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Insights into the Metabolites Conferring Pathogenicity of *Xanthomonas oryzae* and Its Inhibition by *Trichoderma longibrachiatum* EF5

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Authors' contributions

This work was carried out in collaboration among all authors. Author SU has received research grants from MHRD, Government of India and conceptualized the idea. APS executed most of the experiments, Authors KG and ST supervised the works. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present study was aimed to evaluate the effect of volatile and soluble metabolites of *Trichoderma longibrachiatum* EF5 against *Xanthomonas oryzae* pv. o*ryzae*, and to identify the metabolites produced by *Xanthomonas oryzae* pv. *oryzae* in culture filtrate.
Study Design: *In vitro* bioassay with CRD.
Place and Duration of Study: Biocatalysts laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, 2019-2020.
Methodology: Inverted plate, bipartite plate and agar well method was done under *in vitro* to observe the efficacy of *T. longibrachiatum* EF5 VOC against *X. oryzae* pv. *oryzae.* Analysis of *Xanthomonas oryzae* pv. *oryzae* metabolites by using Gas Chromatography and Mass Spectrometry (GC-MS).
Results: *T. longibrachiatum* EF5 VOC and metabolites completely inhibited the growth of *X. oryzae* pv. *oryzae* in inverted plate assay, whereas in bipartite and agar well diffusion assays unmeasurable growth of *X. oryzae* pv. *oryzae* was observed. The metabolites or Diffusible signal

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factors such as butyrolactone, propionic acid derivatives, phenyl acetic acid, hydrofurans, picoxystrobin, benzoic acid derivatives were produced by *X. oryzae* pv. *oryzae* in the growing medium. The role of these metabolites revealed that they are involved in pathogenicity, virulence, quorum sensing and the synthesis of antioxidant.

Conclusion: *T. longibrachiatum* EF5 volatile and soluble metabolites can be used as biocontrol agent against *X. oryzae* pv. *oryzae.*

Keywords: Volatile organic compounds; trichoderma longibrachiatum; Xanthomonas oryzae pv. Oryzae; diffusible signal factors.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the major food crops which was infected by different pathogens such as fungal, bacterial and viruses at all stages that affect the yield and grain quality. Among them, the most common disease, both at nursery and main field is bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* [1]. The pathogen *Xanthomonas oryzae* pv. *oryzae* causes infection at all phases of growth with characteristic symptoms. It invades plants through natural pores and wounds, causing water-soaked lesion. The lesion extends from leaf tip as V shaped wavy margin and cause yellowing and results in death of the plant [2]. *X. oryzae* pv. *oryzae* has different mechanisms to cause infection such as hypersensitive response and pathogenicity (*hrp*) genes, secondary metabolites and toxins, type II and III secretory system, extracellular enzymes, polysaccharides and diffusible signal factors. Different management strategies have been implemented to combat the disease such as use of chemicals, resistant varieties and biocontrol agents [3], (Rajeswari *et al*., 2005).

Plant growth promoting rhizospheric microorganisms were identified and employed as antagonist against *X. oryzae pv. oryzae* [4]. Fungal and bacterial microorganisms either directly kill the pathogen or indirectly induce defense in plants. These organisms produce antibiotics, lytic enzymes and other mechanisms by which they reduce the pathogen growth [5]. Previous studies have shed light on bacterial antagonists such as *Bacillus* and *Pseudomonas* against *X. oryzae* pv. *oryzae* and *Serratia* sp. etc. that induced systemic resistance[6].

