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INVITRO STUDIES ON PRODUCTION OF IAA, PHOSPHATE SOLUBILIZATION AND VOLATILE ORGANIC COMPOUNDS BY *TRICHODERMA* SPECIES

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ABSTRACT

Trichoderma is a potential biocontrol agent that is widely used by farmers worldwide. In this study, we selected four stress-tolerant and chitinase-producing Trichoderma isolates (VT-8, VT-14, VT-15 & VT-17) to analyses their ability to produce plant growth promoters, their phosphate solubilizing ability, and their volatile organic compound availability. We used qualitative and quantitative methods to analyses the invitro production of plant growth promoters. The formation of dark red to pale yellow colour indicated the production of indole acetic acid. Among the four Trichoderma isolates, VT-17 exhibited a dark red colour, indicating the highest indole acetic acid production. Quantitative analysis confirmed that VT-17 produced 79.86±0.12 µg/ml of indole acetic acid. All four isolates were analyzed for their phosphate solubilizing ability using qualitative and quantitative methods. The formation of a clear zone indicated the presence of phosphate solubilizing activity. Trichoderma isolate VT-17 showed the highest activity with a clear zone of 1.17 cm and a quantitative analysis result of 9.25 µg/ml. We selected Trichoderma isolate VT-17 for GC-MS analysis based on their antifungal activity studies. The analysis revealed the presence of seven volatile organic compounds that have been reported to exhibit biocontrol activity. Therefore, Trichoderma isolate VT-17 could be used as a biocontrol agent.

Key words: Trichoderma, Biocontrol agent, Indole acetic acid, Phosphate solubilizer.

INTRODUCTION

Trichoderma is a widely used genus of filamentous fungi that plays an important role in agriculture. Itis a fast-growing plant beneficial microbe found mostly in the soil with excellent survival ability in unfavorable conditions. This fungus was known for its ability to promote plant growth, decompose organic matter, and control plant diseases (Benítez *et al*., 2004; Harman, 2006; Woo *et al*., 2023). It is also an effective antagonist against plant pathogenic fungi. Studies have shown that *Trichoderma* can be effectively used for bioremediation purposes as well (Oskiera *et al*., 2017).

Volatile organic compounds (VOCs) are small carbon-based chemicals with low molecular weight, polarity, and high vapor pressure. They are produced by beneficial fungi and have significant biological and ecological potential. *Trichoderma* species can produce VOCs, which are associated with anti-microbial activity, defense response against plants, and promotion of plant growth (Wonglom *et al*., 2020; Phoka *et al*., 2020; Mukherjee *et al*., 2022; Vicente *et al*., 2022). The volatile compounds produced by *Trichoderma* have a direct inhibitory effect on the growth of pathogens and cause abnormal variations in their interaction with pathogens by mechanisms such as mycoparasitism, antibiosis, and competition. Therefore, the identification and screening of novel *Trichoderma* strains that produce volatile compounds are considered important.

Our previous studies have shown that out of 18 *Trichoderma* species isolates, the four most active ones (VT-8, VT-14, VT-15, and VT-17) exhibit effective biocontrol activity against *Rhizoctonia solani* (Muthuvaduganathan et al., 2023). Therefore, we have selected these four species isolates for further analysis of their volatile organic compounds, their ability to promote the plant growth, and their *invitro* biocontrol activity against *R. solani*.

MATERIALS AND METHODS

Estimation of indole acetic acid

Qualitative analysis

The production of IAA *invitro* is measured using the procedure developed by Yadav et al (2011). *Trichoderma* isolates are cultured on Czapek-Dox broth media that are supplemented with L-tryptophan at a concentration of 1ml/L and then incubated at room temperature for 7 days. The culture broth (5 ml) centrifuged at 3000 rpm for 5 minutes, 1 ml of the supernatant is added to 3 ml of Salkowski reagent and incubated for 30 minutes. If there is a color change from yellow to red, it indicates the presence of IAA.

Quantitative analysis

To measure the production of IAA under *invitro* conditions, we followed the procedure used by Yadav et al. (2011), with slight modifications. *Trichoderma* isolates were cultured on Czapek-dox broth media and incubated at room temperature for 7 days. The filtrate from the fungal culture was used as a crude enzyme extract. The reaction was measured at wavelength of 530 nm by mixing 1.5 ml of the enzyme extract with 3 ml of Salkowski reagent, and then incubating for 30 minutes.

