Novel Metabolites Identified From *Bacillus Safensis* and Their Antifungal Property Against *Alternaria Alternata*

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Research Article

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Abstract

Plant growth promoting rhizobacteria offer an effective and eco-sustainable solution to protect crops against phytopathogens. In the present study, *Bacillus safensis* STJP (NAIMCC-B-02323) from the rhizospheric soil of *Stevia rebaudiana* showed strong biocontrol activity against phytopathogen, *Alternaria alternata*. *B. safensis* STJP produced antifungal volatile organic compounds (AVOC). In the presence of AVOC, there was no conidia germination, mycelium growth was inhibited, and hyphae ruptured as observed by scanning electron microscopy (SEM). When mycelium of the fungus from bacterial treated plate was transferred into fresh potato dextrose agar plate, *A. alternata* could not grow. Extracted AVOC from *B. safensis* STJP were identified by thin-layer chromatography (TLC), fourier-transform-infrared spectroscopy (FT-IR) and gas-chromatography-mass spectrometry (GC-MS). In total 25 antifungal metabolites were identified by GC-MS analysis having alcohol, alkane, phenol, alkyl halide and aromatic compounds. Five compounds (phenol, 2,4-bis (1,1-dimethylethyl)-, 3-hexadecanol, pyrrolo(1,2-a)pyrazine-1,4-dione, 5,10-diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo(1,2-a:1',2'-d)pyrazine and hexadecanoic acid) completely inhibited the mycelium growth, controlling spore formation and conidia germination of *A. alternata*. This study concluded that AVOC producing *B. safensis* can be used as a green-fungicide against *A. alternata*. Bacterial metabolites could pave the way for the development of next generation biopesticide. This can be a reliable technology to enhance the quality and reliability of biopesticides.

1. Introduction

According to estimates, the world population is expected to reach 9 billion by 2050 (FAO 2012). Hence, the demand for food is rising continuously so as to feed the ever growing population. Currently, the total cost of annual food production is USD 1300 billion, out of which 500 billion is lost every year due to biotic stresses such as weeds, insects, fungus, virus and bacterial diseases (Watts et al. 2018). Among all, phytopathogens (including bacteria and fungi) account for yearly loss of USD 120 billion during crop production and after harvesting (Shuping et al. 2017). Decrease in the crop yields and impact on economy due to phytopathogens are often more severe in developing and poor countries, particularly where economy largely depends on agriculture (Danial et al. 2020).

Deadly fungal phytopathogen *Alternaria* spp. is commonly responsible for the reduction of crop productivity all around the globe (Chakraborty and Newton 2011; Müller, et al. 2019). It is one of the common fungal genus, reported around the globe with endophytic, saprophytic and pathogenic properties and also responsible for reduction in crop productivity (Müller, et al. 2019). *Alternaria* spp. are opportunistic pathogens, causing widespread damage to a number of crops and results in up to 80% reduction of yield in affected fields (Nowicki et al. 2012). *A. alternata* is known to cause leaf spot disease in several crops including *Stevia rebaudiana* (Meena et al. 2017). The leaf spot disease is identified by the presence of dark brown color or necrotic lesions on the leaf (Danial et al. 2020). The symptoms of this disease appear after infection and during pathogenesis, when fungal metabolites impact the plant during active phase of growth (Thomma 2003). The spores of *A. alternata* are dispersed ubiquitously 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 (Meena et al. 2017).

Crop rotation, cultural practices, or use of disease-resistant varieties are commonly employed for the management of soil borne pathogens (Roy et al. 2018), but these methods are not satisfactory for control of phytopathogens. Although, chemical pesticides are efficient way to control fungal pathogens and enhance crop productivity, but their application is being increasingly limited or banned due to negative impact on the soil fertility, microbial diversity, and ecosystem and causes toxicity to animals and humans (Alori and Babalola 2018; Prakash, and Arora, 2019). Beside these, green house emission and environmental pollution are also caused due to production and application of pesticides (Harman and Uphoff 2019). Hence, this situation calls for management strategies. Biopesticides are being used to control fungal diseases along with enhancing crop yields in an eco-friendly manner. Microorganisms with the abilities of biocontrol of phytopathogens are being used as biopesticides to realize the target of sustainable agriculture (Mishra et al. 2016). Low input biotechnology utilizing biocontrol agents for suppression of diseases and increased plant growth has now become a needful sector for

research so as to obtain quality products which are reliable and equal or even more effective than chemical pesticides (Shumaila and Khan 2016).

