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Plant Growth Promotion Efficacy of Indole Acetic Acid (IAA) Produced by a Mangrove Associated Fungi-*Trichoderma viride* VKF3

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A B S T R A C T

The microorganisms have been widely used for phytohormone production and in bio-control of fungal pathogens. In the present study, *T. viride* VKF3, a mangrove isolate was used for production of auxin IAA under in vitro condition. *T. viride* VKF3 yielded a high IAA production of 115 µg mL^{-1} with 0.5% L-tryptophan. The IAA produced by the isolate was confirmed through Thin layer chromatography (TLC) and High performance liquid chromatographic (HPLC) analysis by comparison with standard IAA. The isolate was also capable of producing extracellular enzymes like cellulase, xylanase along with siderophore production which conditions soil and bioavailability of nutrients. The culture filtrate exhbibited plant growth promotional activity along with enhanced germination percentage. *T. viride* VKF3 exhibited a maximum inhibition of 82 and 94% was achieved by against fungal pathogens *F. oxysporum* and *A. niger* respectively. This report shows a high IAA production using *T. viride* VKF3 with bio-control potential.

Introduction

Indole acetic acid (IAA) is one of the major physiologically active auxins, a product of L-tryptophan metabolism produced by several microorganisms including Plant Growth Promoting Rhizobacteria (PGPR) (Lynch, 1985). The physiological function of IAA includes the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake (Datta and Basu, 2000). Apart from these, IAA is also the principle agents that regulate plant response to changes in environmental conditions (Tuteja, 2007; Malhotra and Srivastava, 2009). The IAA is widely produced by many microorganisms which are mainly dwelling in the rhizosphere of plants. Although IAA in yeast extracts was discovered, its production by filamentous fungi is relatively new. Moreover, reports on the production of phytohormones using fermentation are sparse especially the IAA synthesized by fungi (Yürekli *et al.*, 1999; Chung *et al.*, 2003). Development of biotechnology had evolved new prospects in production of plant growth hormones using fungi (Berry, 1988). Recently, there are many reports upcoming on fungal IAA production. Ünyayar *et al.*, (2000) and Ünyayar (2002), reported exogenous IAA production by *Phanerochaete chrysosporium* strain ME446

and *Funalia trogii*. IAA production is mainly dependent on L-tryptophan which acts as a physiological precursor. The microorganism such as Streptomyces, Pseudomonas and Bacillus were found capable of synthesising IAA by utilizing L- tryptophan through indole -3- pyruvic acid pathway (Patten and Glick, 1996; Shanmugaiah *et al.,* 2009; Charulatha *et al.,* 2013). Though advancement in researches finally obtained many fungal isolates with IAA production ability, their physiological role remains unclear. One of the roles suggested for production of IAA by fungus is to mediate fungal–plant interaction. A high concentration of IAA inhibited the hypersensitive response (Robinette and Matthysse, 1990; Jouanneau *et al.,* 1991) and suppressed the expression of plant defense genes (Yamada *et al.,* 1985; Shinshi *et al.,* 1987). The present study focuses on production and characterization of IAA produced by the isolate *T. viride* VKF3. The plant growth promotional activity as well as its biocontrol efficacy against the fungal pathogens were also evaluated.

Materials and Methods

Isolation and screening for IAA production

Sediment samples were collected along with plant debris from Valanthakad Mangrove ecosystem, Kerala, India. Serial dilution was performed (upto 10^{-6}) and plated onto potato dextrose agar medium. The inoculated plates were incubated at 28±2°C for 3-5 days. Distinct fungal colonies were purified using spot inoculation. The IAA producing ability of the isolates were screening using the basal medium supplemented with 0.5 $mgmL^{-1}$ of L –tryptophan. Positive strains were cultured in submerged fermentation culture and incubated at 28±2°C under shaking condition (120 rpm) for 3 days and the culture broth was retrieved after incubation period for IAA quantification.

IAA production using *Trichoderma viride* VKF3

Following the incubation time, culture was centrifuged and the supernatant was mixed with Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled water, 7.5 ml of 0.5 M$FeCl_3 \cdot 6H_2O$) with a 1:2 (v/v) ratio, and was allowed to stand at room temperature for 20 min. The pink color developed, indicating IAA production, was measured at 530 nm with a spectrophotometer (Double beam UV-Vis Spectrophotometer 2022). Concentration of IAA was calculated using a standard curve prepared with standard IAA. The experiment was carried out in triplicate and values were expressed as mean ± S.D unless otherwise mentioned. The effect of L-tryptophan concentrations on IAA production was studied using potato dextrose broth medium supplemented with L-tryptophan at concentrations of 0.1, 0.5, 1, and 1.5%. The culture was incubated at 28°C in a shaker at 120 rpm for 7 days.

