbohydrate, and flavonoid content.

### 3.6 Stevioside content

Combine treatment with P-WBF and mycorrhiza showed significantly higher stevioside content (120.62%) in *S. rebaudiana* in the presence of phytopathogen (Figure 67). Only P-WBF treatment resulted in better stevioside quantity (95.39%) in *Stevia* plants in the presence of phytopathogen. However, mycorrhiza treated plant also showed good result and resulted in 29.97% increment in stevioside content as compared to control (*A. alternata*). Overall results suggested that combine treatment P-WBF and mycorrhiza was a more effective method for augmentation of stevioside content in *S. rebaudiana*. This result was confirmed and compared by HPLC.

### 3.7 Impact of bioformulation on nutrients uptake in *S. rebaudiana*

Combine treatment of P-WBF and mycorrhiza was applied on *Stevia* plants and resulted in 94.22% enhancement of P (in plant) when compared with control. P-WBF and mycorrhiza positively affected the P content as compared to uninoculated plants and resulted in 71.52%, and 24.39% enhancement in the uptake of P, respectively as compared to control (*A. alternata*). Zn uptake in *S. rebaudiana* was significantly enhanced when applied with P-WBF+mycorrhiza (111.11%) in comparison with control plants. The quantity of Zn was enhanced by 93.70% and 48.14% respectively, by P-WBF and mycorrhiza treatment alone in comparison to uninoculated plants. P-WBF along with mycorrhiza enhanced K (132.39%) concentration in the comparison with the control plant. Mycorrhiza and P-WBF treated plants showed 34.57% and 87.22% increment in the uptake of K when it was compared with control plants.

Overall, combined treatment (P-WBF+mycorrhiza) was found to be better for uptake of nutrients (P, K, & Zn) by *S. rebaudiana*.

### 3.8 Impact of bioformulation on soil health

After harvesting of *S. rebaudiana*, soil nutrients in the soil were checked and it was observed that K, P, Zn, C, and N of combine (P-WBF and mycorrhiza) treated soils were 100.89, 39.16, 366.66, 67.10, and 74.72% higher than without any treatment. Soil treated with P-WBF increased K, P, Zn, C, and N at the level of 72.19, 20.97, 280.95, 43.66, and 56.31% in comparison with control (Table 23). Mycorrhiza treated soil was 17.48, 2.09 and 114.25% higher in K, P, and Zn content in the presence of phytopathogen (*A. alternata*). However, mycorrhiza did not significantly affect the carbon and nitrogen content when result was compared with control (Table 23). The experimental field soil was alkaline. In this regard, pH of all treated soils were recorded in between 6.7-7.8. Overall, P-WBF and mycorrhiza was found to be a good method in the improvement of soil fertility (K, P, Zn C, and N).

### 3.9 Quantification of steviol glycosides

Transcript accumulation profiling of candidate genes exhibited two groups, 1) upregulation 2) down-regulation in all the treatments (Figure 68). All genes were calculated to transcript levels with the reference gene of act and 18S rRNA. Among all three genes, UGT76G1 and UGT85C1 showed up-regulation expression by P-WBF treated plants and this result was confirmed by accumulation of SGs, whereas UGT74G1 showed down-regulation by P-WBF treatment (means it was unable to enhance the accumulation of SGs in plants). UGT76G1 and UGT85C1 genes showed 4.3 and 2.4 folds enhancement of SGs (by P-WBF) in comparison with control. Overall, two genes (UGT76G1 and UGT85C1) were found to be involved in the up-regulation of SGs.

## 4 Discussion

*B. safensis* STJP showed plant growth promoting and biocontrol ability as described in the previous chapter. *B. safensis* STJP was further taken for the development of solid and liquid bioformulation. Among both bioformulations, liquid bioformulation was taken for further study. Four types of liquid carriers (paneer whey, molasses, potato, and rice broth) were chosen on the basis of economy, availability and eco-friendly

properties. Excess of C/N ratio and the presence of other nutrients is a problem associated with the carriers used in the production of bioinoculants (Arora et al. 2010; Kalita et al. 2015). The pH of bioformulation is also a key factor for survival of microorganisms. The low or high pH can affect the bacterial population. The minimum bacterial cells were recorded in rice broth and molasses based bioformulation may be due to very high pH of the prepared bioformulation. However, slightly acidic or basic pH is appraised to be beneficial for bacterial liquid formulation (Kalita et al. 2015).

Bacterial cell viability in bioformulation is a serious issue worldwide, in this regard rice broth and potato broth bioformulations could not maintain cell population as per Bureau of Indian Standards (BIS) (2017). However, molasses based bioformulation maintained good bacterial population ( $10^8$  cell/ml) after 9 months storage but it was alkaline and use of such bioformulation may increase the cost by the involvement of neutralization process (Pandey and Maheshwari 2007). In P-WBF, the bacterial population was maintained at  $10^8$  cells/ml at neutral pH even after ninth month 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 mho-0.81 mho. According to Biofertilizers and Organic Fertilizers in Fertilizer (Control) Order (1985), EC of bioformulation should not be more than 0.37 mho, because that can decrease the bacterial population of the bioformulation. Pathogenic microorganisms such as *E. coli*, *Shigella* and *Salmonella* were not observed in prepared bioformulations. Non-pathogenic property of bioformulation is a good for handling and storage and for end users. Among all the tested bioformulations, P-WBF recorded good quantities of K and P and the carrier containing a good quantity of nutrients are very essential for soil health, growth and productivity of the crops and maintaining the population of introduced microbe (Arora and Mishra 2016). In addition, carriers contained an adequate quantity of C/N ratio, thus resulting in a better opportunity for soil fertility, maintain soil microflora, and improve crop productivity (Whalen 2014). C/N ratio of 20:1 is considered as ideal for rhizobacteria growth, but excess or lesser C/N ratio can create difficulty for the bacterial population. The excess C/N ratio was detected in potato, rice and molasses based bioformulations due to composition of the carriers. Overall the paneer-whey was found to be an excellent liquid carrier as per bacterial shelf-life, viability, availability, and eco-friendly interest.

According to Panghal et al. (2017), paneer whey production worldwide is 3,625,200 tonnes per year. It is expected that approximately 100,000 tons of whey is annually produced as a byproduct from the dairy industries (Baba et al. 2016). Paneer whey constitutes high amount of milk solids, milk sugar (lactose), milk proteins and minerals and almost all the water-soluble vitamins originally present in milk (Das et al. 2010). The large quantity of whey disposal in water bodies can cause serious environmental pollution and health hazards, toxicity in 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 the 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 (<http://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 mainly important for the development of the bioformulation from it. If small biofertilizer units are established as ancillary or nearby to the dairy industries this can further cut the cost of the formulation. This technology will also be very effective in enhancing the earning of farmers involved in the 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 plant biomass (fresh and dry) was observed in *S. rebaudiana* by the treatments of P-WBF + mycorrhiza, which might be associated with solubilization of minerals, production of hormones, or secretion of various metabolites by *B. safensis* STJP and these nutrients are translocated from interior root to shoot by mechanism of nutrients-transpiration xylem pathway as suggested by Atkins and Smith (2007). Jorjani et al. (2011) reported that *Bacillus coagulans* based bioformulation applied on sugar beet by root treatment method recorded high fresh biomass due to solubilization of minerals such as P, K and Zn. However, some nutrients such as Zn, K, and P directly

help in the increment of fresh biomass and productivity of super Basmati rice and Basmati-385 (Shakeel et al. 2015). However, use of phytohormones like IAA and gibberellic acid producing bacteria is also an effective method for increasing the growth of plants. Kalita et al. (2015) developed bioformulation utilizing *Bacillus* sp. and sprayed over the tomato (*Solanum lycopersicum*), cauliflower (*Brassica oleracea*), chilli (*Capsicum annum*), and brinjal (*Solanum melongena*) crops resulting in increase of biomass and yield. Besides the growth of plant, several *Bacillus* spp. also enhanced the yield and nutrient uptake (K, P, and Zn) in apple and tomato plant (Pirlak et al. 2007; Walpola and Yoon 2013). Until now, there are limited reports available on liquid bioformulations 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 liquid P-WBF. It is also for the first time that paneer whey with glycerol is being used as a carrier for developing bioformulation of *B. safensis* STJP for enhancing stevioside content and growth enhancement of *S. rebaudiana*. Developed P-WBF using *B. safensis* STJP was also capable in controlling of leaf spot disease in Stevia and promote the growth, yield and stevioside content. The biocontrol agent *Bacillus* sp. has reported to control of leaf spot disease caused by *A. alternata* in coriander plant (Mangwende et al. 2019). The combine application of *Bacillus* with *Trichoderma* showed better growth, disease management and yield of plant as compared to single application (*Bacillus* or *Trichoderma*) (Mangwende et al. 2019).

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 in the *S. rebaudiana* plants was observed due to application with *Bacillus gladioli* strain 10216. They reported high stevioside concentration due to an increase in P nutrient uptake in Stevia plant. However, in the present study combined treatment P-WBF and mycorrhiza showed the best increment in stevioside content (120.53% as compared to control) may be due to P, K, and Zn solubilization and availability to the plants. *Bacillus*-based bioformulation 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). *Bacillus* sp. was also known to control leaf spot disease (*A. alternata*) of *Aloe vera* and also improved metabolite content (Ghosh et al. 2018). Kumar et al. (2016) reported that the use of PGPR (*Pseudomonas*

*fluorescens* CL12) on *Curcuma longa* increased the growth and metabolite 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 plants. Similarly *B. safensis* based bioformulation showed P, K, Fe and Zn solubilization properties and its application on the Stevia plant showed enhanced growth, management of disease, nutrient uptake and increased stevioside content. PGPR and their volatile metabolites may have great potential as excellent agents for controlling of various fungal pathogens including *A. alternata* and increase the metabolite content in plants (Vurukonda et al. 2018).

The fertility of soil mainly depends on nutrients availability and microbial population (Chowdhary et al. 2018). The soluble nutrients are easily uptaken by the plants and enhance growth and biomass (Mangwende et al. 2019). 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 bacterium *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*. In this study, the application of P-WBF in soil resulted in boosted nutrient availability in soil and it offers a chance to elevate the plant growth performance by upturned nutrient content in the plants.

The fertility of soils mainly depends on the availability of nutrients, and microbial communities (Chowdhary et al. 2018). Rhizobacteria have ability to solubilise nutrients. In this regard, P-WBF treatment alone or with mycorrhiza increased the availability of nutrients (P, K, Fe and Zn) to the plant. These nutrients play an important role in the increment of biomass, nutrients uptake, and metabolite content of the plant (Nieves-Cordones et al. 2016) as confirmed by this study.

In current study, genes of SGs biosynthesis pathway in *S. rebaudiana* were checked. There are two kinds of transcripts accumulation patterns observed i.e., upregulation and down-regulation. UGT76G1 and UGT85C1 genes showed up-regulation and UGT74G1 gene was found to be down regulated. Modi et al. (2014) observed that transcriptional profiling of genes UGT76G1, UGT85C1 and GT74G1

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involved in the SGs biosynthesis of *S. rebaudiana*. However, UGT76G1 gene was found to be upregulated in biosynthesis of SGs. Kumar et al. (2012) reported SrUGT74G1 gene was upregulated in biosynthesis pathway when was treated with gibberellic acid in *S. rebaudiana*. Beside UGT76G1, UGT85C1 and GT74G1 genes, some other genes SrDXS1 and SrKAH were overexpressed (Zheng et al. 2019).

Overall, *B. safensis* is an eco-friendly tool for promoting plant growth and use as biofertilizer. P-WBF 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 and 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 for farmers through organic farming of *S. rebaudiana*. The exploitation of such PGP strains as a cheap and effective bioformulation based on organic wastes 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.

