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ANTIFUNGAL PROPERTIES OF *BURKHOLDERIA CENOCEPACIA* STRAIN VIMP 01 (JQ867371) AGAINST *CERATOCYSTIS PARADOXA* AND *ALTERNARIA ALTERNATA*

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Abstract: Soil-borne pathogens *Ceratocystis paradoxa* and *Alternaria alternata* are the causative agents of pineapple and leaf spot diseases of sugarcane. Antifungal activity of phosphate solubilizing bacterium- *Burkholderia cenocepacia* strain VIMP 01(JQ867371) was tested by employing techniques viz., dual culture and agar well diffusion. Culture filtrate as well as ethyl acetate extract showed prominent antifungal activity against soil borne pathogens under study. HPLC analysis confirmed presence of different organic acids including lactic, acetic, oxalic, pyruvic and formic acids. GC-MS analysis of culture extract revealed presence of 14 potential antimicrobial compounds including heptadecane 9-hexyl; 1, 2 benzenedicarboxylic butyl 2-methyl propyl ester; 9-octadecane 1, 1-(1,2-ethanediyl(oxy) bis; 1, 3, 5, 7, 9-pentaethylbicyclo (5, 3, 1) pentasiloxane and phenol-, alcohol- and phtalic acid- derivatives. Hence the culture of *B. cenocepacia* strain VIMP 01(JQ867371) can be explored as aspirant biocontrol agent.

Key words: Soil borne pathogens, *Burkholderia cenocepacia*, antifungal, HPLC, GCMS.

INTRODUCTION

Sugarcane is one of the most popular cash crops and is being cultivated in around 4.50 million hectares in India. This crop has served as a major driving force in boosting the rural economy and hence in uplifting poor farmers of several states including Uttar Pradesh, Maharashtra, Tamil Nadu, Karnataka and others. Management of sugarcane diseases and pests has become imperative as losses due to diseases are estimated to be about 10-15% [1]. Among various diseases of sugarcane, sett rot or pineapple disease caused by *Ceratocystis paradoxa*, is reported unremitting and severe in low lying areas and poorly drained soils even after chemical fungicide treatment. Mild leaf spot incidence related to *Alternaria alternata* is commonly noticed in the states of Kerala, Maharashtra and Karnataka while in Gujarat pineapple disease is commonly reported [2,3].

Many researchers have investigated the bioactive metabolite production in microorganisms which have the potential to promote plant growth. Talukder *et al.,* [4] assessed antagonism of *Trichoderma harzianum* against *C. paradoxa* and evaluated its efficiency in controlling pineapple disease and forecasted the role of *T. harzianum* produced metabolite 6-phenyl-a pyrone (6-p-p) in inhibition of *C. paradoxa*. *Trichoderma viride* and *T. polysporum* significantly reduced the growth of *C. paradoxa* [5]. Plant growth promoting rhizobacterial *Burkholderia* species have been demonstrated to reduce plant diseases by suppressing soil-borne pathogens through antibiosis [6]. Based on GC-MS analysis, antagonistic attributes and responsible metabolites of *Burkholderia* from different origins were studied [7, 8, 9]. *Burkholderia cenocepacia* strain VIMP 01(JQ867371) was isolated by Mahamuni and Patil [10] from sugarcane rhizosphere having phosphate solubilizing potential. The present study was intended to evaluate antifungal activity of *B. cenocepacia* strain VIMP 01 (JQ867371) especially against soil-borne pathogens such as *C. paradoxa* and *Alternaria alternata* and to determine the antifungal GC-MS profile using ethyl acetate culture extract.

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MATERIALS AND METHODS

Cultures

The plant growth promoting rhizobacterial culture used for the present investigation was *B. cenocepacia* strain VIMP 01(JQ867371), isolated by Mahamuni and Patil from sugarcane rhizosphere, basically as phosphate solubilizer using Pikovskaya's agar and it was characterized as Gram negative, non-spore forming, non-capsulated rod shaped, motile bacterium and having composting ability also [10]. Phytopathogenic cultures- *Sclerotium rolfsii*, *C. paradoxa* and *A. alternata* were obtained from the Plant Pathology Section of Vasantdada Sugar Institute, Manjari Bk., Pune.