Endophytes encompassed of fungal and bacteria which live inside the plants without causing harm to the plant [7]. Such endophytes can be isolated from internal parts of plant without contaminating with epiphytes. They enter plants through natural pores and wounds and colonize the plants acropetally to all parts [8]. Further, they assist the host for uptake of nutrients, inducing defense and production of plant growth hormones [9]. In the same way, as direct application of biocontrol agents, these organisms produce volatile organic compounds (VOCs) which act as a medium for disease suppression by direct inhibition of plant pathogens and indirectly by inducing defense response and plant growth promotion [10]. These VOCs from microorganisms are produced naturally and alters during interaction with other organisms such as microbes, nematodes and plants. Ryu et al. [11] reported the first VOC compound 2, 3-butanedial which induce defense against *Pectobacterium carotovorum* ssp. *carotovorum* in *Arabidopsis thaliana.* Many synthetic VOCs were exploited against plant pathogens for their antimicrobial activity such as dimethyl disulfide, 1-undecene, benzaldehyde, benzothiazole, dimethyl trisulfide, cyclohexanol, decanal, 2-ethyl-1-hexanol, methyl pyrazine and some mid- and long-chain alkanes, alkenes and alcohols [12]. VOCs from *B. subtilis* reduced *Escherichia coli* motility and increased resistance to antibiotics [13]. *Bacillus subtilis* D13 volatiles reduced the motility of *X. oryzae* pv. *oryzae* and altered the surface morphology with concentrated cytoplasm. Among the 12 VOCs profiled in GC-MS, 0.48 mg decyl alcohol and 2.4 mg 3,5,5-trimethylhexanol inhibited the growth of pathogens [14]. However, despite a large number of reports on this topic, there are no available reports on endophytic fungal strains for controlling rice bacterial blight. The study was aimed to exploit the virulence factors of *Xanthomonas oryzae* pv. *oryzae* and the volatiles and metabolites mediated inhibition by fungal antagonist *Trichoderma longibrachiatum* EF5 .

2. MATERIALS AND METHODS

2.1 Microorganisms and Culture Conditions

Endophytic fungus *Trichoderma longibrachiatum* EF5 isolated from rice leaves was obtained from

Biocatalysts Lab, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo) was collected from Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. The fungus and bacteria were maintained in Petri plates containing potato dextrose agar (PDA) and nutrient agar (NA) at 28 ± 2 ºC, respectively.

2.2 Profiling Soluble Metabolites of *x. oryzae* pv. *oryzae* Responsible for Pathogenicity

The pathogen**,** *X. oryzae* pv. *oryzae (Xoo)* was grown in 250 ml Potato Dextrose (PD) broth. Three replications were maintained and the set up was kept at 28 ± 2 ºC for 3 days. Potato Dextrose broth without inoculation served as a control. After incubation the culture was centrifuged at 6000rpm for 20 min at 4^{o}C. The filtered broth was mixed with equal quantity of ethyl acetate and kept in shaker for overnight, concentrated using vacuum flash evaporator. Consequently, the crude metabolite extracted in 1 ml methanol was further used for GCMS analysis after passing through a 0.2µm syringe filter [15].

The purified crude methanolic extract was subjected to GC-MS analysis in a Perkin Elmer GC-MS Clarus® SQ 8 equipped with DB-5MS (Agilent, USA) capillary standard non-polar column with dimensions of 0.25mm OD x 0.25 µm ID x 30 m length. The instrument was set to an initial temperature of 40˚C, and the injection port temperature was ensured at 220˚C, interface temperature set 250°C, source kept at 220°C, oven temperature-programmed as 75°C for 2 min, 150°C at 10°C/min, up to 250°C at 10°C per min. The GC conditions were as followed: 1:12 split, helium carrier at 20 psi. The MS conditions were: positive ion mode, electron impact spectra at 70 eV. The mass spectral scan range was set at 50 to 600 Da. The MS peaks were determined by their scatter pattern. The linear regression coefficient was used to calculate the concentrations in the samples from peak areas obtained in the chromatographs. The bioactive molecules were identified by comparison of mass spectra with NIST 08 Mass Spectra Library (National Institute of Standards and Technology). The name, molecular weight, and structure were ascertained from NIST, Pub Chem, and HMDB databases [16].

