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Determination of potential plant growth promoting compounds from *Ochrobactrum* sp. (MH685438) by thermal desorption-gas chromatography

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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 *Ochrobactrum* sp. (MH685438) on *Nicotiana tabacum*. Volatile organic compound (VOCs) emitted from the *Ochrobactrum* sp. was evaluated under *in vitro* were resulted in significant growth promotion on tobacco plants. VOCs emitted by *Ochrobactrum* sp. was identified through gas chromatography/mass spectrometry- thermal desorption (GC-MS-TD) analysis. These results suggest that a volatile compound released from *Ochrobactrum* sp. significantly enhances the plant growth promotion.

Keywords: *Ochrobactrum* sp., volatile organic compounds (VOC), GC-MS-TD, PGPR

Introduction
The PGPR interact with plants through direct pathways which involve the release of phytohormones and organic substances that contribute to growth stimulation and nutrient availability. Indirect pathways comprise substances that prevent phytopathogens attack through the production of hydrolytic enzymes, antibiotics, siderophores and hydrogen cyanide (Goswami *et al*., 2016; Vejan *et al*., 2016) [5, 7]. However, a new mechanism of plant growth promotion mediated by volatile organic compounds (VOCs) was reported for the first time by Ryu *et al*. (2003) [14].
PGPRs, or products derived by PGPRs, usually require physical contact with plant parts for the stimulation of plant growth (Xie *et al*., 2014) [18]. However, many types of bacteria can regulate plant growth from a distance without any contact, suggesting the possibility that these bacteria emit invisible volatile compounds that promote or inhibit plant growth. Volatile organic compounds (VOCs) produced by plant growth promoting rhizobacterium (PGPR) has been proved to have the potential to control plant pathogens, to stimulate plant growth and to induce systemic disease resistance (Ryu *et al*., 2003; Lee *et al*., 2012; Park *et al*., 2015; Raza *et al*., 2016; Tahir *et al*., 2017) [14, 7, 13, 16].
Microorganisms produce wide range of secondary metabolites which are main components of plant growth and most of them are volatile compounds (Farag *et al*., 2013) [4]. Bacterial volatiles are found to be a rich source for new natural chemical compounds (Marco *et al*., 2009) [9]. Nearly 350 bacterial species have been reported to produce around 846 different potential VOCs, with 5431 synonyms (Lemfack *et al*., 2014) [8]. Bacteria having potential growth-promoting activity include species of Pseudomonas, Bacillus, Stenotrophomonas, Serratia, and Arthrobacter (Ryu *et al*., 2003; Bailly and Weisskopf, 2012; Kai and Piechulla, 2014; Park *et al*., 2015; Raza *et al*., 2016) [1, 6, 12, 13, 14]. Studying the comprehensive chemical profile of PGPR volatiles further showed that more than 30 different volatiles are emitted from *Bacillus spp*.(Farag *et al*. 2006; Lee *et al*. 2012) [3, 7].
The volatiles compounds emitted from *Bacillus subtilis* GB03 induced growth on *Arabidopsis thaliana*, without physical interaction with plant roots was the first evidence that volatile organic compounds can modulate growth, stress, nutrition, and health processes in plants (Pare *et al*., 2005; Ryu *et al*., 2003, 2004) [10, 14]. Furthermore, Ryu *et al*. (2004) [14] reported that several airborne chemicals from certain soil bacteria which are the physically separating PGPR from their host plant have been identified as effective signals for triggering plant growth and ISR.

Ryu *et al*. (2003) [14] first reported two VOCs (2,3- butanediol and acetoin) that triggered growth promotion. *Bacillus megaterium* XTBG34 produces 2-pentylfuran which promoted the growth of *Arabidopsis thaliana* plants by two fold after 15 days of treatment (Zou *et al*., 2010) [19]. More recently, Park *et al*. (2015) [12] reported that 13-tetradecadien-1-ol, 2-butanone, and 2-methyl-n-1-tridecene, produced by *Pseudomonas fluorescens* SS101, enhanced the growth of *Nicotiana tabacum*. The main objective of this present study was to explore the growth promoting activity of VOCs produced by *Ochrobactrum* sp. in partition plate. VOCs emitted by *Ochrobactrum* sp. increased plant biomass as compared to the water control.