Culture media

Pikovskaya's broth [11] was used for the cultivation of phosphate solubilizing bacterium- *B. cenocepacia* strain VIMP 01(JQ867371). It contained (g litre^{-1}) Dextrose 10; Ca_3 $(PO_4)_2$ 5; yeast extract 0.5; KCl 0.2; $(NH_4)_2SO_4$ 0.5; $MgSO_4.7H_2O$ 0.1; $MnSO_4.H_2O$ 0.0001; NaCl 0.2. The pH of medium was 7.0 (± 0.2).

Potato dextrose agar (PDA) was used to check *in vitro* antagonistic activity. It contained (g litre^{-1}) Dextrose 20; potato infusion 200; Agar 20. The pH of medium was 5.6 (± 0.2).

Chitinase activity was detected by means of colloidal chitin agar (pH 7.0± 0.2). It contained (g litre^{-1}) KH_2PO_4, 0.3; $FeSO_4.7H_2O$ 0.01; $MgSO_4.7H_2O$, 0.5; $ZnSO_4$, 0.001; $MnCl_2$, 0.001; colloidal chitin, 20 and agar 20. Colloidal chitin was prepared as per the protocol used by Shanmugaiah *et al.,* [12]. Milk agar was employed to check protease activity. It had ingredients (g litre^{-1}) Pancreatic digest of casein, 5; Dextrose, 1; Yeast extract, 2.5; Skim milk, 100ml and agar, 20. The pH of culture media was adjusted using 1N NaOH or 1N HCl. Media were sterilized by autoclaving at 120^0 C for 15 min.

Chitinase and protease producing abilities

Both chitinolytic and proteolytic abilities of *B. cenocepacia* strain VIMP 01 (JQ867371) were tested qualitatively by spot inoculation on chitin and milk agar, respectively. Incubation temperature was 30^{0}C. Chitinolytic (chitinase producing) activity was examined as positive after 5 days incubation, if there was clear zone around the growth [12] while proteolytic (protease producing) activity was detected after 48hrs as positive, if there was clear zone around the growth against opaque background.

HPLC Organic acid profile of culture filtrate

B. cenocepacia strain VIMP 01(JQ867371) culture was first cultivated in Pikovskaya's broth. Organic acids from broth were detected by high performance liquid chromatography (HPLC). Bacterial culture broth was filtered through 0.2µm filter (Millipore) and 20µl of filtrate was injected to HPLC (Model Waters alliance Company) equipped with a UV Detector. Organic acid separation was carried out on organic acid (Prevail) column (Make Grace) with specification 150cm length and 4.6mm internal diameter and 25mM KH_2PO_4 as mobile phase. Retention time of each signal was recorded at a wavelength of 210 nm.

***In vitro* antagonistic activity**

Soil borne pathogens *C. paradoxa*, *Sclerotium rolfsii* and *A. alternata* were used to check *in vitro* inhibition [13, 14] trend along with *B. cenocepacia* strain VIMP 01(JQ867371). Dual culture *in vitro* assay technique was used. The bacterial culture was spot inoculated at one end of the Potato dextrose agar. After two days incubation at room temperature, 6 mm agar disc impregnated with freshly grown fungal pathogen from the PDA agar culture, was placed at the other marginal side of the plate and incubated at room temperature for seven days. The radii of the fungal colony towards and away from the bacterial colony were noted. The per cent growth inhibition was calculated using formula,

Per cent inhibition = (R-r / R) × 100

Where "r" is the radius of the fungal colony opposite the bacterial colony and 'R' is the maximum radius of the fungal colony away from the bacterial colony.

Antagonist activity of culture filtrate

The culture filtrate of the VIMP isolate grown in Pikovskaya's broth for seven days at 30^{0}C was collected by centrifugation at 3000rpm for 10 min and sterilized by passing it through millipore membrane filter (0.45µm pore size). The sporulated cultures of fungi were separately inoculated into sterile molten PDA medium (45°C) and poured into sterile Petri dishes. Antagonistic activity of culture filtrate (100µl) was detected in triple sets by agar well diffusion technique.

