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# Antibiotic-producing *Pseudomonas fluorescens* mediates rhizome rot disease resistance and promotes plant growth in turmeric plants

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ABSTRACT

Rhizome rot of turmeric caused by *Pythium aphanidermatum* is a major threat to turmeric-cultivating areas of India. This study intends to evaluate the performance of fluorescent pseudomonads against Rhizome rot disease and understand the resistance mechanism in Turmeric plants. Fluorescent pseudomonads were screened against *Pythium aphanidermatum* using dual culture. Selected strains were evaluated for the performance of growth promoting attributes and the presence of antibiotic genes through PCR analysis. Strain FP7 recorded the maximum percent inhibition of *P. aphanidermatum* under *in vitro* conditions. Strains FP7 and TPF54 both increased plant growth in turmeric plants *in vitro*. Strain FP7 alone contained all the evaluated antibiotic biosynthetic genes. Talc and liquid-based formulations were prepared with effective strain and tested for its biocontrol activities under both glasshouse and field conditions. Enzymatic activities of the induced defense enzymes such as PO, PPO, PAL, CAT and SOD were estimated and subjected to spectrophotometric analysis. A combination of rhizome dip and soil drench of FP7 liquid formulation treatment remarkably recorded the minimum disease incidence, higher defense enzymes, maximum plant growth and yield under glasshouse and field conditions. Application of strain FP7 increased the defense molecules, plant growth and yield in turmeric plants thereby reducing the incidence of rhizome rot disease. Moreover, this study has a potential to be adopted for sustainable and eco-friendly turmeric production.

## 1. Introduction

Turmeric is the golden spice of India. In world trade, India ranks first in both production as well as export. Turmeric cultivation is severely afflicted by various diseases. Amongst them, rhizome rot disease caused by *Pythium aphanidermatum* leads to extensive yield loss in India (Ravindran et al., 2007; Selvan et al., 2007). Its management has been exclusively based on the use of chemical fungicides. Although chemicals show promising results in controlling the disease, there are several major issues concerning pesticide residues, human health predicaments and environmental pollution. Therefore, an alternative for the management of rhizome rot disease is essential. Plant Growth Promoting Rhizobacteria (PGPR) are well-known beneficial microbes residing in the rhizosphere of different crop plants (Vacheron et al., 2013). They induce plant growth-promoting hormones which support plant growth and provide protection against phytopathogens. Among them fluorescent pseudomonads acclaim major attention due to their broad spectrum of antagonistic activity against various plant pathogens (Liu et al., 2016).

PGPR displays two major mechanisms comprising antagonism (Beneduzi et al., 2012) as a direct mechanism and indirectly by induced systemic resistance (ISR) (Kloepper et al., 2004). Antagonism of fluorescent pseudomonads can be determined by their ability to produce antibiotic metabolites, such as pyoluteorin, pyrrolnitrin, phenazine, 2, 4 diacetylphloroglucinol (DAPG), hydrogen cyanide, kanosamine, pyocyanin and viscosinamide, to suppress soil-borne fungal pathogens (Park et al., 2011; Saraf et al., 2014; Meyer et al., 2016). ISR by biocontrol agents against diseases has been established as a potential tool in crop protection through which the plants defend themselves from pathogen attack (Manikandan and Raguchander, 2014). These induced defense responses are regulated by interconnected signal transduction pathways *viz*., salicylic acid (SA), jasmonic acid (JA) and ethylene (ET); which play a crucial role in activating the defense related genes encoding peroxidase (PO), polyphenol oxidase (PPO), chitinase, β-1,3-glucanase, catalase (CAT), superoxide dismutase (SOD), proteinase inhibitors, lipoxygenase (LOX) and phenylalanine ammonia lyase (PAL) (Van Loon et al., 2008). For example some strains of *Pseudomonas* sp. elicited a systemic resistance against multiple plant diseases

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(Ramamoorthy et al., 2002). In addition, PGPR enhances plant growth through a wide range of mechanisms including various phytohormone secretion (Santner et al., 2009), enzyme production which alters plant growth and development (Penrose and Glick, 2003), nutrients supplication through biological nitrogen fixation (Bhattacharjee et al., 2008), easy phosphorus uptake (Yazdani et al., 2009) and siderophores production (Rosenblueth and Martinez-Romero, 2006). PGPR possess two different mechanisms: biocontrol and growth promotion, which are closely related to each other. A single bioagent executes both activities administering multiple mechanisms. Previous studies demonstrated that a single potential strain exhibits multiple traits related to biocontrol activity and growth promotion both *in vitro* and *in vivo* (Praveen Kumar et al., 2014; Liu et al., 2016).

Though several researchers reported the efficiency of biocontrol agents against various diseases, relatively very few researchers have attempted to study the effectiveness of the biocontrol agent against rhizome rot disease and its multiple functions related to turmeric production. Against this background, objectives of this study were to 1) select an effective bioagent based on the results of antagonism to *P. aphanidermatum in vitro*, growth promotion activity and presence of antibiotic biosynthetic genes 2) prepare a bioformulation with an effective bioagent from objective 1 to evaluate against rhizome rot disease in turmeric under both glasshouse and field conditions 3) understand the mechanism of induced systemic resistance to turmeric rhizome rot disease.

