

Antagonistic potential of *Trichoderma* strains isolated from *Musa paradisiaca* cv. Malnad Rasbale grown farmyards against Foc race 4 pathogen.

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Research Article

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Abstract

Foc race 4 is a causative pathogen for Panama wilt disease of *Musa Paradisiaca* cv. Malnad Rasbale. The cost-effective measure to control rather than the usage of agrochemicals is still not available for this cultivar. *Trichoderma* strains act as an antagonistic agent against different phytopathogenic fungi, including many pathogenic races of Panama wilt-causing pathogens. An attempt has been made to recognize the mode of action of this antagonistic agent in *in-vitro* conditions, interaction between six *Trichoderma* strains and Foc race 4 was first investigated by dual plate culture method on PDA medium. This study revealed the potential of native strain KUVKU-TH02 for the biological control of Foc race 4 pathogen affected Malnad Rasbale cultivar in *in-vivo* conditions rather than native isolates KUVKU-TH01, KUVKU-TV01, and KUVKU-TV02. Observations revealed the lysis of hyphal ends in inhibited colonies of the fungal pathogen. Pure culture of isolated fungal strains incubated on Potato dextrose broth made a path to isolate DNA for identification and molecular characterization studies. Upon DNA sequencing native isolates sequences were deposited to NCBI genebank to gain accession IDs. The phylogenetic tree built showed the evolutionary relationship between the isolates and also the potency of native biocontrol isolates against procured isolates.

1. Introduction

Musa Paradisiaca cv. Malnad Rasbale (silk AAB) banana fruits are well known for their good flavor and taste. It is an endemic cultivar of Malnad regions which are geographically recognized as the Central Western Ghats of Karnataka, India. The susceptibility of this cultivar to Panama wilt disease is identically high which is instigated by the pathogen *Fusarium oxysporum* f.sp. *cubense* tropical race 4 (Foc race 4) (Prasanna et al., 2022). Panama wilt is perplexing to manage, it is one of the supreme destructive disease to all banana cultivars of India and it lasts in soil for many years, which averts the planting of banana cultivars again (Stover 1962). This pathogen can be eradicated with the application of chemicals and biocontrol agents (Ploetz 2015). Banana cultivars affected by Foc race 4 include Rasthali, Nanjanagudu Rasbale, Malnad Rasbale, Karpuravalli, Ney Poovan, Monthan, Hill banana, and Pachanadan in India (Thangavelu and Mustaffa 2010, Prasanna et al., 2022).

In current years extreme practice of agrochemicals and pesticides for enhancement of plant growth and pest protection has made an impact on human health. Considerable destruction to the ecosystem, humans, and animals are caused by excessive use of chemical fertilizers and pesticides. To interchange hazardous agrochemicals, promote plant growth, and survive against pathogens many biological solutions are existing for plants through microbes. Usage of biocontrol agents like *Trichoderma* eco-friendly fungus is a substitute method for plant treatment against pathogens (Pilkington et al., 2010). *Trichoderma*, a filamentous fungus has made extensive attention worldwide (Harman and Bjorkman 2014). These species are acknowledged as biocontrol militants because of their enthusiastic attributes which include antibiosis mycoparasitism, supply of nutrient elements to plants for growth promotion, and increased nutrient and water uptake (Sharma et al., 2018, Vos et al., 2015, Chet 1987, Sharma et al., 2013 and Salwan et al., 2019).

Worldwide distribution of this biocontrol agent is extraordinary and also has enlarged high biodiversity (Jiang et al., 2016). For many years most species of the genus *Trichoderma* have been recognized and its application for plant pathogenesis resistor and growth promotion is a success until today (Topolovec-Pintaric 2019, Harman et al., 2019). Apart from *Trichoderma* used as a biocontrol agent it also subsidizes enhancing soil fertility and improves the availability of nutrition in soil (Singh et al. 2018; Zhai et al. 2019; Zhang et al. 2018). Many recent investigations have revealed that native *Trichoderma spp.* are effective biocontrol agents, more productive, and have good biocontrol activities than artificially induced strains because they have previously adapted to local agricultural and environmental conditions (Joshi and Misra 2013, Wang et al., 2021). Henceforth, exploration of native *Trichoderma spp.* is important to develop biocontrol agents for local agricultural and environmental conditions. Panama wilt of banana is a highly destructive disease in tropical countries and also in India. As biological control is an alternative method for Foc race 4 to control through *Trichoderma* strains, which are particularly active in plants and have been colonized near the rhizospheric part of banana plants as an active controller of the pathogen. Our investigation aimed at evaluating the ability of four native *Trichoderma* strains isolated from rhizospheric parts of *Musa Paradisiaca* cv. Malnad Rasbale banana grown farmyards of Malnad region of Karnataka, India to check the antagonistic potential against resident Foc race 4 pathogen in *in-vitro* conditions and to detect the evolutionary relationships between them.