Qualitative analysis of phosphate solubilization

To determine the ability of *Trichoderma* isolates to solubilize phosphate, they were cultured on Pikovskaya (PVK) media as described by Zehra et al. (2017). Four isolates were grown on PVK media without glucose at room temperature. The formation of a clear zone within 2 to 10 days was used to determine the phosphate solubilizing activity of *Trichoderma* isolates. The solubilization index was calculated using the following formula:

PSI = (Colony diameter + Halo diameter)/Colony diameter

Quantitative analysis of phosphate solubilization

The quantitative estimation of phosphate solubilization by *Trichoderma* isolates were carried out using the ascorbic acid method developed by Watanabe and Olsen in 1965. The isolates were grown on PVK media for 14 days. Phosphatase was extracted from the culture by treating it with ammonium bicarbonate-diethylene triamine penta acetic acid. The enzyme reaction was initiated by adding 1 ml of *Trichoderma* extract to 9 ml of water and 1.6 ml of a coloring agent consisting of 5N sulfuric acid, 0.324 g of K(SbO)C4H4O6.1/2H2O, 4 g of (NH4)2 Mo7O24, and 1.76 g of ascorbic acid. The reaction mixture was then incubated for 5 minutes and the activity was measured using spectrophotometer at a wavelength of 880 nm. The amount of phosphorus in the growth media was determined relative to potassium dihydrogen phosphate, as described by Desbois and Smith in 2010.

GC-MS analyses of volatile compounds

Plugs of *Trichoderma* isolates (VT-17) measuring around 6 mm were added to a 250 ml Erlenmeyer flask containing 100 ml of PDB medium. The mixture was then incubated in a shaker for 7 days at 25 ±1°C with 150 rpm. To prevent the escape of VOC, the conical flask was sealed with tin foil. To identify the fungal volatile organic compounds, a 65-µm PDMS/DVB fiber tip was used. The incubated culture flask was placed in a water bath at 40°C, and the sample was adsorbed and extracted for 30 minutes. After extraction, the fiber head was retracted, and the needle was immediately removed and inserted into the gasification chamber in the gas chromatograph (Agilent 7000B, USA). The high-temperature chamber was used to identify the volatile organic compounds for 3 minutes. The GC-MS conditions included a Rtx-5 quartz capillary column, helium as the carrier gas, 230°C inlet temperature, initial temperature of 40°C for 3 minutes, increased at 10°C per minute to 95°C, increased at 30°C /minute to 230°C, and maintained for 5 minutes. Electron energy of 70eV was used, and the spectrum search was conducted at Nist 05 and Nist 05 library (Kong et al., 2020).

STATISTICAL ANALYSIS

RESULTS

In this study, four *Trichoderma* isolates (VT-8, VT-14, VT-15, and VT-17) were analyzed for their ability to produce the plant growth hormone, indole acetic acid. The study also examined their phosphate solubilizing ability using both qualitative and quantitative methods under *invitro* conditions. The most active *Trichoderma* isolate, based on its indole acetic acid production and phosphate solubilizing activity, was further used to analyze its volatile organic compounds by GC-MS analysis.

Indole acetic acid production

The study revealed that all four *Trichoderma* isolates exhibited different concentrations of indole acetic acid production, which was represented by varying shades of brown color. The darker the color, the higher the production of indole acetic acid is inferred. Among the four isolates, VT-17 showed the highest production of indole acetic acid, which was represented by the darkest brown color. The other *Trichoderma* isolates, VT-14, VT-15, and VT-8, exhibited moderate and mild color changes.

The quantity of growth-promoting hormone indole acetic acid produced by four different *Trichoderma* isolates (VT-8, VT-14, VT-15, and VT-17) was analyzed using an *invitro* spectrophotometer method. The study found that all four *Trichoderma* isolates exhibited variations in the concentration of indole acetic acid production. *Trichoderma* isolate VT-17

produced the maximum concentration of 79.86±0.12 µg/ml of indole acetic acid, while *Trichoderma* isolates VT-15, VT-14, and VT-8 produced 49.59±0.21 µg/ml, 32.75±0.19 µg/ml, and 9.51±0.15 µg/ml of indole acetic acid, respectively.