Materials and Methods

Bacterial Strain, Plant Material, and Plant Growth Conditions

Ochrobactrum sp. was originally isolated from paddy rhizosphere soil (Paiyur, Tamil Nadu, India). In order to store for a long time, bacterial cultures were maintained at -80 in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA) that contained 20% glycerol. *Ochrobactrum* sp. were streaked onto tryptic soy agar plates and incubated for 24 h in darkness at 28^0c. Water agar (WA) media was used as control. Tobacco seeds were surface-sterilized (2-min, 70% ethanol soaking followed by a 20-min, 1% sodium hypochlorite soaking), rinsed (four times) in sterile distilled water, placed on petri dishes containing half-strength Murashige and Skoog salt (MS) medium containing 0.8% agar and 1.5% sucrose, adjusted to pH 5.7, and vernalized for 2 days at 4°C in the absence of 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 per cent. Germinated seedlings were transferred after 4 days to partition plates for the experimental uses described below.

Volatile mediated Plant- microbial interaction

Ochrobactrum sp. obtained from rice rhizosphere was tested for volatile mediated plant growth promotion using passive diffusion method. Tobacco (*Nicotiana tabacum*) var Abirami was taken for this study. *Ochrobactrum* spp., (MH685438), was used against test plant tobacco.

In vitro study for plant growth promotion by bacterial volatiles using Passive Diffusion Systems

Partition petri dish (Hi Media, Bangalore) in which a center barrier separates the dish into two compartments in order to ensure a physical separation of rhizobacteria and plants, were used to test the volatile emitted from *Ochrobactrum* sp. (MH685438) and their impact on plant growth promoting activity. Ten microliter of *Ochrobactrum* sp. (MH685438) suspensions (10^6 cells/ml) was inoculated in one compartment contained TSA media and pre-germinated tobacco seedlings (3 seedlings per plate) were transferred to another side of the I-plates that containing half-strength Murashige and Skoog salt (MS) medium (Ryu *et al*., 2003) [14]. Absolute control was maintained against tobacco seedlings using Sterile distilled water (SDW). Plates were tightly sealed with parafilm to avoid gaseous exchange and arranged in a completely randomized design in growth cabinet with 12-h-light_12-h-dark cycle under 40-W fluorescent lights; the temperature was maintained at 22 ± 1^0C with a relative humidity of 50 – 60 per cent. Two weeks after inoculation, the total fresh weights were recorded.

VOC analysis by Gas Chromatography Mass Spectrometry Thermal Desorption (GC–MS- TD) for *Ochrobactrum* sp. (MH685438)

To identify the volatiles emitted from bacterial culture *Ochrobactrum* sp. GC MS-TD analysis was performed. The *Ochrobactrum* sp. was inoculated in tryptic soy broth (TSB) of 500 ml conical flask were closed with rubber cork. The pre-conditioned stainless steel desorbing tubes coated with tenax TA were decapped at one end and were fitted in the center of rubber cork of culture flask and again tightly sealed with parafilm were kept for 24hrs at 28^0c for volatile collection. Volatiles trapped in desorption tube were detached and recapped were used for further GC-MS-TD analysis. All the above said procedures were done aseptically.

The thermal desorper (TD) coupled with TurboMass quadrupol mass spectrometer (Perkin Elmer, Clarus SQ8C MS) was used for the non targeted analysis of volatile organic compounds from the rhizobacterial isolates. Volatiles emitted from the *Ochrobactrum* sp. 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 analyzed 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 ^{0}C which was subsequently heated to 280 ^{0}C to transfer the VOCs into the GC capillary column. Mass spectra were recorded at 2 scans with the m/z of 50–500 scanning range. The transfer line temperature was set to 280^0C, the ion source to 200^0C, the filament at 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, at the scanning rate 20 scans/s and the total experimental time was 30 minutes.

Results

Plant growth promotion by bacterial volatiles

The emission of VOCs with specific profile depends strongly on the environment in which the microorganism grows. The experiments performed under controlled conditions have shown that a single bacterial strain might induce growth depending on the medium it grows. To investigate the positive effects of volatiles emitted from *Ochrobactrum* sp. on plant growth, we co-cultured tobacco seedlings with *Ochrobactrum* sp. on an I-plate containing TSA culture media as nutrient rich medium and water agar (WA) were used as positive control. As shown in Fig. 1, all seedlings co-cultured with the *Ochrobactrum* sp., displayed growth promotion compared to WA.

Culture medium used for microorganism's growth (TSA) enhances the production of volatile compounds. Interestingly, results revealed that the co-cultured seedlings with *Ochrobactrum* spp., on TSA showed the highest average fresh weight of tobacco seedling as 0.184 g, but seedlings on WA was recorded as 0.03g, 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 *Ochrobactrum* sp. mediated tobacco plant growth promotion.