2. Materials and methods

2.1 Isolation of *Trichoderma* strains: *Trichoderma harzanium* and *Trichoderma viride* were isolated from Malnad Rasbale plants grown in farmyards near Bhadra Wildlife Sanctuary (13°43'26" N75°38'21" E), Karnataka, India. Well-grown Malnad Rasbale plant was observed and soil was collected from the rhizosphere part of the plantlet. The collected soil sample was carefully transferred to a polythene bag and instantly transferred to a thermocol container supported with ice packs and carried to the lab. For isolating fungi serial dilution method was carried out (Elad Y. *et al.* 1981). For acquiring pure culture, emerged hyphae were constantly subcultured on potato dextrose agar (PDA) medium. Isolated colony morphologically notifying *Trichoderma* strains were selected for further work.

2.2 Isolation of Foc race 4: Fresh tissue of Malnad Rasbale presenting emblematic Foc race 4 signs was collected from disease-infected farmyards of Malnad region of Karnataka, India near Bhadra Wildlife Sanctuary (13°43'26" N 75°38'21" E). Tissue samples from the pseudostem part of the pathogen-infected plant were torn vertically and the tissues were collected by cutting apart into 2-3 cm in length and were carefully transferred to a polythene bag and immediately transferred to a thermocol container supported with ice packs and was carried to the lab. Samples were then washed with running-tap water. Again samples were washed with 70% ethanol for up to 20-30sec and washed with distilled water to remove the traces of ethanol. Disinfected plant samples were then kept between sterile tissue paper to remove the outer water content. Tissue samples were inoculated on PDA under aseptic conditions and incubated for up to 72 hrs at room temperature (Venkatesh *et al.*, 2014).

2.3 Dual-culture of *Trichoderma* strains against Foc race-4: For dual-culture here 9cm diameter autoclaved Petri plates were incubated with PDA, Foc race 4 was placed 2.5 cm away from the center of Petri plate followed by *Trichoderma* strain in opposite direction and retained under *in-vitro* conditions for up to 6 days at 26 °C with a photoperiod of 6-8 hours under fluorescent light. After the progression in colonies growth expansion of fungi was noted to isolate the dominant *Trichoderma* strain for future investigations (Wang *et al.*, 2021). *Trichoderma harzianum* (NFCCI-3464) and *Trichoderma viride* (NFCCI-2552) gained from NFCCI- Agarkar Research Institute was also used to compare the efficiency of wild strains isolated. For the investigation of two strains that showed eminent antagonistic activity was selected (Savani *et al.* 2020, Venkatesh *et al.*, 2014). Observation of antagonistic action of *Trichoderma* against pathogen was witnessed day by day. Further, two *Trichoderma* strains that showed the highest antagonistic activity against pathogen were noted (Savani *et al.*, 2020).

2.4 Molecular credentials of *Trichoderma* and Foc race 4: To isolate DNA, pure fungal cultures were cultured in PDB for 6 days at 28 °C, on the 6th day fungal mat was separated using whatsmann filter paper and ground in liquid nitrogen. Extraction of DNA from mycelia was executed using the CTAB method of DNA isolation. ITS fragments in rDNA of fungal strains were amplified through polymerase chain reaction (PCR) via primer pairs ITS1 (5'-TCCGTAGGTGAA CCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATAT GC-3') (Kullnig- Gradinger *et al.*, 2002, Samuels 2006). PCR programming for *Trichoderma* strains was set to 94 °C for 2 min, 30 cycles of 94 °C for 30 s, and 55 °C for the ITS fragment. For Foc race 4 initial thermal cycling, condition denaturation was made at 95°C for 3 min, followed by 35 cycles consisting of denaturation at 95°C for the 30s, annealing temperature at 55°C for 45s, and extension at 72°C for 1 min, followed by a finally 72°C for 10 min extension (Venkatesh *et al.*, 2014). PCR products for all fungal strains were analyzed by electrophoresis on 2% (w/v) Agarose gel dissolved with Ethidium bromide (5µg/ ml) in 1xTris Borate-EDTA buffer and visioned in UV transilluminator by Alpha imager EP (Alpha Innotech Corporation, USA). The amplified DNA product was eluted from the gel and sequenced by Applied Biosystems DNA Analyzer 3037xl (Bio Serve Technologies, Hyderabad) (Venkatesh *et al.*, 2014). Through ITS primers and amplified PCR products were achieved. BLAST algorithm helped to search for sequence resemblances. (Saitou *et al.*, 1987, Tamura *et al.*, 2004, Tamura *et al.*, 2013).