Phosphate solubilization

The phosphate solubilizing index was observed for 2, 4, 6, 8, and 10 days of incubation. The highest solubilization was recorded as 1.17±0.04 cm on the 6th day of incubation for VT-17, whereas the lowest solubilization was observed as 1.09±0.12 cm on the 6th day of incubation for VT-8. The zone of solubilization increased with the duration of incubation and decreased once the maximum zone of solubilization was attained **(Fig. 1)**. The phosphate solubilizing activity of four *Trichoderma* isolates (VT-8, VT-14, VT-15, and VT-17) was measured using the colorimetric molybdate blue method. The analysis was conducted after 14 days of incubation in Pikovskaya media. The results showed that all four *Trichoderma* isolates had a phosphate solubilizing activity ranging from 0.92µg/ml to 9.25µg/ml. The highest phosphate solubilizing activity was observed in *Trichoderma* isolate VT-17 with 9.25µg/ml, followed by VT-15, VT-14, and VT-8 with 5.12µg/ml, 3.51µg/ml, and 0.92µg/ml, respectively.

Identification of volatile organic compounds by GC-MS chromatography

The GC-MS analysis identified chemical constituents, such as volatile organic compounds, in *Trichoderma* isolate VT-17. Seven compounds were found in the isolate, namely limonene, isobutyric acid, beta-farnesene, caryophyllene, pentacosane, 6-pentyl-2H-pyran-one, and beta-myrcene, with different peak percentages and retention times. All these constituents are reported to exhibit biocontrol activity against various fungal pathogens (Fig.2 Table 1)

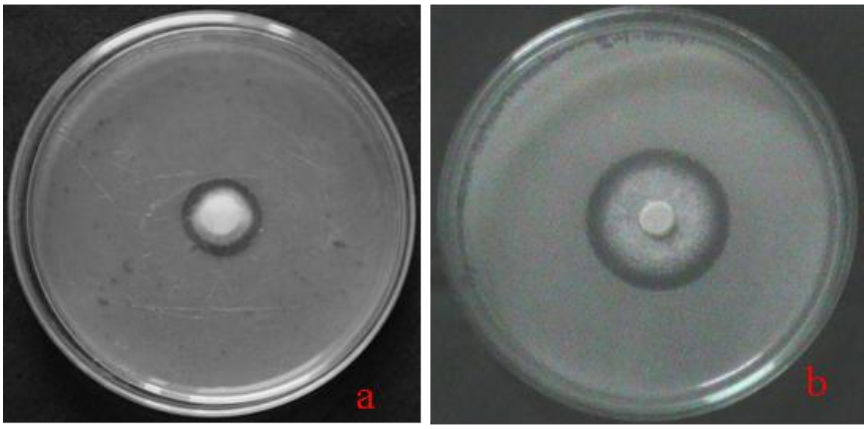

Figure 1: Phosphate solubilizing activity of *Trichoderma* isolate VT-15 (a) & VT-17 (b)

