

# MOLECULAR CHARACTERIZATION OF *TRICHODERMA* SPP. AGAINST *COLLETOTRICHUM GLOEOSPORIOIDES* PENZ. WITH RAPD AND ITS-PCR

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## KEY WORDS

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## ABSTRACT

Genetic variability among the isolates of *Trichoderma* spp. against *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. was studied using molecular techniques like RAPD and ITS-PCR. The RAPD banding pattern reflected the genetic diversity among the isolates with the formation of three clusters. ITS region of rDNA amplification with genus specific ITS1 and ITS4 universal primers produced products that varied in size from 566 to 657 bp in all the isolates. These results indicated polymorphism at ITS region of rDNA among the isolates of *Trichoderma*.

## INTRODUCTION

Mango (*Mangifera indica* L.) is considered as one of the most popular fruits among millions of people grown throughout the tropics and subtropics worldwide. India is the world's largest producers, shares around 56 per cent of total global production (Chadha, 2001) and Andhra Pradesh ranks first in production (45.89 lakh tonnes). Mango is affected by large number of fungal pathogens. Among which, *Colletotrichum gloeosporioides* causes anthracnose, which is the most important biological constraint to mango production in Southeast Asia resulting in substantial yield loss (Dodd *et al.*, 1991). *Trichoderma* species have been investigated as biological control agents (BCAs) for over 70 years (Hjeljord *et al.*, 1998), but it is only recently that strains have become commercially available. Knowledge concerning the behaviour of these fungi as antagonists is essential for their effective use since they can act against target organisms in several ways (Jeffries *et al.*, 1994) by producing extracellular enzymes (Haran *et al.*, 1996) antifungal antibiotics (Ghisalberti *et al.*, 1993) and also be competitors to fungal pathogens (Simon *et al.*, 1989). Of late, molecular techniques are gaining importance in characterization and diagnosis of microbial population. Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. has been reported by several researchers (Hermosa *et al.*, 2000 and Venkateswarulu *et al.*, 2008). These techniques are not influenced by environment, independent of growth stage and reproducible when compared to conventional methods and helps in identification of antagonists which can be used as markers in

diagnosis of potential biocontrol agents in future. Thus, the present study was undertaken to assess the significant genetic variations by nucleic acid based marker techniques using RAPD and ITS-PCR to distinguish the genetic variability in nine isolates of *Trichoderma* spp. evaluated against *Colletotrichum gloeosporioides*.

## MATERIALS AND METHODS

The molecular variability among the isolates of *Trichoderma* spp. having different degrees of antagonistic activity against *C.gloeosporioides* incitant of mango anthracnose was studied by using random amplified polymorphic DNA (RAPD) and ITS region of rDNA.

### Fungal cultures

Mycelial discs of 5 mm diameter *Trichoderma* isolates were cut from periphery of an actively growing 7 day old culture on PDA and inoculated on 250 mL conical flask containing 100 mL of sterile potato dextrose broth. The flasks were kept rotated constantly at a speed of 125 rpm in an orbital shaker at  $28 \pm 2$  C. The resultant growth of mycelial mat was harvested and excess moisture was completely removed through sterile blotting paper and used for DNA extraction.

### DNA extraction from *Trichoderma* isolates

The total genomic DNA from different isolates of *Trichoderma* was extracted from vegetative mycelium using the procedure adopted by Reader and Broda (1985). Mycelial mat of 200 mg was grounded in the pre-sterilized pestle and mortar with liquid nitrogen until fine powder was obtained. About 50 mg of

freeze-dried ground mycelium in an eppendorf tube was resuspended in 500  $\mu\text{L}$  of extraction buffer. To this 350  $\mu\text{L}$  of phenol and 150  $\mu\text{L}$  of chloroform was added and thoroughly mixed. The suspension was centrifuged at 13000 rpm for 1 hr. The upper aqueous phase was transferred to a new eppendorf tube. To this, 25  $\mu\text{L}$  of RNase (10%) was added and incubated to 37°C for 30 min. The solution was extracted with chloroform isoamylalcohol (24:1). The upper phase was transferred to an eppendorf tube and 0.54 volume (270  $\mu\text{L}$ ) of isopropanol was added and mixed thoroughly by inverting the tubes. DNA precipitated visibly into lump was observed. The tube was spun at 12,000 rpm for 10 min to pellet the DNA and the remaining liquid was removed. The pellet was rinsed with 70 percent ethanol two times, dried under vacuum and resuspended in 100  $\mu\text{L}$  TE buffer (pH 8.0). The DNA samples were stored at -20 C for further studies.