2.5 Phylogenetic analysis: The phylogenetic tree was constructed based on PCR amplified product, generated by ITS1 and ITS4 primers. Investigation of similar sequences was conducted through the BLAST algorithm. MEGA 11 was used to build a phylogenetic tree that detected the evolutionary relationships of the isolated fungal strains. Neighboring joining method and bootstrapping analysis for 1000 replicates were performed to detect the confidence level at the inner nodes of topology (Saitou *et al.*, 1987; Tamura *et al.*, 2004; Tamura *et al.*, 2013).

3. Result and Discussion

3.1 DNA sequencing of *Trichoderma* and Foc race 4: At starting period after inoculation white hyphae appeared. Aggressive growth started after 72 hrs of incubation. After this, the whole culture turned dark

green after 24 hrs. Greenish circle-like patterns were formed on PDA measuring up to 3–4 cm (Fig. 1A & 1B). Observation revealed that the strains were transparent. Conidia were spherical in shape and green in color. Observed morphology of fungi matched *Trichoderma harzianum* and *Trichoderma viride* as described in ISTH.12.10 days old inoculated infected pseudostem part on PDA (Fig. 1C), produced a white mat with mycelium again after a few days this mycelium produced a pinkish colony on PDA (Fig. 1D) (Groenewald et al., 2006). The presence of microconidia, more branches of hyphae, and sickle-shaped macroconidia revealed that the isolate was Foc race 4(Beckman 1987).

DNA isolated from mycelia executed quality DNA on 0.8% agarose gel. DNA was visualized under a transilluminator (Kullnig-Gradinger et al., 2002). DNA concentration ranged from 50–150 ng/μl. For PCR amplification DNA bands with outstanding molecular weight and brightness were selected. DNA sequencing was carried out. For confirmation of fungal strains, the molecular methodology was followed by cloning, sequencing through ITS (620 bp), and deposited to GeneBank by accession IDs as mentioned in Table 1. Gained ITS sequences were submitted to NCBI nucleotide BLAST. Obtained results of fungal strains revealed that they were 100% matched to *Trichoderma* strains. Finally, isolated fungal strains after molecular characterization revealed that they were *Trichoderma harzianum*, *Trichoderma viride*, and Foc race-4.

Table 1
Isolated Fungal strains, procured fungal strains with NCBI genebank accession ID.

Sl No.	Fungi name	Genebank assesion ID
1	<i>Trichoderma harzianum</i> -KUVKU-TH01	ON319115.1
2	<i>Trichoderma viride</i> -KUVKU-TV01	ON408308.1
3	<i>Trichoderma harzianum</i> -KUVKU-TH02	ON319118.1
4	<i>Trichoderma viride</i> -KUVKU-TV02	ON319120.1
5	<i>Trichoderma harzianum</i>	NFCCI-3464
6	<i>Trichoderma viride</i>	NFCCI-2552
7	<i>Fusarium oxysporium f.sp. cubence</i> – KUVKU-FOC01	ON334167.1

3.2 Inhibitory activity of *Trichoderma* strains against pathogen: After 72 hrs of dual culture inoculation, mycelia of Foc race-4 was surrounded by *Trichoderma*, and growth of pathogen was limited. Suggesting micoparasitism of the *Trichoderma* inhibition zone was formed as shown in Fig. 1E and 1F by native isolated *Trichoderma harzanium* and *Trichoderma viride* respectively. (Atanasova et al. 2013). When compared to reference strains wild isolated variety showed a high growth rate and inhibitory activity. When observed after 7 days of dual-culture mycelia of the pathogen was sternly suppressed by *Trichoderma* strains compared to procured strains from NFCCI- Pune.

3.3 Phylogenetic analysis: Phylogenetic trees were constructed based on ITS rDNA sequences of *Trichoderma harzanium*, *Trichoderma viride*, and Foc race 4 strains obtained from Malnad Rasbale grown farmyards illustrating the phylogenetic relationships by intraspecific level. The trees generated were considered because of uniformity in topology. It was constructed depending on the nucleotide sequences of isolated 5 fungal strains. The evolutionary relationship of *Trichoderma* strains and Foc race 4 are shown in Fig. 5A, 5B, and 5C respectively. The evolutionary history was incidentally using the neighbor-joining method. Evolutionary distances were computed by the maximum composite likelihood method. The analysis involved 5 nucleotide sequences. Positions of codon containing gaps and data missing were eliminated. Evolutionary data analysis was performed using MEGA11 software. Molecular characterization of isolated fungal strains revealed the existence of Panama wilt causing pathogen Foc race 4 in *Musa Paradisiaca* cv. Malnad Rasbale and also *Trichoderma* strains in banana farmyards.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.02570557 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps were eliminated. There were a total of 509 positions in the final data set. Evolutionary analyses were conducted in MEGA11 software (Saitou N, and Nei M 1987, Felsenstein 1985, Tamura et al., 2004, Kumar et al., 2016).

The evolutionary history was inferred using the Neighbour-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 156 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 software (Saitou N, and Nei M 1987, Felsenstein 1985, Tamura et al., 2004, Kumar et al., 2016).