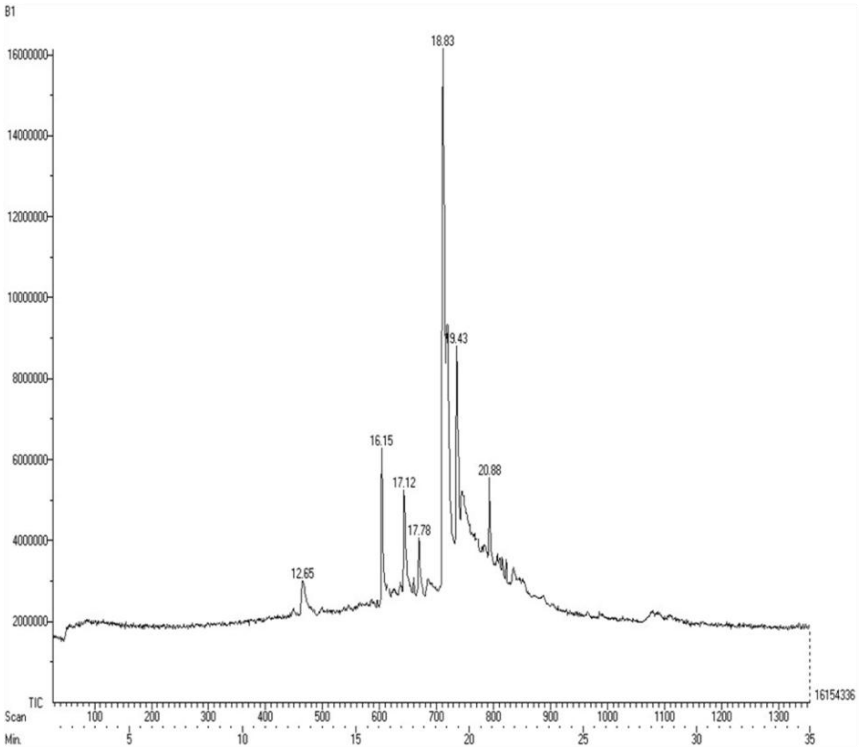

Figure. 2 GC-MS analysis of volatile compound of *Trichoderma* VT-17

Peak	Chemical name	Retention time	Molecular formula	Activity	Reference
1.	Limonene	12.65	$C_{10}H_{16}$	Biocontrol activity against *Fusarium Graminearum*	Yunqing Jian *et al*., (2023)
2.	Isobutyric acid	16.15	$C_4H_8O_2$	Mycelial growth inhibition against various fungal pathogens	Saxeena *et al*., (2014)
3.	Beta farnesene	17.12	$C_{15}H_{24}$	Acts as an alarm pheromone in aphids	Stoppacher *et al*., (2010)
4.	Caryophellene	17.78	$C_{15}H_{24}$	Attracts nematodes that preys on insect larvae	Rasmann *et al*., (2005)
5.	Pentacosane	18.83	$C_{25}H_{52}$	Antibacterial activity	Kaplan *et al*., (2020)
6.	6-pentyl-2H-pyran-2-one	19.43	$C_{10}H_{14}O_2$	Mycelial growth inhibition against *Fusarium oxysporum*	Rao *et al*., (2022)
7.	Beta myrecene	20.88	$C_{10}H_{16}$	Mycelial growth inhibition against various fungal pathogens	Suwannarach *et al*., (2013)

Table 1: Volatile organic compounds observed by GC-MS analysis of *Trichoderma* isolate VT-17

DISCUSSION

Eighteen *Trichoderma* isolates were gathered from arid and semi-arid regions across sixteen districts in India. These isolates were put to the test for their biocontrol activity against various fungal pathogens and their ability to tolerate stress factors such as temperature, pH, salinity, and drought. While high temperature tolerant *Trichoderma* isolate is also the need of the hour for successful application in crop protection (Poosapati et al. 2014). In this regard, *T. asperellum* BHUT8 was showed Despite significant advances, challenges remain, such as the pH sensitivity of myclial growth upto 38 ºC while 30 ºC most favorable temperature of its growth. *Trichoderma*, which limits enzyme activity (Liliana Villao-Uzho *et al*., 2024). Salinity induces nutrient deficiencies, membrane disfunction, dehydration and oxidative stress which cause tissue damage and early senescence (Essah *et al*. 2003). *T. asperellum* BHUT8 has a great potential to adapt under challenging condition such as high salinity and temperature. Adaptation of a biocontrol agent to a variety of stressful conditions is useful for managing crop health under stressed environment. abiotic stresses such as extreme light, drought, high salinity and temperature cause heavy losses in crop productivity throughout the globe (Jithesh *et al*., 2006; Chitara *et al*., 2017). Similarly, reported *Trichoderma* strains with highest growth rate at 37 ºC They were also screened for their effect on non-volatile compounds and chitinase activity. Based on the study, isolates VT-8, VT-14, VT-15, and VT-17 were found to be the most efficient *Trichoderma* isolates by exhibiting the best activities mentioned above (Muthuvaduganathan *et al*., 2023). These four isolates were chosen for further analysis based on a previous report, which included their ability to produce the plant growth promoter IAA, phosphate solubilization, and the presence of volatile organic compounds.

IAA production

A plant growth-promoting hormone called auxin indole acetic acid was found at higher concentrations in *Trichoderma* isolates VT-17. This was determined through a qualitative analysis that showed the production of a dark brown color. A similar result was observed in another study where *T. virens* was found to have the ability to produce indole acetic acid through a qualitative method in Czapek dox broth media (Inayathi et al., 2021). In terms of quantitative analysis, VT-17 produced 79.86±0.12 µg/ml of indole acetic acid. A similar result was observed in another study reported by Inayathi et al., (2021), where *T. virens* (TV-7) produced a maximum of 71.5 µg/ml. Bader et al., (2020) reported a maximum concentration of 21.14 µg/ml and a minimum of 7.19 µg/ml IAA. Among the *Trichoderma* spp. Ng et al., (2015)reported maximum production of 93.73 µg/ml of indole acetic acid.

