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Research Article

PROFILING OF VOLATILES EMITTED BY *PSEUDOMONAS FLUORESCENS* KRB7 USING GC-MS-TD AND THEIR EFFECT ON PLANT GROWTH PROMOTION OF TOBACCO (*NICOTIANA TABACUM* L.)

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Abstract- Volatile organic compounds (VOCs) produced by plant growth promoting rhizobacteria (PGPR) has emerged as a mode of communication between bacteria and plants. In current study *in vitro* experiment was conducted to investigate the effect of volatile organic compounds (VOCs) produced by the plant growth promoting rhizobacterium Pseudomonas *fluorescens* KRB7(was originally isolated from paddy rhizosphere soil) on *Nicotiana tabacum*. Volatile organic compound (VOCs) emitted from the *Pseudomonas fluorescens* KRB7was evaluated under *in vitro* were resulted in significant growth promotion on tobacco plants and also has potential to inhibit the mycelium growth of pathogen *Fusarium oxysporum*. VOCs emitted by *Pseudomonas fluorescens* KRB7 was identified through gas chromatography/mass spectrometry- thermal desorption (GC-MS-TD) analysis. These results suggest that a volatile compound released from *Pseudomonas fluorescens* KRB7significantly enhances the plant growth promotion and immunity.

Keywords- *Pseudomonas fluorescens KRB7, Fusarium oxysporum, Volatile organic compounds, GC-MS-TD, Induced systemic resistance*

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Introduction

Plant growth-promoting rhizobacteria (PGPR) are a group of root-colonizing bacteria in the rhizosphere of many plant species that enhance plant productivity and often elicit plant immunity against multiple plant pathogens [1-3], a process referred to as induced systemic resistance (ISR). Microbes can impart over long distances inside the root framework, both among microscopic organisms and with plant host, where they inspire prompted induced systemic resistance (ISR) and plant growth [4]. VOCs produced by various soil microbes can influence the development, anti-microbial generation [5]. Attributable to these multi-utilitarian roles of VOCs, they have a vast potential for biotechnological applications [6]. Antagonistic microorganisms harbor a vast potential to produce active biomolecules for direct activity against pathogens but also for mediators in various interactions, *e.g.*, pathogen defense, quorum sensing, microorganism-host-interaction. The biological control of plant soil-borne diseases has emerged as an attractive alternative after decrease in the use of chemicals due to risks of environmental pollution and increase in the demand for organic food. Among different biocontrol agents, Pseudomonas strains especially *P.* fluorescens have their own importance. *P. fluorescens* strains have versatile metabolism, inhabit many environments, including plant, soil and water surfaces and have been successfully used to control different plant soil-borne pathogens [7]. *P. fluorescens* BE8 showed 55% control of *Fusarium oxysporum* f. sp. cucumerinumin a pot experiment [8]. Another *P. fluorescens* strain inhibited *Xanthomonas oryzae* pv. oryzae, the bacterial leaf blight pathogen in rice [9]. The understanding of biocontrol mechanisms of action is very important to develop commercially efficient and successful biocontrol strategies against plant pathogens. The biocontrol mechanisms include production of antibiotics, hydrolytic enzymes and volatile organic compounds (VOC), competition for space and nutrients and induction of systematic resistance in plants [10-12].

The most ignored part is the production of VOCs by *P. fluorescens* strains and their role in the control of plant soil-borne disease. The VOCs, which are low molecular weight and high vapor pressure compounds, are generally produced by microbes through catabolic pathways, including glycolysis, proteolysis and lipolysis [13]. The VOCs produced by soil microbes have been reported to promote plant growth, exhibit antimicrobial and nematocidal activity and induce systemic resistance in crops [14]. Volatile organic compounds (VOCs) emitted by two *Bacillus* spp. were shown to be novel determinants of both plant growth promotion and elicitation of ISR in *Arabidopsis* [15]. *P. fluorescens* UM270 produced VOCs that showed antifungal and plant growth promoting activity [16]. In this study, the VOCs produced by a *Pseudomonas fluorescens* KRB7were evaluated for their effects on the growth of tobacco and antifungal activity of *F. oxysporum* using Passive Diffusion System. The VOCs produced by *Pseudomonas fluorescens KRB7* were identified using GC-MS-TD. This study extends our knowledge about the interactions of VOCs in nature, which would be helpful to develop safer fumigants to control plant diseases.