#### Qualitative and quantitative verification of DNA

The quality and quantity of DNA was analyzed by running 2  $\mu\text{L}$  of each sample mixed with 2  $\mu\text{L}$  of 10x loading dye in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands in one percent agarose gel indicating the good quality of DNA. The DNA has been quantified by comparing with the marker (Genei, Bangalore).

#### RAPD profiles through polymerase chain reaction (PCR)

Five different random primers viz., OPA-03, OPA-05, OPA-08, OPA-09 and OPA-10 (Operon technologies Inc.) were screened for generating polymorphism among the isolates under the study. The experiment was repeated thrice and results were reproducible. The primer sequence used in RAPD technique are given as below:

S.No.	Name of the primer	Sequence
1.	OPA-03	5' – AGT CAG CCA C – 3'
2.	OPA-05	5' – AGG GGT CTT G – 3'
3.	OPA-08	5' – GTG ACG TAG G – 3'
4.	OPA-09	5' – GGG TAA CGC C – 3'
5.	OPA-10	5' – GTG ATC GCA G – 3'

PCR amplifications were carried out in 0.2 mL eppendorf tubes with 25  $\mu\text{L}$  reaction mixture which consists of 2.5  $\mu\text{L}$  of 1 x Taq buffer, 2  $\mu\text{L}$  of 25 mM  $\text{MgCl}_2$ , 4  $\mu\text{L}$  of primer (25 picomolar /  $\mu\text{L}$ ), 0.5  $\mu\text{L}$  of 100 mM dNTP mix, 0.6  $\mu\text{L}$  of Taq polymerase enzyme (conc. 3U  $\mu\text{L}^{-1}$ ) and 13.4  $\mu\text{L}$  of sterile PCR water (Genei, Bangalore) and 2  $\mu\text{L}$  (20-25 ng) of DNA sample. Amplification was carried out by 5 minutes of initial denaturation of 94°C followed by 45 cycles (Sreenivasaprasad *et al.*, 1992) of denaturation of 94°C for 1 minute; annealing at 37°C for 1 minute; extension at 72°C for 2 minutes with final elongation at 72°C for 5 minutes. Amplified PCR products were subjected to 1.0 per cent agarose gel electrophoresis with 1.0 x TBE as running buffer. The banding patterns were visualized under UV trans-illuminator with ethidium bromide (1 mg  $\text{mL}^{-1}$ ) staining. The DNA banding profiles were documented in the gel documentation system (Alpha Innotech) and compared with 1 kb DNA ladder (Genei, Bangalore).

#### Scoring and data analysis

Each amplified band was considered as RAPD marker and recorded for all samples. Data were entered using a matrix in which all observed bands or characters were listed. The RAPD

pattern of each isolate was evaluated, assigning character state '1' to all the bands that could be reproducible and detected in the gel and '0' for the absence of band. The data matrix thus generated was used to calculate Jaccard similarity coefficient for each pair wise comparison. The coefficients were calculated In Silico following Jaccard (1908). The similarity coefficients were subjected to Unweighted Pair-Group Method on Arithmetic Average (UPGMA) cluster analysis to group the isolate based on their overall similarities. Statistical Package for Social Science (SPSS) package was used for the cluster analysis and subsequent dendrogram preparation.

#### PCR amplification of ITS region

PCR amplification of ITS (Internal Transcribed Spacers) region of rDNA was performed using universal primers ITS-1 (5' – TCC GTA GGT GGA CCT GCG G – 3') as forward primer and ITS-4 (5' – TCC TCC GCT TAT TGA TAT GC – 3') as reverse primer (White *et al.*, 1990) in eppendorf PCR master cycler. Amplification was carried out in 0.2 mL eppendorf tubes with 25  $\mu\text{L}$  reaction mixture containing 2.5  $\mu\text{L}$  of 10 x Taq buffer, 2  $\mu\text{L}$  of 25 mM  $\text{MgCl}_2$ , 0.5  $\mu\text{L}$  of ITS-1 primer (25 picomolar/  $\mu\text{L}$ ), 0.5  $\mu\text{L}$  of ITS-4 primer (25 picomolar/  $\mu\text{L}$ ), 0.5  $\mu\text{L}$  of 100 mM dNTP mix, 0.5  $\mu\text{L}$  of Taq polymerase (3 U/ $\mu\text{L}$ ) and 15.5  $\mu\text{L}$  of sterile PCR water (Genei, Bangalore) and 3  $\mu\text{L}$  (30-40ng) of DNA sample. The PCR amplification was carried out by 35 cycles, of which denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute and extension at 72°C for 1.5 minutes with initial denaturation at 94°C for 4 minutes before cycling and final extension at 72°C for 6 minutes after cycling. Amplified PCR products were observed in 1.0 per cent agarose gel in 1% TBE buffer and visualized under trans-illuminator with ethidium bromide staining. The size of the PCR product was estimated by comparison with known DNA marker of 1 kb DNA ladder. The banding profiles of ITS-PCR products were documented in gel documentation system (Alpha Innotech).