2.3 Antagonistic Assay of Volatile Organic Compounds (vocs) of Ef5 against *xoo*

The VOC mediated antagonistic assay was performed both by inverted and bipartite plate assays. An 8 mm disc of *T. longibrachiatum* EF5 was inoculated in bottom plate containing PDA and incubated for 3 days at 28±2º C. After 3 days, 48 h old culture of *Xanthomonas oryzae* pv. *oryzae* was streaked on another bottom plate and placed above to the antagonist plate for the exposure of VOC and sealed with parafilm to prevent the VOC from escaping. A plate without antagonist but containing PDA alone was used as a control. Three replications were maintained and it was completely randomized. The plates were incubated for 3 days at 28±2º C to examine the effect of VOC [15]. While in bipartite plate, 8 mm disc of *T. longibrachiatum* EF5 was placed at periphery of one compartment and incubated at 28±2º C for 2 days. After incubation, 48 h old culture of *X. oryzae* pv. *oryzae* was streaked on another compartment, sealed and incubate at 28±2º C for 2 days. Three replications with completely randomized design were maintained.

2.4 Soluble Metabolites (sms) of Ef5 Against *xoo*

The antagonistic effect of SMs against *Xoo* was assayed by seeded agar plate technique [15]. *T. longibrachiatum* EF5 was grown in PD broth for 10 days and the filtrate was obtained by separating the mycelial mat through Whatman filter paper. The filtrate was then passed through 0.2µm filter. The non-volatile metabolite was mixed with warm PDA medium at 25 per cent concentration and plated in Petri plate. The pathogen load was adjusted to 0.1 OD_{600} and 100µl was poured on each agar well. PDA medium without metabolites served as control. These plates were maintained at 28±2º C till colony in control plate was visible. Three replications with completely randomized design were maintained.

2.5 Efficacy of Extracted Crude Metabolites

T. longibrachiatum EF5 was grown in PD broth for 10 days and the filtrate was obtained by separating the mycelial mat through Whatman filter paper. The filtrate was then passed through 0.2µm membrane filter. Then it was mixed with equal volume of ethyl acetate and kept in shaker

overnight. The mixture was placed in a separating funnel, and allowed to stand until the aqueous and organic phases are separated. The organic phase was collected from the separating funnel. Crude metabolite was separated in a vacuum flask evaporator using methanol. Approximately, 100µL of *X. oryzae* pv. *oryzae* culture (OD_{600}=0.1) was spread on a PDA plate. Four uniforms wells were drilled using sterile cork borer and 50 µl of crude metabolite was placed in each agar well, incubated at 28±2º C. The formation of zone around the well was measured [17].

3. RESULTS AND DISCUSSION

3.1 Diffusible Soluble Metabolites Conferring *Xoo* Pathogenicity

In the present study, we profiled the soluble metabolites and diffusible soluble factors responsible for virulence in *Xanthomonas oryzae* pv. *oryzae.* The soluble metabolites or diffusible soluble factors (DSF) responsible for pathogenicity were investigated in GC-MS. More than 40 compounds produced by *Xoo* cultured in PDB medium were detected; nevertheless, PDB medium can produce many compounds likewise. The same compounds produced by *Xoo* and PDB medium were deducted, and 33 compounds specifically produced by *Xoo* were identified, including acids, alcohols, ketones, benzene derivatives, and esters. The metabolites present in *Xoo* were indicated in Table 1.