Extraction and Characterization of IAA

Crude IAA was extracted using extraction method described by Harikrishnan *et al.,* (2014). The fractions (10 - 20 micro litter) and standard IAA were placed on TLC plates (silica gel G f254 thickness 0.25mm). TLC was run by using solvent system benzene: n-butanol: acetic acid in 70:25:5 proportion and spots were detected by spraying the plates using Salkowaski reagent. Rf value of the standard and IAA produced by the isolate was calculated. Partial purification of Indole Acetic Acid from crude extract was done by using silica gel column chromatography and fractions were collected with solvent system ethyl acetate and hexane (20:80 v/v). Each fraction was tested in thin layer chromatography and then developed with Salkowski reagent. HPLC analysis of IAA was carried out usig the method described

earlier by Harikrishnan *et al.*, (2014). Briefly, on a C18 column (5µm; 25 x 0.46cm) by using HPLC grade acetonitrile- water system containing 0.1% trifluoroacetic acid was programmed over 30 min at a flow rate of 0.5mL/min with UV detector at 220 nm at 40°C. Mobile phase consisted of methanol and water (80: 20 v/v) run at flow rate was analysed by comparison with the elution profiles of those authentic IAA injected separately.

Screening for extracellular enzyme and siderophore production

All fungal isolates were checked for their ability to produce cellulase enzyme on PDA media supplemented with 2% carboxy methyl cellulose (CMC) (Lingappa and Lockwood, 1962)**.**Cellulolytic fungi exhibited a clearing zone around the colony when medium was supplemented with 0.2% congo red and destained with 1M NaCl solution after incubation for 3 to 5 days. Assay for positive isolates were performed to select the isolate with highest cellulolytic activity using potato dextrose broth under similar growth conditions using the standard method described in the following section. Xylanase screening for fungal isolate was performed on PDA plates supplemented with 2% birch wood xylan (BWX) and the incubated plates were flooded with iodine solution to observe clear zone around positive colonies (Charitha and Sunil, 2013).

Lipase screening for fungal isolates was performed using PDA plates supplemented with 1% olive oil and 0.5% phenol red indicator. Lipase enzyme degrades oil to release free fatty acids which changed the medium pH and the medium colour changed from red to yellow. Laccase activity was assessed by a modified method proposed by Hankin and Anagnostakis (1975). The fungi were grown on PDA agar medium amended with 0.5% tannic acid and incubated for 3-5 days at 28±2ºC. On oxidation of tannic acid by laccase, the medium changed a reddish brown. Siderophore production was screened by method described by Harikrishnan *et al.*, (2014).

Plant growth promotion activity

Seeds of *Vigna radiata*, *Vigna mungo* and *Sesamum indicum* were treated with culture supernatant containing IAA for 30 min. These seeds were air dried and sown into pots containing sterile soil. Seeds treated with distilled water served as control. Various parameters like germination percentage, shoot and root length as well as dry and fresh of the plantlets after 1 week growth was recorded.

Antagonistic Effects

For understanding the antagonistic effect against the pathogenic fungi, two discs (6mm in the diameter) of one week old culture on (PDA), one carrying the stock of the antagonistic agent (*Trichoderma viride*)and the other the pathogenic agent were then placed at the periphery of Petri plate at the same distance on PDA medium. One disc of each pathogenic agent was maintained as control. Each replicates has three plates. The inoculated plates were incubated at 25°C for four days, and measurement of radial mycelia of the fungus were taken every 24 hrs. The percentage growth inhibition (I) was calculated using the formula I (%) = (1 – T /C) x 100, where: I=Percentage inhibition of pathogen by antagonists. C=Radial growth in control, T=Radial growth in the treatment.