The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 0.06658330 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and the units are in the number of base substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 480 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 software (Saitou N, and Nei M 1987, Felsenstein 1985, Tamura et al., 2004, Kumar et al., 2016).

4. Conclusion

Our investigation revealed the clear presence of *Trichoderma* strains, popularly used as a bio-control agent for Panama wilt disease in *Musa Paradisiaca* cv. Malnad Rasbale farmyards near Bhadra Wildlife Sanctuary. First-ever reporting, that this cultivar is also affected by Foc race 4 pathogen which is a common pathogen for many banana cultivars worldwide (Thangavelu and Mustaffa 2010, Prasanna et al., 2022). Dual plate culture method results showed that the potency to inhibit the pathogenesis of Foc race 4 from native *Trichoderma* strains was high when compared to procured strains from NFCCI-Pune and here on 7th day of comparison i.e, on 168th hours of investigation revealed that KUVKU-TH02 *Trichoderma harzanium* strain showed the highest growth rate and NFCCI-2552 *Trichoderma viride* strain showed the lowest growth rate against the pathogen. This gave a clear idea for us to investigate further by using these native strains to treat Foc race 4 pathogenesis in *in-vivo* conditions considering Multi Seasonal Trials (MST), Plant Growth Promotion (PGPR) activities, Innate Immune Response (IIR) by developing the biocontrol formulations and obtained data may reveal the inhibition of Panama wilt disease in *Musa Paradisiaca* cv. Malnad Rasbale plants which are grown in the Central Western Ghats of Karnataka, India.

Declarations

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Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