Gram-positive *Bacillus* spp. are one of the front runners for use as biopesticides because of their ability to produce a number of secondary metabolites with very different structure and nature and also broad spectrum antifungal potential (Kapetanakou and Skandamis 2016; Kai 2020). Rhizospheric *Bacillus* spp. are widely known to produce secondary metabolites such as bacitracin, polymyxins, lipopeptide surfactin, subtilosin, lantibiotic, iturin and gramicidin (Stoica et al. 2019; Kaspar et al. 2019). There is a continuous need to extract and identify biocontrol metabolites to develop biopesticides with effective potential against fungal pathogens such as *A. alternata*. The objective of the present study was to investigate the biocontrol potential of *B. safensis* strain against *A. alternata*. Extracellular antifungal metabolites produced by *B. safensis* were identified and checked for inhibition of *A. alternata* so as to check the mechanism of biocontrol agent (BCA) and determine the effective anti-fungal biochemicals.

2. Materials And Methods

2.1. Microorganisms

B. safensis STJP NAIMCC-B-02323 taken for the study has already been reported as an effective plant growth promoting rhizobacteria (PGPR) (Prakash and Arora 2019; Prakash and Arora 2020). *B. safensis* STJP showed plant growth promoting (PGP) characters viz. solubilization of phosphate, zinc, potassium, production of siderophore, and indole acetic acid (IAA) as reported in earlier studies by the authors (Prakash and Arora 2019; Prakash and Arora 2020). *A. alternata* isolate P1 (accession number: KX494864) was procured from Laboratory for Rhizospheric Microbiology and Sustainable Agriculture, School for Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow and grown (at 28°C) and maintained on potato dextrose agar (PDA) and Czapek dox agar (CDA) at 4 °C for further use and experiments. The fungal isolate has also been already reported as a phytopathogen in an earlier study (Prakash and Arora 2019).

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, fungal mycelia and hyphae were cut with the help of cork borer (from an already cultured PDA plate and kept in the centre of prepared PDA plate and STJP was spotted and streaked (on separate plates) around the fungal pathogen (Schoeman et al. 1994). Subsequently, Petri plate were incubated for 120 h at 28°C and calculated for fungal growth inhibition rate as per Schoeman et al. (1994).

2.3. Dual culture method

Dual culture test was performed on PDA (Himedia, Mumbai) for screening of biocontrol potential as per Arora et al. (2001). Briefly, the fungal mycelium was cut (5 mm) by cork borer (from a PDA plate grown with *A. alternata* after 5 days incubation period) and placed on the center of PDA plate. Afterwards, the fresh log phase culture of *B. safensis* STJP (on the basis of rapid screening) was streaked on two edges of Petri plate (Arora et al. 2001). Further, Petri plates were sealed with parafilm and then incubated at 28°C for 120 h. After incubation period, the inhibition of fungal growth was observed and inhibition zone was calculated by using the formula: $100 \times (C-B)/C$; where; C is the diameter of fungal colony in control plate and B diameter of fungus in the dual culture plate.

2.4. Screening of bacteria for antifungal volatile compound production by seal plate method

Bacterial strain *B. safensis* STJP was checked for the production of antifungal volatile compounds as per Fernando et al. (2005). Actively growing mycelia (disc) of *A. alternata* (5 mm) were kept at the center of Petri plate containing PDA. The overnight grown bacterial culture was spread on another Petri plate containing nutrient agar (NA). Petri dish containing

fungal mycelia was inverted over the NA and both plates were immediately wrapped with parafilm. The plates were further incubated for 120 h at 28°C. Every 24 h, growth inhibition of *A. alternata* by bacterial isolate was observed in comparison to control plate (contained only mycelia) as per Fernando et al. (Kersten and Kirk 1987). After 120 h, fungal mycelia were detached from the plate and further confirmed for viability in a fresh Petri plate containing PDA. The experiments were repeated three times with five replicates taken each time.