**Table 17.** Characteristics of carriers used for the development of solid bioformulations

<b>Physiochemical properties</b>	<b>Tea compost</b>	<b>Wheat and Potato Peel</b>	<b>Wheat Bran + Mustard Cake</b>	<b>Talc</b>
pH	9.2	7.9	8.9	6.8
Electric conductivity (mho)	0.75	0.12	0.24	0.26
Salmonella	–	–	–	–
<i>E. coli</i>	–	–	–	–
Shigella	–	–	–	–
Total K (%)	0.14	0.121	1.14	Nd
Total P (%)	0.56	1.60	4.44	Nd
C/N (%)	102:1	120:1	287.2	Nd
Shelf life (after 270 days)	10 <sup>3</sup>	10 <sup>6</sup>	10 <sup>5</sup>	10 <sup>7</sup>

**Table 18.** Characteristics of carriers used for the development of liquid 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	–	–	–	–
<i>E. coli</i>	–	–	–	–
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 (after 270 days)	10 <sup>4</sup>	10 <sup>6</sup>	10 <sup>8</sup>	10 <sup>8</sup>

**Table 19.** Plant growth parameters of *S. rebaudiana* after application of P-WBF (after 30 days)

Treatments	Growth parameters of <i>S. rebaudiana</i> (30 days)						
	Plant length (cm)		Number of leaves	Fresh weight (gm)		Dry weight (gm)	
	Shoot length	Root length	-	Shoot	Root	Shoot	Root
Control	13.30±2.28d	3.20±0.56d	45.66±2.10d	20.10±1.24d	5.24±0.86d	9.30±0.86d	2.10±0.04d
STJP	22.50±2.21b	5.00±1.02a	85.00±2.14b	35.24±2.40b	8.62±0.96a	12.45±1.24b	5.00±0.05b
Mycorrhiza	18.30±2.45c	5.10±0.28c	80.42±2.36c	26.42±1.42c	6.86±0.80c	10.24±1.12c	4.20±0.70c
Mycorrhiza + STJP	28.83±2.04a	5.14±1.60ab	90.66±4.12a	40.12±1.24a	8.44±0.94ab	14.20±1.60a	6.20±1.02a

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 20.** Plant growth parameters of *S. rebaudiana* after application of P-WBF (after 60 days)

Treatments	Growth parameters of <i>S. rebaudiana</i> (60 days)						
	Plant length (cm)		Number of leaves	Fresh weight (gm)		Dry weight (gm)	
	Shoot length	Root length	-	Shoot	Root	Shoot	Root
Control	18.08±1.78d	4.32±0.86d	85.33±2.84d	35.72±2.60d	7.46±0.86d	15.10±2.20d	3.98±0.86d
STJP	31.60±4.48b	6.80±2.46b	152.46±2.80b	58.46±2.45b	10.24±0.48b	26.10±1.24b	8.62±0.80ab
Mycorrhiza	28.63±4.56c	5.96±0.80c	142.00±4.24bc	51.84±2.45c	9.52±0.48c	22.50±1.10c	7.62±0.54c
Mycorrhiza + STJP	36.34±5.45a	7.24±1.89a	164.88±5.24a	62.34±2.47a	12.54±1.14a	30.12±3.24a	8.96±1.21a

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 21.** Plant growth parameters of *S. rebaudiana* after application of P-WBF (after 90 days)

Treatments	Growth parameters of <i>S. rebaudiana</i> (90 days)						
	Plant length (cm)		Number of leaves	Fresh weight (gm)		Dry weight (gm)	
	Shoot length	Root length	-	Shoot	Root	Shoot	Root
Control	23.43±4.25d	5.90±1.14d	135.44±6.10d	62.45±2.84d	9.64±1.12d	25.36±2.12d	5.96±0.98d
STJP	40.26±4.15b	8.20±1.50b	224.18±6.88b	118.42±4.42b	20.56±1.48b	60.12±3.46b	12.24±2.24b
Mycorrhiza	30.12±2.46c	7.00±0.84c	180.60±6.50c	74.52±1.86cd	12.54±0.84c	50.24±2.42c	10.58±1.70c
Mycorrhiza + STJP	58.46±4.46a	9.1±2.42a	245.45±5.20a	142.46±4.86a	24.89±1.88a	74.56±4.56a	14.46±1.12a

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 22.** Plant components after application of P-WBF after 90 days

Treatments	Plant components/ parameter (90 days)					
	Chlorophyll a	Chlorophyll b	Protein	Carbohydrate content	Flavonoids	Antioxidants
Control	80.12±4.46d	78.86±4.46d	1.96±0.90d	3.12±0.12d	2.46±0.10d	80.12±2.60d
STJP	126.36±6.36b	96.46±4.48b	4.96±0.64b	4.82±1.12b	3.86±0.86b	120.50±4.80b
Mycorrhiza	96.36±12.42c	79.12±2.14cd	2.62±0.96c	3.92±0.10c	2.86±0.86c	90.24±3.46c
Mycorrhiza + STJP	186.56±2.36a	110.25±4.89a	6.42±1.20a	6.36±1.12a	4.60±1.12a	140.30±2.60a

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

**Table 23.** Nutrients content in *S. rebaudiana* after application of P-WBF (90 days)

Treatments	Potassium uptake (mg/plant)	Phosphorus uptake (mg/plant)	Zinc uptake (mg/plant)
Control	3.21 $\pm$ 0.07 d	129.01 $\pm$ 6.84 d	1.08 $\pm$ 0.08 d
STJP	6.01 $\pm$ 0.41 b	221.28 $\pm$ 5.76 b	2.12 $\pm$ 0.10 b
Mycorrhiza	4.32 $\pm$ 0.63 c	160.48 $\pm$ 5.68 c	1.60 $\pm$ 0.10 c
Mycorrhiza + STJP	7.46 $\pm$ 0.30 a	250.57 $\pm$ 12.79 a	2.28 $\pm$ 0.04 a

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

**Table 24.** Nutrients content in soil after application of P-WBF (90 days)

Treatments	Nutrients content in soils					
	Potassium uptake (mg/kg)	Phosphorus uptake (mg/kg)	Zinc uptake (mg/kg)	Carbon	Nitrogen	pH
Control	4.46±0.86d	2.86±0.42d	0.42±0.20d	8.45±0.40d	3.60±0.40c	7.8
STJP	7.68±2.12b	3.46±0.84b	1.60±0.10b	12.14±0.60b	5.69±0.12b	7.2
Mycorrhiza	5.24±0.47c	2.92±0.89cd	0.90±0.10c	08.46±0.84cd	3.64±0.84c	6.7
Mycorrhiza + STJP	8.96±1.12a	3.98±0.86a	1.96±0.12a	14.12±1.10a	6.36±0.64a	6.9

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 25.** Details of genes and primers used

Name of the genes	Corresponding enzymes	Accession number	Primer Sequence		In silico product size
<i>ACT</i>	Actin	AF548026	R- F-	5-CCGTTCGGCGGTGGTGGTAA-3 5-CGCCATCCTCCGTCTTGATCTTGC-3	100
18S rRNA	Ribosomal enzyme	-	R- F-	AGGCCACTATCCTACCATCGAA CCGGCGACGCATCATT	59
UGT85C2	UDP glucosyltransferase 85C2	AF548026	R- F-	5-CTCTGATTGGGATGCTCGCT-3 5-CATCGGGCCCACATTGTCTA-3	99
UGT74G1	UDP glucosyltransferase 74G1	AF548026	R- F-	5- TCCAAATATGATTCTCCTGCACTCA-3 5-ACCACAGTAACACCACCACC-3	97
UGT76G1	UDP glucosyltransferase 76G1	AF548026	R- F-	5-ATGCGTTCGTCTTGTGGGT-3 5-CACCATCTTTCACACCAACTTCA-3	91



**Fig. 64** Different bioformulation developed during the study



**Fig. 65** Growth of *S. rebaudiana* at 30 days (in the field)



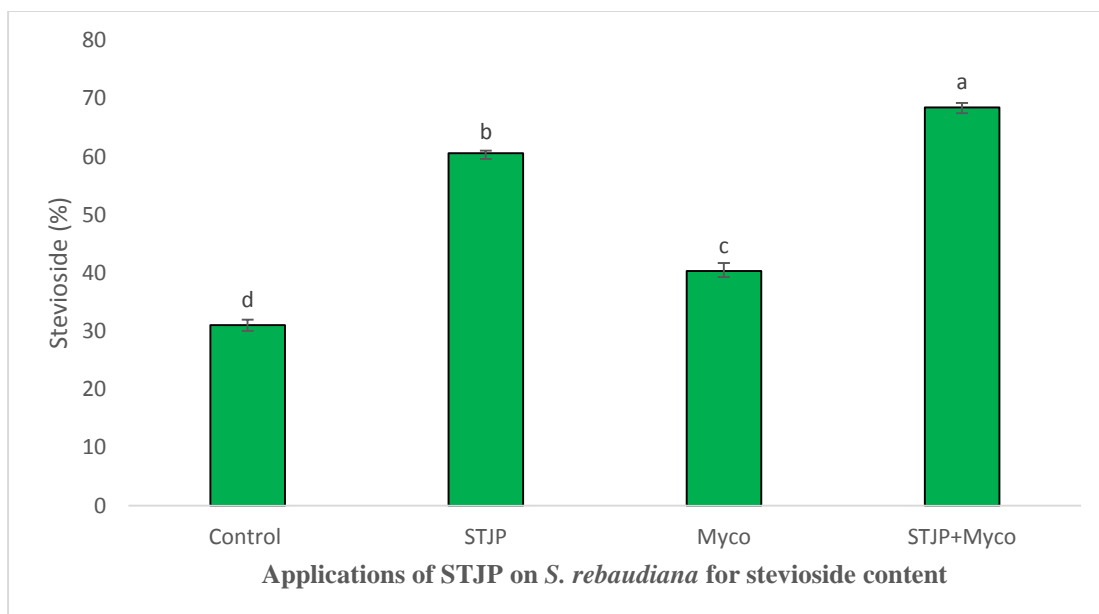
**Fig. 65** Growth of *S. rebaudiana* at 60 days (in the field)