Antifungal activity of ethyl acetate extract

Antifungal principles from the cell free filtrate were extracted by ethyl acetate. Ethyl acetate extract was evaporated at room temperature and concentrated. Evaporation reduced the volume of 500ml ethyl acetate extract to 15ml. Antifungal activity of concentrated ethyl acetate extract was detected in triple sets using phytopathogen *Alternaria* and *Ceratocystis* by agar well technique using 100µl of the extract.

GC-MS profile of ethyl acetate extract

Analysis was conducted using thermo gas chromatography coupled with ITQ 1100 mass detector and X-Caliber software and NIST Spectral data (GCMSMS, Thermo Fisher Scientific). Analytical conditions were as follows: 2µl sample injected, a DB-5 MS capillary column with specification 30 X 0.25µm internal diameter and 0.25µm film thickness; carrier gas helium @ 1 ml per min; Injector and transfer line temperatures used were 250^{0}C and 280^{0}C respectively; over temperature program included- 60^{0}C - 2min hold, 15^{0}C per min rate, increased to reach 160^{0}C remained at temperature for 0 min hold, 3^{0}C per min rate, increased to reach 200^{0}C remained at temp for 1 min hold, 2^{0}C per min rate, increased to reach 230^{0}C remained at temp for 1 min hold, 8^{0}C per min rate, increased to reach 285^{0}C remained at temp for 6 min hold; split ratio = 1:50; ionization energy 70 eV; mass range 50- 650. The components were identified using NIST Library data.

RESULTS AND DISCUSSION

Antifungal properties of *B. cenocepacia* strain VIMP 01(JQ867371) was appraised against selected sugarcane and sugar beet pathogens viz., *C. paradoxa*, *A. alternata* and *S. rolfsii*. Results of dual culture *in vitro* assay technique revealed that the growth of *S. rolfsii* was not inhibited by the strain VIMP 01. *B. cenocepacia* strain VIMP 01 (JQ867371) inhibited *C. paradoxa* with 46 per cent inhibition and *Alternaria alternata* with 58 per cent inhibition. Kadir *et al.,* [15] reported 41 to 81.7 per cent mycelial growth inhibition by *B. cepacia,* which is also in good agreement with results of present investigation. Variations regarding per cent inhibition may be explained on the basis of culture, media, incubation and experimental differences. Culture *B. cenocepacia* strain VIMP 01(JQ867371) showed protease activity by inducing clear zone around the colony on milk agar medium and showed chitinase activity by developing clear zone around the colony on chitin agar medium.

Antagonist activity of culture filtrate and ethyl acetate extract from culture supernatant inhibited the growth of *A. alternata* prominently by showing zone of inhibition (ZOI) 31mm (Standard Deviation (SD) ±1.73) and 22mm (SD ±2.65), respectively and 17mm (SD ±2) and 13mm (SD ±1.73), respectively growth inhibition zones (ZOI) were recorded with the use of *C. paradoxa*. Standard deviation (SD) for zone of inhibition was ranged in between ±1.73 to ±2.65. Regarding *A. alternata* 29.03 per cent less inhibition (ZOI) was recorded by crude ethyl acetate extract as compared to the aqueous culture filtrate while 23.53 per cent less inhibition (ZOI) was recorded by crude ethyl acetate extract as compared to the aqueous culture filtrate in case of *C. paradoxa* (**figure 1**)

Figure 1: Antagonist activity

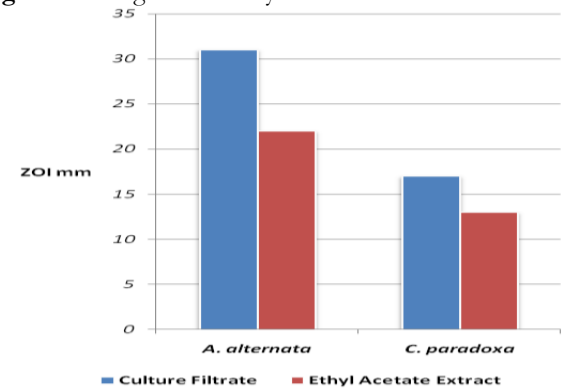

Figure 1a: Comparative antagonist activity of culture filtrate and ethyl acetate extract