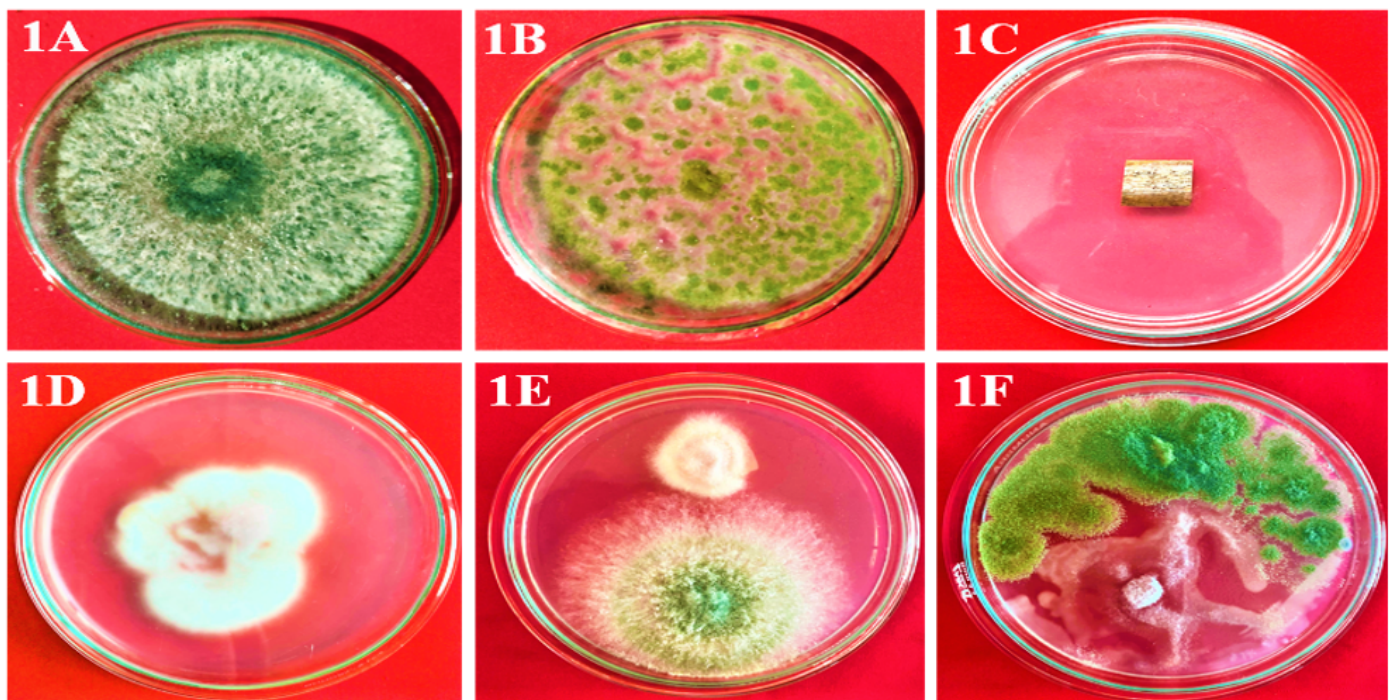


Figure 1

A represents 7 days old pure culture of *Trichoderma harzanium*, **B** represents 7 days old pure culture of *Trichoderma viride*. Both the strains were isolated from Malnad Rasbale grown farmyards as mentioned above. **C** represents the small piece of the infected pseudostem part of the Malnad Rasbale plantlet. **D** represents the pathogen Foc race 4 grown isolated from the pseudostem part. **E** represents the dual plate