Among the DSF compounds mentioned above, butyrolactone involved in quorum sensing and biofilm formation. Compounds such as 1H-indene [18], nonanal [19] and phenyl acetic acid [20] whereas Picoxystrobin is commercially using as a fungicide. Hence the pathogenicity and virulence factors of *Xoo* have to be quenched down by an appropriate antagonist. The results expedited key metabolites such as benzoic acid and other benzene derivatives. The yellow pigmentation of *Xoo* was due to xanthomonadiins which is produced from 3-Hydroxybenzoic Acid and 4-Hydroxybenzoic acid. Previous reports suggest that hydroxyl benzoic acid is one of the virulent factors [21] for *Xanthomonas* and it also protects the bacteria from photooxidative damage [22]. The results of the investigation also confirm the presence of Xanthomonadiin biosynthetic pathway and the strain was virulent. During pathogenesis, *Xanthomonas campestris* pv. *manihotis* produced blight inducing toxin 3(methylthio) propionic acid (MTP acid) in cassava leaves. The necrotic tissues also contain sulphur containing compounds thiopropionic acid is more volatile than MTP acid [23]. Likewise, the present study reported that identification of Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), in the metabolites of Xoo expected that these compounds may be derivative to MTP. Similar compounds identified by Noda et al. [24] in ethyl acetate extract of Xoo were 3-methylthioproprionic acid, trans-3-methylthioacrylic acid, phenylacetic acid, isovaleric acid, succinic acid and fumaric acid. When the culture suspension was treated on rice leaves it induced necrosis and chlorosis at higher concentration (2000 µg/ml). The results, further confirmed the presence of succinimide, phenyl acetic acid and other phenolic derivatives.

Another phytotoxic compound produced by *X. albilineans* was albicidin which is the major pathogenicity factor to cause symptoms in sugarcane [25]. Similar to previous reports on virulence factors, *Xoo* also registered Picoxystrobin. Few soluble compounds such as 2is (2S, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate may act in signal transduction pathway and in inter and intra species communication as reported in *Vibrio harveyi* [26], whereas in *S. typhimurium* it is (2R,4S)- 2-methyl- 2,3,3,4-tetrahy droxytetrahydrofuran acts as auto inducer [27]. The results also revealed butyrolactone and other fatty acid derivatives, which are involved in cell to cell communication and bio-film formation. This fatty acid family regulates QS signaling and involved in DSF synthesis [28]. Numerous reports are available on fatty acid derived AHL 3-hydroxypalmitic acid methyl ester by Gram negative bacteria *Ralstonia solanacearum* [29]. Phenylacetic acid produced in *Xoo* culture is argued for depressive growth of young rice seedling roots as supported by Egawa *et al*., 1967. It can be concluded that the metabolites produced by *Xoo* were utilized for its growth, survival, cell-cell communication, signaling, virulence and pathogenicity [28].

In our investigation, we attempted to control *Xoo* pathogenicity by an endophytic antagonistic fungus *T. longibrachiatum* EF5.

3.2 *Trichoderma longibrachiatum* Ef5 on the Virulence of Xoo

The effect of strain EF5 volatiles on *Xoo* virulence was evaluated (Fig.1a, b). The results showed that the VOC blends emitted by EF5

exerted strongest inhibition on *Xoo* cells as evident by inverted plate assay. The pathogen was not grown in the VOC exposed plate whereas in control, yellow colony was observed. While, when the VOC exposure was stopped and kept for further incubation period, Xoo growth was initiated. Further, bipartite plate assay also demonstrated the VOC mediated growth inhibition of *Xoo*, where minute growth was observed. VOCs of *T. longibrachiatum* EF5 expressed strong inhibition activity in inverted plate assay. In the bipartite assay considerable reduction in growth was noticed. Since, both the methods were evaluated for its volatile mediated inhibition, this change in inhibition might be due to the diffusion of volatiles within the plate and its interaction with pathogen. In inverted plate, VOC from *T. longibrachiatum* EF5 directly interacted with the pathogen which was placed ventrally whereas in bipartite, the VOC has to diffuse through the plate to another compartment. The inhibition might be also due to changes in the VOC production by *T. longibrachiatum* EF5 on due course of time during the interaction. These volatiles inhibit the normal growth of pathogen with change in morphology and color of the pathogen. Similar result was revealed by Xie et al. [14] in which *B. ceres* D13 strongly inhibited *X. oryzae* pv. *oryzae* growth and reduced the motility and virulence. Co-cultivation of *Xanthomonas* sp. with D13 modified the cytoplasm with transformed exterior morphology under ultramicroscopic study. This may cause leakage of cell content and disrupt the normal physiological process. When *Xanthomonas* sp. was exposed to VOCs produced by other microorganisms in the soil might reduce the disease incidence and motility of the pathogen [30-33]. The high vapor pressure of volatiles made it to pass through the soil pores and air allows communicating in short and long distance. Research states that volatiles of *Trichoderma* have both antifungal [15,34] and antibacterial activity [35]. Our previous study stated that *Trichoderma longibrachiatum* EF5 produced VOCs such alcohols, esters, aldehydes, ketones and terpenes, more specifically longifolene, cedrane, caryophyllene and cuprenene (Sridharan et al. 2020). The result suggested that the synergistic action of VOC blend inhibited the growth of *X. oryzae* pv. *oryzae*. Nevertheless,