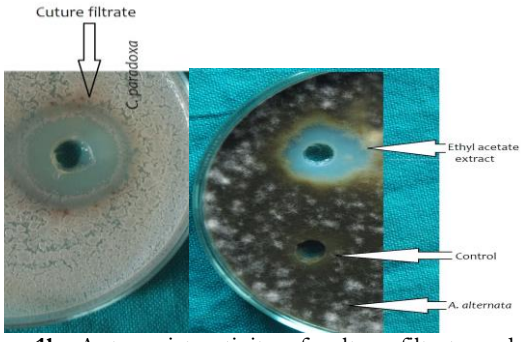

Figure 1b: Antagonist activity of culture filtrate and ethyl acetate extract (Agar well diffusion technique)

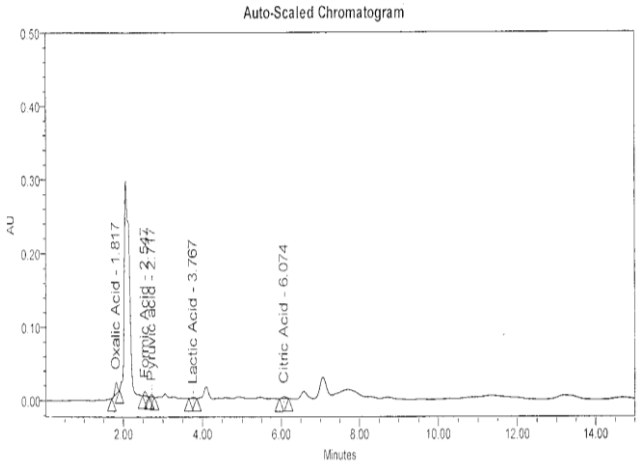

Figure 2: High performance liquid chromatogram of organic acids from *B. cenocepacia* strain VIMP 01 (JQ867371)

The HPLC analysis of culture filtrate as shown in **figure 2** revealed the presence of five organic acids. The highest amount of organic acid produced by *B. cenocepacia* strain VIMP 01(JQ867371) was formic acid (3.15 mg / 100 ml) and it was followed by citric acid (1.23 mg / 100 ml), lactic acid (1.12 mg / 100 ml), oxalic acid (0.5 mg / 100 ml) and pyruvic acid (0.10 mg / 100 ml). Organic acid profile of *B. cenocepacia* strain VIMP 01(JQ867371) is presented in **table 1.**

Table 1: Organic acids detected by HPLC

Name of Organic acid	Retention Time (Min)		Amount of organic acid in culture filtrate (mg /100 ml)
	Standard	Culture Filtrate	
1. Oxalic acid	1.774	1.817	0.5
2. Formic acid	2.275	2.547	3.15
3. Pyruvic acid	2.61	2.717	0.1
4. Lactic acid	3.694	3.767	1.12
5. Citric acid	6.108	6.074	1.23
6. Gibberelic acid	3.093	X	X
7. Acetic acid	4.243	X	X

Note: "X" Not detected

For the identification of biologically active organic compounds, GC-MS method is considered fast and undeviating logical approach. The ethyl acetate extract from culture supernatant of *B. cenocepacia* strain VIMP 01(JQ867371) was further studied by GC-MS to investigate antifungal active principles. GC-MS analysis of the ethyl acetate extract revealed the presence of 15 compounds that could be responsible for the antifungal activity of the *B. cenocepacia* strain VIMP 01(JQ867371). List of compounds detected by GC-MS is presented in **table 2** along with retention time (RT) in minutes, molecular formula and molecular weight and corresponding gas chromatogram is presented in **figure 3.** The first compound identified with less RT (12.19min.) was cyclotetradecane, whereas tetratetracontane was the last compound which took longest RT (33.34min.) to identify. Compounds identified showed molecular weights (M.W.) ranging from 196 to 618. **Table 2** reveals that cyclotetradecane was compound having the least molecular weight (M.W. 196) while tetratetracontane was compound having the highest molecular weight (M.W. 618). Other important compounds detected were 9-octadecane 1, 1-(1,2-ethanediylbis (oxy) bis (M. W. 562), 1, 3, 5, 7, 9-pentaethylbicyclo (5, 3, 1) pentasiloxane (M.W.384), Cyclohexane (1-hexyltetradecyl) (M.W. 364), Cyclohexane 1, 1-dodecylidenebis(4-methyl) (M.W. 362), Cyclohexane (6-cyclopentyl-3-(3-cyclopentylpropyl) hexyl (M.W.346), heptadecane 9-hexyl (M.W.324) and 1, 2 benzenedicarboxylic butyl 2-methyl propyl ester (M.W. 278). In addition to this phenol, alcohol and phtalic acid derivatives were also detected in the crude ethyl acetate extract under study. Among these 1, 3, 5, 7, 9-pentaethylbicyclo (5, 3, 1) pentasiloxane (M.W.384) and Phenol 2, 4 di-t-butyl-6-nitro (M.W. 251) were reported as silicon and nitrogen containing organic compounds.