**Fig. 65** Growth of *S. rebaudiana* at 90 days (in the field)

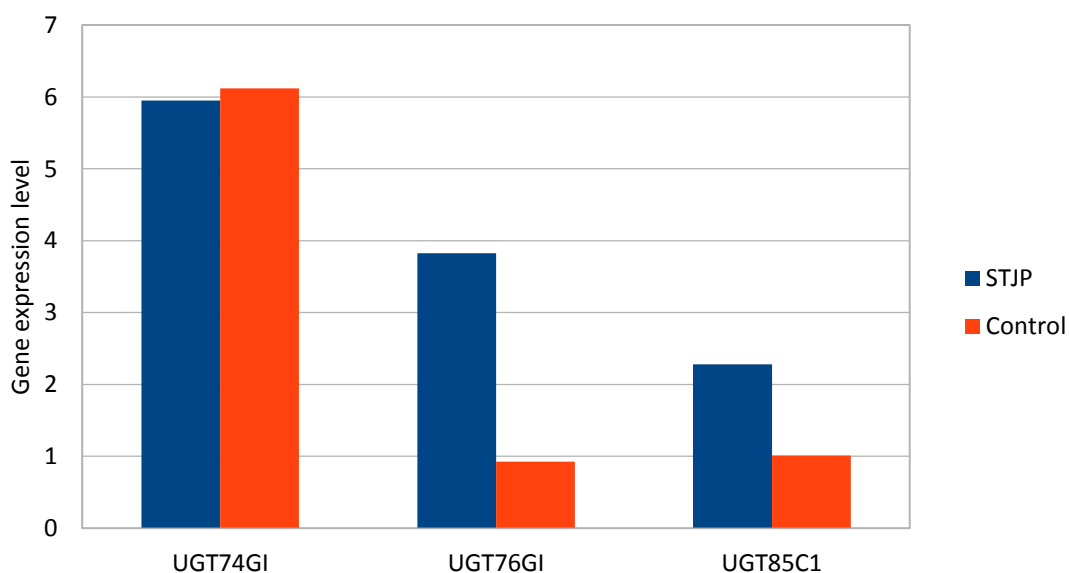


**Fig. 66** Fresh plants by different treatments after 90 days



**Fig. 67** Effect of P-WBF and other treatments on stevioside content of *S. rebaudiana* (90 days)

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



**Fig. 68** Transcript accumulation profiling of candidates genes

Data are mean of standard deviation with three replicas

## Melt Curve (Derivative Reporter)

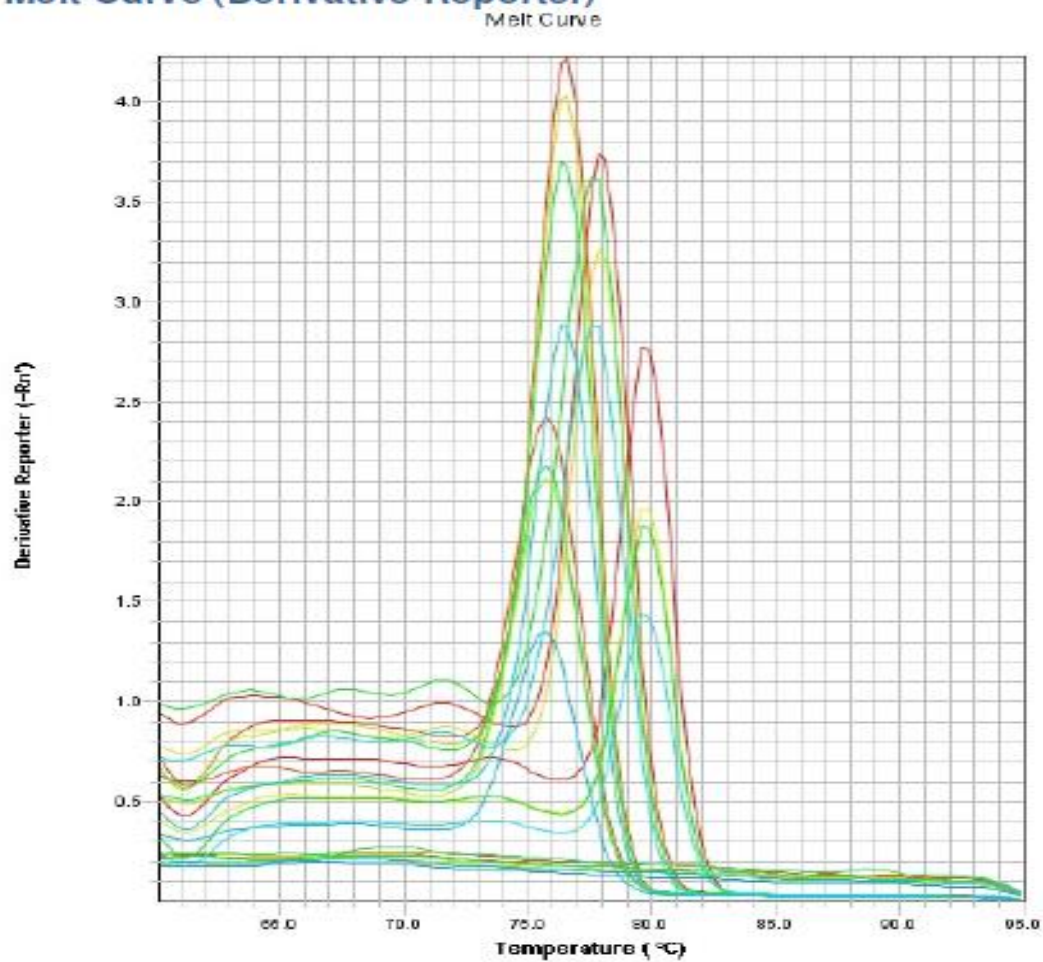


Fig 69. Melting curve of different primers used in the study

*Chapter-10*  
*Conclusions and Summary*



*Stevia rebaudiana* is an ayurvedic herb and known by several common and local names such as sweet leaf or candy leaf or honey leaf or sweet herb or Meethi Patti. It is rich in nutrients and has several potential biomedical applications viz. anti-inflammatory, anti-hyperglycaemic, anti-tumor, hepatoprotective activity, immunomodulation, control diabetes, hypertension reduction and in the treatment of skin disorders. In addition to high medicinal value, it is also a natural sweetener with zero calories and low glycemic index. Its sweetening property mainly depends on the alkaloid and diterpene glycosides (DGs). DGs consist of several compounds like stevioside, steviolbioside, rebaudioside A, B, C, D, E and dulcoside, which have been extensively explored in many applications. Among them, stevioside is an important compound which is slightly bitter in taste, non-fermentable, non-caloric and 300 times sweeter than normal/table sugar with a long shelf life. The leaves of *S. rebaudiana* contain a very high content of stevioside (approx. 6-18%) and gradually it decreases in other parts of the plants. The priceless medicinal properties of stevioside have lead to an upturn in *S. rebaudiana* cultivation all over the world. *S. rebaudiana* suffers from some common phytopathogens including *Alternaria alternata* resulting in the reduction of crop productivity. *A. alternata* causes leaf spot disease in *S. rebaudiana* and the symptoms of this disease are dark brown color or necrotic lesions. The symptoms of this disease appear during pathogenesis due to toxicity of fungal metabolites during active phase of plant growth.

In the present study, a total of 11 bacteria were isolated from rhizosphere of different medicinal plants including *S. rebaudiana*. Among them, the selected bacterium isolate STJP (on the basis of PGP and biocontrol traits) was identified as a *Bacillus safensis* STJP. The potential isolate was submitted in National Bureau of

Agriculturally Important Microorganisms Culture Collection (NAIMCC), Uttar Pradesh, India (a national culture collection center) with accession number; NAIMCC-B-02323. *B. safensis* STJP produced significant quantity of indole 3-acetic acid (30.59 µg/ml), gibberellic acid (8.22 µg/ml), carboxylate (16.06 µg/ml), and hydroxymate (3.21 µg/ml) types of siderophores. In addition, isolate STJP was able to solubilize K (4.41 µg/ml), Zn (ZnO: 5.40 µg/ml, ZnSO<sub>4</sub>: 3.20 µg/ml, ZnCO<sub>3</sub>: 3.80 µg/ml), and phosphate (610.33 µg/ml). Isolate STJP showed 81.45 U/ml phosphatase activity after 96 h which reduced with further incubation. Regarding biocontrol traits, *B. safensis* STJP produced significant amount of salicylic acid (12.80 µg/ml), lytic enzymes viz. chitinase (75.58 nmol/min/mg), β-1, 3 glucanase (148.50 nmol/min/mg), and cellulase. These enzymes help in the breakdown of fungal cell wall.

The selected strain was checked as biocontrol against *A. alternata* P1 (KX494864). *B. safensis* STJP showed significant antagonistic activity against *A. alternata* (14.8 mm) after 120 hrs when checked by dual culture technique. The growth reduction of *A. alternata* by *B. safensis* STJP was confirmed (no new conidia formation, destruction, and ruptured hyphae of fungus) by SEM. *B. safensis* STJP did not show any pathogenic activity and this is a safe candidate for the development of bioformulation to fight the current challenge of food security.

*B. safensis* strain STJP produced several volatile metabolites as characterised through thin layer chromatography, FT-IR, and GC-MS. These organic compounds contained a wide range of functional groups viz. fatty acid, alcohol, and phenol. Among all, 2, 4-bis (1, 1-dimethylethyl)/2, 4-di-tert-butylphenol was identified by GC-MS and showed 50% growth reduction of *A. alternata* at 40 ppm concentration; further increase in the concentration of metabolite enhanced the rate of inhibition. The production of

volatile compounds by *B. safensis* could be effective in controlling the fungal pathogens such as *A. alternata*.

The cocktail of PGP and biocontrol characters of *B. safensis* STJP alone or in combination with mycorrhiza were checked for enhancing the nutrient uptake, biomass, and stevioside content of *S. rebaudiana* as well as soil health in the presence and absence of phytopathogen (*A. alternata*). In the greenhouse conditions, treatment with STJP significantly increased plant growth parameters viz. number of leaves, fresh and dry weight, and plant length of *S. rebaudiana* as compared to control. Combination of STJP and mycorrhiza treatment showed highest plant growth parameters as compared to control. Chlorophyll (a and b), carbohydrate, flavonoid content, and antioxidant activity were also enhanced by STJP inoculation. STJP treatment and in combination with mycorrhiza was also able to significantly enhance nutrients uptake in *S. rebaudiana* and enhanced soil nutrient levels as compared to control. Stevioside is a key metabolite of *S. rebaudiana* having wide applications in various industries. In this regard, *B. safensis* STJP alone and combination with mycorrhiza enhanced stevioside content in Stevia plant in the presence or absence of phytopathogen (*A. alternata*). Enhancement of stevioside yields that too through eco-friendly biological approach can be a major breakthrough to enhance stevioside production in an eco-friendly biological and organic manner.

After successful pot trials, a suitable bioformulation was prepared using various carriers materials (including both solid and liquid) and the selected strain *B. safensis* STJP. Among all paneer-whey (a dairy waste) based bioformulation (P-WBF) was found to be most feasible and effective on the basis of shelf-life, ease of availability, nutrient characteristics, and being economical. The P-WBF was inspected for the ability to enhance the growth, nutrients uptake, and stevioside content in *S. rebaudiana* in the

field conditions. A combined P-WBF and mycorrhiza treatment of *S. rebaudiana* enhanced fresh and dry weight by 89.02 and 184.22% after 90 days, in comparison with control plants. Further, P-WBF + mycorrhiza treatment also resulted in maximum shoot length and number of leaves (30.34 and 81.22% enhancement respectively, as compared to the control) 90 DAS. Similarly, as in the chlorophyll, carbohydrate, flavonoid and antioxidant activity were maximally enhanced in Stevia plant, when treated with P-WBF and mycorrhiza. The treatment with STJP + mycorrhiza also showed significantly high stevioside content in *S. rebaudiana* as compared to control. STJP treatment also resulted in better stevioside quantity in Stevia plants when compared with control and mycorrhizal treated plants even in presence of phytopathogen. Overall results suggested that P-WBF along with mycorrhiza is a reliable method for enhancing growth and yield of *S. rebaudiana*.

P-WBF with mycorrhiza resulted in 80.86% enhancement of P in the plant when compared with control. Zn and K nutrients uptaken in *S. rebaudiana* were significantly enhanced when applied with P-WBF + mycorrhiza. The quantity of Zn and K was enhanced by 44.54% and 122.70% respectively, by P-WBF and mycorrhiza treated *S. rebaudiana* in comparison to control. Mycorrhiza and P-WBF (when applied alone i.e., without combination) also recorded increment in Zn (19.62%, 38.62%) and K content (56.11% and 95.63%) as compared to control.

After harvesting of *S. rebaudiana*, soil nutrients was checked and it was observed that K, P, Zn, C, and N of P-WBF and mycorrhiza treated soils was significantly higher than without any treatment (control soils). Overall, combination of P-WBF and mycorrhiza was found to be best method for the enhancement of soil.

Transcript accumulation profiling of candidates genes exhibited two groups, 1) upregulation 2) down-regulation (in all the treatments). All genes were calculated to

transcript levels with the reference gene of act and 18S rRNA. Among all three genes, UGT76G1 and UGT85C1 showed up-regulation expression in P-WBF treated plants and this result was confirmed by more accumulation of SGs. UGT76G1 and UGT85C1 showed more than 4.3 fold and 2.4 fold changes in comparison with control, respectively. Overall, two genes (UGT76G1 and UGT85C1) were found to be involved in up-regulation or transcript accumulation.

The novel approach of utilizing an excellent PGPR *B. safensis* NAIMCC 02323 (isolated, identified and selected during this study) in a new paneer whey based bioformulation can be a clean and low input green biotechnology for enhancement of yield of a very important MAP, *S. rebaudiana*. *B. safensis* NAIMCC 02323 having a bouquet of PGP characters and biocontrol activities (against fungal phytopathogens) developed as a cheap and effective bioformulation can bring a change in the future cultivation methodology of medicinally and commercially important crops such as *S. rebaudiana*. This will simultaneously lead to the production of healthy food, reducing health problems related with chemical pesticides, and providing green metabolites to pharmaceutical industry along with remediation of chemically contaminated fields.

*Chapter-11*  
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# *Appendices*



Simmon's citrate agar

<b>Ingredients</b>	<b>gm/liter</b>
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulphate	0.2
Bromothymol blue	0.08
Agar	20.00
Distilled water	1000 ml

Urea agar

<b>Ingredients</b>	<b>gm/liter</b>
Peptone	1.0
Sodium chloride	5.0
Potassium monohydrogen (or dihydrogen phosphate) phosphate	2.0
Glucose*	1.0
Phenol red solution**	6.0 ml
Urea (20% aqueous solution)	100 ml
Agar	20.00
Distilled water	1000 ml

\*Add glucose and phenol red to the molten base and steam for 1 hour, cool to 50°C.

\*\*Add 100 ml urea (filter standardized solution) to the basal medium.

Tryptone water

<b>Ingredients</b>	<b>gm/liter</b>
Tryptone	10.0
Sodium chloride	5.0
Calcium chloride- Previously sterilized CaCl <sub>2</sub> was added after autoclaving	1.0ml
Distilled water	1000ml

## Skim milk agar

<b>Ingredients</b>	<b>gm/liter</b>
Skim milk powder	100
Peptone	5.0
Agar	15.0

## Starch agar

<b>Ingredients</b>	<b>gm/liter</b>
Starch	20.0
Peptone	5.0
Beef Extract	3.0
Agar	15.0
Distilled water	1000ml

## MR-VP broth

<b>Ingredients</b>	<b>gm/liter</b>
Peptone	7.0
Potassium phosphate	5.0
Dextrose	5.0
Distilled water	1000.00 ml

## Tween 80 agar

<b>Ingredients</b>	<b>gm/liter</b>
Peptone	10.0
Sodium chloride	5g
Calcium chloride dihydrogen	0.1g
Tween 80	10g
Agar	20
Distilled water	1000ml
Ph	6

## Glucose peptone agar

<b>Ingredients</b>	<b>gm/liter</b>
Glucose	40
Peptone	5
Agar	20
Distilled water	1000ml

## Hoffer's alkaline medium

<b>Ingredients</b>	<b>gm/liter</b>
Mannitol	10
Dipotassium phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Yeast extract	1
Bromothymol blue	0.016
Distilled water	1000ml

## Yeast extract glucose agar

<b>Ingredients</b>	<b>gm/liter</b>
Glucose	10
Dipotassium hydrogen phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Calcium carbonate	4
Yeast extract	1
Agar	20
Bromothymol blue	25mg

## Peptone water

<b>Ingredients</b>	<b>gm/liter</b>
Peptone	10.0
Sodium chloride	5.0

## Pikovskaya's agar

<b>Ingredients</b>	<b>gm/liter</b>
Yeast extract	0.5
Dextrose	10.0
Calcium phosphate	5.0
Ammonium sulphate	0.5

Potassium chloride	0.2
Magnesium sulphate	0.1
Magnesium sulphate	0.0001
Ferrous sulphate	0.0001
Agar	20
Distilled water	1000 ml

## Water agar

Ingredients	gm/liter
Agar	8
Distilled water	1000 ml

## Real-Time PCR

S.No	Ingredients
1	Fast SYBR Green Master Mix
2	cDNA template
3	Gene specific primers
4	Nuclease-free water.
5	Fast plate.
6	Plate seal.