Materials and methods

Preparation of microbial culture and tobacco seedlings used

Pseudomonas fluorescens KRB7was originally isolated from paddy rhizosphere soil (Tirunelveli,Tamil Nadu, India). In order to store for a long time, bacterial cultures were maintained at -80 in tryptic soy broth (TSB) that contained 20% glycerol. *Pseudomonas fluorescens* KRB7were streaked onto tryptic soy agar plates and incubated for 24 h in darkness at 28°C. Water agar (WA) media was used as control. Tobacco seeds were surface-sanitized (2-min, 70% ethanol splashing pursued by a 20-min, 1% sodium hypochlorite dousing), washed (multiple times) in sterile refined water, put on petri dishes containing half-quality

Murashige and Skoog salt (MS) medium containing 0.8% agar and 1.5% sucrose, changed in accordance with pH 5.7, and vernalized for 2 days at 4°C without light. Seedlings were placed in growth cabinets (Pooja Lab, Mumbai) set to a 12-h-light and 12-h-dark cycle under 40-W fluorescent lights; the temperature was maintained at 22 ± 1°C with a relative humidity of 50-60 percent. Germinated seedlings were transferred after 4 days to partition plates for the experimental uses described below.

In vitro study for evaluation of Tobacco Growth Promotion by Volatiles Produced by _Pseudomonas fluorescens_ KRB7using Passive Diffusion System

Split Petri dish/Partition petri dish was used to evaluate the volatile producing rhizobacterial isolate *Pseudomonas fluorescens* KRB7 based on their plant growth promoting activity, in which a barrier separates the dish into two compartments in order to ensure a physical separation of rhizobacteria and plants (tobacco). Rhizobacterial isolate *Pseudomonas fluorescens* KRB7 were inoculated in one compartment containing TSA medium and tobacco seedlings were transferred to another compartment of Partition petri dish containing half-strength Murashige and Skoog salt (MS) medium [15].

Assessing antibacterial activity of _Pseudomonas fluorescens_ KRB7against pathogen _Fusarium oxysporum_

Volatile mediated antimicrobial activity of rhizobacterial isolates against selected rice pathogen (*Fusarium oxysporum*) was assessed using partition plate assay. The isolated bacterial cultures were selected and further evaluated for volatile-mediated antifungal activity against *F. Oxysporum* using the partition plate system, which consisted of centrally partitioned plastic Petri dishes (85 × 15 mm) with no physical contact between the two microorganisms grown on either side. One compartment of the divided plates containing modified TSA medium (containing 1.5% agar, 1.5% sucrose, and 0.4% TSA) was inoculated with *Pseudomonas fluorescens* KRB7and another compartment containing PDA medium was used to test the ability of the VOCs to inhibit mycelial growth of fungi (*F. oxysporum*). The plates were double sealed and incubated at 28°C for three days. The control without the inoculation of rhizobacterial isolates was also maintained. The diameters of the mycelium growth of fungus were measured after 3 days of incubation at 28°C.

Profiling of volatiles Produced by _Pseudomonas fluorescens_ KRB7using GC- MS-TD Preparation of samples for VOC analysis

The *Pseudomonas fluorescens* KRB7culture was grown overnight in Tryptic Soybroth. The inoculums in 500 ml conical flask were kept for 24 hrs with tight sealing using rubber cork and tightly wrapped by parafilm. After 24hrs of rhizobacterial inoculation, the stainless steel pre-conditioned desorbing tubes coated with tenax TA were decapped at one end and it were fitted in the center of rubber cork of culture flask containing rhizobacterial cultures and again tightly sealed with paraffin. Volatiles were collected at different time intervals by detaching the desorption tube from cork and recapped at another end of desorption tube and used for further GC-MS-TD analysis. Above said procedures were done aseptically.