Analysis of mass spectrum of GC-MS was done using the NIST database. In the present investigation the limited characteristics of the culture filtrate as well as ethyl acetate extract of the culture supernatant pointed out that antifungal activity was likely to be due to production of potential organic substances.

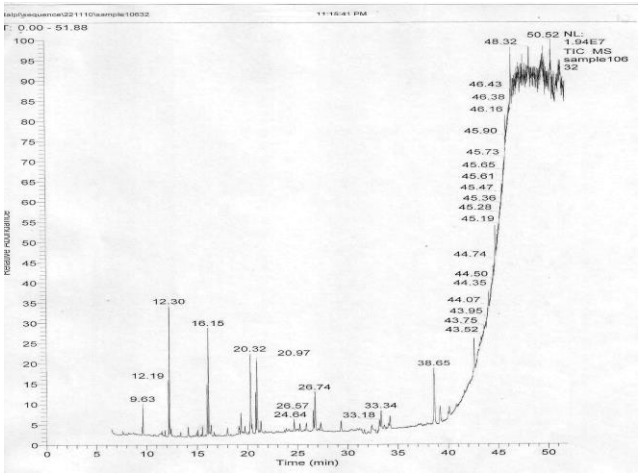

Figure 3: Gas chromatogram of ethyl acetate extract from *B. cenocepacia* strain VIMP 01(JQ867371)

Various types of chemical fungicides are employed in the management of soil borne diseases of sugarcane and sugar beet caused by different fungi such as *C. paradoxa*, *Sclerotium rolfsii*, *Alternaria* spp., *Rhizoctonia solani*, *Aphanomyces chochlioides* and *Phytium* spp. Biocontrol agents having antifungal activity are also useful in the management of diseases [16, 17]. Earlier studies have reported antifungal activities of biocontrol agents including actinomycetes, yeast, bacteria as well as fungi [18, 19, 20, 21, 13, 22, 23, 24, 25, 26 and 14]. The use of lactic acid bacteria as an antifungal agent was reported by Gerez *et al.,* [27]. Lavermicocca *et al.,* [28] purified and characterized novel antifungal compounds from *Lactobacillus plantarum* and the compounds identified in the inhibitory fraction by them were phenyl lactic acid, palmitic acid and p-hydroxy phenyl lactic acid. They also reported that lactic and acetic acid produced by bacteria played the most important role in antifungal activities. **Table 1** elucidating organic acids from *B. cenocepacia* strain VIMP 01(JQ867371) culture filtrate is only one of its kinds due to variety. Presence of variety of organic acids along with proteolytic and chitinolytic enzymes might be the reason that their cell free extract showed more antifungal activity as compared to ethyl acetate extract of the culture under study.

Table 2: GC-MS profile of ethyl acetate extract from *B. cenocepacia* strain VIMP 01(JQ867371)