This study found that the production of indole acetic acid (IAA) depends on the type of strain used and on external factors associated with IAA production. L-tryptophan is a major precursor and plays a crucial role in IAA production (Inayathi et al., 2021). Other external factors such as media composition (carbon, nitrogen, temperature, and pH sources), also affect IAA production by *Trichoderma*.

IAA is a major plant hormone that supports the growth and development of roots in plants. It is produced by both plants and microbes. IAA produced by microbes activates plant growth directly or indirectly by regulating the expression of gibberellic acid in plants. Based on the above studies, it is possible that *Trichoderma* isolates VT-17 can produce IAA and could express its biocontrol activity against fungal pathogens, as well as enhance the growth and development of plants.

Phosphate solubilization

The ability of *Trichoderma* isolates to solubilize phosphate was evaluated through qualitative and quantitative analysis. The highest solubilization of phosphate was observed on the 6th day of incubation of *Trichoderma* isolate VT-17 with 1.17±0.04 cm. The study also found that supplementing inorganic phosphate in PVK media enhances the utilization of phosphate by *Trichoderma* isolates. *Trichoderma* species and other microbes can utilize phosphate by producing phosphatase and phytase, which can hydrolyze organic phosphate in the medium and convert them into soluble inorganic forms of phosphate as ortho phosphate. This makes phosphate readily available and easily absorbed by root plants. (Alam et al., 2022; Chagas et al., 2017; Hidayat et al., 2006; Promwee et al., 2011).

The ability of *Trichoderma* isolates to solubilize phosphate varies depending on the type of *Trichoderma* strain used and the length of the incubation period. Incubating for a shorter period showed higher phosphate solubilization activity. In a study by Bonini et al. (2020), a 10 mm zone of clearance was exhibited with a shorter incubation time. It also found to solubilize 9.25 µg/ml of phosphate. Thus, *Trichoderma* isolate VT-17 was found to be an efficient phosphate solubilizer through both qualitative and quantitative methods. However, there is still no explanation of the mechanism of phosphate solubilizing microbes and their relationship with the suppression of pathogen survival. Several reports suggest a positive correlation between phosphate solubilizing ability and the production of plant growth promoters in plants, as well as the effective reduction of pathogen survival (Chagas et al., 2017; Bader et al., 2020).

Identification of volatile organic compounds by GC-MS

Trichoderma isolate VT-17 has been found to produce seven volatile organic compounds that exhibit *in vitro* fungal growth inhibition against soil-borne, air-borne and postharvest pathogens. These low molecular weight volatile organic compounds produced by the growing VT-17 isolate are diversely distributed and exhibit anti-fungal activity. One of the compounds identified in *Trichoderma* isolate VT-17, named 6-pentyl-2H-pyran-2-one was found to arrest the mycelial growth of fungal pathogens. An endophytic fungus *T. asperelloides* PSU-P1 released a major volatile compound, 6-PP, which exhibits antifungal activity (Phoka et al., 2020). Pyrone derivatives were observed in *Trichoderma viride, T. atroviride, T. harzianum,* and *T. koningii,* which were reported to exhibit antifungal activity against *R. solani, F. oxysporum, Botrytis* spp., *Penicillium* spp., *Aspergillus fumigatus, Candida albicans*, and *Cryptococcus neoformans* (Garnica-Vergara et al., 2015). Beta-caryophyllene and beta-farnesene were two sesquiterpene derivatives observed in *T. virens* and *T. atroviride* (Kunert et al., 2005; Rasmann et al., 2005). The activities of all seven compounds observed in *Trichoderma* VT-17 were tabulated (Table 1). Volatile organic compounds observed in *Trichoderma* VT-17 were found to possess biocontrol and other pharmacological activities.

CONCLUSION

According to this study, among the four isolates of *Trichoderma*, VT-17 and VT-15 are the most effective producers of plant growth hormone, IAA. Additionally, they can solubilize and utilize phosphate.Seven volatile compounds recorded in the study were found to have the biocontrol ability. Further greenhouse and field studies are needed to explore the potential benefits of using the VT-17 isolate. These findings could be used to promote the beneficial effects of *Trichoderma* consortium treatment in farmers' fields.

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