## 2. Material and methods

### 2.1. Plant material, biocontrol agents and pathogen

Turmeric cultivar BSR2 was utilized as a constituent of entire investigation. Sixty fluorescent pseudomonads were isolated from the turmeric rhizosphere soil samples of Tamil Nadu, India using Kings' B (KB) medium. The isolates were confirmed as per Bergey's manual of systematic bacteriology (Krieg and Holt, 1984). *P. fluorescens* strains FP7 and Pf1 were obtained from Plant Pathology Division, Tamil Nadu Agricultural University (TNAU), Coimbatore, India. The pathogen *P. aphanidermatum* was isolated from infected pseudostems and rhizomes using potato dextrose agar medium (PDA). The pathogen was identified based on its morphological characters as described by Middleton (1943) and molecular level using the specific primer combination of Paph54F-ITS2 (Prabhukarthikeyan et al., 2015).

### 2.2. Screening of fluorescent pseudomonads under in vitro conditions

The antifungal effectuality of fluorescent pseudomonads was tested by the dual culture technique (Dennis and Webster, 1971) using PDA medium. A mycelial disk of the pathogen (9 mm diameter) *P. aphanidermatum* was placed at one end of the plate and bacterial antagonists were streaked opposite the pathogen. The plates had only *P. aphanidermatum* without bacterial antagonist served as a control. The plates were incubated for 72 h at 28 ± 2 °C. The mycelial growth of the pathogen was measured after the incubation period. Effective fluorescent pseudomonads were identified at molecular level using the 16S-23S rRNA intervening sequence ITS1F (5′-AAGTCGTAACAAGGTAG-3′); ITS2R (5′GACCATATATAACCCCAAG- 3′) primers (Rameshkumar et al., 2002). DNA was extracted following the procedure described by Gomes et al. (2000). PCR reactions were carried out in 20 µl reaction mixture containing 10× buffer (with 2.5 mM $MgCl_2$), 2 µl; 2 mM dNTP mixture, 2 µl; 2 M primer, 5 µl; *Taq* DNA polymerase, 3 U; $H_2O$, 8 µl and 50 ng of template. DNA samples were amplified on DNA thermalcycler (Eppendorf Master Cycler Gradient, Westbury, New York) using the PCR conditions 92 °C for 4 min, 55 °C for 1 min and 72 °C for 2 min. The total number of cycles was 40 with the final extension time of 10 min. The PCR products were resolved on 1.5% agarose at 50 V stained with ethidium bromide (0.5 µg $ml^{-1}$) and photographed and analyzed using gel documentation system (Alpha Innotech Corporation, San Leandro, California). Amplified PCR products were purified using a QIA quick gel extraction kit (Qiagen, Inc., Chatsworth, California) according to the instructions of manufacturer. The DNA sequencing was performed at Chromos Biotech Pvt. Ltd. Bangalore, India.

### 2.3. Plant growth promotion

Fluorescent pseudomonad strains were inoculated into KB broth and kept at room temperature (28 ± 2 °C) with shaking at 150 rev $min^{-1}$ for 48 h. The bacterial suspension was then centrifuged at *4000g* for 15 min and the pellets were resuspended in sterile water. The concentration was later adjusted to approx. $10^8$ CFU $ml^{-1}$ ($OD_{595}$ = 0.3) and used as the bacterial inoculum (Thompson et al., 1996). Healthy rhizomes were cut into small pieces with single buds weighing 4–6 g. The rhizome pieces with single buds were treated with fluorescent pseudomonads for 30 min before planting. The bacterized rhizomes were planted in a pro-tray (98 well) containing sterilized coir pith and vermicompost mixture (75:25). The pro-trays were maintained under shade-net conditions. Frequent need-based irrigation with a rose cane was provided. The turmeric rhizomes without fluorescent pseudomonads served as a control. Three replications were maintained for each treatment. The root length and shoot length of individual rhizome seedlings were measured one month after planting. The germination percentage of the rhizome was also calculated. The vigour index was calculated using the formula given by Abdul-Baki and Anderson (1973) as: Vigour index = Germination Percentage × (Mean of root length + Mean of shoot length).

### 2.4. PCR detection of antibiotic biosynthesis genes

The antibiotic genes of phenazine, 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin, pyoluteorin and hydrogen cyanide were amplified using the appropriate primers provided in Supplementary file 1 (Raaijmakers et al., 1997; Jorge and Raaijmakers, 2003; Ramette et al., 2003). 20 µl reaction mixture contained approximately 50 ng of total DNA, 5 mM each dNTPs, 20 pmol each of both forward and reverse primers and 0.5 U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler applying the conditions determined in the Supplementary file 1.

### 2.5. Talc and liquid based bioformulations of P. fluorescens (FP7)

A loopful of FP7 culture was inoculated into the King's B broth and incubated for 72 h at room temperature. The broth containing $9 \times 10^8$ CFU $ml^{-1}$ of the culture was used for preparation of the bioformulation. To 400 ml of bacterial suspension, 1 kg purified talc powder, 15 g calcium carbonate and 10 g carboxy methyl cellulose were added under sterile conditions following the method described by Saravanakumar et al. (2009). At the time of application, the bacterial population was monitored to be around 2.5–3 × $10^8$ CFU $g^{-1}$.

The liquid formulation of FP7 was prepared according to the procedure described by Manikandan et al. (2010). For developing a liquid formulation of FP7, nutrient broth was prepared accordingly with the addition of 2% glycerol. 1 ml log phase culture of FP7 ($3 \times 10^{10}$ CFU $ml^{-1}$) was added to the nutrient broth and incubated at room temperature (25 ± 2 °C) until further use.

### 2.6. Glasshouse study

Glasshouse studies were carried out at glasshouse PL480, Plant Pathology Division, TNAU, India. In our study, individual and combined application of rhizome dip (RD) and soil drenching (SD) were evaluated for both talc and liquid-based formulations of *P. fluorescens* (FP7). The virulent strain of *P. aphanidermatum* was mass multiplied in a sand–maize medium and mixed with sterilized potting medium at 5%