2.5. Growth inhibition of *A. alternata* by extracellular or intracellular metabolites

The overnight grown bacterial culture was inoculated in nutrient broth and incubated (28°C, 48 h). After incubation, broth was centrifuged at 5,000 rpm for 15 min to get the supernatant and pellet. Subsequently, two wells were made on the prepared PDA plate with the help of sterilized cork borer and the obtained supernatant and pellet were transferred in the well of Petri plate (separately), with *A. alternata* at centre point to check whether the biocontrol activity is by extracellular or intracellular metabolites (Kersten and Kirk 1987). Besides, control plate of *A. alternata* was also incubated simultaneously. All the plates were incubated for 120 h at 28°C.

2.6. Growth inhibition of *A. alternata* checked by scanning electron microscopy

For sample preparation of scanning electron microscopy (SEM), the fungal mycelia were picked up from dual culture and control plates and fixed with modified Karnovsky's fixative (2% glutaraldehyde and 2% paraformaldehyde in sodium cacodylate buffer (0.05 M; pH 7.2) and incubated for 12 h at 4°C (Kaminskyj and Dahms 2008). 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 an ethanol solution of concentration of 30%, 50%, 70%, 80%, and 90% (5 min), and lastly, absolute (100%) ethanol was used (three times) for dehydration of cell for 2 min (Hennequin et al. 2006). Afterwards, the specimen was further dried by using hexamethyldisilazane and with the help of a sputter coater, and specimen was finally coated with gold (MSC-101, JOEL). The prepared sample of the specimen was observed under SEM (Model: JSM-7610F, JEOL, Japan).

2.7. Extraction and purification of metabolites

For extraction of antifungal metabolites, 24 h culture of bacterium STJP (selected on the basis of PGP traits) was inoculated in NB and inoculated at 120rpm, 28°C, 7 days (Model: Labtech LS1-3016R). After the incubation period, the broth was centrifuged (rpm: 10,000rpm, 15 min, 4°C) (Centrifuge Model: Sigma 3-18KS) to obtain supernatant. The pH of the obtained supernatant was adjusted at 7.0 and subsequently it was dissolved in the solvent; chloroform + ethyl acetate + dichloromethane (DCM) in a ratio of 1:2:1 as per Hennequin et al. (Hennequin et al. 2006). Further, 50 ml of solvent-supernatant mixture was filled in a separating funnel and mixed vigorously for 15 min. The funnel was then hanged on a stand and this process was done for 24 h at 28°C. The transparent layer of the reaction mixture 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, HiMedia, Mumbai) as per Hennequin et al. (2006). The obtained sample containing antifungal metabolites was kept in a sterilized beaker, and finally, solvent was evaporated by using a rotatory vacuum evaporator (Model: UTS: 1.53, Thermo-scientific).

2.8. Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was done for the separation of different compounds present in the crude metabolite sample as per Reddy et al. (Reddy et al. 2009). Briefly, TLC was done on freshly prepared silica plate (20 cm 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 as per Reddy et al. (2009). The running lane was then dried thoroughly and the fraction of the compound was analyzed under UV light

(254nm). Different bands of metabolites were scraped separately into microcentrifuge tubes and further centrifuged with ethyl acetate as per Gocan (2002). After centrifugation, the solvent was evaporated through a rotatory vacuum evaporator (Model: UTS: 1.53, Thermo-scientific) and finally supernatant was taken for further experiment.

2.9. Fourier Transform Infrared Spectroscopy (FT-IR)

To analyze the functional groups of purified metabolites of bacterium STJP (obtained after TLC), the sample was dried in a hot air oven at 40°C and subsequently analyzed as per Reddy et al. (2009). Briefly, the dried sample of metabolites was mixed with analytical grade potassium bromide (KBr) (IR Grade; purity $\geq 99\%$) (1:30 ratio) and the reaction mixture was thinly ground and further fused into a thin pellet (13mm X 1mm) by PCI hydraulic pressure with a 15 tons capacity under vacuum pressure. Afterwards, spectra absorbance was observed by FT-IR (Model: Nicolet™ 6700, Thermo Fisher Scientific, USA). Further, the result was analyzed in the mid-infrared region from 4000 to 450 cm^{-1} with a resolution of 4 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 OMNIC™ (v7.4) software.