Results and Discussion

Isolation and screening of actinomyctes

In this study, about 15 different fungal colonies were obtained from mangrove

sediment samples. Among the same, 4 isolates were positive for IAA production. Based on the secondary screening (quantification of IAA), the VKF3 isolate was found to be potential for IAA production. The isolate VKF3 was identified as *Trichoderma viride* based on lactophenol cotton blue (LCB) mount staining, colony morphology and molecular identification using D1-D2 region amplification (Nathan *et al.,* 2014). The fungal isolates produced many phytohormones which are essential for plant – fungus interaction including the mechanism of pathogenesis or symbiotic strategies, leading to plant growth promotion and basal plant defense mechanism modification (Fu *et al.,* 2015). Many *Trichoderma* species were able to produce the auxin phytohormone indole-3-acetic acid (IAA), and its production has been suggested to promote root growth. Nieto-Jacobo *et al.,* (2017) reported *Trichoderma sp*. namely *T. virens* Gv29.8, *T. atroviride* IMI206040, *T.* sp. "*atroviride B"* LU132, and *T. asperellum* LU1370 with ability to synthesize volatile plant gwoth promoting factors. Similarly, *T. harzianum* was found to enhance root growth and plant development which was attributed to the unidentified growth-regulating factors by the fungus (Windham *et al.,* 1986; Harman *et al.,* 2004).

Effect of L- tryptophan concentration on IAA production

IAA production in microbes is mainly mediated through indole-3-pyruvate acid and tryptamine which uses tryptophan as substrate (Tudzynski and Sharon, 2002). Tryptophan supplementation enhanced the biosynthesis of IAA by many folds by the increased availability of substrate. To evaluate the effect of tryptohan on IAA production, different concentrations of L- tryptophan between 0.1 to 1.5% were supplemented in the medium. The spectrophotometric analysis showed that gradual increase in the IAA production with respective L-tryptophan concentration (Fig. 1). The maximum IAA production observed by *T. viride* VKF3 was 115µgmL^{-1} when the medium was amended with 0.5% L-tryptophan. However, the very high concentration of L- tryptophan lowered IAA production which was supported by Harikrishnan *et al.,* (2014) who observed an adverse effect on IAA production by the isolate *Streptomyces sp* VSMGT1014 with high tryptophan concentrations. Difference in IAA production among fungal isolates was observed by many researchers. Yadav *et al.,* (2011) reported, IAA production using various fungal isolates like *Aspergillus niger* (85 µg mL^{-1}) and *T. harzianum* (68 µg mL^{-1}) and *Penicillium citrinum* (52 µg mL^{-1}) with 3 days of incubation at 30° C. The results observed in the present study were very high compared to these reports. The better IAA production was achieved at 28°C which was supported by Gunasekaran, (1978) and Hasan, (2002) who also observed a similar high IAA production trend at this incubation temperature. Similarly, IAA production using *Streptomyces sp* was also high at the optimal temperature range of 25-30° (Aldesuquy *et al.,* 1998; Khamna *et al.,* 2010). Moreover, Gravel *et al.,* (2007), also yielded a maximum IAA of only 6.2 µg mL^{-1} in the presence of 200 µg mL^{-1} tryptophan using *Trichoderma atroviride*. The tryptophan induced enhancement of IAA production was supported by previous reports also (Patten and Glick, 1996; Shanmugaiah *et al.,* 2009; Charulatha *et al.,* 2013).

Characterization of IAA

The IAA produced by *T. viride* VKF3 was characterized by TLC and HPLC analysis. As shown in Figure 2b, when the TLC plate was treated with Ehmann reagent, the ethyl acetate extract from culture filtrate showed a clear pink colour spot on the TLC plate at the Rf

value corresponding to standard IAA (0.62). Thin layer chromatography findings were in agreement with the previous reports (Ahmad *et al.*, 2005). Similarly, HPLC analysis was conducted to identify and quantify the IAA production precisely. As shown in Figure 2a, ethyl acetate extract from the culture filtrate of the strain and corresponding reference authentic standard showed peak at the similar retention time (4.967 min).

Extracellular Enzyme Production by Fungus

Extracellular enzymes produced by the fungal strains also help in conditioning of the soil for the better plant growth. Hence, we screened the extracellular enzyme production (qualitative). *T.viride* VKF3 was protential for cellulase, xylanse and laccase production (Table 1). However, there was no lipase activity observed. This hypothesis was in hand with a previous study (Jog *et al.*, 2012), where the extracellular enzyme produced by the Actinomycete isolates were found to be capabale of utilizing the nutrients and in turn exhibit PGP activities that were extremely beneficial for plants. Moreover, the isolate *T.viride* VKF3 was capable of siderophore production also. Anke *et al.*, (1991) reported the efficiency of *T. longibrachiatum* and *T. Pseudoko ningii for* producing siderophores under iron deficienct conditions. This was also supported by reports of Vinale *et al.*, (2013), who identified harzianic acid, a novel siderophore from *Trichoderma harzianum.*

Fig.1 Effect of L-tryptophan concentration on IAA production by *Trichoderma viride* VKF3