## RESULTS AND DISCUSSION

Total genomic DNA extracted from the isolates produced clear sharp bands indicating the good quality of DNA.

#### Characterization of *Trichoderma* isolates by RAPD

Five random primers viz., OPA-3, OPA-5, PA-8, OPA-9 and OPA-10 generated reproducible polymorphism among the nine isolates of *Trichoderma*. Amplified products with all the primers showed polymorphic and distinguishable banding pattern indicating the genetic diversity among all isolates of *Trichoderma*. A total of 119 reproducible and scorable polymorphic bands ranging approximately as low as 75 bp to as high as 2500 bp were generated with five primers among the nine *Trichoderma* isolates characterized.

The *Trichoderma* isolates  $T_1$  and  $T_7$  were identified as *Trichoderma koningii* ( $T_1$ ) (accession no. 6623) and *Trichoderma fasciculatum* ( $T_7$ ) (accession no. 6624) respectively at IARI, New Delhi. All the five primers produced unique bands in case of *T.koningii* (Isolate  $T_1$ ). Primer OPA-3 amplified a single fragment of approximately 300 bp in case of  $T_2$  isolate and 710 in case of  $T_6$  isolate (Fig 1A). All the five primers amplified similar fragments in case of  $T_3$  and  $T_4$  isolates, except for an 850 bp fragment by OPA-3 in case of  $T_4$  indicating