2.10. Gas Chromatography-Mass Spectrophotometry (GC-MS)

GC-MS was performed using a flame ionization detector (Model: Varian Star 3400 CX, OH, USA). Briefly, 15 m DB-1 megabore column of 100% dimethylpolysiloxane was used for the separation of the crude metabolite sample (Gocan 2002). The sample was injected into a fused silica column [(25m x 0.22mm x 0.25 μm (length x width x thickness))] at 22°C which was focused cryogenically with chilled acetone (time: 2 min). This column was previously joined with a Hewlett-Packard 5890 gas chromatograph, linked with 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 from 35-200°C at 4°C /min ramp rate. The result obtained in the form of unknown compounds were compared with mass spectra (MS) using NIST/EPA/NIH mass spectroscopy library (Version 17).

2.11 Examination of antifungal activity of volatile organic compounds

The metabolites produced by *B. safensis* STJP and detected through GC-MS analysis were purchased (mentioned in Table 3) from Sigma Aldrich, USA (synthetic chemicals) to antifungal activities against phytopathogen (*A. alternata*) and individually evaluated using seal plate method (Fernando et al. 2005). Briefly, a pure individual compound (purchased) with volume of 100 μl was prepared and spread onto sealed Petri plate in one compartment and a 5 mm mycelium disk of *A. alternata* was placed in another compartment. Afterwards, plates were incubated for 5 days at 28°C for observation of result. For control, dimethyl sulfoxide (DMSO) was used as per Fernando et al. (2005).

Table 1
FT-IR spectral data of bacterial metabolites with their corresponding peak assignments

Frequencies observed (cm-1)	Tentative peak assignments	Tentative functional groups
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

Table 2
Bacterial metabolites identified by GC-MS from extracted sample

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 tetradecanoate	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	Heptadecane	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		

Table 3

Inhibition of mycelial growth and conidia germination by volatile organic compounds identified through GC–MS analysis and procured in pure form

Metabolites	Procured compounds (Sigma Aldrich)	Inhibition of mycelia and conidial germination by metabolite from <i>B. safensis</i>
Nonadecane	Yes	No
Phenol, 2,4-bis (1,1-dimethylethyl)-	Yes	Yes (12.8 mm)
Docosane	Yes	No
Octadecane	Yes	No
3-hexadecanol	Yes	Yes (10.8 mm)
1-proline, n-valeryl, heptadecyl ester	Yes	No
Isopropyl tetradecanoate	Yes	No
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Yes	Yes (9.6 mm)
Heptadecane	Yes	No
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	Yes	No
Eicosane	Yes	No
5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine	Yes	Yes (8.5 mm)
3-ethyl-3-undecanol #	Yes	No
1,2-benzenedicarboxylic acid, ditridecyl ester	Yes	No
Hexadecanoic acid	Yes	Yes (8.9 mm)
Cyclohexanol, 1-butyl-	Yes	No
Dotriacontane	Yes	No
Tetratetracontane	Yes	No
Hexacosyl acetate	Yes	No
Hexatriacontane	Yes	No
1,3,5-trisilacyclohexane	Yes	No
D-Ribose, 2-deoxy-bis(thioheptyl)-dithioacetal	Yes	No
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Yes	No
4,4'-((p-Phenylene)diisopropylidene)diphenol	Yes	No
2-tert-Butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol	Yes	No
Figure mentioned in brackets are the zone of inhibition due to the pure metabolite by well plate method.		

Each treatment had five replications and all the experiments were performed in triplicates. Data were analyzed using means and using standard deviation as per Gomez and Gomez (1984).

3. Results And Discussion

3.1 Biocontrol activity

B. safensis STJP showed biocontrol activity against *A. alternata* *in-vitro* conditions as confirmed by rapid screening and dual culture methods and the zone of inhibition was 14.8 ± 1.34 mm (Fig. 1) and percentage inhibition of $86.24 \pm 2.12\%$. *Bacillus* spp. are well known to be safe microorganisms for application in agriculture, holding incredible abilities for synthesizing a wide range of plant beneficial metabolites (Lateef et al. 2015; Prakash and Arora 2019). This genera has potential abilities for improving plant growth and productivity either by direct action such as phytohormones production, solubilization of phosphate, zinc, potassium, and production of siderophore or by controlling phytopathogens (by production of antibiotics, HCN, siderophores and lytic enzymes such as chitinases, glucanases) even under stress conditions (Kapetanakou and Skandamis 2016; Prakash and Arora 2019). The principal mechanisms of phytopathogen control include production of volatile or non-volatile extracellular or intracellular metabolites (Roy et al. 2018). However, there are very few studies available on *B. safensis* as a biocontrol agent against phytopathogen(s). These reports are mainly available on growth inhibition of *B. safensis* against phytopathogens including *A. alternata* by dual culture method, mechanisms till now not be investigated. Some researchers have reported that *B. safensis* produces metabolites such as growth regulators, hormones, lipopeptides, bacilomycins, fengycins, surfactins and other bioactive compounds and their roles have been considered mainly in plant growth and productivity (Lateef et al. 2015; Van Lenteren et al. 2018). Apart from agriculture sector, secondary metabolites from *Bacillus* spp. are being used in several other industries (pharmaceutical, food, and dairy) (Srihong et al. 2018). However, useful metabolites from this very diverse genus and in particular from non-pathogenic species such as *B. safensis* can turn out to be very important for managing phytopathogens and organic agriculture production. These metabolites can also be used for development of novel bioformulations which are far more efficient and reliable (Arora and Mishra 2016).