## cDNA preparation

S.No	Ingredients
1	Template RNA.
2	DEPC-treated water.
3	Reverse transcriptase.
4	dNTPs.
5	Buffer.
6	MgCl <sub>2</sub> .
7	Oligo d(T) 18 primers.
8	RiboLock RNase inhibitor.
	Template RNA.

## RNA extraction

S.No	Ingredients
1	Autoclaved water.
2	Extraction buffer
3	Chloroform.
4	Isoamyl alcohol.
5	Absolute alcohol.
6	3 M sodium acetate.
7	0.3× TE Buffer.
8	Autoclaved tips (10, 100, and 1000 µl) and vials (1.5 and

**STAINS, INDICATORS, AND REAGENTS****a) Gram staining**

Crystal violet solution: Dissolve 2.0 gm crystal violet in 20.0 ml of 95% ethyl alcohol

Gram's Iodine solution: Mixed 1.0 gm Iodine, 2.0 gm potassium iodide in 300.0 ml of distilled water

Safranin: Dissolved 10.0 ml of safranin into 100.0 ml of distilled water

**b) Indole production test**

Kovac's reagent: Dissolve the 5.0 gm of diaminobenzaldehyde in the 75.0 ml of amyl alcohol. Then add 25.0 ml of hydrochloric acid to the above preparation. Store the reagent in the refrigerator.

**c) MR-VP test**

Methyl red indicator: Dissolve methyl red (0.1 gm) in 500 ml of 95% ethyl alcohol. Add distilled water and filter the preparation.

VP reagent I: 5.0 gm alpha-naphthol was weighed and dissolved in 95.0 ml of absolute ethyl alcohol

VP reagent II: 40% potassium hydroxide (KOH)

**d) IAA production**

Salkowski reagent : 2 ml 0.5M FeCl<sub>3</sub> and 49 ml water and 49 ml 70% perchloric acid.

**e) Ammonia production**

Nessler's reagent : Dissolve 50.0 gm of potassium iodide in 35 ml of distilled water and added saturated solution of mercuric chloride. Added 400 ml of potassium hydroxide. Dilute to 1000 ml by addition of distilled water. Allow to settle for one week. Stored in tightly stopper brown bottles.

**f) HCN production**

2% Sodium carbonate solution: Dissolve 2 gm of  $\text{NaCO}_3$  in 100 ml of distilled water. 0.5% Picric acid solution Mix 0.5 gm picric acid in 100 ml of water.

**g) Amylase production**

Gram's Iodine: 1.0 gm Iodine and 2.9 gm of Potassium iodide was mixed and add water to make total of 300 ml.

**h) Cellulase production**

Congo red dye (1 mg/1 ml solution) is used for staining.

1M NaCl solution is used for destaining.



*Scientific Publications*



## **Publications**

### **a) Research articles**

1. Jai Prakash and Naveen Kumar Arora (2020). Development of *Bacillus safensis*-based liquid bioformulation to augment growth, stevioside content, and nutrient uptake in *Stevia rebaudiana*. **World Journal of Microbiology and Biotechnology**. 36(1) pp.1-13  
(IF: 2.867)
2. Jai Prakash and Naveen Kumar Arora (2019). Phosphate-solubilizing *Bacillus* sp. enhances growth, phosphorus uptake and oil yield of *Mentha arvensis* L. **3 Biotech**. 9(4) pp.126-138 (IF: 1.786)
3. Jai Prakash and Naveen Kumar Arora (2019) Study of biocontrol activity of fluorescent pseudomonads against *Alternaria alternata*, isolated from rhizosphere locale of *Stevia rebaudiana*. **Journal of Emerging Technologies and Innovative Research**. 6(3) pp. 1-7
4. Jitendra Mishra, Jai Prakash and Naveen Kumar Arora (2016). Role of beneficial soil microbes in sustainable agriculture and environmental management. **Climate Change and Environmental Sustainability**. 4(2) pp.137-149

### **b) Book Chapter**

1. Naveen Kumar Arora, Maya Verma, Jai Prakash, Jitendra Mishra (2016) Regulation of Biopesticides: Global Concerns and Policies. **In book: Bioformulations: for Sustainable Agriculture**, Edition: 1, Chapter: 16, Publisher: Springer India, Editors: Naveen Kumar Arora, Samina Mehnaz, Raffaella Balestrini, pp.283-299, 2016.

### **c) Magazine articles**

1. Jai Prakash, Rachna Singh, Naveen Kumar Arora (2015) *Stevia rebaudiana*: A herbal medicine & alternative source of calorie free sweetener. **Microbiology World**. Issue 9: Jan-Feb-2015, ISSN 2350-8774
2. Jai Prakash, Adarsh Kumar, Naveen Kumar Arora (2015) Biofertilizers: an alternative sources of chemical fertilizer for sustainable crops in 21st century. **Microbiology World**. Issue 10, March-April, 2015, ISSN 2350-8774

#### **d) Workshops**

- 1) ICAR–Sponsored Short Course on “ **Molecular Approaches for Plant disease diagnostics and Resistance mechanism**” organized by **Department of agricultural Biotechnology, Assam Agricultural University, Jorhat-13, Assam** (3<sup>rd</sup> -12<sup>th</sup> November, 2016)
- 2) “**Workshop on Chromatography**” A technique for analysis of biomolecules from natural resources and agricultural crops (A training programme on **HPTLC, HPLC, GC, GC-MS, SPME-GC-MS**) organized by **Department of Botany (Centre for Advanced Study), University of Calcutta, India** (5<sup>th</sup> -8<sup>th</sup> January, 2016)
- 3) ICAR – Sponsored Short Course on “**Recent Advances in Mass Production on Biological Control Agents**” at **ICAR- National Centre for Integrated Pest Management, IARI, Pusa, New Delhi** (1<sup>st</sup> -10<sup>th</sup> September, 2015)
- 4) Hands on training on “**SEM, FTIR, FPLC, and Ion chromatography**” **University Science Instrumentation Centre, Babasaheb Bhimrao Ambedkar University, Lucknow** 18<sup>th</sup> -20<sup>th</sup> February, 2015)

#### **e) Poster presentation in National and International Conferences**

1. Jai Prakash and Naveen Arora (2018). Role of *Bacillus safensis* for controlling *Alternaria alternata*, a phytopathogen of *Stevia rebaudiana*. **Association of Microbiologists in India (AMI) 2017**
2. Jai Prakash and Naveen Arora (2015). Developing PGPR based bioformulation for enhanced production and protection of Agricultural crops. **6<sup>th</sup> India Japan Science Seminar, International conferences on contemporary advances of science and technology (IC-CAST)-7-9 August, 2015**



# 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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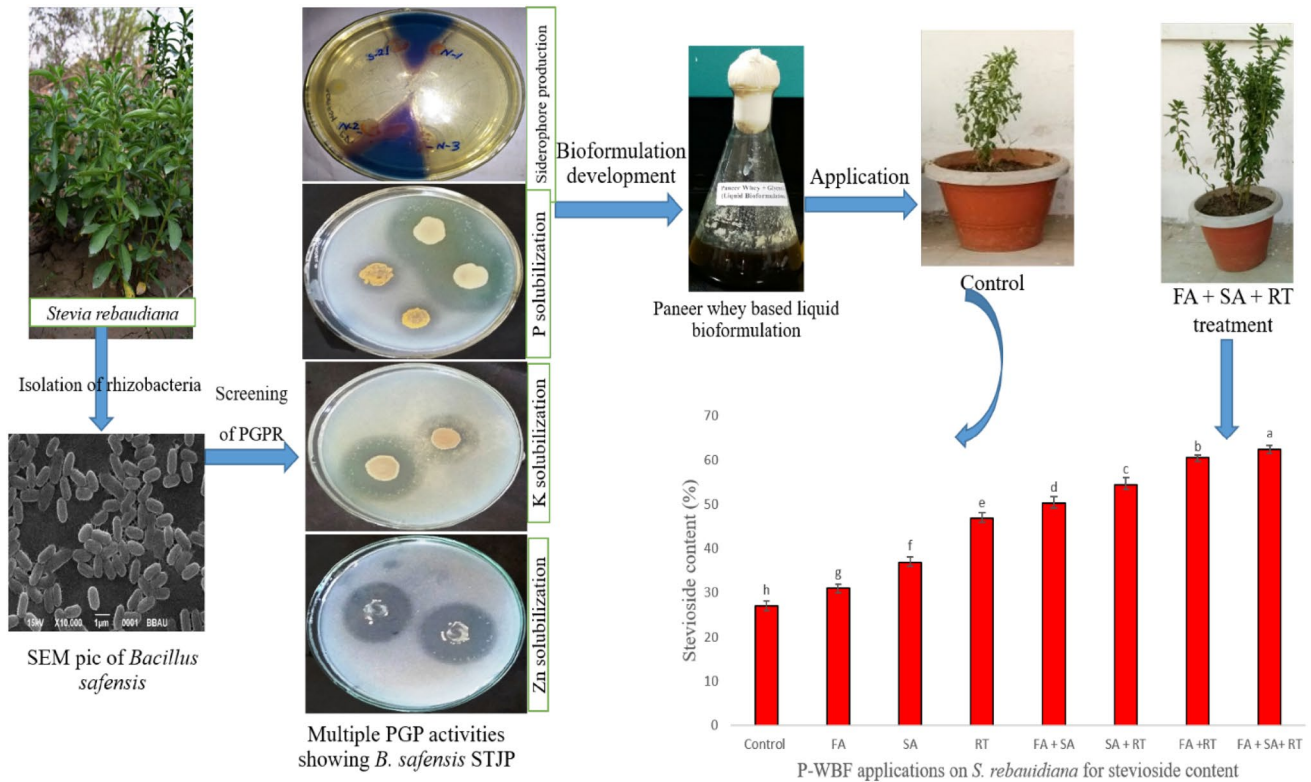
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## Graphic abstract



**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 (Gawel-Beben 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 (WHOEF) 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 comparison 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 ( $\text{g}^{-1}$ ;  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ : 0.2,  $\text{CaPO}_4$ : 0.2,  $\text{CaCO}_3$ : 0.01,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ : 0.05,  $\text{C}_6\text{H}_{12}\text{O}_6$ : 0.5,  $\text{FeCl}_3$ : 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 ( $\text{g}^{-1}$ ); KCl: 0.2,  $\text{K}_2\text{HPO}_4$ : 0.1,  $\text{MgSO}_4$ : 0.2,  $(\text{NH}_4)_2\text{SO}_4$ : 1, dextrose: 10], and inorganic Zn compound ( $\text{ZnO}$ : 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:  $78 \pm 2$  °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<sup>-1</sup>) 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<sub>2</sub>SO<sub>4</sub> and HClO<sub>4</sub> (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  $\pm$  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.