culture of *Trichoderma harzanium* against Foc race 4, **F** represents the dual plate culture of *Trichoderma viride* against Foc race 4 pathogen. As shown in **Fig. 1E** and **1F** both were 7 days old. All the cultures were inoculated on Potato Dextrose Agar medium and incubated at 28°C and 6 hrs of photoperiod and maintained in aseptic conditions.

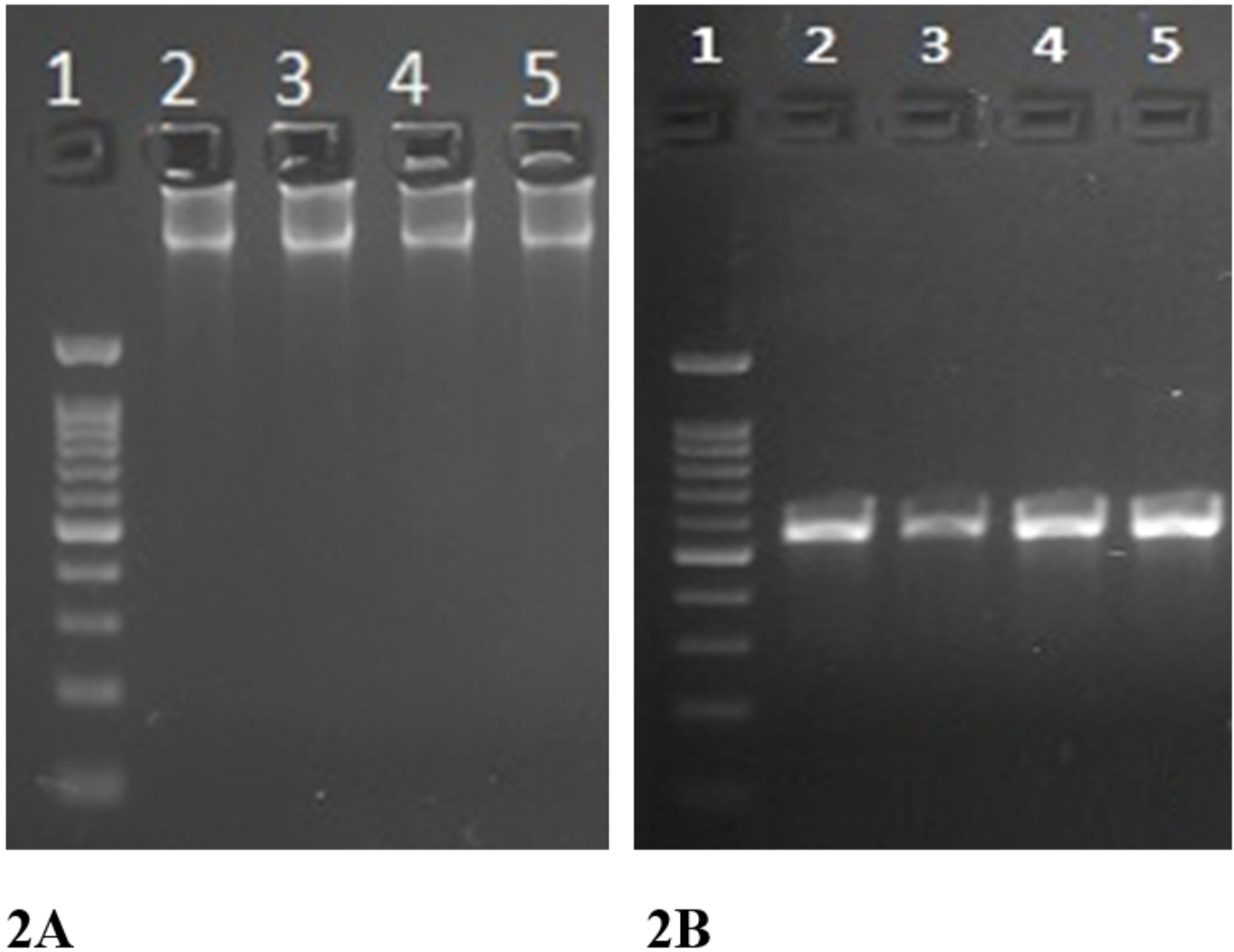
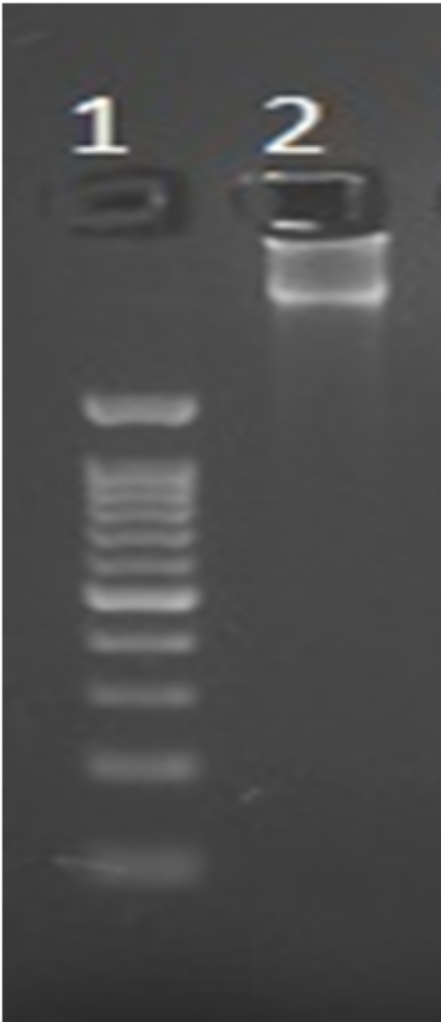
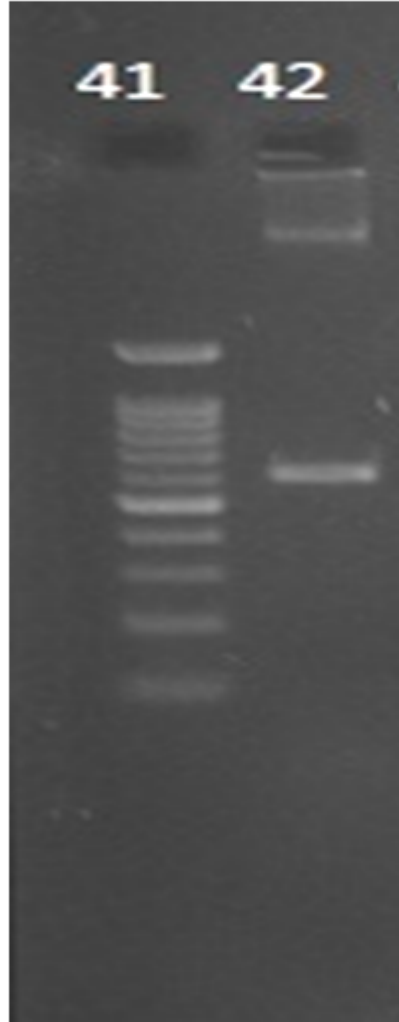