Profiling of VOC emitted by _Pseudomonas fluorescens_ KRB7using GC-MS-TD

The thermal desorper (TD) coupled with TurboMassquadrupol mass spectrometer (Perkin Elmer, Clarus SQ8C MS) was used for the non-targeted analysis of volatile organic compounds from the *Pseudomonas fluorescens* KRB7. Volatiles emitted from the rhizobacterial isolate KRB7 were trapped in preconditioned desorbing tubes and were thermally desorbed at 225°C into the packed liner of Thermal Desorper (Perkin-Elmer Clarus 60). Before the sample analysis, empty and sorbent-only tubes were also analysed as a quality assurance procedure. Solvent venting mode was used to transfer the sample to the packed liner (filled with Tenax TA) and held at −55°C which was subsequently heated to 280°C to transfer the VOCs into the GC capillary column. Mass spectra were recorded at 2 scans with a m/z of 50–500 scanning range. The transfer line temperature was set to 280°C, the ion source to 200°C, the filament to 70eV. The mass spectrometer was run in the TIC mode from 40 to 620 amu in electron ionization mode at 70 eV, with a scan range of m/z 29–400 Da, scanning rate 20 scans/s and the experimental time was 30 minutes. The MS of the peaks were determined by their scatter pattern. Internal standards were used to normalize the peak areas. In addition, the program is provided with a few trial target libraries extracted from the NIST databases. Compounds were identified by comparison of spectra obtained from the *Pseudomonas fluorescens* KRB7with those from a reference library (NIST 08 Mass Spectra Library, National Institute of Standards and Technology). GC–MS- TD analysis was conducted in triplicates for each treatment [17].

Results

Enhancement of tobacco growth by _Pseudomonas fluorescens_ KRB7 volatiles

To investigate the positive effects of volatiles emitted from *Pseudomonas fluorescens*KRB7 on plant growth, tobacco seedlings were co-cultured with *Pseudomonas fluorescens* KRB7on an I-plate containing TSA culture media and water agar (WA) was used as positive control. As shown in [Fig-1], all seedlings co-cultured with the *Pseudomonas fluorescens* KRB7displayed growth promotion compared to control (water agar). Culture medium used for microorganism's growth (TSA) enhances the production of volatile compounds. Results revealed that the co-cultured seedlings with *Pseudomonas fluorescens* KRB7on TSA showed the highest average fresh weight of tobacco seedling as 0.26 g, but seedlings on WA was recorded as 0.05g, reaching the best yield with 10 µl of applied inoculums [Fig-1]. These results suggested that unidentified volatile signals would be the key factors in *Pseudomonas fluorescens* KRB7mediated tobacco plant growth promotion.

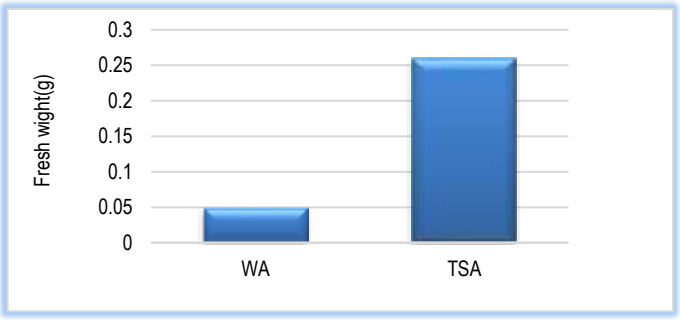


Fig-1 *In vitro* plant growth promotion in tobacco seedlings by exposure of VOCs from *Pseudomonas fluorescens* KRB7 cultured on different laboratory media using I-plate assay.