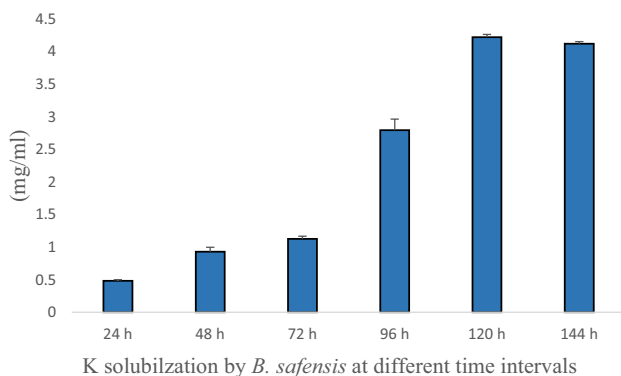
## Results

### K and Zn solubilization traits

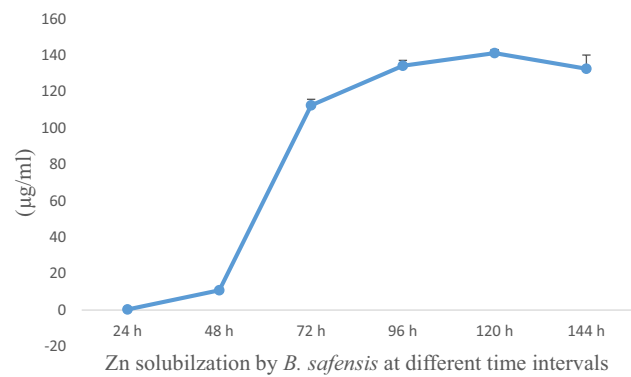
In this study, the ability of *B. safensis* STJP was checked for solubilization of K (Fig. 1) and Zn at different time intervals. Isolate STJP showed maximum K solubilization (4.4 mg/L) after 5 days of incubation. *B. safensis* STJP maximally solubilized Zn (5.4 mg/L) after 5 days of incubation (Fig. 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  $\pm$  standard error of means



**Fig. 2** Zn solubilization by *B. safensis* STJP at different time intervals. Data are mean of five replica  $\pm$  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	–	–	–	–
<i>E. coli</i>	–	–	–	–
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 <sup>4</sup>	10 <sup>6</sup>	10 <sup>8</sup>	10 <sup>8</sup>

**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

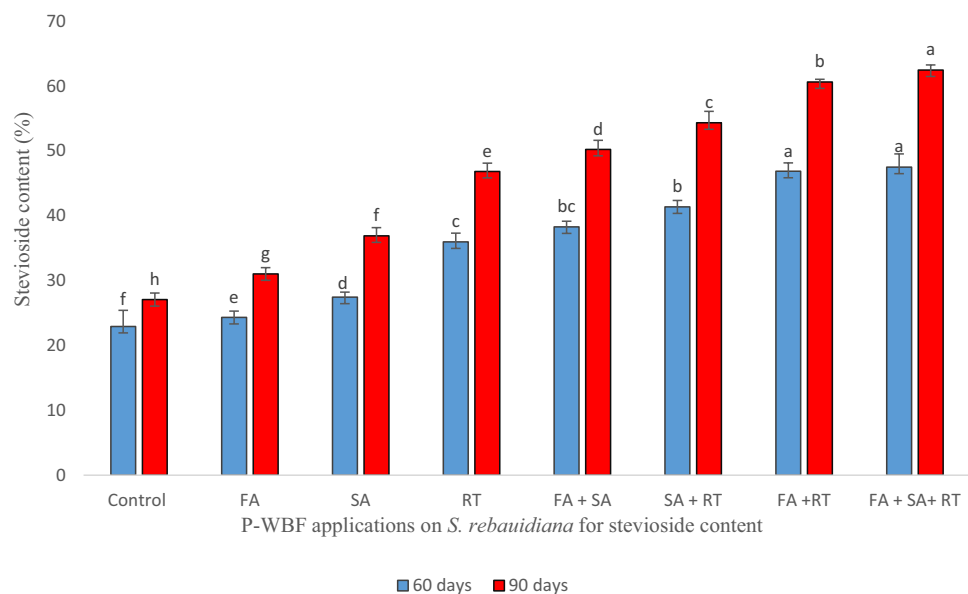
(for 60 days) and in this case, the single treatment of RT, SA and FA on *S. rebaudiana* increased dry weight by 26.23%, 13.68% and 3.04%, respectively (Table 3). When RT + SA, RT + FA and SA + FA treatments were applied 73.04%, 61.44%, and 17.68% enhancement in dry weight was observed as compared to 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-sterilized 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).

**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  $\pm$  standard error of means, followed by the same letter in a column are not significantly different by Duncan's multivariate test (DMRT)

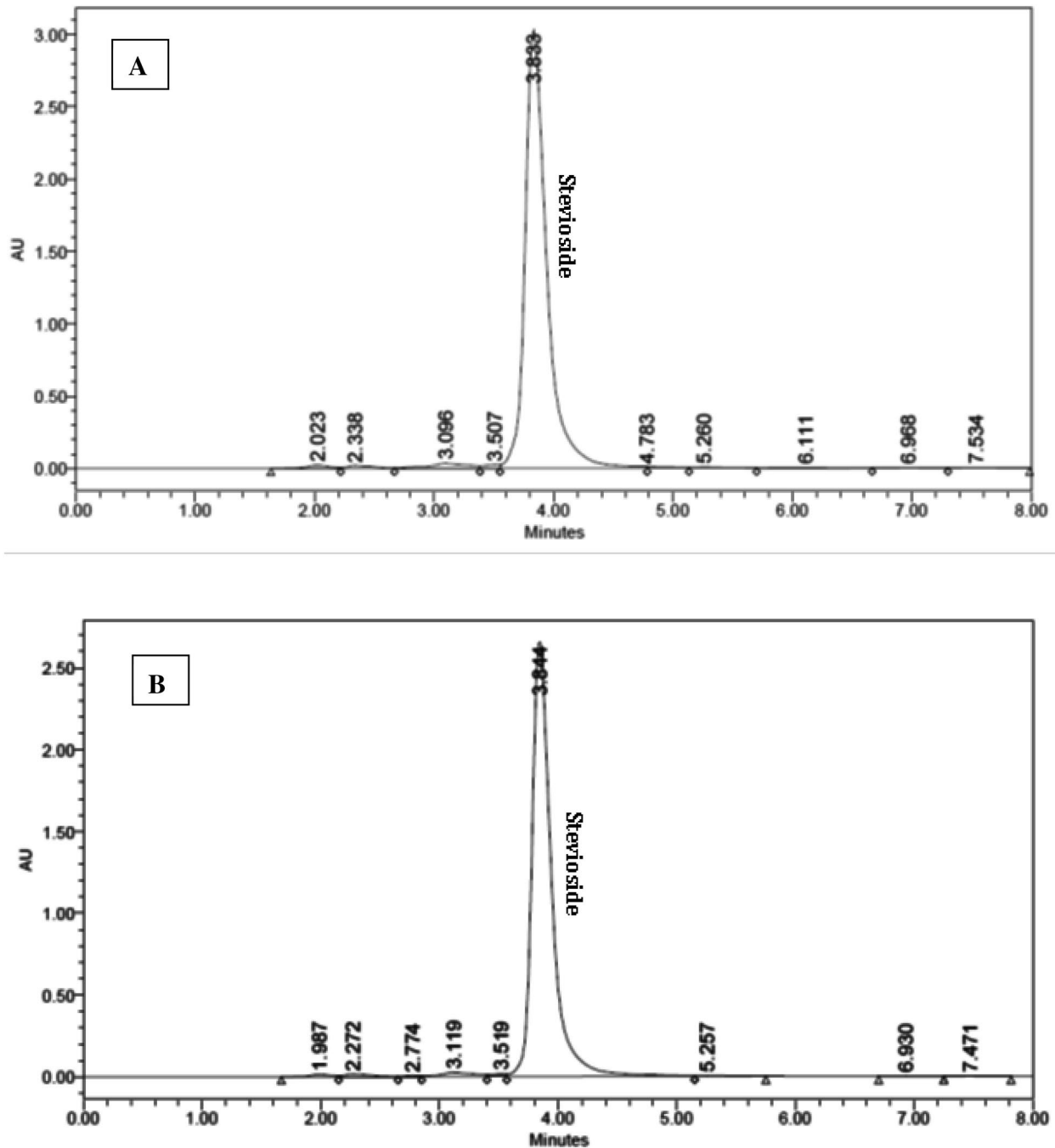


### Bioformulation enhances nutrients uptake in *S. rebaudiana*

Application with RT + SA + FA treatment resulted in  $\sim 2.8$  (sterilized soil) and  $\sim 2.37$  (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. 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 Fakhru 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 ratio 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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# Phosphate-solubilizing *Bacillus* sp. enhances growth, phosphorus uptake and oil yield of *Mentha arvensis* L

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## Abstract

In the present study, phosphate solubilizing rhizobacterial isolate STJP from the rhizosphere of *Stevia rebaudiana* was identified as a *Bacillus* sp. on the basis of phenotypic, biochemical, and 16S rRNA gene sequencing. In addition to phosphate solubilization ability, isolate *Bacillus* sp. STJP produced a significant quantity of siderophore (16.06 µg/ml) and indole 3-acetic acid (30.59 µg/ml). In the greenhouse experiment, treatment with STJP along with tricalcium phosphate (TCP<sup>200</sup>) showed significant increase in the plant growth parameters, oil yield and P uptake in *M. arvensis* as compared to the control plants. Amongst all the treatments, highest oil yield and menthol content were observed when treated with *Bacillus* sp. STJP + TCP<sup>200</sup>. Hence, an integrated approach of using *Bacillus* sp. STJP along with TCP can be used to increase the production of menthol and oil yield of *M. arvensis*. This approach of using fertilizer along with phosphate solubilizing *Bacillus* sp. worked very well and was more effective in comparison with individual treatment of fertilizer or plant growth promoting rhizobacteria. A combined use of efficient phosphate solubilising bacteria loaded with plant growth promoting characters along with TCP can thus be far effective way for enhancing the yield of crops in a sustainable manner.

**Keywords** *Bacillus* · *Mentha arvensis* · Menthol · Plant growth promoting rhizobacteria · Sustainable agriculture

## Introduction

Medicinal and aromatic plants (MAPs) have proved to be the major sources of medicines in the world. They are extensively used for the treatment of several diseases and boost the economy as well as the living standard of farmers in several countries including India (WHO 2013). But nowadays, assurance of the efficacy, quality, and supply of MAPs and their products has become a major issue around the globe. Day by day, we are losing the natural reserves of MAPs mainly due to degradation of habitats (Stutte 2016). Hence, the need has arisen to cultivate the MAPs in an eco-friendly

manner so as to maintain their continuous supply to get the useful products. For the cultivation of MAPs, the use of biological methods, so as to enhance the productivity is better and this can reduce the load of chemicals in the agroecosystems (Singh and Arora 2016). The use of plant growth promoting rhizobacteria (PGPR) can thus be more useful and good for the environment.

*Mentha arvensis* (Japanese mint or menthol mint) of family Lamiaceae, is widely used for the treatment of several diseases such as abdominal pain, cough, diarrhoea, fever, headache, nausea, sunburn, skin irritation and vomiting (Alankar 2009). The leaves of *M. arvensis* contain high levels of flavonoids and antioxidants. In addition, *M. arvensis* also contains an important secondary metabolite known as menthol which has potential applications in several industries including pharmaceutical, flavoring, food, agrochemicals and cosmetics. The worldwide production of menthol is estimated as 20,000 metric tons every year (Srivastava et al. 2002). India is the chief menthol producer (more than 80% of the total produce in the world) and its leading exporter as well (Bakry et al. 2016). Approximately, 0.145 million hectares (ha) of agricultural land is being used for growing *M. arvensis* in northern India's Indo-Gangetic plains

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(Kumar et al. 2011). Day by day the demand for *M. arvensis* and its essential oil is increasing; hence it is important to increase the yields without affecting the quality and purity of agro-ecosystems.

Nutrient deficiency in soils is one of the major issue for agricultural production around the globe affecting the quantity and quality of crops (Tewari and Arora 2016). For the improvement of crop yields, farmers have commonly used chemical fertilizers for agricultural production causing an adverse effect on autochthonous organisms and deteriorating the quality of agro-ecosystems and aquatic resources (Arora et al. 2016). Most of the phosphate fertilizers remain unused and accumulate in the soil as precipitates. Apart from this, the accumulated phosphates (in soil) may also reach water bodies causing eutrophication. Therefore, the use of phosphate solubilising microbes along with the applied phosphate can provide a solution to the menace of accumulation of phosphates in the fields and water bodies. *Bacillus* sp. are well-known rhizobacteria which facilitate the plant growth either by solubilization of minerals like phosphorus or production of metabolites such as siderophores and phytohormones, and are excellent colonizers of the roots (Ahmad et al. 2018). In addition, *Bacillus* spp. have added advantage as their communities are found in the rhizosphere of wide varieties of crop plants and can survive stress condition very well (Abd-Allah et al. 2018). Use of *Bacillus* sp. has become a very important practice for eco-friendly and sustainable agriculture for enhancing crop production and control phytopathogens (Alori et al. 2017). An integrating approach of utilizing *Bacillus* spp. having multiple capabilities (including phosphate solubilization) along with phosphate fertilizers can enhance the growth of plants and maintain soil fitness even in presence of biotic and abiotic stresses (Mishra et al. 2016; Tewari and Arora 2018; Gouda et al. 2018).

Phosphate solubilizing bacteria (PSB) are emerging as important biofertilizers with the ability to utilize precipitated phosphorus (P) in the soil, leading to eco-friendly agriculture and maintaining the quality of agro-ecosystems (Pereira et al. 2015; Alori et al. 2017). PSB based biofertilizers are cheaper and can be of particular interest in nutrient (phosphorus) deficient soils (Shen et al. 2011). Although, in last few years, the role of PSB as attractive biofertilizers is on the rise, still, there are negligible reports on their utilization as biofertilizers with commercial use for enhancing the growth of *M. arvensis* and improving its oil content. With this aim, in the present study, PSB were isolated and checked for their plant growth promoting (PGP) characters followed by evaluation of the ability to enhance the growth, oil yield, and quantity of menthol in *M. arvensis*.