Figure 2

A represents genomic DNA QC and **B** represents amplification QC of 4 *Trichoderma* strains in 100bp ladder DNA.



3A



3B

Figure 3

A represents genomic DNA QC and **B** represents amplification QC of Foc race 4 strain in 100bp ladder DNA.

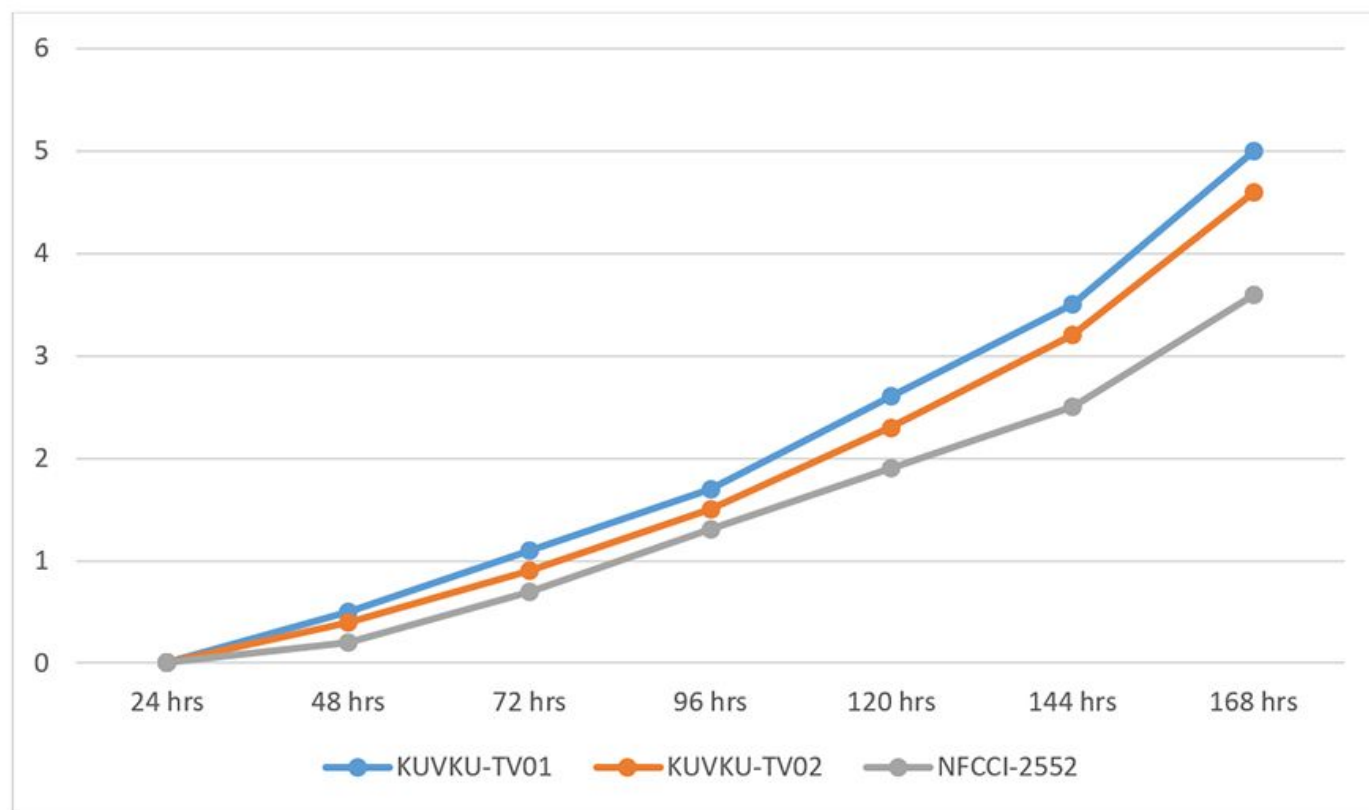
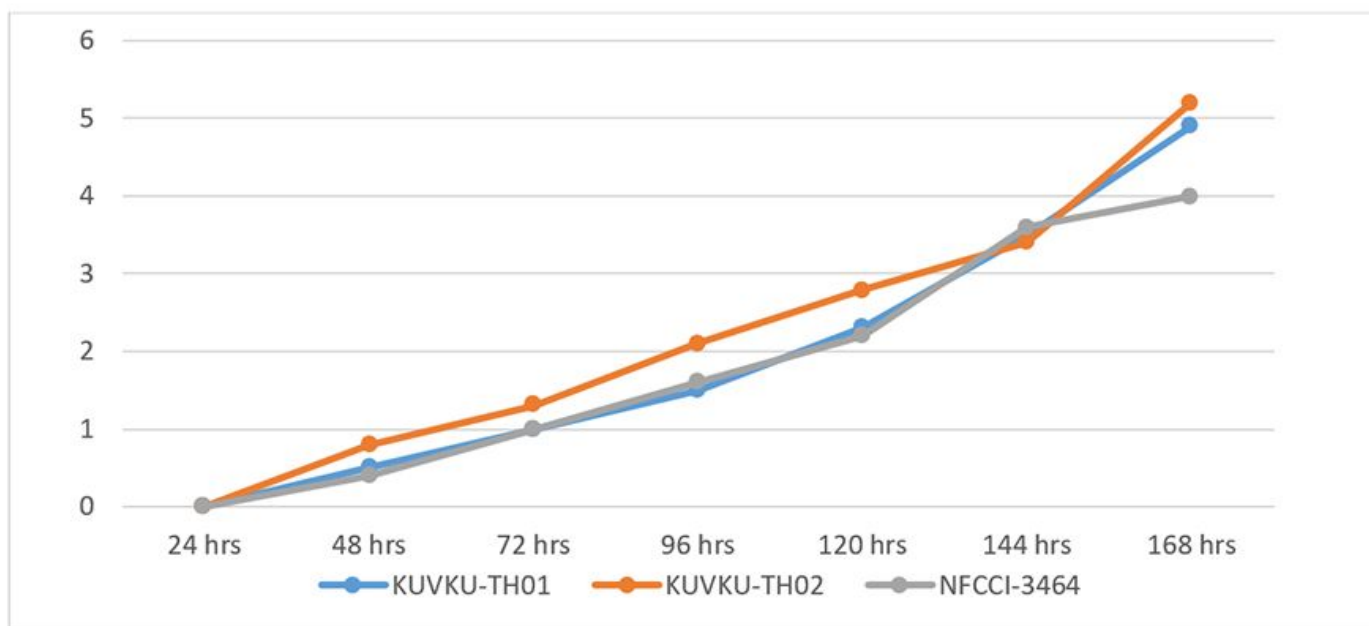


Figure 4

A: It represents the growth rate of native and procured *Trichoderma harzanium* strains against the Foc race 4 pathogen in cms from a time interval of 24 hrs to 168 hrs i.e. from day 1 to day 7. KUVKU-TH01 and KUVKU-TH02 are the native strains and NFCCI-3464 is the procured strain. Investigation revealed that KUVKU-TH02 showed the highest inhibition activity when compared to other strains as shown in **Fig. 1E** and the Graphical representation is shown below.