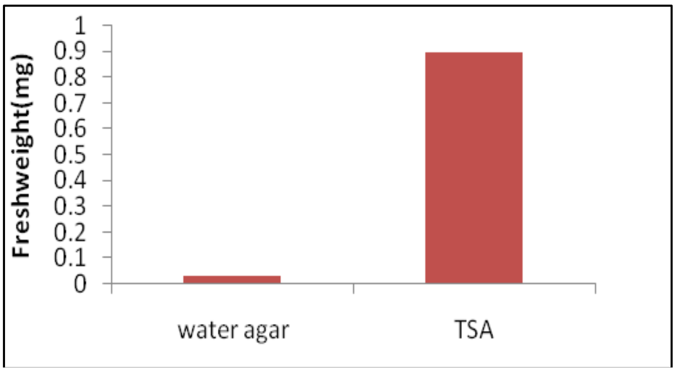

Fig 1: *In vitro* plant growth promotion in tobacco seedlings by exposure of VOCs from *Ochrobactrum* sp. cultured on different laboratory media using I-plate assay.

Profiling of volatiles released by *Ochrobactrum* sp. using GC MS TD

Currently, GC-MS is the dominant method used to characterize volatile profiles from soils, housing materials, as well as microbial and plant samples. The bacterial isolate *Ochrobactrum* sp. were grown in TS broth in 500 ml flask fitted with desorption tube for volatile trapping were kept for 24 hrs. After incubation period, the desorption tube were detached from flask and used for GC-MS analysis. Compounds were identified by comparison of spectra obtained from the bacterial samples with those from a reference library (NIST 08 Mass Spectra Library, National Institute of Standards and Technology). Our results revealed that volatile signals emitted by *Ochrobactrum* sp. were identified, which are 1-Methyl-1-silacyclobutane, Disulfide, dimethyl, Nicotinic acid, 3-Methylsalicylic acid, Valeric acid, 3-Hydroxy-2-butanone and Benzaldehyde and other potential compounds which are listed in table 1. We found that 3-hydroxy-2-butanone (acetoin) and 2,3- Butanediol compounds emitted from *Ochrobactrum* sp. which related to plant growth promotion and induced systemic resistance on various plants.

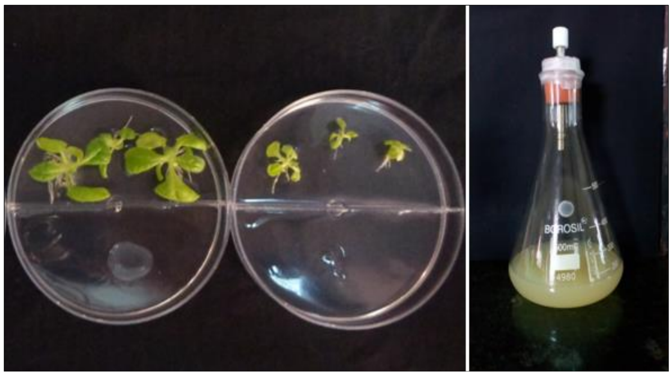

Fig 2: *In vitro* plant growth promotion in tobacco seedlings by exposure of VOCs from *Ochrobactrum* sp cultured on different laboratory media using I-plate assay. The plant growth promotion effects of strain PPB-8. Bacterial suspension of *Ochrobactrum* sp (10 6 cells/ml) were dropped on the one side of I-plate containing different laboratory media.

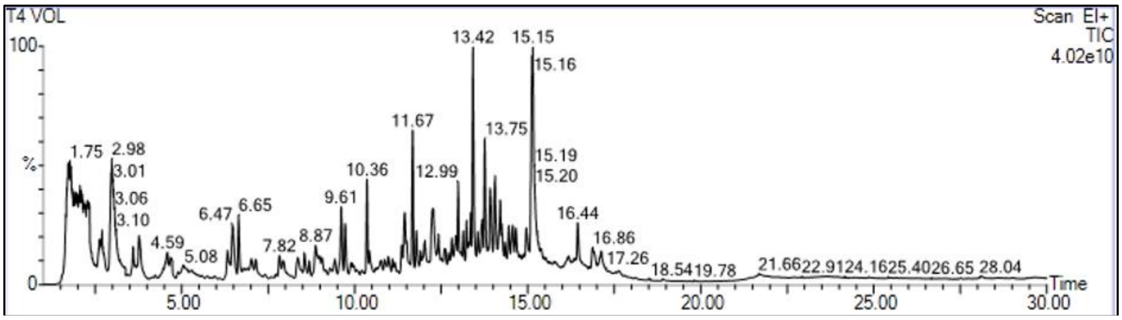

Fig 3: Chromatogram of VOC of *Ochrobactrum* spp., by GC/MS/TD. The volatile compounds were identified by comparing the compounds of the reference data library (NIST 08).

