

We cannot accept this manuscript in its current form albeit with some minor content changes. Authors need to address following points and can resubmit. The observations made in the manuscript is very interesting and encouraging. But manuscript fails to adhere some basic scientific norms at many places. Ignoring such basic scientific norms make the manuscript non impressive. Therefore, scientific language and content needs to be improved in terms of Introduction, methodology, results and conclusion and some more recent literature/ referencing can improve the quality of the paper.

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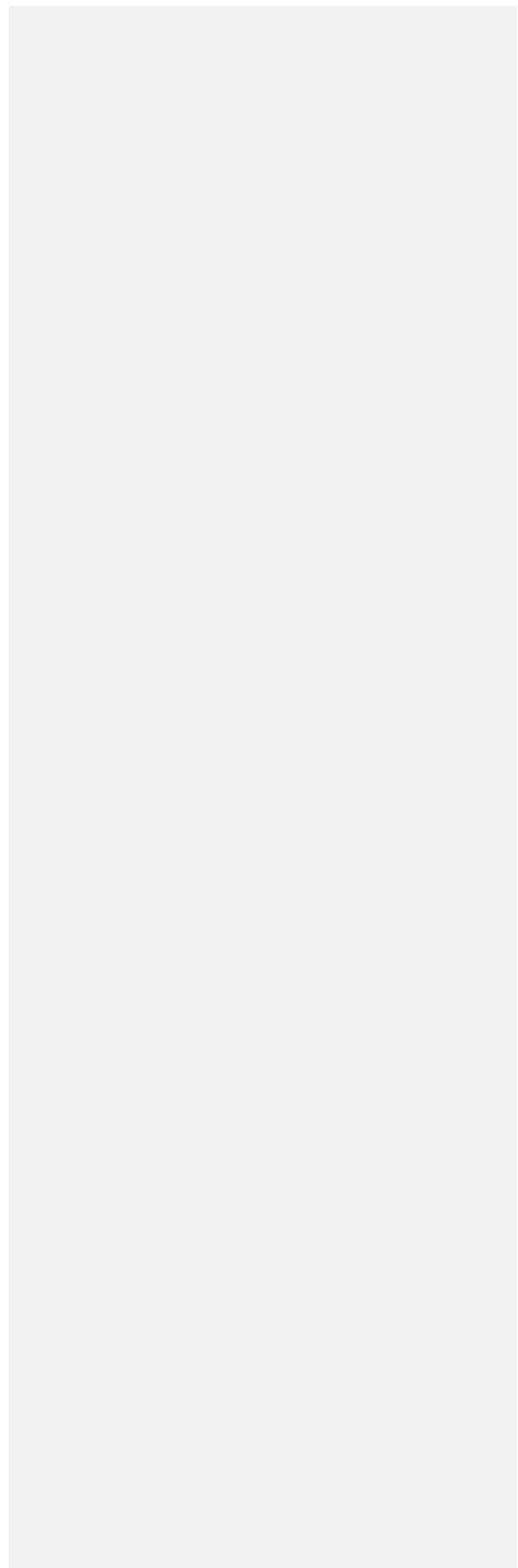
## Efficacy of biocontrol and antifungal agents on leaf spot and blight pathogen *Fusicladium pongamiae* in *Millettia pinnata*: An in vitro approach

### Abstract:

*Millettia pinnata* is a valuable tree species, commonly planted for its economic and ecological contribution. Despite this, it is susceptible to a number of foliar diseases, of which leaf spot and blight, induced by *Fusicladium pongamiae*, are major hazards in nursery and plantation conditions. The current research was conducted to isolate, identify, and determine the pathogenicity of *F. pongamiae*, and to test the effectiveness of chosen biocontrol agents (*Trichoderma viride* and *Pseudomonas fluorescens*) and antifungal agents (Pongam oil and Carbendazim) under in vitro conditions. Infected leaf samples were obtained and the pathogen was purified on Potato Dextrose Agar (PDA). Morphological identification under a stereomicroscope proved the identity of *F. pongamiae*. Pathogenicity was established by artificial inoculation on healthy seedlings, which proved to cause typical symptoms. In dual culture assays, the maximum inhibition of *F. pongamiae* was caused by *P. fluorescens* (68.46%), followed by the combination of *T. viride* and *P. fluorescens* (61.46%), and *T. viride* (53.79%). Using poisoned food technique assays, 0.1% Carbendazim strongly inhibited mycelial growth (83.82%), followed by 5% Pongam oil (41.28%). The research concludes that *P. fluorescens* has high biocontrol activity against *F. pongamiae*, while Carbendazim is the most efficient antifungal agent. These results justify the combination of biological and chemical approaches for sustainable control of leaf spot and blight disease in *M. pinnata*, resulting in better plant health and productivity in afforestation schemes.