Single VOC is not much effective than VOC blends. The bacterial pathogen *Xoo* was significantly inhibited by the soluble metabolites. Since, no growth was visible in the agar well showed that soluble metabolites suppressed the growth of the pathogen by direct interaction (Fig.1c). The crude metabolite of *T. longibrachiatum* EF5 expressed a halo zone of 8 mm around the agar well by suppressing the growth of pathogen. The halo region is the indication of direct action of crude metabolite against *Xoo* (Fig. 1d). Many antagonistic bacteria inhibited the *X. oryzae* pv. *oryzae* such as fluorescent *Pseudomonas* [36], *Bacillus* sp., *B. subtilis*, *Pseudomonas putida* and *Enterobacter* sp. [37].

Table1. Non-volatile compounds profiled in *Xanthomonas oryzae* pv. *Oryzae*

RT	Compound	Area	RT	Compound	Area
3.314	Butyrolactone	0.716	7.265	1H-Indene, 1-methylene-	1.75
3.514	Desulphosinigrin	0.267	7.375	Dodecane	4.289
3.556	Galacto-heptulose	0.268	12.297	2,4-Di-tert-butylphenol	1.632
3.779	Ethyl cyanoacetate	0.275	12.572	Benzoic acid	0.331
3.959	Benzaldehyde	0.482	12.687	2(3H)-Furanone, dihydro-5-phenyl-	0.483
4.123	Phenol	0.381	13.132	Pentadecane, 5-methyl-	0.366
4.469	Decane	0.469	16.514	3-Methyl-1,4-diaza bicyclo[4.3.0]nonan-2,5-dione,N-acetyl-	0.334
5.134	Benzyl methallyl sulphide	0.368	17.919	Heptadecane, 3-methyl-	0.391
5.134	Benzeneethanamine	0.371	20.756	Diethyltrisulphide	1.264
5.314	Ethanone, 1-(1H-pyrrol-2-yl)-	0.348	20.921	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	2.112
5.377	3-Acetyl-1H-pyrroline	0.501	21.645	l-(+)-Ascorbic acid 2,6-dihexadecanoate	2.513
5.975	Nonanal	0.528	21.661	n-Hexadecanoic acid	2.45
6.140	Benzaldehyde dimethyl acetal	0.304	24.852	Picoxystrobin	0.928
6.205	Phenylacetic acid, cyclobutyl ester	0.622	25.013	L-Ascorbic acid, 6-octadecanoate	0.791

RT	Compound	Area	RT	Compound	Area
6.405	Pyrimidine	0.321	25.027	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	0.494
6.424	Succinimide	0.345	25.407	Dasycarpidan-1-methanol, acetate (ester)	0.787
6.725	Cycloheptatrienone	0.639	34.756	Cyclohexanol	0.862