## Materials and methods

### Isolation and characterization of phosphate solubilizing rhizobacteria

PSB were isolated from the rhizosphere soil of *S. rebaudiana*, a medicinal plant growing in farmer's field at Lucknow (26.7679°N, 80.9263°E), Uttar Pradesh, India. For isolation, 1 g of sample was dissolved in 9 ml of sterile water (pH 7.1). From fifth dilution ( $10^{-5}$ ), 0.1 ml of soil sample was spread on Pikovskaya's (PVK) agar (Pikovskaya 1948) containing 0.5% tri-calcium phosphate and incubated at 28 °C for 72–120 h for checking phosphate solubilization. Isolates were also grown at 30 °C for 120 h in National Botanical Research Institute Phosphate (NBRIP) broth medium to check the amount of phosphate solubilization. The soluble P content ( $\mu\text{g/ml}$ ) was determined by spectrophotometer at 600 nm (Nautiyal 1999). Selected isolate STJP (on the basis of phosphate solubilization ability) was identified by phenotypic (flagellated or non-flagellated, shape and size) and biochemical features such as oxidase, urease, catalase, gelatinase, indole,  $\text{H}_2\text{S}$ , citrate, and nitrate production as per Bergey's Manual of Systematic Bacteriology (Garrity 2005). The cell shape of the bacterium was confirmed by scanning electron microscopy (SEM) performed according to the method of Golding et al. (2016). Isolate STJP was further identified by 16S rRNA sequencing and sequence analysis (Maidak et al. 2000). The evolutionary distance of isolate STJP and the closely related taxa was calculated using MEGA (version 6.00) software.

### Phosphatase enzyme assay

Phosphatase enzyme assay was performed as per the method of Tabatabai and Bremner (1969). Briefly, 100  $\mu\text{l}$  bacterial culture was inoculated in NBRIP broth (50 ml) and incubated at 28 °C for 7 days. After incubation (24 h), the broth culture was centrifuged (speed: 10,000 rpm, time: 10 min, temp: 4 °C) and supernatant was collected. One ml of supernatant was mixed with modified universal buffer (4 ml; pH 6.8) and subsequently, disodium *p*-nitrophenol phosphate (1 ml; 0.025 mM) was added in supernatant for quick analysis of phosphate enzyme activity and incubated at 28 °C for 60 min. After incubation, 1 drop of toluene was added and further incubated (time: 1 h; temp: 37 °C) and finally 0.5 M  $\text{CaCl}_2$  and 0.5 M NaOH (ratio 1:4) was mixed for inhibition of reaction. The obtained sample was filtered via the Whatman filter paper no 42 and the quantity of enzyme were examined in 1  $\mu\text{mol}$  of *p*-nitrophenol/ml/min through spectrophotometer (420 nm). For standard, *p*-nitrophenol was used for the quantification of phosphatase.

## Investigation of plant growth promoting characteristics

Bacterial isolate STJP was inoculated in tryptic soy broth (TSB) comprising (g/l); tryptone:15, NaCl: 8, soy peptone: 5, tryptophan: 1 (HiMedia, Mumbai), 1000 ml sterilized water; pH 7.0 in 50 ml Erlenmeyer flask (EF) and incubated at 30 °C for 72 h (120 rpm) for IAA production. After incubation, 5 ml culture broth was centrifuged (speed: 5000 rpm, time: 10 min, temp: 4 °C). The obtained supernatant was mixed with Salkowski reagent in the ratio of 3:2 and was left in the dark (20 min). Afterwards, sample was analyzed spectrophotometrically at 530 nm for quantitative estimation of IAA ( $\mu\text{g/ml}$ ) (Brick et al. 1991). For quantifying of siderophore, isolate (STJP) was inoculated in nutrient broth (NB) and incubated for 72 h at 28 °C (130 rpm). After incubation, the culture was centrifuged to obtain supernatant (speed: 5000 rpm, time: 15 min, temp: 4 °C) and the collected sample was taken to examine the siderophore production (Reeves et al. 1983). The siderophore quantity was expressed in  $\mu\text{mol}$  benzoic acid/ml. The ability to fix nitrogen was checked as per Line and Loutit (1971) and production of hydrogen cyanide (HCN) was evaluated according to Millar and Higgins (1970).

## Effect of isolate STJP on the growth of *M. arvensis*

The pot experiment was performed in University Greenhouse Horticulture Research Farm (HRF) for 90 days. Selected isolate STJP was grown in NB in a shaker (speed: 120 rpm, time: 48 h, temp: 28 °C). Afterwards, growth culture was centrifuged (speed: 5000 rpm, time: 10 min, temp: 4 °C) for collecting the pellet. Further, sterilized deionized water was mixed in the pellet to obtain population density of  $10^8$  CFU/ml (Maheshwari et al. 2012).

Sandy-loam soil [pH: 7.69, EC: 0.48 dS/m, N: 108 (kg/ha), K: 89 (kg/ha), and P: 35.26 (kg/ha)] was collected from HRF and 6 kg was filled in clay pots (20 cm  $\times$  20 cm  $\times$  20 cm). Rootlets of seedling of *M. arvensis* (var. CIMAP SARYU) were disinfected by ethanol (70%, time: 2 min) followed by sodium hypochlorite (2%, time: 5 min) and subsequently washed with deionized water for 10 min (Arora et al. 2001). The seedling was then dipped in inoculum suspension ( $10^8$  CFU/ml: time: 20 min) and allowed to dry (time period: 30 min). Subsequently, the seedlings (five per pot) were sown as per following sets: T-1 control (*M. arvensis*); T-2 STJP; T-3 TCP<sup>100</sup> (100 mg/kg in soil); T-4 STJP + TCP<sup>100</sup>; T-5 TCP<sup>200</sup> (200 mg/kg in soil); T-6 STJP + TCP<sup>200</sup> as per Mamta et al. (2010). The work was done with five replications for each set in a completely randomized design (CRD). Irrigation was done using tap water (in equal amount) for each pot as per requirement. The plants were uprooted 90 days after sowing (DAS) and

checked for the shoot and root length, number of leaves, fresh and dry weight.

## Examination of oil yield from *M. arvensis*

The dry *M. arvensis* plants were used for extraction of oil by Clevenger's apparatus in hexane solvent. Before extraction, the fresh shoots (leaves and stem) were harvested and separately dried (temp: 45 °C, time: 96 h). Completely dry shoots were crushed, samples were dissolved in *n*-hexane (50 ml) for 10 h and oil was extracted as per method described by Langenau (1948). The obtained oil was filtered via Whatman filter paper no. 42. The filtered oil was evaporated using rotatory evaporator (temp: 40 °C, speed: 50 rev/min, vacuum pressure: 80 mbar) to obtain pure oil. The oil yield was calculated (g/pot) as per the method of Singh et al. (2013).

## Effect of isolate STJP on menthol content

To determine menthol content, 5 ml of the methanol and water in a ratio of 75:25 v/v were properly mixed with mentha oil obtained from plants. Further, the samples were filtered through 0.45 M Millipore filter membrane. The filtered sample was injected in the high-pressure liquid chromatography (HPLC) (Model No. WATER—2489) with C-18 column for determination of menthol content. Menthol: water (HPLC grade) (75:25) with  $2 \times 10^{-5}$  M *n*-heptyl-*p*-aminobenzoate was used as mobile phase and pressure and flow rate was set 800 psi, and 1.5 ml/min (Perkin 1984). The wavelength was monitored at 290 nm and compared with standard menthol (Sigma-Aldrich, USA).

## Examination of P uptake in *M. arvensis*

For examination of P uptake in *M. arvensis*, roots, stem, and leaves were harvested (90 DAS) from each pot and dried separately. Further, dried sample was digested and analyzed using atomic absorption spectroscopy (Model: AA240FS Fast Sequential AAS, USA) for quantification of P as described by Koenig and Johnson (1942).

## Statistical analysis

Pot experiments were organized in a complete randomized design (CRD) method. One-way analysis of variance (ANOVA) was chosen for data analysis. Duncan's multiple range test (DMRT) was used for comparison of means (Gomez and Gomez 1984) using the software of Statistical Package for Social Sciences (SPSS) version 15.

## Results

### Characterization of phosphate solubilizing rhizobacteria

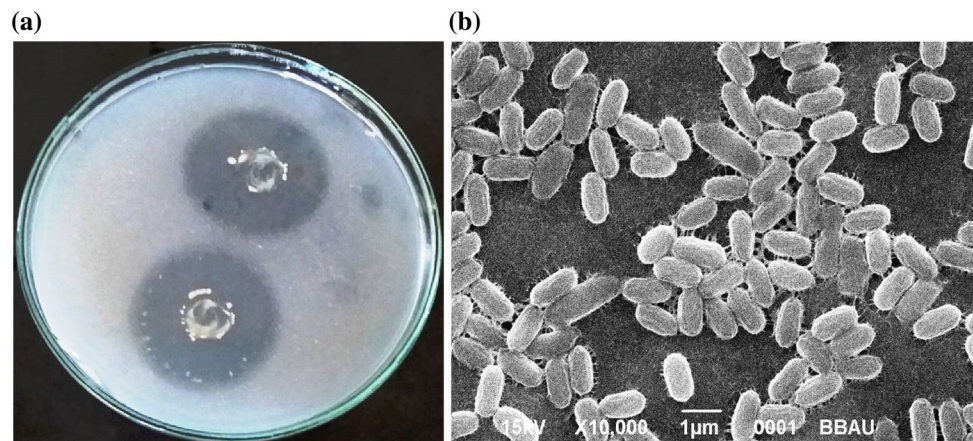
Eleven PSB were isolated from rhizospheric soil of *S. rebaudiana*. Among them, six isolates showed greater than 6 mm zone on PKV agar plates (Fig. 1a). Isolates were further screened for the amount of phosphate solubilization in liquid NBRIP medium. All isolates were able to carry out phosphate solubilization ranging from 8 to 610.33 µg/ml (Table 1). Among them, isolate STJP showed maximum P-solubilizing activity (610.33 µg/ml) in NBRIP medium and hence was selected for further study. Isolate STJP was found to be flagellated bacterium with peritrichous arrangement and cell size of  $1.0\text{--}1.28 \times 0.5\text{--}0.7$  µm (Fig. 1b). Isolate STJP was found positive for oxidase, urease, catalase, and gelatinase, but negative for indole, H<sub>2</sub>S, citrate, and nitrate production. On the basis of phenotypic

and biochemical traits, isolate STJP was found to be a member of genus *Bacillus* sp. on the basis of Bergey's Manual of Systematic Bacteriology (Garrity 2005). The comparison of the whole 16S rRNA gene sequence of a neighbour isolate of STJP showed 99% similarities with *Bacillus safensis* strain NBRC 100820. The gene sequence data is submitted in NCBI database with Accession number KX372540 and can be said to belong to *B. safensis* clade. However, further studies are required to confirm the species of the strain.

### Phosphatase assay

The production of phosphatase by isolate STJP was checked every 24 h up to 7 days in NBRIP medium. Isolate STJP produced maximum phosphatase (81.45 U/ml) after 96 h which reduced with further incubation (data not shown).

**Fig. 1** **a** Phosphate solubilization by isolate STJP on Pikovskaya agar. **b** Cell shape of the bacterium STJP



**Table 1** Quantitative examination of phosphate solubilization by different isolates (µg/ml) (1–5 days)

PSB isolates	1st day	2nd days	3rd day	4th day	5th day
B1TNP	48.33 ± 2.35	70.66 ± 2.49	122.33 ± 2.05	189.33 ± 2.49	188.33 ± 2.35
B2NPT	70.00 ± 4.08	80.66 ± 2.49	88.33 ± 2.35	234.33 ± 3.29	161.66 ± 2.35
B1PPoNP	10.00 ± 4.08	27.66 ± 2.05	68.33 ± 4.64	138.00 ± 1.63	134.66 ± 3.39
NPSP	89.33 ± 2.49	102.33 ± 6.12	31.00 ± 2.94	21.33 ± 1.88	8.00 ± 1.63
NPPsP2	83.33 ± 2.35	113.33 ± 4.71	159.33 ± 0.94	192.00 ± 1.63	185.66 ± 3.29
<b>STJP</b>	<b>139.00 ± 2.94</b>	<b>188.33 ± 6.23</b>	<b>347.66 ± 2.05</b>	<b>610.33 ± 2.05</b>	<b>604.00 ± 4.10</b>
STJP-12	052 ± 2.12	75.66 ± 3.29	101.66 ± 2.35	162.00 ± 1.63	235.00 ± 4.08
STJP-18	31.33 ± 1.88	53.33 ± 3.39	91.66 ± 1.24	161.33 ± 1.88	132.66 ± 1.88
STJP-20	89.66 ± 3.68	135.00 ± 4.08	237.66 ± 2.05	241.66 ± 2.35	208.33 ± 2.35
STJP-22	036 ± 561	092.00 ± 2.16	38.00 ± 1.63	132.66 ± 2.49	114.33 ± 3.29
STJP-30	37.66 ± 2.05	52.50 ± 1.87	88.33 ± 2.35	181.66 ± 2.35	132.33 ± 2.05

Bold values are for the selected isolate STJP which showed maximum phosphate solubilization on all the days of incubation

Data are mean of three replicates ± standard error of means

## Plant growth promoting characteristics

The intensity of pink color production within 30 min is the first visible confirmation of IAA production. Isolate STJP showed intense pink color and recorded a significant amount of IAA (30.59 µg/ml). Further, isolate STJP showed a good amount of siderophore production (16.06 µg/ml) and observed a high affinity for iron chelation. The color change of the filter paper from yellow to brown is a key for detection of HCN. STJP did not show HCN production as confirmed by the absence of any color change of the filter paper laden with picric acid. Isolate STJP was unable to grow on nitrogen-free medium when tried repeatedly confirming its inability to fix nitrogen.