**Keywords:** *Millettia pinnata*, *Fusicladium pongamiae*, *Pseudomonas fluorescens*, *Trichoderma viride*, leaf spot, blight.

UNDER PEER REVIEW



## Introduction:

*Millettia pinnata*, while it was raised as avenue plantations and on degraded lands it attracts many pests and diseases are being recorded that attacks the leaves. There are many adverse effects of diseases *i.e.*, root rot, leaf spot and blight, rust on the nursery seedlings and trees in the plantations (Hossain, 2007; Mridha *et al.*, 2007). One of the major diseases occurring on *M. pinnata* was leaf spot and blight disease which was caused by various agents such as *Fusicladium*, *Colletotrichum*, and *Dothiorella*. The parasitic fungus, *Fusicladium pongamiae* is usually observed on the lower leaf of *M. pinnata* in the form of brown-colored patches, and often resulting in curled leaf appearance. *Fusicladium* is a genus of ascomycete fungi in the family Symptentariaceae. The leaf spot disease of *Pongamia pinnata* caused by an aspersorium-like asexual morph, which is usually referred to as *Aspersorium pongamiae*, is quite common during monsoon seasons in India. Microscopic observations revealed that long spores of fungus assuming one or two- capsule shape (Pathak *et al.*, 2015) known to get infected by many foliar diseases, which affect the growth and development of the trees. Which were described further and also these fungal pathogens due to optimum moisture and temperature were found to be mostly affecting the nursery seedlings.

Leaf spot and blight symptoms were evident on both tender and matured leaves, but blight symptoms were mostly confined to matured leaves. On the upper surface of the leaves, water-soaked yellow spots first appeared singly, which over time coalesced to form big patches and leaves turned yellow (Chlorosis). On the lower surface of the leaves, it caused black patches and occasional leaf curling was associated with heavy conidiation. Later, all these yellow spots progressively turned brown and showed necrotic symptoms with dry leaves falling off early. Fungal and bacterial antagonists play an important role as microbial biocontrol agents in managing plant diseases and can be delivered as bio fungicides (Kohl *et al.*, 2019; Thambugala *et al.*, 2020). Bio-fungicides used for the biological control of plant pathogens are microorganisms-based formulations, which include antagonistic fungi and bacteria (Morandi *et al.*, 2009). Among fungi-based formulation, species from the genus *Trichoderma* are the most common biocontrol agents (Silva *et al.*, 2015; Yu and Luo, 2020). In comparison, fluorescent species from the genus *Pseudomonas* are the most common antagonists among bacteria-based formulations. *Trichoderma* spp. due to its biocontrol activity against various aerial, root and soil pathogens of economic importance, it has attained scientific attraction (Elad 2000; Chaube *et al.*, 2002).

Considering the pressing need for a sustainable management strategy to control of leaf spot and blight of *Millettia pinnata* caused by *Fusicladium pongamiae* and to test the efficacy of the biocontrol agents against the fungal pathogen, the current study was formulated.

## 2. Materials and Methods:

Laboratory studies on isolation and identification of pathogen associated with leaf spot and blight of *M. pinnata* were carried out in the Plant Pathology laboratory, Department of Forest Resource Management, Forest College and Research Institute, Mulugu. The details of materials and methodology followed are described here under.