B. safensis STJP was found to be inhibitor of *A. alternata*, causal agent of several diseases in diverse crops (Shafi et al. 2017). The bacterium produced volatile organic compounds and inhibited mycelial growth, and conidia formation of *A. alternata*. Mycelia and conidia of the fungus (taken from seal plate experiment inverted over NA with bacterial growth) failed to germinate, when plated on fresh PDA plate. Overall, STJP was found to be an effective bacterium for volatile compounds production. Abdelmoteleb et al. (2017) observed that *Bacillus* sp. considerably reduced the growth of *A. alternata* by production of volatile compounds. However, biocontrol potential by *B. safensis* STJP against *A. alternata* (~86%) was found to be better than the earlier reported isolates of *Bacillus* spp. against phytopathogens including *A. alternata* (60%), *Macrophomina phaseolina* (50%), and *Fusarium oxysporum* f.sp. *cubense* (Foc) (79%) (Pane et al. 2015; Torres et al. 2016; Abdelmoteleb et al. 2017; Bubici et al. 2019)

3.3. Extracellular or intracellular metabolites production

B. safensis STJP inhibited mycelia growth of *A. alternata* as confirmed by inhibition zone when supernatant was poured, whereas no inhibition zone was observed around the well in which pellet was added. This confirmed that the isolate STJP produced extracellular metabolites which were involved in inhibition of phytopathogen. Further, these extracellular metabolites were found to be volatile in nature as confirmed by seal plate method (Khare and Arora 2011; Islam et al. 2018). Volatile compounds can be far more effective in soil because these can transcend through the layers and soil aggregates and inhibit the phytopathogens hiding in such habitats (Gao et al. 2018). The extracellular metabolites that are secreted by bacterial cells also known as exometabolome are very effective AVOC against fungal phytopathogens including *A. alternata* as compare to intracellular metabolites as reported previously as well (Pinu et al. 2017; Celik et al. 2020).

3.4. Scanning electron microscope (SEM) analysis

For SEM analysis, fungal mycelium was taken from dual culture plate in which *B. safensis* STJP inhibited the growth of *A. alternata*. Fungal mycelia of *A. alternata* were also taken from control plate. From the control plate, straight large catenate conidia chains with transverse septa of *A. alternata* were observed (Fig. 3a), whereas from bacterial treated (dual) plate, the destruction, and ruptured hyphae of fungus could be seen (Fig. 3b). Through SEM images it was confirmed that STJP showed strong antagonistic activity against *A. alternata*. On the basis of SEM analysis, it was proved that volatile nature of antifungal compounds was able to prevent conidia germination of *A. alternata*. Volatile compounds produced by *B. safensis* STJP as soil amendments, foliar, or root applications can be used for the control of *A. alternata*. SEM observations suggested that the mycelia of *A. alternata* was deformed, ruptured and disintegrated. In the presence of AVOC, reduced apical growth, cell disruption and irregular distortions in the phytopathogen *Aspergillus flavus* with reduction in the thickness of mycelium and conidia were observed by Horak et al. (2019) and Muhialdin et al. (2020).