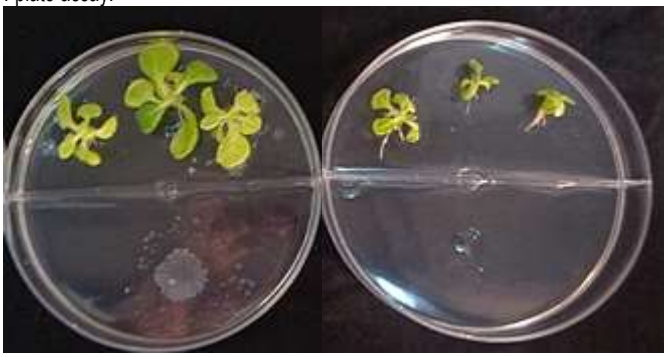


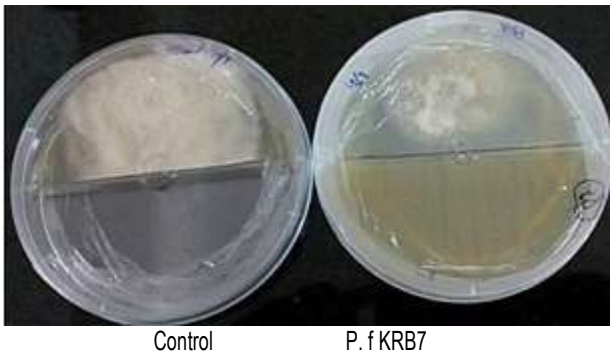
Fig-2a *In vitro* plant growth promotion in tobacco seedlings by exposure of VOCs from *Pseudomonas fluorescens* KRB7cultured on different laboratory media using I-plate assay. Bacterial suspension of *Pseudomonas fluorescens* (108cells/ml) were dropped on the one side of I-plate containing different laboratory media.

Antogonistic activity of rice rhizobacterial _Pseudomonas fluorescens_ KRB7 VOCs against _F. oxysporum_

Potential VOC producing *Pseudomonas fluorescens* KRB7 were evaluated based on mycelia growth inhibition of *Fusarium oxysporum* by co-inoculation using partition plate assay. *Pseudomonas fluorescens* KRB7 were considerably reduced the mycelial growth of *F. oxysporum comparison* with the control plates [Fig-2b].

Table-1 *Volatiles collected from 24hrs old Pseudomonas fluorescens KRB7 and Identification of volatile compounds by GC-MS-TD*

Retention time	Compounds identified	Potential use
2.324	3(2H)-Furanone, dihydro-5-isopropyl-	BCA, Signaling molecule
2.484	2-Butenoic acid, 2-methyl-, 2-methylbutyl ester,	Disease resistance, Involved in fungal pathogenesis
2.799	4-Decenal	Antifungal activity, Plant growth promotion
4.525	Benzene, 1,3-dimethyl-	Activation of enzyme system
4.695	2-(2-Hydroxyphenoxy)-1-phenylethanol	Flavanoid phytoalexin
5.040	1,3,5,7-Cyclooctatetraene	Control post-harvest disease
5.760	1,5,5-Trimethyl-6-methylene-cyclohexene Cyclopropane, trimethyl(2-methyl-1-propenylidene)-	Antifungal properties
6.461	Decane, 2-methyl-Nonane, 5-(1-methylpropyl)-	Plant growth promotion
6.446	Disulfide, dimethyl,	Plant growth promotion
6.656	3-Methylsalicylic acid, 2TMS derivative	Enhanced accumulation pathogen related protein
5.015	Methyl valerate	Antifungal properties
6.330	Benzaldehyde	Signaling compound
15.364	Palmitoleic acid	Antifungal properties



Control P. f KRB7

Fig-2b *In vitro* antifungal activity by exposure of VOCs from *Pseudomonas fluorescens* KRB7 using I-plate assay. Bacterial suspension of *Pseudomonas fluorescens* were streaked on the one side of I-plate containing TSA media and other side of compartment containing PDA media inoculated with *F.oxysporum* pathogen. Control plate maintained without inoculation of KRB7.

Profiling of volatiles emitted by *Pseudomonas fluorescens* KRB7 by Gas Chromatography –Mass Spectrometry –Thermal Desorption

In this study, we elucidated the potential compounds produced by *Pseudomonas fluorescens* KRB7and its effect on plant growth promotion. GC-MS-TD revealed that, potential VOCs produced from *Pseudomonas fluorescens* KRB7, were act as an active signal molecule for influencing plant growth promotion [Fig-2]. Three isolates *Pseudomonas fluorescens* KRB7which were shown to promote growth in tobacco plants were used for subsequent GC-MS-TD profiling. Isolate-specific VOCs were identified by overlays of total ion chromatograms [Fig-2]. A total of 13 compounds (Table 1) were found to be unique. Compound identification indicated that *Pseudomonas fluorescens* KRB7emitted plant growth promoting compounds like, 4-Decenal, Decane, 2-methyl-, Nonane, 5-(1-methylpropyl)-, Disulfide, dimethyl and also emitted potential antifungal compounds *viz*., Furanone, 2-Butenoic acid, Benzene, 2-(2-Hydroxyphenoxy)-1-phenylethanol, Cyclooctatetraene, cyclohexene, 3-Methylsalicylic acid, Methyl valerate, Benzaldehyde and Palmitoleic acid.