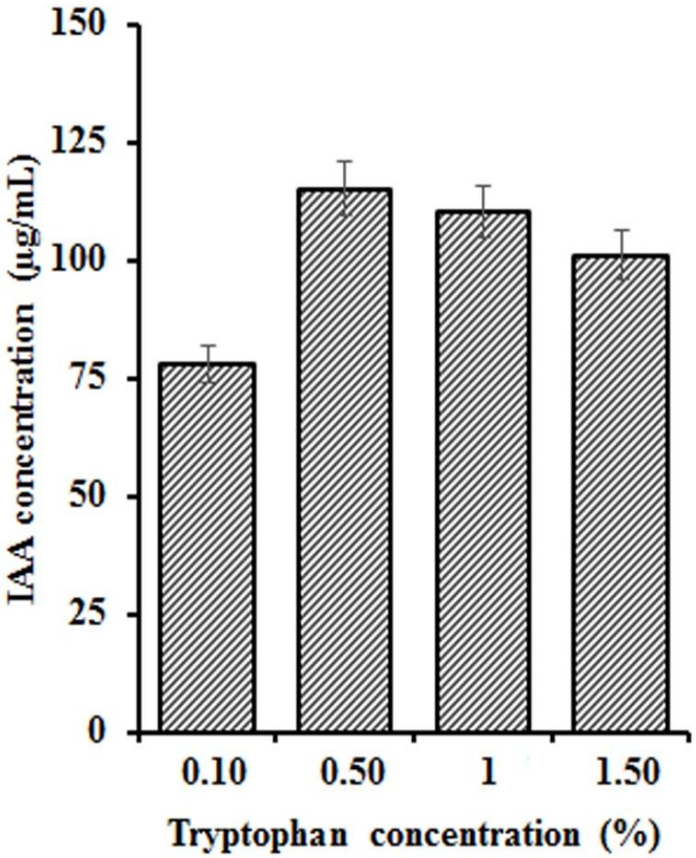
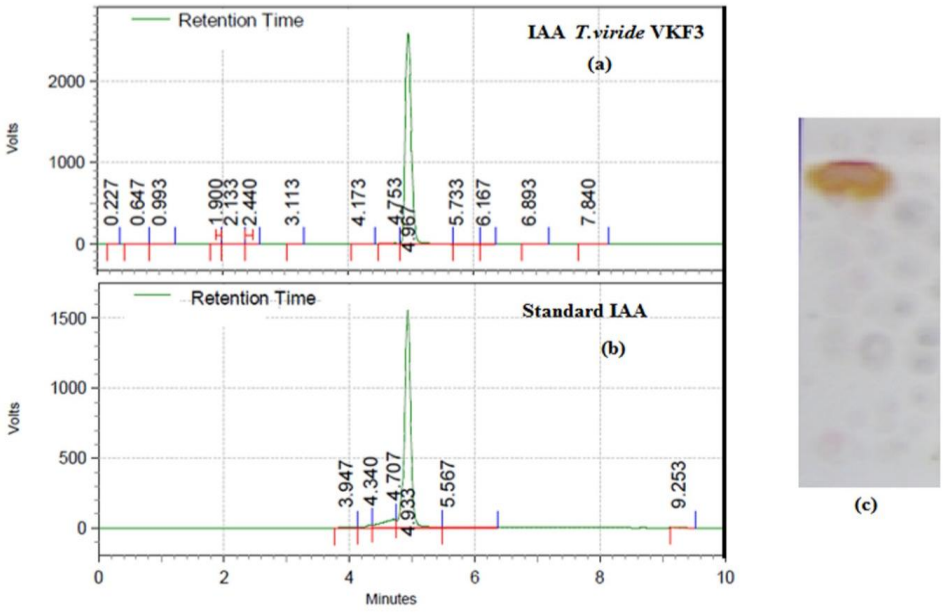
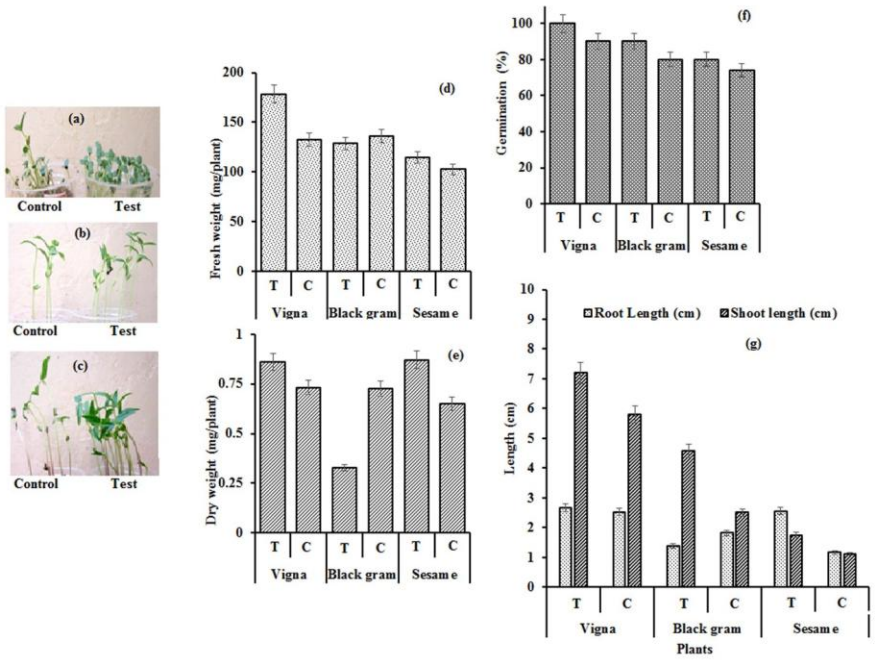