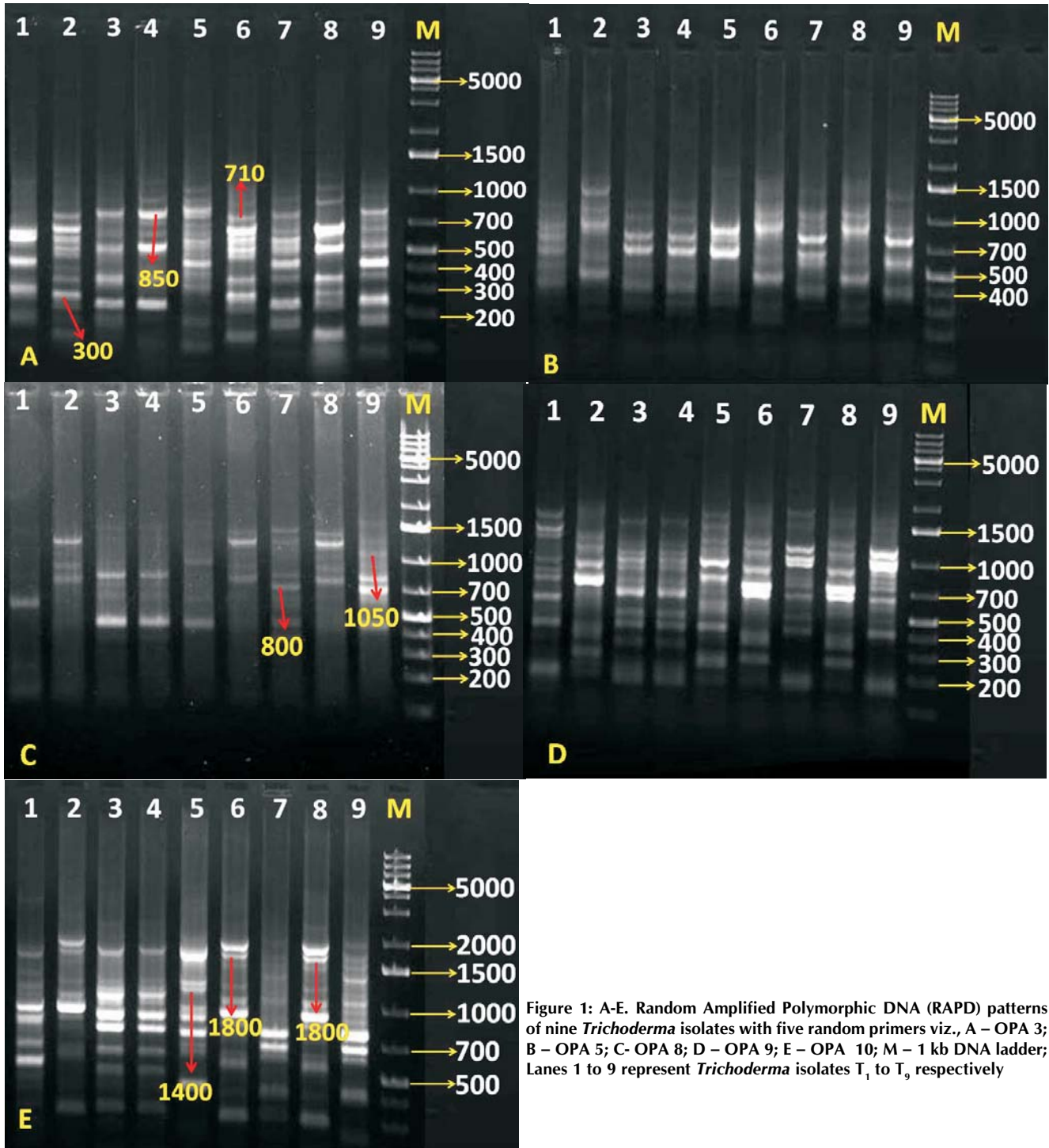


Figure 1: A-E. Random Amplified Polymorphic DNA (RAPD) patterns of nine *Trichoderma* isolates with five random primers viz., A – OPA 3; B – OPA 5; C- OPA 8; D – OPA 9; E – OPA 10; M – 1 kb DNA ladder; Lanes 1 to 9 represent *Trichoderma* isolates  $T_1$  to  $T_9$ , respectively

the homogeneity of their genomes. primer OPA-8 amplified a unique band of 800 bp in case of *T.fasciculatum* (Isolate  $T_7$ ) and 1050 bp in case of  $T_9$  and 850 bp in case of  $T_6$  and  $T_8$  isolates (Fig 1C). Primer OPA-10 amplified a unique band of approximately 1400 bp in case of  $T_5$  and an 1800 bp band in case of  $T_6$  and  $T_8$  (Fig 1E).

Relationship among the isolates were evaluated by cluster analysis of data based on similarity matrix. The dendrogram (Fig 2) was generated using UPGMA package based on Ward's

Squared Equalidean Distance method. Based on the results obtained, all the nine isolates were grouped into three main clusters. Cluster I contains four isolates viz.,  $T_3$ ,  $T_4$ , *T.fasciculatum* and  $T_9$  of which the first two and last two forms two separate sub-clusters viz., Ia and Ib. Clusters II contains four isolates viz.,  $T_2$ ,  $T_5$ ,  $T_6$  and  $T_8$ , of which the first three forms a separate sub-cluster viz., Ia and  $T_8$  isolate alone forms a separate sub-cluster Ib. Cluster III contains only one isolate *T.koningii* indicating its genetic difference with other isolates.

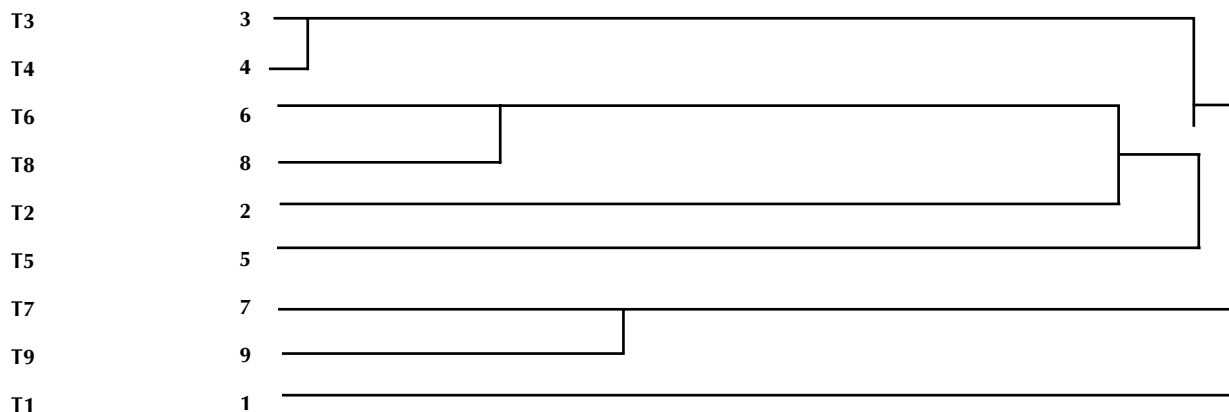


Figure 2: Dendrogram generated using UPGMA analysis showing polymorphism between isolates of *Trichoderma* spp. using RAPD markers