B: It represents the growth rate of native and procured *Trichoderma viride* strains against Foc race 4 pathogen in cms from a time interval of 24 hrs to 168 hrs i.e; from day 1 to day 7. KUVKU-TV01 and KUVKU-TV02 are the native strains and NFCCI-2552 is the procured strain. Investigation revealed that KUVKU-TV01 showed the highest inhibition activity when compared to other strains as shown in **Fig. 1F** and the Graphical representation is shown below.

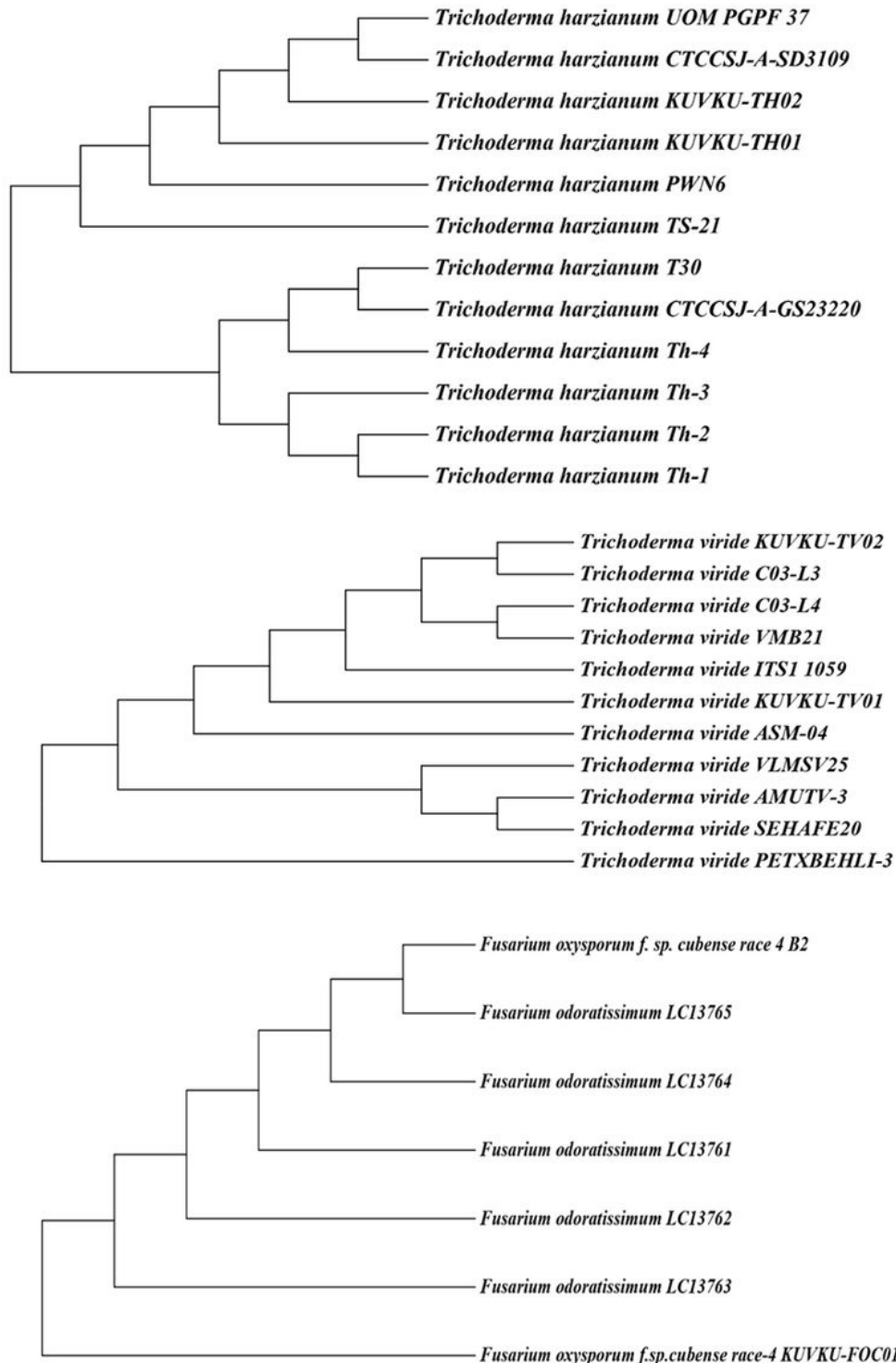


Figure 5

A: Evolutionary relationships of native *Trichoderma harzanium*.

B: Evolutionary relationships of native *Trichoderma viride*.

C: Evolutionary relationships of native *Fusarium oxysporum f.sp. cubense* race-4