INTRODUCTION

Phytopathogenic fungi such as Alternaria alternata, Rhizoctonia solani, Aphanomyces chohlioides, Sclerotium rolfsii, and Ceratocystis paradoxa are the major causes for the soilborne diseases of sugarcane, sugar beet, coconut, pineapple, and other crops. C. paradoxa is a causative agent of pineapple disease or black rot of sugarcane. The C. paradoxa enters through cut ends and proliferates rapidly in parenchymal tissues of internodes developing brown-black cavities. It thus damages cane setts and alters the quality of pineapple juice by decreasing the level of sucrose and glucose [1-3].

Most of the Burkholderia species produce bioactive compounds for the suppression of plant diseases, especially antibiotics, siderophores, organic acids, biocidal volatile organic components, phenolic and phthalate derivatives, aromatic complexes, and enzymes including chitinase, cellulase, and protease. Bioactive components were well documented after agar well diffusion, high-performance liquid chromatography (HPLC), and gas chromatography-mass spectroscopy (GC-MS) studies by a number of investigators in extracts obtained from different plants as well as bacterial and fungal cultures [16,20-27]. In the present investigation, antifungal characterization of B. gladioli strain VIMP03 (JQ867372), an isolate from sugar beet rhizosphere, was carried out by dual culture, agar well diffusion, HPLC, and GC-MS studies by a number of investigators in extracts obtained from different plants as well as bacterial and fungal cultures [16,20-27]. In the present investigation, antifungal characterization of B. gladioli strain VIMP03 (JQ867372), an isolate from sugar beet rhizosphere, was carried out by dual culture, agar well diffusion, HPLC, and GC-MS methods.
Culture media
Pikovskaya's broth and agar [29] media were used to cultivate *B. gladioli* strain VIMP03 (JQ867372) at 30°C for 4 days. The Pikovskaya's agar contained (g/L) dextrose 10; NaCl 0.2; Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>; Na<sub>2</sub>SO<sub>4</sub> 0.5; KH<sub>2</sub>O 0.2; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1; MnSO·H<sub>2</sub>O 0.0001; yeast extract 0.5; and Agar 18. The pH of medium was 7.0 (±0.2). The Pikovskaya's broth contained the same components without agar.

Potato dextrose agar (PDA) was used to cultivate phytopathogenic culture of *C. paradoxa* and also to check in vitro antagonistic activity at room temperature for 4-7 days. The PDA contained (g/L) potato infusion 200; dextrose 20; agar 20, and the pH of medium was 5.6 (±0.2).

The pH of both culture media was adjusted using 1N NaOH or 1N HCl.

Media were sterilized by autoclaving at 120°C for 15 minutes.

Cellulase activity
On cellulose agar plate containing cellulose as sole carbon source and pH 7.5 (±0.2), the bacterial culture *B. gladioli* strain VIMP03 (JQ867372) was spot inoculated and incubated at 30°C (±0.2°C) for 48 hrs. After incubation plate was repeatedly treated with 0.5% Congo red for 15 min at room temperature and was counterstained with intermittent thorough washing by 1M NaCl solution. The nonappearance of clear zone around the colony was considered cellulase negative activity [30].

Protease activity
For protease test, culture under study was spot inoculated on milk agar having pH of 7 (±0.2) and incubated at 30°C (±0.2°C) for 48 hrs. The development of clear zone around the colony against opaque background was considered protease positive [31].

Chitinase activity
The culture of *B. gladioli* strain VIMP03 (JQ867372) was spot inoculated on colloidal chitin agar and incubated at 30°C (±0.2°C) for 5 days, and chitinase activity was examined as positive if there was zone of clearance around the colony [32].

HPLC analysis for organic acids
Organic acids were detected by HPLC in Pikovskaya's broth, in which *B. gladioli* strain VIMP03 (JQ867372) was cultivated at 30°C (±0.2°C) for 4 days. The culture broth was filtered through 0.2 µm filter (Millipore), and 20 µl of filtrate was injected to HPLC (Model - Waters Alliance Company) equipped with an ultraviolet detector. Organic acid (ID) 4.6 mm and 25 mM KH<sub>2</sub>PO<sub>4</sub> as mobile phase. At a wavelength of 210 nm, retention time (RT) of each signal was recorded. Organic acid content in mg per 100 ml (or mg %). In the culture filtrate profile of the bacterial culture is presented in Table 1 along with RT and HPLC (peaks) of organic acids is shown in Fig. 1. The organic acid chromatographic peaks with those of reference standards.