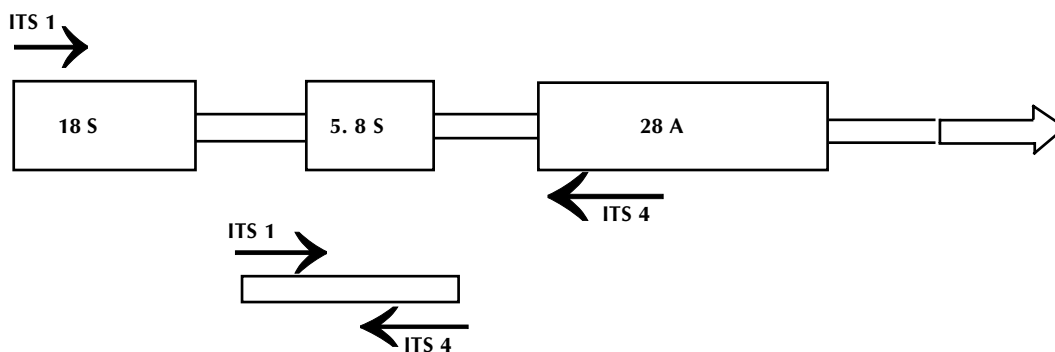


Figure 3: Structure of rDNA cluster and the position of ITS primers used in the PCR amplification of *Trichoderma* isolates

Jaccard's similarity co-efficient among the nine *Trichoderma* isolates were calculated to establish their genetic relationships. The similarity index values ranged from 0.00 to 100% indicating the presence of a high range of variability at nucleic acid level among the nine *Trichoderma* isolates. It is worthwhile to screen more number of RAPD primers belonging to different operon series in order to generate more polymorphism among the isolates. This study may help in tagging of the RAPD fragment to its antagonistic activity.

#### Characterization of *Trichoderma* isolates by ITS-PCR

Genus specific target primers viz., ITS-1 and ITS-4 were used for PCR amplification of ITS region of rDNA cluster which includes ITS-1, 5.8S and ITS-2 regions of all the nine isolates. The structure of rDNA and the expected amplified products with primers are shown in Fig 3. Amplified products size varies approximately in the range of 566 to 657 bp in all the isolates under the study. However, in case of *T.koningii* (Isolate T<sub>1</sub>) and *T.falsicatum* (Isolate T<sub>7</sub>) the size of the band obtained was 657 bp and 603 bp respectively (Fig 4). The results were in accordance with Latha *et al.*, (2002) who reported the size of bands obtained with ITS-1 and ITS-4 primer pairs in case of all *Trichoderma* isolates was around 600 bp. These results confirmed that all the isolates belong to the genus *Trichoderma*. Similar results were reported by Muthumeenakshi *et al.*, (1994), Ospina (1999) and Lieckfeldt (1999), who observed that the amplified rDNA fragment of approximately 500 to 600 bp by ITS-PCR in *Trichoderma*.

The ITS-PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma* isolates. But based on these results it is not possible to conclude that all the isolates are the same. Digestion analysis of amplified ITS region with different restriction enzymes may reveal more polymorphism. Finally, sequencing of the amplified ITS region will help to know the differences at rDNA level among the isolates under study. The sequence information allows in developing a SCAR marker for *Trichoderma koningii* and *Trichoderma fasciculatum*, the

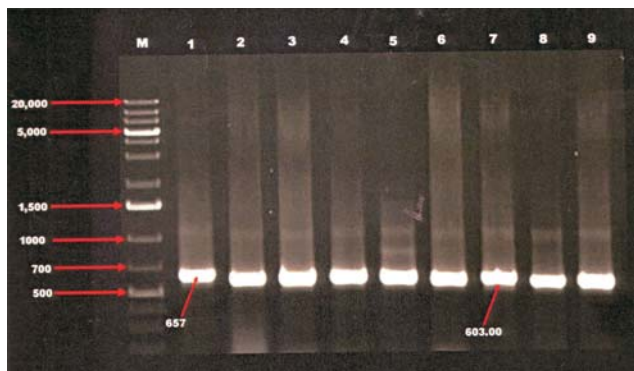


Figure 4: Amplification product of Internal Transcribed Spacer (ITS) with ITS-1 and ITS-4 ribosomal DNA primers. M-1 kb DNA Ladder; Lanes 1 to 9 represent *Trichoderma* isolates T<sub>1</sub> to T<sub>9</sub> respectively. The arrows indicate the ITS-PCR bands specific for *T.koningii* and *T.fasciculatum*

potential native antagonist against *Colletotrichum gloeosporioides* causing mango anthracnose. In this direction the future line of research work will be focused.

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