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## 2.1 Isolation and Identification of microbe associated with blight disease of *M. pinnata*

### 2.1.1 Pathogen identification:

Infected leaves were brought to the laboratory using paper envelopes (Figure 1). The infected leaves were surface sterilized using 95% ethanol and dissected for the identification of the ~~organism based~~ organism based on conidial morphology by observing under stereo microscope. Same leaf samples were used to isolate the pathogen and pure culture of the associated pathogen was grown on Potato Dextrose Agar (PDA) medium was maintained.

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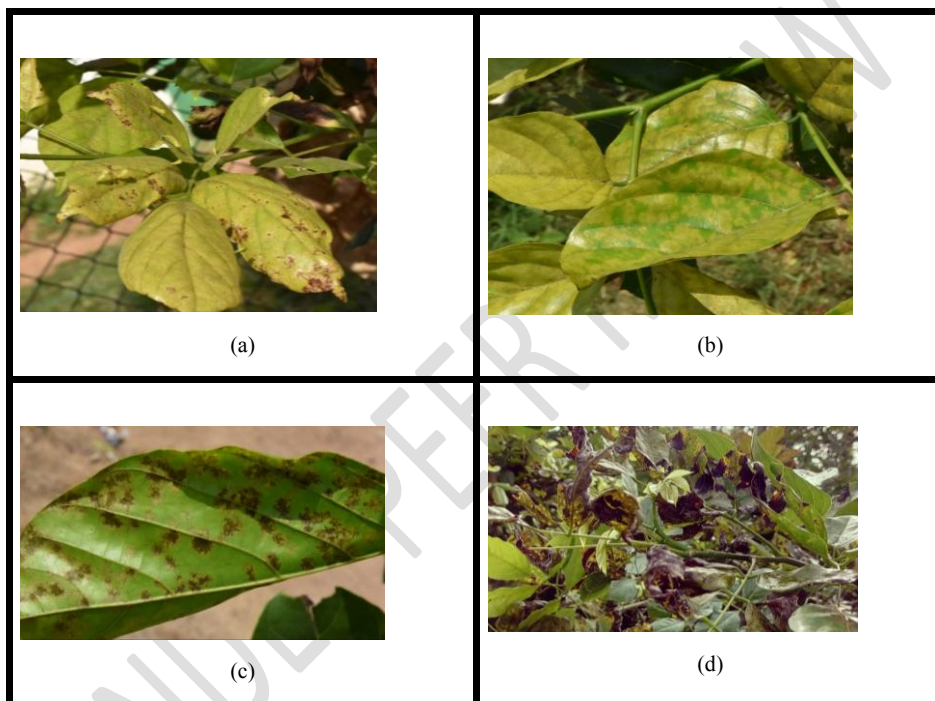


Figure 1: Symptoms of Leaf spot and blight of *Millettia pinnata* (a) Chlorosis, (b) Yellow spots on the leaf, (c) Black patches on the ventral portion of leaf, (d) Curling, drying and falling of diseased leaves.

Isolation of pathogen was carried out from fresh infected leaves of *M. pinnata*. The leaf sample that showed typical leaf spot and blight disease symptoms were cut into small sections of 1 cm diameter and were washed with sterile distilled water for 30 sec. The leaf sections were then surface sterilized with 1% sodium hypochlorite and 70% ethanol. The sections were placed on a microscopic slide and were further chopped with the help of a sterilized blade. 1-2 drops of sterilized distilled water were added onto the slide and the suspension was observed under stereo microscope for the presence of conidial spores. Based on the colony morphology, mycelium, and spore morphology, the species was identified as *Fusicladium pongamiae*.

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### 2.1.2 Preparation of pure culture

A pure culture of the disease-causing pathogen was obtained using a microscope and laminar air flow. Sterilized PDA was used as a growth medium. The fungal pathogen's pure cultures were obtained by extracting actively growing mycelium in the form of an 8 mm disc using a cork borer and an inoculation loop. The disc was then placed in the centre of a PDA plate to facilitate maximum growth. The Petri dishes were incubated in a BOD incubator for a week, with daily observations made until the pure culture fully developed (Figure 2). Observations were made using both a stereo microscope and a trinocular microscope, confirming that the morphological characteristics of the spores grown on PDA matched the initial observations of spores from the diseased leaf samples.