Dual culture method
Primary antifungal activity of *B. gladioli* strain VIMP03 (JQ867372) was checked by the dual culture in vitro assay method [33,34]. The culture was spot inoculated at one end of the PDA. After 2 days incubation at room temperature, 6 mm agar disc, using growth of fungal pathogen *C. paradoxa* from fresh PDA agar culture, was placed at the other marginal side of the plate and incubated at room temperature for 7 days. The radii of the fungal colony toward and away from the bacterial colony were noted to calculate percent growth inhibition by the following formula:

\[ \text{Percent inhibition}=\frac{(A-a)}{A}\times100 \]

Where, "a" is the radius of the fungal colony opposite to the bacterial colony and "X" is the maximum radius of the fungal colony away from the bacterial colony.

Antifungal activity of culture filtrate and ethyl acetate extract
The culture *B. gladioli* strain VIMP03 (JQ867372) grown in Pikovskaya's broth for 4 days at 30°C (±0.2°C) was centrifuged at 3000 rpm for 10 minutes, and the supernatant was sterilized by passing it through a millipore membrane filter (0.45 µm of pore size). The sporulated culture of *C. paradoxa* was inoculated into sterile molten PDA medium (45°C) and poured into sterile Petri dishes. Antagonistic activity of culture filtrate was detected by agar well diffusion technique.

A bioassay-directed practice was followed in the process of isolating and fractionating ethyl acetate extract. Antifungal principles from the cell-free filtrate were extracted by solvent ethyl acetate. Ethyl acetate extract was evaporated at room temperature and concentrated. About 500 ml of ethyl acetate extract was reduced to 15 ml. Antifungal activity of concentrated ethyl acetate extract was detected qualitatively by agar well technique using 100 µl of the extract.

GC-MS analysis of ethyl acetate extract
The GC-MS analysis was done with thermo GC coupled with ITQ 1100 mass detector and X-Caliber software and the National Institute of Standards and Technology (NIST) Spectral data (GCMSMS, Thermo Fisher Scientific). A DB-5 MS capillary column having 30 mm × 0.25 mm ID and coated with 0.25 µm film thickness was injected with 2 µl sample. The carrier gas helium (99.99%) was used at a flow rate of 1 ml/minute in split mode (1:50). The temperature of the column was programed at 60-280°C. The injection port and transfer line temperatures were used 250°C and 280°C, respectively. The temperature program initiated at 60°C for 2 minutes hold, then it was raised at 15°C/min to 160°C, which was held for 0 min, and then at 3°C/min to 200°C, which was held for 1 minute, and again at 6°C/min to 280°C, which was held for 6 minutes. The mass spectrum of compounds present in the sample was recorded with electron impact ionization energy 70 eV over mass range of 50-650 Da amu. The chemical components from ethyl acetate extract of culture filtrate were identified by comparing RT of chromatographic peaks with those of reference standards from database of the NIST library.

RESULTS

The culture under studies, *B. gladioli* strain VIMP03 (JQ867372), was protease and chitinase positive as clear zones were developed around the colonies (growth) on milk agar and chitin agar, respectively; while the culture was avowed cellulose negative due to non-development of clear zone around its colony.

HPLC (peaks) of organic acids is shown in Fig. 1. The organic acid profile of the bacterial culture is presented in Table 1 along with RT and organic acid content in mg per 100 ml (or mg %). In the culture filtrate obtained from Pikovskaya’s broth, four organic acids were detected. The highest amount of organic acid produced by the *B. gladioli* strain VIMP03 (JQ867372) was acetic acid (17.22 mg %), which was followed by formic acid (1.15 mg %), oxalic acid (0.27 mg %), and pyruvic acid (0.11 mg %).

| Table 1: Organic acid profile based on HPLC |
|---------------------------------------------|
| **S. No.** | **Organic acids** | **RT (minutes)** | **Content (mg %)** | **Percent area** |
| 1          | Oxalic acid      | 1.855           | 0.27              | 22.39           |
| 2          | Formic acid      | 2.433           | 1.15              | 6.06            |
| 3          | Pyruvic acid     | 2.609           | 0.11              | 7.94            |
| 4          | Lactic acid      | –               | –                 | –               |
| 5          | Citric acid      | –               | –                 | –               |
| 6          | Gibberellic acid | –               | –                 | –               |
| 7          | Acetic acid      | 4.178           | 17.22             | 63.60           |