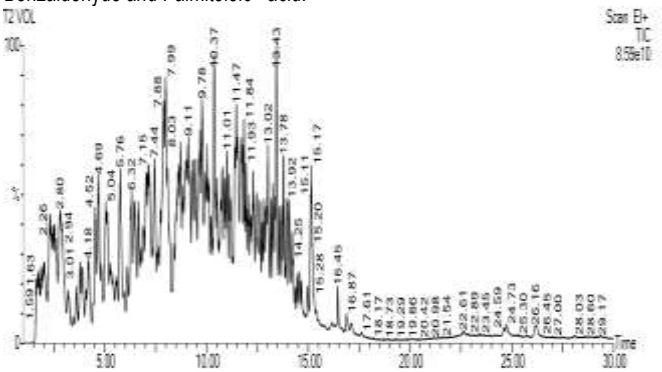


Fig-3 Chromatogram of VOC of *Pseudomonas fluorescens* KRB7by GC/MS/TD. The volatile compounds were identified by comparing the compounds of the reference data library (NIST 08).

Discussion

VOCs differ significantly in structure and function where a single compound can affect numerous aspects of an organism's growth and development. The most potent chemical species should grant inhibitory effects even at very low amounts [18]. Volatiles such as 2, 3-Butanediol and 3- hydroxy-2-butanone (acetoin) was already reported as a beneficial volatile compound produced from a PGPR strain *Bacillus* sp. Other volatile metabolites such as acetoin, some Indole, 1-hexanol and pentadecane are also appeared to promote plant growth [19]. These lead to speculate that the plant growth promoting effects by *Bacillus* sp. are commonly induced by similar active metabolites, and these signal molecules are recognized and activated by diverse plant species. It is possible that the bacterial emitted volatiles are commonly recognized by similar signaling pathways and components. *P. polymyxa* E6 81, uncover that long-chain bacterial VOCs, *i.e*., the C13 hydrocarbon tridecane, likewise can induce ISR, as can C4 alcohols, for example, 2,3-butanediol [20]. *Paenibacillus polymyxa* strain E681 of barley root act as potential biocontrol agent that inhibit soilborne pathogens *Fusarium oxysporum, Rhizoctonia solani* and *Pythium ultimum* which causing damping-off in cucumber and sesame [21,22] and E681 also elicit plant immunity by emitting diverse class of VOCs. The volatile 1-undecene emitted from *Pseudomonas* had abundant inhibitory ability to inhibit the growth of *P. infestans.* Although1-undecene certainly contributes to the total activity of the whole volatile blend, the doses required to achieve significant *P. infestans* growth inhibition were very high [23,24]. The dimethyl disulfide, which is produced by plants and microorganisms, has multiple functions as an insect attractant, elicitor for plant systemic resistance, and suppressor of pathogenic fungi [25-27]. In present investigation, the *Pseudomonas fluorescens* KRB7, showed strong plant growth promotion and immunity in tobacco under *in vitro*. Even though our study discovers the plant growth promotion by treatment with *Pseudomonas fluorescens* KRB7, is due to the production of volatiles, many details of the molecular basis of plant responses to bacterial-released metabolites including VOCs remains to be explored in future.

Conclusion

Plant microbiota emit diverse group of volatile organic compounds (VOCs) has to potential to inhibit the phytopathogens growth and also improve the plant growth. Overall, the results provide new evidence that signaling molecules, emitted from *Pseudomonas fluorescens* KRB7, can promote plant growth and immunity.

Application of research: Current study also suggested that volatile compounds produced by rhizobacteria act as a precursor/signaling molecule of pathways leads to plant growth hormone production and might have direct influence on growth promotion of crops. Therefore, these molecules are prospective alternatives to synthetic chemicals and the determination of their bioactivities against plant threats could contribute to the development of control strategies for sustainable agriculture.

Research Category: Plant Pathology

Abbreviations:
VOCS-Volatile organic compounds
GC-MS-TD- Gas Chromatography –Mass Spectrometry –Thermal Desorption
WA-Water Agar
TSA-Tryptic Soy Agar
ISR-Induced systemic resistance
PDA-Potato dextrose agar

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Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Tirunelveli, Tamil Nadu, India

Cultivar / Variety / Breed name: Tobacco (*Nicotiana tabacum* L.)

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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