Fig.2 Characterization of IAA produced by *Trichoderma viride* VKF3



a) HPLC chromatogram of IAA by *T.viride* VKF3 and b) standard IAA
c) Thin layer chromatogram of IAA produced by *T. viride* VKF3

Fig.3 Evaluation of plant growth promotional activity of IAA produced by *Trichoderma viride* VKF3



Experimental setup: a) *Sesamum indicum* b) *Vigna radiate* and c) *Vigna mungo*
d) Effect of IAA treatment on fresh weight e) Effect of IAA treatment on dry weight
f) Effect of IAA treatment on Germination percentage and g) Effect of IAA treatment on shoot and root length

Table.1 Screening for extracellular enzymes production by *T. viride* VKF3

Parameters	Results
Siderophore	++
Cellulase	++
Xylanase	++
Laccase	+
Lipase	-

Plant Growth Promotion Efficiency

Vigna radiata, *Vigna mungo* and *Sesamum indicum* were used for the evaluation of plant growth promotion efficiency. The Figure 3a-c depicted the experimental setup of plant growth promotional activity, where there was enhanced growth in IAA treated plants compared to control. The fresh weight and dry weight of the three plants were noted and was also found to be increased during IAA treatment (Fig. 3d and e). Germination percentage also was found to increase by IAA treatment (Fig. 3f). Maximum germination was observed in case of *V. radiata* followed by *V. mungo* and *S. indicum.* Root and shoot length of treated plants were found to have an increasing trend. Shoot length of *V. radiata* was enhanced during the treatment (Fig. 3g). However, no much change was observed in case of root length. In case of *S. indicum*, both root and shoot length was increased compared to control plant treated with distilled water. The present report was in agreement with the previous report of Harikrishnan *et al.,* (2014), who observed that IAA from *Streptomyces sp* VSMGT1014 substantially increased seed germination, root length and shoot length. Moreover, dry weight and vigor index of the tested rice plants were also significantly increased by the isolate VSMGT1014 compared to control.

Antagonistic Activity of *T. viride* VKF3

Antagonistic effect of *T. viride* VKF3against two fungal plant pathogens, *Fusarium oxysporum* and *Aspergillus niger* was evaluated. Maximum inhibition of 82 and 94% was achieved by *T. viride* VKF3 against *F. oxysporum* and *A. niger* respectively. Harikrishnan *et al.,* (2014) also reported the efficacy of microorganisms for biocontrol of fungal pathogen with IAA production. Similar reports were available on production of IAA and antagonistic mechanism of five streptomyces strain isolated from rice rhizosphere having biocontrol potential against Fusarium wilt disease in chickpea and also having Plant growth promoting traits (PGPT) such as IAA and siderophore production (Gopalakrishnan *et al.,* 2011).

T. viride VKF3, a mangrove isolate was effeicient for high IAA production upto was 115 µg mL^{-1} with 0.5% L-tryptophan. The IAA produced by the isolate was confirmed through TLC and HPLC analysis by comparison with standard IAA. The isolate was also capable of producing extracellular enzymes like cellulase, xylanase along with siderophore production. The culture filtrate exhibited plant growth promotional activity along with enhanced germination percentage. *T. viride* VKF3 exhibited a maximum inhibition of 82 and 94% was achived by against fungal pathogens *F. oxysporum* and *A. niger* respectively. This report shows a high IAA production using *T. viride* VKF3 with bio-control potential.

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