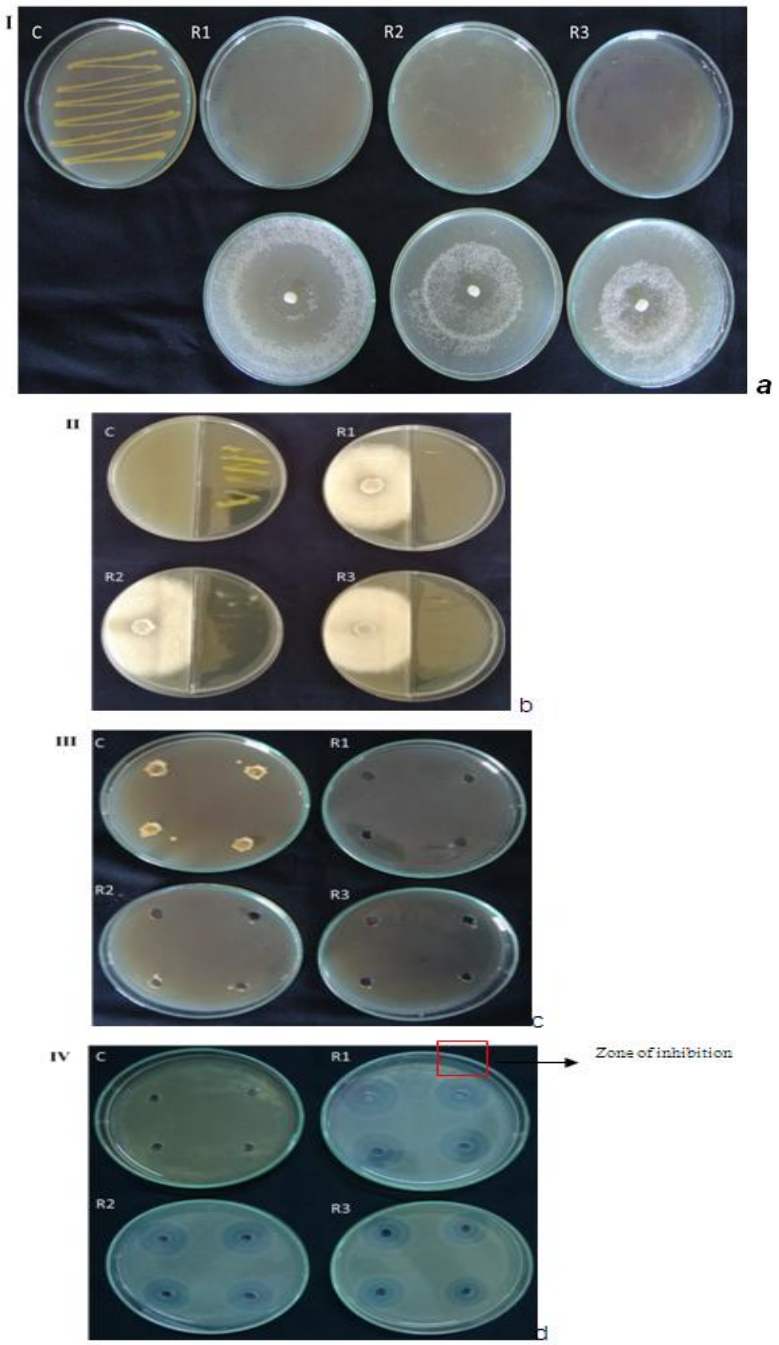

Fig. 1. Effect of volatile and soluble metabolites of *Trichoderma longibrachiatum* EF5 against *Xanthomonas oryzae* pv. *Oryzae*

Inhibition of Xanthomonas oryzae pv. oryzae growth in (a) inverted plate assay – complete growth inhibition by VOCs, (b) bipartite plate assay – restricted growth mediated by VOCs, (c) seeded agar assay –growth inhibition by soluble metabolites, (d) crude metabolite well assay – growth inhibition and formation of halo zone

4. CONCLUSION

In the present investigation, Thus, volatile and soluble metabolites from *T. longibrachiatum* EF5 might act as fumigant, thereby suppressing Xoo growth. Based on the metabolite concentration the growth of the pathogen was completed inhibited in crude metabolite but unmeasurable growth was observed in culture filtrate. Hence it can be concluded that, there is great potential of developing biological bactericide with the endophytic fungi *T. longibrachiatum* EF5 and its metabolites for management of bacterial leaf blight disease of rice.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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