## Effect of STJP on growth promotion of *M. arvensis*

Bacterial isolate STJP positively affected the plant growth parameters as observed after 90 days. STJP showed stimulatory effect, in term of growth parameters as compared to the control (Table 2). Among all treatments, STJP + TCP<sup>200</sup> recorded maximum enhancement in vegetative parameters as compared to the control.

STJP treatment of *M. arvensis* enhanced fresh and dry weight by 46.27% and 49.57% respectively, in comparison with the control plant. The combined application of TCP<sup>100</sup> and STJP corresponded to 46.65% and 70.37% rise in fresh and dry weight respectively, in comparison with un-inoculated plants. Moreover, STJP + TCP<sup>200</sup> treatment resulted in maximum fresh and dry weight enhancement (by 70.13% and 82.11%, respectively) as compared to the control plants (Table 2). The plant length of *M. arvensis* treated with STJP, STJP + TCP<sup>100</sup>, STJP + TCP<sup>200</sup> recorded an increase of 55.12%, 74.36%, and 86.57%, respectively, as compared with the control plants. However, treatment with 100 mg TCP and 200 mg TCP only, did not affect significantly in the length of the plant. Although, *M. arvensis*

treated with STJP showed significant increase in the leaf number (39.84%) as compared to the control plants but STJP + TCP<sup>200</sup> application showed maximum increment in leaf number as compared to all other treatments. The number of leaves did not increase significantly after treatment with 100 mg and 200 mg TCP applications in comparison with the control plants.

## Effect of isolate STJP on oil yield and menthol content in *M. arvensis*

*M. arvensis* grown in soil amended with TCP showed higher oil yield as compared to without TCP amended soil (Fig. 2). A significant rise in oil yield of *M. arvensis* was observed after inoculation of STJP (56.71%) as compared to the control (without any treatment). There was 107.68% and 79.85% enhancement in oil yield by the treatment with STJP + TCP<sup>200</sup> and TCP<sup>100</sup> + STJP, respectively, in comparison with the control plant. Beside oil yield enhancement, the menthol content also increased significantly by the combined treatment of TCP and STJP (figure not shown). However, as in case of vegetative parameters, greatest enhancement in menthol content was shown when *M. arvensis* was treated with STJP + TCP<sup>200</sup>, as this treatment showed a maximum spike in peak (as compared to the control) when checked by HPLC.

## Effect of STJP on P uptake in *M. arvensis*

Total P uptake in *M. arvensis* was studied after harvesting. The increase in P levels in roots, stem, and leaves were found to be 232%, 343.75%, and 327% respectively, by the STJP + TCP<sup>200</sup> treatment which was maximum in comparison to any other treatment when compared with the control plant (Fig. 3).

**Table 2** Effect of isolate STJP on growth promotion of *M. arvensis*

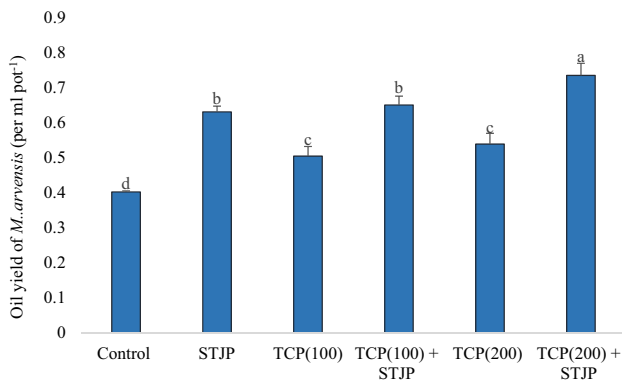
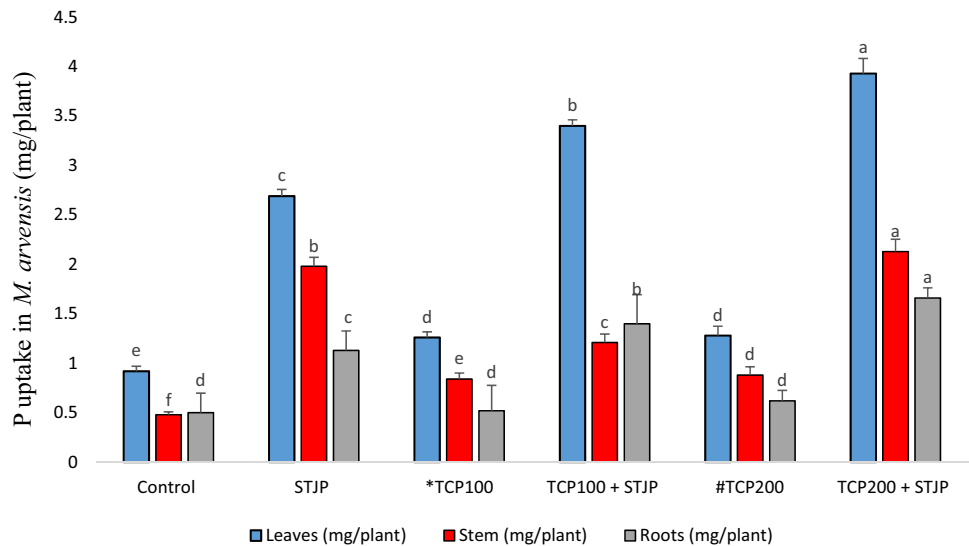
Treatments	Plant shoot length (cm)	Plant root length (cm)	Number of leaves	Total fresh weight (g)	Total dry weight (g)
Control	18.00 ± 3.20 <sup>c</sup>	09.03 ± 1.66 <sup>c</sup>	42.66 ± 1.88 <sup>c</sup>	28.83 ± 1.24 <sup>d</sup>	13.03 ± 1.23 <sup>d</sup>
STJP	27.20 ± 2.08 <sup>b</sup>	14.73 ± 0.55 <sup>ab</sup>	59.66 ± 1.69 <sup>b</sup>	42.17 ± 2.10 <sup>b</sup>	19.49 ± 1.52 <sup>bc</sup>
TCP <sup>100A</sup>	18.93 ± 1.23 <sup>c</sup>	09.63 ± 0.91 <sup>c</sup>	44.00 ± 1.63 <sup>c</sup>	35.61 ± 2.15 <sup>c</sup>	16.87 ± 1.22 <sup>c</sup>
STJP + TCP <sup>100</sup>	29.53 ± 0.80 <sup>ab</sup>	17.60 ± 1.62 <sup>a</sup>	61.66 ± 2.62 <sup>ab</sup>	42.28 ± 1.18 <sup>b</sup>	22.20 ± 0.98 <sup>ab</sup>
TCP <sup>200B</sup>	19.93 ± 0.66 <sup>c</sup>	10.13 ± 0.66 <sup>bc</sup>	44.33 ± 1.69 <sup>c</sup>	36.01 ± 2.12 <sup>c</sup>	17.22 ± 1.15 <sup>c</sup>
STJP + TCP <sup>200</sup>	32.20 ± 2.01 <sup>a</sup>	18.23 ± 1.97 <sup>a</sup>	66.66 ± 3.39 <sup>a</sup>	49.05 ± 2.28 <sup>a</sup>	23.73 ± 1.81 <sup>a</sup>

Data are mean of three replicates ± standard error of means. Means, followed by the same letter in a column are not significantly different ( $P=0.05$ ) by Duncan's multivariate test (DMRT)

<sup>A</sup>TCP<sup>100</sup> (100 mg tricalcium phosphate in per kg of sterilized soil)

<sup>B</sup>TCP<sup>200</sup> (200 mg tricalcium phosphate in per kg of sterilized soil)

**Fig. 2** Effect of isolate STJP on P uptake in *M. arvensis* [data are mean of three replicates  $\pm$  standard error of means. Means, followed by the same letter in a column are not significantly different ( $P=0.05$ ) by Duncan's multivariate test (DMRT) ( $P\leq 0.05$ )]



**Fig. 3** Effect of isolate STJP on the yield of mentha oil in *M. arvensis* [data are mean of three replicates  $\pm$  standard error of means. Means, followed by the same letter in a column are not significantly different ( $P=0.05$ ) by Duncan's multivariate test (DMRT)]

## Discussion

Menthol is one of the key plant metabolites with a wide range of applications in medicine and industries. According to an industrial report, the estimated production of menthol was 36,000 metric tonnes in 2017, which is much lower than the current global demand of approximately 45,000 metric tonnes (<http://www.aosproduct.com/MARKET-REPORT/Menthol-Crystals-and-Mentha-oil>). Hence, there is an urgent requirement to raise the menthol production which can be achieved in a better and sustainable manner if done through biological approaches. PGPR can thus play a key role in the enhancement of productivity of menthol producing plants. Among PGPR, *Bacillus* spp. are omnipresent in nature, and are widely used for enhancement of growth, development, and yield of plants

by producing bioactive metabolites and nutrients (Radhakrishnan et al. 2017). These bacteria have the ability to solubilize phosphate in the soil and also the ability to produce a number of metabolites such as IAA and siderophores which are directly involved in nutrients uptake and plant growth (Ribeiro et al. 2018).

P is the second most important macronutrient which is also a limiting factor for plant growth and development apart from nitrogen. Most of the P in soil is found in an insoluble form such as aluminium phosphate ( $AlPO_4$ ) or ferric phosphate ( $FePO_4$ ) which cannot be taken up by crops (Chen et al. 2017). The ability of PSB to solubilize insoluble P and make it available to the plants by one of the several mechanisms such as producing phosphatase enzyme, releasing organic acids (gluconic, oxalic and succinic acids), ion exchange and chelation (Wei et al. 2018) are well known. *Bacillus* spp. and in particular, *B. subtilis* are widely reported as PSB (Radhakrishnan et al. 2017; Ahmad et al. 2017). In the current study, phosphate solubilizing isolate STJP from the rhizosphere of a medicinal plant and identified to be *Bacillus* sp. was tested as a potent PGPR for *M. arvensis*. Mukhtar et al. (2017) reported *B. safensis* and *Bacillus megaterium* as phosphate solubilizers, however in their study, *Bacillus megaterium* was found to be better phosphate solubilizing agent as compared to *B. safensis*. In the present study *B. safensis* isolate STJP was found to be effective phosphate solubilizer. The ability to solubilise phosphate by STJP was found to be better than reported earlier for *B. safensis* (Mukhtar et al. 2017).

Mechanisms of phosphate solubilization by PSB are very well known (Sharma et al. 2013; Behera et al. 2017). Phosphatase enzyme has been commonly reported for solubilization of inorganic phosphate (Wei et al. 2018). In our study, isolate STJP produced 81.25 U/ml phosphatase enzyme (after 96 h). There are many reports available on the

production of phosphatase by *Bacillus* spp. (Parhamfar et al. 2016; Behera et al. 2017). The phosphatase enzyme cleaves the insoluble phosphate into phosphate ions and thus helps in uptake of ions by the plants. Maximum growth enhancement of *M. arvensis* was observed in the presence of STJP in combination with TCP<sup>200</sup> treatment. This might be associated with the presence of a phosphatase enzyme produced by *Bacillus* sp. STJP. Isolate STJP produced 8.64 U/ml phosphatase after 24 h which increased with incubation period up to 96 h (81.45 U/ml). Similarly, Swain et al. (2012) reported that *B. subtilis* produced 30.2 U/ml phosphatase at 12 h incubation period but further increase in incubation period resulted in higher production of the enzyme.

P content was also increased to the maximum level in *M. arvensis* when treated with STJP + TCP<sup>200</sup> as compared to the addition of TCP only. Treatment of STJP + TCP<sup>200</sup> also resulted in maximum enhancement of plant growth parameters. Pereira et al. (2015) examined that *Arthrobacter nicotivanovans* EAPAA inoculated in TCP amended soil enhanced biomass and uptake of P in *Zea mays* plants.

In addition to phosphate solubilization, isolate STJP also showed a significant amount of IAA and siderophore production. Phytohormone IAA stimulates the lateral root formation, cell elongation, and also helps in cell division and differentiation in plant cells (Goswami et al. 2016). Siderophore plays a significant role in chelation of micro-nutrients such as iron even under limiting conditions (Arora and Verma 2017). Earlier studies have reported *Bacillus* spp. to be good producers of IAA and siderophores (Walpolia and Yoon 2013). Researchers have also reported *B. safensis* as effective producer of IAA (Mukhtar et al. 2017) and siderophore (Chakraborty et al. 2013). Production of siderophore in the rhizosphere also helps in phosphate solubilisation and availability of phosphate ions to the plant through chelation of iron from precipitated forms (FePO<sub>4</sub>) (Sharma et al. 2013). *B. safensis* is a well-reported PGPR which has the ability to enhance the growth and productivity under biotic and abiotic conditions (Lateef et al. 2015). However, in the present work *Bacillus* sp. STJP capable of production of IAA, siderophore and phosphate solubilisation, significantly enhanced the growth of *M. arvensis*.