*– Not detected*
The percent growth inhibition estimated by dual culture method, exhibited by the *B. gladioli* strain VIMP03 (JQ867372), against *C. paradoxa* was 41%. This finding is in accordance with that of Kadir et al. [35] who reported 41-81.7% fungal growth inhibition by *B. cepacia*. Both ethyl acetate and culture filtrate extracts under the present investigation inhibited the fungal pathogen growth with zone of inhibitions (ZOIs) of 19 mm (standard deviation [SD] ±2) and 24 mm (SD ±2), respectively.

The GC-MS technique was used to identify the probable antifungal compounds of *B. gladioli* strain VIMP03 (JQ867372) extract that possessed antifungal activity, and 9 compounds were identified. GC of ethyl acetate extract is shown in Fig. 2.

The number of peaks, the RT, and area and matching factor of the compounds present were compared with those of in the NIST database. The compounds identified are shown in Table 2 along with RT, molecular formula (MF), molecular weight (MW), and their chemical structures. The first compound identified with less RT (12.20 minutes) was E-2-octadecadecen-1-ol, whereas the last compound which took longest RT (33.33 minutes) to identify was tetracontane.

**DISCUSSION**

Different solvents have various degrees of solubility for different antimicrobial compounds and also organic solvents have more powerful antimicrobial activity as compared to aqueous extracts [22].

The present study outcomes were not in harmony with the findings of Bhuvaneswari and Gobalakrishnan [22] as more ZOI was recorded by the aqueous culture filtrate extract. Antibiosis is generally mediated by specific or non-specific metabolites of microbial origin, by lytic agents, enzymes, volatile compounds, or other toxic substances [36]. Lavermicocca et al. [37] purified and characterized novel antifungal compounds from *Lactobacillus plantarum* and also reported that lactic and acetic acid produced by bacterium played the most significant role in antifungal actions. The VIMP strain produced a variety of organic acids as shown in Table 1 and lytic enzymes such as chitinase and protease. These might be the reasons that the culture exhibited antifungal activities as supported by percent inhibition by dual culture method and ZOIs as shown by both ethyl acetate and culture filtrate extracts. The ZOI exhibited by culture filtrate extract was 26.31% more than the ZOI revealed by the ethyl acetate extract. This might be due to the presence of more amounts of lytic enzymes, volatile compounds, and other metabolites in concentrated and active states in culture filtrate rather than in the ethyl acetate extract. Mahamuni [38,39] also reported large ZOI for aqueous culture filtrate extracts against fungal phytopathogens such as *A. alternata* and *C. paradoxa*. Degree of solubility for all antimicrobial components in ethyl acetate extract might not be so much extensive.

Many previous reports stated antifungal activities of ethyl acetate extracts obtained from the supernatants of different microbial cultures as well as of different plant extracts and listed the identified possible antifungal compounds based on GC-MS analyses. Many researchers reported the antimicrobial role of phenolic, alkaloid, carboxylic acid, hydrocarbon, ketone, ester, and phthalate derivatives [17]. Presence of tetracontane and diethyl phthalate having antimicrobial
Fig. 3: Mass spectra (MS) of the compounds. (a) MS of E-2-octadecadecen-1-ol, (b) MS of cyclopentane 1, 1(3-(2-cyclopentyl ethyl) 1, 5-pentadiyl, (c) MS of 7, 9-Di-ter-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione, (d) MS of phthalic acid butyl 2-pentyl ester, (e) MS of 9-nonadecene, (f) MS of heptadecane 9-hexyl, (g) MS of erucic acid, (h) MS of cyclohexane 1, 1-dodecylidenebis (4-methyl), (i) MS of tetratetracontane

| Sr. No. | Name and chemical structure of the compound                                      | MF       | MW   | RT (minutes) |
|---------|---------------------------------------------------------------------------------|----------|------|--------------|
| 1       | E-2-octadecadecen-1-ol                                                          | C₁₈H₃₆O₂  | 268  | 12.20        |
| 2       | Cyclopentane 1, 1(3-(2-cyclopentyl ethyl) 1,5-pentadiyl                        | C₁₀H₁₈O₂  | 304  | 16.01        |
| 3       | 7,9-Di-ter-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione                    | C₁₅H₂₄O₂  | 276  | 19.35        |
| 4       | Phthalic acid butyl 2-pentyl ester                                              | C₁₇H₂₄O₄  | 292  | 20.32        |