Table 1: Identification of volatile compounds from *Ochrobactrum* spp., using GC-MS-TD

Compounds identified	Retension Time	Potential use
2-Heptanone, 7,7,7-trichloro- 1-Methyl-1-silacyclobutane16.45 2-Pentanone, 4-amino-4-methyl-	(1.799)	Inhibition of mycelial growth and sporangial germination
1,5-Dicyano-2,4-dimethyl-2,4-diazapentane	(2.099)	Bio-control
6-Azidotetrazolo(b)pyridazine	(2.299)	Herbicide derivative and Plant growth regulator
3-Methyl-2-butanol	(2.624)	To control post harvest plant diseases
Acetoin	(2.624)	Promotes plant growth
2,3-Butanediol	(3.599)	Induction of ISR
Cyclopentanol, 2-methyl-, trans Cyclobutanol, 2-ethyl-Hexanal	(3.619)	Expression of defense genes Resistant to insects and fungal attack Anti- nematode
Benzene, 1,3-dimethyl-Ethylbenzene	(4.685)	Activation of enzyme system in plants
Isonicotinic acid, 2-phenylethyl ester Nicotinic acid, 2-phenylethyl ester	(5.045)	Improves plant growth
ethanone, 1,1'-(1,2,3,4-tetramethyl-3-cyclobutene-1,2-diyl)bis-Oxime-, methoxy-phenyl-4-Ethylbenzoic acid, 4-hexadecyl ester	(5.275)	Stimulating natural defense mchanim in plant
Benzaldehyde	(6.330)	Signalling compound
3-Methylsalicylic acid	(6.656)	Regulatory role in inducing multiple stress tolerance

Discussion

Recently, the great mystery of pathogenic, parasitic and commensal interactions among microbe and higher eukaryotic organisms has been explored. In plants, many receptor genes for microbe pattern recognition have been cloned and their downstream signaling components are also exclusively studied. Since the effects of PGPR on plant growth and immunity have been discovered, many scientific research groups have exerted to decipher the molecular mechanisms of interactions between host plants and PGPR. Surprisingly, it has been reported that micro-organisms produce over 20,000 metabolites, and these rich sources are sometimes used for influencing the survival of themselves or the growth controls. In this study, we elucidated the beneficial effects of *Ochrobactrum* sp. (MH685438), producing VOCs on plant growth promotion and immunity. GC-MS-TD revealed that, potential VOCs produced from *Ochrobactrum* sp. (MH685438), were act as an active signal molecule for influencing plant growth promotion (Fig. 2). Interestingly, the 2,3-Butanediol and 3- hydroxy-2-butanone (acetoin) was already reported as a beneficial volatile compound produced from a PGPR strain *Bacillus* sp. (Ryu *et al*. 2003; 2004) [14]. As well as an acetoin, some other volatile metabolites such as Indole, 1-hexanol and pentadecane are also appeared to promote plant growth (Blom *et al*., 2011). 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. Although we here identified an active volatile metabolite to promote plant growth and immunity from a well-known PGPR, *Ochrobactrum* sp. (MH685438), we could not exactly investigate the downstream signaling pathways of the volatile-mediated plant growth promotion. It is possible that the bacterial emitted volatiles are commonly recognized by similar signaling pathways and components. Plant hormone Brassinosteroids are broadly involved in plant growth promotion and various stress tolerances. Auxin acts also as an essential factor for plant growth and organogenesis. These support some possibilities that bacterial-derived metabolites could effect to plant growth and immunity through directly influencing the signaling or metabolic pathways of plant hormones. The strain *Ochrobactrum* sp. (MH685438), showed strong plant growth promotions in various crops under field conditions. Even though our study discovers the plant growth promotion by treatment with *Ochrobactrum* sp. (MH685438), is due to the production of 2,3-Butanediol and acetoin, many details of the molecular basis of plant responses to bacterial-released metabolites including VOCs remains to be explored in future.

Conclusion

Volatile organic compounds (VOCs) produced by the plant microbiota have been demonstrated to elicit plant defenses and inhibit the growth and development of numerous plant pathogens and also promote plant growth. Current study 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**.**

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