To the best of our knowledge, this is the first report on a PSB strain (*B. safensis* STJP) which has been employed successfully to significantly enhance the menthol content of *M. arvensis* simultaneously with plant growth and P content. Treatment with STJP + TCP<sup>200</sup> (in pot trials) resulted in a maximum increment of oil content and it was approximately 37% higher than treatment with TCP<sup>200</sup> alone. It has been informed earlier that the application of IAA increased the yield of menthol in *Mentha piperita* (Khan et al. 2015). Similarly, Hazzoumi et al. (2014) studied that essential oil content of Basil increased on the application of IAA. Isolate STJP is also a prolific IAA producer and this study

also suggests the role of this growth hormone in enhancing the oil content of the plants might be associated with better growth of roots and shoots. P plays an important role in the enhancement of total biomass, and yields of bioactive components in the medicinal plants (Sharma et al. 2013). The deficiency of P in soils/plants causes a reduction in the net photosynthesis rate (PN) resulting in a decrease of biomass and oil content in crops (Xu et al. 2007). P also induces the synthesis of secondary metabolites and highly modulates the oil synthesis in medicinal plants via a nutrient-plant growth enhancement mechanism (Malusà et al. 2006; Nell et al. 2009) and menthol is a type of metabolite which is produced by *M. arvensis*. Also, IAA production results in improved root hair formation. Simultaneously release of IAA and phosphate solubilization activities by STJP most probably resulted in enhanced P uptake by the plant. This may justify the maximum increase in root weight in case of plants treated with PGPR and TCP<sup>200</sup>. It has been reported earlier that PSB having the capability to produce other metabolites such as IAA and siderophore are far more efficient in enhancing the growth and yields of crop plants (Koo and Cho 2009). In fact, Gupta et al. (2012) also reported the role of IAA and siderophore produced by PSB strains along with TCP in enhancing the growth and Aloin-A content of *Aloe barbadensis*. Hence, using *B. safensis* as a biofertilizer in an integrated approach with TCP could be an effective tactic for improvement of soil health, growth enhancement and oil yield in *M. arvensis*. When applied alone most of the TCP gets precipitated in the soil and causes stresses such as salinity and eutrophication as runoff to the water bodies (Tewari and Arora 2018). By the way of integrated approach of utilizing an efficient PGPR with multiple growth traits including efficient phosphate solubilizing abilities we can reduce the precipitation of phosphates in the soil and simultaneously diminish the eutrophication of water bodies like ponds and lakes which are integrated for water supply in agro-ecosystems (Arora et al. 2016; Gouda et al. 2018). PSB possessing PGP characters such as siderophore and IAA production can thus not only enhance the growth and metabolite content of medicinal plants but also protect from unnecessary pollution due to the addition of phosphate fertilizers.

## Conclusion

The present study reports *B. safensis*, an efficient PGPR with phosphate solubilizing abilities, for growth promotion and increasing the oil content of *M. arvensis*. The experiments clearly show the efficiency of an integrated approach wherein fertilizer TCP and the PSB are co-inoculated to enhance the productivity of a very important medicinal and commercial plant. *Bacillus* sp. STJP is also an efficient producer of IAA and siderophore. The cocktail of PGP

characters helped in enhancing the yield and oil content of *M. arvensis*. This is the first report suggesting the use of phosphate solubilizing *Bacillus* in enhancing the menthol content of *M. arvensis*. Menthol is a key plant metabolite having wide applications in various industries. Enhancement of oil yields that too through eco-friendly biological approach can be a major breakthrough for menthol production and development of the country's economy.

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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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# Role of Beneficial Soil Microbes in Sustainable Agriculture and Environmental Management

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**Abstract** Rapidly increasing human population is expected to make food security a big issue in the future. Agriculture is facing severe challenges of land degradation, lesser productivity and susceptibility towards abiotic and biotic stresses. Sustainability in the agricultural sector is proving a formidable task because the current trend involves excessive use of chemical fertilisers and pesticides. Besides these, anthropogenic activities such as urbanisation and industrialisation are generating more waste, posing a severe threat to the ecosystems. Various approaches were tried to eradicate waste and restore ecosystems, but the success was limited. Beneficial soil microbes (BSMs) are identified as suitable candidates that may help in the sustainable management of the environment. These microorganisms possess several mechanisms that can be exploited at the commercial level in developing biotechnology for solving the key environmental issues. Beneficial microbe-based products currently used in agroecosystems have shown remarkable success. Their proper use in agroecosystems is changing the scenario of present-day agriculture. In the future, utilisation of such microbes in the clean-up of pollutants, waste eradication and combating climate change can provide substantial aid in on-going-greener campaign towards environmental sustainability. In this review, we have summarised the role of BSMs particularly the plant-growth-promoting bacteria and fungi in sustainable agriculture and also addressed their role in the management of environmental problems.

**Keywords** Beneficial soil microbes, Pollutants, Climate change, Agroecosystems, Sustainability, Agriculture, Plant Growth Promoting Rhizobacteria

## 1. Introduction

The human population is increasing very rapidly and creating excessive pressure on the existing land area for food,

fuel and raw materials. To supply the demand, agriculture practices are using intensive amounts of chemical fertilisers and pesticides which has resulted in land degradation and loss of biodiversity (Carsten and Mathis, 2014). In many developing countries, agriculture plays a vital role in the economic development and also provides self-employment (Gindling and Newhouse, 2012). According to an estimate, it was found that developing countries such as India, Pakistan, Bangladesh, Iran, Afghanistan, Nepal, Sri Lanka and Bhutan are losing crops, equivalent to at least, US\$10 billion annually due to land degradation (Lal *et al.*, 2010). Several factors have been reported by the researchers, which are responsible for degradation of soils. Among them, anthropogenic activities are directly involved in the land degradation process. The result of land degradation, may be exhaustion, salinisation and desertification of soil (Kesavan and Swaminathan, 2008). Many technological inventions are being carried out for the improvement of crop productivity, but still, we are not in a position to fulfil the global demand for food. Microbes associated with plants can be used to overcome the problems related to soil salinity, fertility, degradation and habitat loss. Soil contains diverse organisms like bacteria, archaea, fungi, algae, insects, annelids and other invertebrates which show an intimate relation to each other and with plants (Glick, 2010). Among them, microbial entities are unique as they are directly involved in increasing soil fertility, plant growth promotion and lowering biotic and abiotic stresses by various mechanisms. BSMs enhance the plant growth by nutrient uptake, form complex soil matrix and help in plant defence response by secretion of various metabolites. In addition, BSMs can show tolerance towards adverse environmental conditions such as salt stress, drought stress, weed infestation, nutrient deficiency and heavy metal contamination. Since past few years, researchers have found that soil microbes play beneficial and harmful roles in the soil ecosystem. However, BSMs gained much importance

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## Abstract

In the last few years, biopesticides have emerged as suitable alternatives to synthetic chemical pesticides. They are cheaper and pose no threat to agroecosystems. This is why their demand and production are also increasing at global level. The law and policies regulating their use and development vary from country to country, and in-depth analysis shows that there is no uniform regulatory model that can simplify their regulation and registration process. Although by the effort of some global agencies such as the International Organization for Biological Control (IOBC), European and Mediterranean Plant Protection Organization (EPPO) and Organization for Economic and Co-operative Development (OECD), some flexibility to biopesticide regulation have been provided, but in comparison to chemical pesticides, which have firm market and established nonoverlapping laws, biopesticides lag behind. This chapter provides comprehensive details of regulation systems adopted around the globe and to address shortcomings of existing system; besides this emphasis is also given to adopt innovative practices that could pave way for regulations which are simpler and more universal.

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# Study of biocontrol activity of fluorescent pseudomonads against *Alternaria alternata*, isolated from rhizosphere locale of *Stevia rebaudiana*

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## Abstract

Florescent pseudomonads are the efficient and environment-friendly microbes that are widely used for biocontrol and growth promotion in the agricultural crops. In a survey of potential antagonistic stains, a total of 11 fluorescent pseudomonads was isolated from the rhizosphere locale of *Stevia rebaudiana*. *S. rebaudiana* is a traditional, ayurvedic herb belonging to the family Asteraceae and famous as a natural high sweetener. Isolate NPSP was identified as a *Pseudomonas* sp. on the basis of morphological, biochemical and 16s rRNA gene sequencing. Isolate NPSP showed highest chitinase activity (72.66 nmol/min/mg) and siderophore production as compared to other isolates. In addition, NPSP has inhibited the growth of mycelial of *Alternaria alternata* as observed by dual culture plate method and their inhibition was recorded as 76.6 % after 3 days of incubation period. Thus, using of *Pseudomonas* sp. as a biocontrol agent could be an economical cheap and eco-friendly method for protection of disease against *A. alternata* in several agricultural crops including *S. rebaudiana* and for sustainable agriculture.

**Keywords:** *Alternaria alternata*, Biocontrol agent, Fluorescent Pseudomonads, *Stevia rebaudiana*

## Introduction

According to United Nation of Department of Economics and social affairs (2013), the world population is expected to be reached 9 billion by 2050. The demand for food is continuous rises with growing population all over the world. However, total annual food production is 1.3 trillion US dollars worldwide, out of which 31–42 % losses food crops and the economy US\$500 billion due to associated with biotic stresses such as weeds, insects, fungus, virus and bacterial diseases (Mishra et al. 2016). Among them, 6–20 % crop yields and US\$120 billion economy are lost every year due to fungal diseases during the crop production and after harvesting (<http://www.fao.org/3/y5800e/y5800e06.htm>). The drastic decrease in the crop yield and economy due to pathogens are often more severe in major developing countries like India (Mishra et al. 2016). Among all phytopathogens, *Alternaria alternata* is a common phytopathogen which is responsible for the reduction of crops productivity especially in *S. rebaudiana* (Prakash and



## ***Stevia rebaudiana*: A herbal medicine & alternative source of calorie free sweetener**

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### **Introduction**

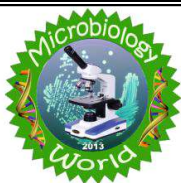
*Stevia rebaudiana* is a traditional, ayurvedic herb belonging to family *Asteraceae* and famous as a natural non-calorie high sweetener. Stevia is native to certain regions of South America such as Paraguay and Brazil (Sivaram and Mukundan., 2003). High medicinal value and sweetener property of stevia is mainly due to presence of alkaloids diterpene glycosides viz. stevioside, steviolbioside, rebaudiosides A-E, and dulcoside but which are not metabolized (Brandle et al., 1998). among these stevioside are most popularized.



**Stevia plants**

### **Propagation of stevia plant**

Stevia can be propagated by seed, tissue culture and vegetative cutting but mostly preferred tissue culture technology because its seedling or seed germinations are very slow while cutting is limited too and not good for high production of stevia crops but the tissue culture technique is only alternative for conserve and rapid growth of rare and endangered medicinal plant (Murashige and Skoog., 1962). The concentration of sweet alkaloids in the leaves of Stevia increases, when the plants are grown under long days. Since its



## **Biofertilizers: an alternative sources of chemical fertilizer for sustainable crops in 21st century**

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### **Abstract:**

Chemical fertilizers use continuously in the last years for the improvements of crops. However, the use of these fertilizers increases the crop yield. But they also affect soil microflora and health hazards of other living organisms. In recent years, an emerging economically attractive and ecofriendly technique developed known as biofertilizer.

### **Introduction:**

Soil has itself a balance of major and minor nutrients, as well as microbial population. It's vastly more complicated than, simply adding nitrogen, phosphorus and potassium (NPK) to the ground and calling it a day known as fertilizer (<http://www.sustainablebabysteps.com/effects-of-chemical-fertilizers.html>). Fertilizers are the substance that provide nutrients in the soil to promote plant growths and increase soil fertility (<http://en.wikipedia.org/wiki/Biofertilizer>). There are broadly two types of fertilizer; (1) synthetic fertilizer (2) biological fertilizer. (1) A synthetic fertilizer containing inorganic material of completely or partially artificial origin that is added to the soil to promote plant growth. It is an inorganic material that increase or decrease soil pH, however, reduce the soil beneficial microorganism and interfere with plant growth. For example: Urea is a popular fertilizer that provides ammonia for plants, but it also contributes to acid rain, groundwater contamination and ozone depletion due to release of nitrous oxide by denitrification process. Moreover, that may be produce many diseases like gastric cancer, goiter, birth malformations, and hypertension testicular cancer, stomach cancer, respiratory ailments, cardiac disease etc. (Gaur and Sharma, 2012). To forget this problem an emerging alternative [www.microbiologyworld.com](http://www.microbiologyworld.com) [www.facebook.com/MicrobiologyWorld](http://www.facebook.com/MicrobiologyWorld)