Sr.No.	Name of compound	Molecular formula	Molecular Weight (M.W.)	Retention time (min)
1	Cyclotetradecane	$C_{14}H_{28}$	196	12.19
2	Phenol 2,4 di-t-butyl-6-nitro	$C_{14}H_{21}NO_3$	251	14.12
3	1-Hexadecanol	$C_{16}H_{34}O$	242	16.01
4	1,2 Benzenedicaboxylic butyl 2-methyl propyl ester	$C_{20}H_{30}O_4$	278	19.13
5	7,9 Di-ter-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276	19.34
6	Phthalic acid butyl 4-octyl ester	$C_{20}H_{30}O_4$	334	20.32
7	n-Decane 1,10 diclohexyl	$C_{20}H_{42}$	306	20.82
8	Heptadecane 9-hexyl	$C_{23}H_{42}$	324	20.97
9	9-Octadecane 1,1 –(1,2-ethanediyl (oxy) bis	$C_{38}H_{74}O_2$	562	21.34
10	Ethanol 2 (octadecyloxy)	$C_{20}H_{42}O_2$	314	25.21
11	Cyclohexane (6-cyclopentyl-3-(3-cyclopentylpropyl)hexyl	$C_{25}H_{46}$	346	26.57
12	Cyclohexane (1-hexyl tetradecyl)	$C_{26}H_{12}$	364	27.30
13	1,3,5,7,9-pentaethylbicyclo (5,3,1) pentasiloxane	$C_{10}H_{28}O_6Si_5$	384	29.34
14	Cyclohexane 1,1-dodecylidenebis(4-methyl)	$C_{26}H_{50}$	362	33.17
15	Tetratetracontane	$C_{44}H_{90}$	618	33.34

Many investigators studied ethyl acetate extracts from supernatants of different cultures as well as of different plant extracts, based on GC-MS analysis [9, 29, 30, 31, 32, 33 and 34] and reported numerous potential antimicrobial compounds. Antimicrobial 1, 2 benzenedicarboxylic acid was reported in methanol extract of *Cassia italica* [35] and ethyl acetate extracts of *Pseudocercospora kaki* and *Penicillium sclerotiorum* [36], *B. cepacia* [9], *Acinobacte*r sp. Strain An 2 [37]. Verma *et al.,* [38] reported antifungal activity of partial crude extract from endophytic fungi due to 1, 2 benzenedicarboxylic acid. Presence of phtalic acid and derivatives were reported as antimicrobial and antioxidants [39]. Phenol and alcohol derivatives were found to be present in the ethyl acetate culture extract of *B. cenocepacia* strain VIMP 01(JQ867371) which are well known potential antimicrobials [37, 31, and 34]. Khairy and El-Kassas [29] reported heptadecane and 1, 2 benzenedicarboxylic acid in the ethyl acetate culture extracts of *Anabaena flos aquae, Anabaena variabilis* and *Oscillatoria angustissima* and octadecane in the ethyl acetate culture extract of *Anabaena variabilis*. Antimicrobial role of heptadecanes were also reported by Abraham *et al.,* [40]. El-Mehalawy [41] reported tetratetracontane and heptadecane 2 as antifungal compounds produced by *Saccharomyces unispora* and *Candida steatolytica*. Similar or slightly different derivatives of cited compounds found to be produced by *B. cenocepacia* strain VIMP 01(JQ867371) which was confirmed after GC-MS analysis of ethyl acetate culture extract. All these compounds may interact with sensitive cells involving cell wall. These findings are in accordance with results of earlier researchers [41, 9, 37, 35, 31, 38, 36, and 34] although GC-MS analysis of ethyl acetate extract explicated many compounds which would be antifungal. GC-MS analysis of most of the plant extracts also revealed diverse metabolites having antimicrobial activities. Few metabolites of microbial origin were found to be alike or slightly similar to plant derived antimicrobial principles. The results revealed in the present study are in close agreement to earlier findings.

CONCLUSION

The outcomes of present investigation highlighted the presence of antifungal bioactive principles in culture filtrate as well as ethyl acetate culture extract of *B. cenocepacia* strain VIMP 01(JQ867371). Many of the bioactive

principles were not reported earlier. The culture under study of *B. cenocepacia* strain VIMP 01(JQ867371) may help in developing biofertilizer-cum-biofungicide for eco-responsive management of minor fungal disease (Alternaria leaf spot) and major fungal disease (pineapple disease). This was the first report of new isolate *B. cenocepacia* strain VIMP 01(JQ867371) elucidating its antifungal role especially against *C. paradoxa* and *A. alternata*. Isolation and purification of bioactive principles with their structural explanations and future field studies should be conducted to assess impact of experimental culture in decreasing incidence of pineapple and *Alternaria* leaf spot disease.

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