Table 2: GC-MS profile of ethyl acetate extract

(Contd...)
activity was recorded based on GC-MS studies of endophytic fungal extracts [40]. Antifungal B. cenocepacia strain VIMP01 ([JQ867371] produced tetratetracontane, heptadecane-9-hexyl, and 7,9-Di-ter-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione [38], whereas B. gladioli strain VIMP02 ([JQ811577] produced compounds such as 9-nonadecene, 7,9-Di-ter-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione, phthalic acid butyl 2-pentyl ester, and tetratetracontane [39]. Usha et al. [26] documented antimicrobial compounds of marine Streptomyces caecul strain SU2 ([JF730119]) in the ethyl acetate extract by GC-MS such as phthalic acid butyl ester and 1 nonadecene derivatives. El-Baz et al. [23] listed the presence of antimicrobial tetratetracontane in ethyl acetate extract of Jatropha curcas leaves. The presence of antimicrobial heptadecane was also reported by Khairy and El-Kassas [41] in ethyl acetate extract from blue green algae such as Anabaena flos-aqua, Anabaena variabilis, and Oscillatoria angustissima. Taha et al. [42] reported antifungal activity of seed oil from Eruca sativa containing erucic acid, a major fatty acid, along with garlic oil against dermatophytes causing hair diseases. Gopalakrishnan et al. [43] reported that ethanolic extract of stem of Ceylon tea contained bioactive tetratetracontane. Rabha et al. [44] detected oxalic acid and extracellular hydrolytic protease and chitinase enzymes in the extract obtained from endophytic fungus, C. gloeosporioides, having antifungal traits. Results of the present investigation are in accordance with metabolites reported by the above researchers. It may be the first documentation highlighting the production of antimicrobial formic acid and erucic acid by any Burkholderia culture. Combination of organic acids, enzymes such as chitinase and protease, and antimicrobial compounds as identified by GC-MS exerted antifungal activity in the present investigation. These components may affect fungal cell wall, proteins, and nucleic acids. However, differences at the level of antifungal activity, HPLC, and GC-MS profiles can be explained on the basis of cultural differences, media composition, growth conditions, and diversity in antifungal metabolites.

CONCLUSION

The present study outcomes highlighted antifungal activity in the culture filtrate and ethyl acetate extract of B. gladioli strain VIMP03 ([JQ867372]) due to the presence of antimicrobial principles. On the basis of biochemical, HPLC, and GC-MS analyses, the number of bioactive principles was detected such as lytic enzymes such as protease and chitinase; organic acids such as acetic, formic, oxalic, and pyruvic acids; and other compounds including erucic acid, 9-nonadecene, heptadecane 9-hexyl, and tetratetracontane. Many of the bioactive principles were not reported earlier for any Burkholderia species. Regarding the culture under the study, the GC-MS and HPLC profiles in combination may be unique. The culture may have agroclinical potential to develop biofertilizers having fungicidal activity, especially against pineapple disease causing C. paradoxa. Field studies should be conducted in the future to access the impact of B. gladioli strain VIMP03 ([JQ867372]) in declining the incidence of pineapple disease.

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Table 2: (Continued…)

| Sr. No. | Name and chemical structure of the compound | MF | MW | RT (minutes) |
|--------|------------------------------------------|----|----|-------------|
| 5      | 9-nonadecene                             | C_{20}H_{18} | 266 | 20.83       |
| 6      | Heptadecane 9-hexyl                      | C_{19}H_{18} | 324 | 20.98       |
| 7      | Erucic acid                              | C_{22}H_{29}O_{2} | 338 | 26.57       |
| 8      | Cyclohexane 1,1-dodecylideneb (4-methyl) | C_{13}H_{10} | 362 | 33.19       |
| 9      | Tetratetracontane                        | C_{40}H_{40} | 618 | 33.33       |

GC-MS: Gas chromatography-mass spectrometry
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