3.5. Thin layer chromatography (TLC)

Crude antifungal metabolites obtained after extraction and evaporation of the solvent reached maximum at 7.5 cm with a retention time of 45 min. On the TLC plate, three major bands were detected under ultraviolet (UV) light (254 nm) in order of decreasing R_f values (0.86, 0.72, 0.14) (figure not shown). TLC is a simple, rapid, and economical method of separation, and tentative identification of metabolites. The R_f values (0.86, 0.72, 0.14) indicate the presence of at least three types of compounds in the extract. Interpretation of results showed aliphatic, aromatic, and phenolic compounds (Rajivgandhi et al. 2019; Celik et al. 2020). However, on the basis of R_f value compounds or their functional groups cannot be identified. Hence further identification of bacterial metabolites was done by FT-IR and GC-MS.

3.6. Fourier transform-infrared spectroscopy (FT-IR)

FT-IR spectrum was obtained of the metabolites (dried extract of the supernatant) from *B. safensis* STJP (Fig. 4). The absorption peaks of the FT-IR spectrum were examined as per Griffith (1996). The absorption peak at 3610.0–3355.4 cm⁻¹ indicated the –OH group (–OH stretching) with strong intensity corresponded to the existence of alcohol, phenol, and carboxylic acid present in the metabolite sample (Fig. 4). Absorbance peak at 3406.5 cm⁻¹ is attributed to –O–H stretching corresponded to the presence of carboxylic and phenolic compounds. The absorbance peak 1620.8–1434 cm⁻¹ indicated the C–C stretching resembling the presence of aromatic compounds such as benzene, phenol, and toluene, and absorbance peak 1115.6 cm⁻¹ indicated the alkyl halide and peak 800.7–662.2 cm⁻¹ showed the presence of aliphatic compounds such as alkane or alkene. The absorbance peak at 601.1 cm⁻¹ attributed to alkyl halide and another peak 465.2 cm⁻¹ showed the alkane group. Overall, the absorbance spectrum of bacterial metabolite from *B. safensis* STJP showed the presence of alcohol, alkane, phenol, alkyl halide, and aromatic ring. Puškárová et al. (2017) reported that aliphatic and aromatic compounds produced by *Bacillus* spp. were more effective in growth inhibition of *A. alternata*. However, Wang et al. (2018) suggested that alcohol and phenolic compounds were also found to be efficient in early and post-harvest leaf spot disease control, by *A. alternata*.

3.7. GC-MS analysis and examination of antifungal activity of volatile organic compounds

The GC-MS analysis of chloroform + ethyl acetate + DCM extract revealed the presence of various organic compounds, fatty acid, alcohol, phenol, and other aliphatic compounds at different RT as identified by using NIST mass spectral library (Fig. 5). The major peaks were detected at 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, 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, docosane, hexadecanoic acid, cyclohexanol, 1-butyl-, l-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 (Table 2), respectively, based on matching with NIST database. However, all compounds detected at major RT peaks (Table 3) did not play important roles in the antagonistic activity against *A. alternata* (Nicolaou et al. 2011; Siddharth and Vittal 2018). The volatile compounds found to be produced by

strain *B. safensis* STJP in the present study, viz., docosane, hexadecanoic acid, cyclohexanol, 1-butyl-, l-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) have been identified to be produced by *Bacillus* spp. and *Pseudomonas* spp. and were reported to show antimicrobial, antioxidant, and medicinal properties (Nicolaou et al. 2011; Oda et al. 2017; Jishma et al. 2017; Siddharth and Vittal 2018).

Different minor peaks 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 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, and 1,3,5-trisilacyclohexane (Table 2), respectively. However, in this study, we did not observe inhibition of fungal pathogen from the above compounds except 3-hexadecanol. The compounds nonadecane and octadecane were observed in bacterial metabolite sample of GC-MS, but their function as antifungal compounds or in biological systems is not reported. Hence, it might be possible that these compounds have come from preparation or running samples.

Out of a total of 25 compounds which were identified by GC-MS (produced by *B. safensis* STJP), 20 synthetic chemical counterparts (based on availability) were purchased from Sigma Aldrich, USA. Among them only 5 compounds (phenol, 2,4-bis (1,1-dimethylethyl)-, 3-hexadecanol, 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, and hexadecanoic acid) in pure form (Sigma Aldrich) inhibited the growth of mycelium and conidia germination completely by well plate method at tested volume of 100 µl (Table 3). Mycelia or conidia of fungus from the antifungal test, when re-plated on the fresh Petri plates, failed to grow indicating fungicidal property of volatile compounds.

Phenol, 2, 4-bis(1,1-dimethylethyl), a member of the class of phenol carrying two tert-butyl substituents at positions 2 and 4 and already reported as a bacterial metabolite, is known to play an important role in biocontrol activity of several phytopathogens (Dharni et al. 2014; Rice et al. 2019). Phenol 2, 4-bis(1,1-dimethylethyl), produced by *B. safensis* STJP in the present study, inhibited the growth of *A. alternata* and is important component that delayed spore formation and conidia germination at 100 µl. Another bacterial metabolite, 3-hexadecanol was detected and their roles are recorded in several industries such as pharmaceuticals, cosmetics, perfumery, detergents and emulsifier (Lou et al. 2013). It is also a type of pesticide but not yet approved by any government agencies and could be involved in the enhancement of plant's immune system as well (Lou et al. 2013). Pyrazine is a heterocyclic compound and its derivatives such as pyrrolo[1,2-a]pyrazine-1,4-dione, and 5,10-diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine were identified as strong antifungal metabolites against phytopathogens such as *Botrytis cinerea*, *Puccinia recondite*, and *Rhizoctonia solani* and useful for prevention against fungal diseases in plants (Oda et al. 2017). The chemical nature of the organic compounds appears to determine their antifungal property. Pyrrole (5,10-diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine) and ketone (pyrrolo[1,2-a]pyrazine-1,4-dione) were more effective than alcohols in the inhibition of growth of *A. alternata* (Lou et al. 2013). Bacterial volatile compounds also promote the growth of plants. Pyrrolo[1,2-a]pyrazine-1,4-dione is reported to enhance the growth of *Vigna radiata* seedlings and also recorded a potent inhibitory effect on bacterial pathogen (*Streptococcus aureus*) and fungal pathogen (Jishma et al. 2017). However, fungal response to rhizobacterial volatiles seems to be environment, species and age-specific. Further, hexadecanoic acid detected in crude metabolite extract, showed antifungal activity against several fungal pathogens including *Alternaria* (Lou et al. 2013). Apart from these metabolites (phenol, 2,4-bis (1,1-dimethylethyl)-, 3-hexadecanol, pyrrolo(1,2-a)pyrazine-1,4-dione, and hexadecanoic acid), another metabolite 5,10-diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine, whose role has not been reported earlier in inhibition of recorded in the inhibition of *A. alternata* before. Overall results of the present study suggested that five AVOC produced by *B. safensis* STJP showed strong biocontrol activity against *A. alternata* and this is the first report on the growth inhibition of *A. alternata* by *B. safensis*. It is also for the first time that AVOC (phenol, 2,4-bis (1,1-dimethylethyl)-, 3-hexadecanol, pyrrolo(1,2-a)pyrazine-1,4-dione, and hexadecanoic acid, 5,10-diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine) were detected to be produced by *B. safensis*. However, other compounds produced by *B. safensis* STJP

might be playing roles in other activities such as plant microbe interactions or development of plant immune response and required to be investigated further.

4. Conclusion

It can be concluded that *B. safensis* can be an eco-friendly tool for promoting plant growth by using as a biocontrol agent against fungal phytopathogens. *B. safensis* produced a bouquet of antifungal volatile compounds against *A. alternata*. The antifungal compounds were mainly volatile in nature. The exploitation of *B. safensis* as a biopesticide would be a good option in for production of healthy food, reducing health problems related with chemical pesticides, and sustainable crop and agro-industry. The variety of extracellular metabolites produced by *B. safensis* may be involved in several other roles related to plant-microbe interactions which need to be explored further.

Declarations

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Figures

Figure 1

Fig. 2a

Fig. 2b

Figure 2

a) SEM image of *A. alternata* showing healthy mycelia and conidia (control)

Figure 3

FT-IR spectra of metabolites sample extracted from bacterial supernatant [peaks showing different frequencies (cm⁻¹)]

Figure 4

Chromatogram of GC showing peaks of different metabolites derived from extracted bacterial supernatant. Each labeled refers to AVOC: 1) phenol, 2,4-bis (1,1-dimethylethyl)- (RT: 21.439), 2) 3-hexadecanol (RT: 27.085), 3) pyrrolo(1,2-a)pyrazine-1,4-dione (RT: 27.783), 4) 5,10-diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo(1,2-a:1',2'-d)pyrazine (RT: 29.374), and 5) hexadecanoic acid (RT: 29.765)