Figure 2: Pure Culture of *F. pongamiae*

### 2.1.3 Pathogenicity studies:

#### a. Spray inoculation

An *in vivo* infection system was employed to reproduce the leaf spot and blight disease symptoms. Conidia were collected by scraping 1-month-old cultures with a sterile spatula and transferring conidia to sterile water. Conidia were then filtered and the concentration determined using a hemocytometer. The concentration of conidia was then adjusted to 10<sup>5</sup> conidia/ml in sterile water with 0.5% Tween 20. Spore suspension (20 ml) was applied by spraying onto the leaves. *M. pinnata* seedlings, 2-month-old and 25 cm in height and presenting young leaves were used for inoculation. Six seedlings were inoculated with *F. pongamiae* conidial suspension, while other six control seedlings were spray inoculated with water and 0.5% Tween 20. All plants were kept in a chamber at 100% relative humidity for 1 week and afterwards moved into a greenhouse at 20°C and ambient relative humidity.

### 2.2 To evaluate anti-fungal agent(s) *in vitro* against the *Fusicladium pongamiae*

The biological control is the tool for reduction in inoculums density or disease producing activities of a pathogen or parasite in its active or dormant state by the activation of one or more organisms, either accomplished naturally or through manipulation of environment, host or antagonist or by mass introduction of one or more antagonists (Baker *et al.*, 1974). The antagonists such as fungi and bacteria can act as powerful means of suppressing or destroying the plant pathogens without disturbing the ecosystem and they do not create the problems of pollution, the development of resistance in pathogen and production of new biotypes. Hence, investigations on

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the biological control of *Fusicladium pongamiae* through potential antagonists were tested in vitro (Table 1).

Table 1: Treatments for evaluation of efficacy of biocontrol and anti-fungal agents

Treatment	Treatment Details
T1	<i>Trichoderma viride</i>
T2	<i>Pseudomonas fluorescens</i>
T3	<i>T. viride</i> + <i>P. fluorescens</i>
T4	Pongam oil (5%)
T5	Carbendazim (0.1%)
T6	Control

### 2.2.1 Preparation of pure culture of biocontrol agents

#### a. *Trichoderma viride*

The mother culture was brought from NIPHM National Institute of Plant Health Management, Rajendra Nagar Hyderabad, and was serial diluted with  $10^{-2}$  on dilution factor and added in PDA at Sterilized Petri dishes of 90mm. (1ml of dilutant solvent was taken with 1000ml of pipette added in PDA media with the help of spreader). Then wrapped the petri plates with para film to avoid contamination and kept in BOD Incubator at room temperature  $24^{\circ}\text{C}$  and allowed to grow for 3-5days.

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#### b. *Pseudomonas fluorescens*

Mother culture was procured from AICRP (All India coordinated Research Project), Rajendra Nagar Hyderabad. Then pure culture was inoculated at Department of Forest Resource Management, FCRI, Mulugu.

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Nutrient agar media was prepared. The mother culture brought from AICRP was serial diluted with  $10^{-2}$  on dilution factor and added in nutrient agar medium in aseptic conditions of Petri dishes of 90mm. (Diluted solvent was taken with inoculation loop and streaked on the medium). The petri plates were wrapped using Para film wax to avoid contamination and kept in BOD Incubator at room temperature  $24^{\circ}\text{C}$  and allowed to grow bacteria for 7days.

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### 2.2.2 Preparation of anti-fungal agents

#### a. Pongam oil (5%)

Pongam seed oil was procured from the Biochemistry laboratory, Forest College and Research Institute, Mulugu. The oil was extracted by High Performed Liquid Chromatography

(HPLC) purity of 67%. Then Pongam seed oil was made into 5% concentration by adding 5ml of Pongam seed oil in 95ml n-Hexane.

#### b. Carbendazim (0.1%)

Carbendazim (systemic fungicide) was procured and used to test their efficacy against the pathogen. 0.1% of carbendazim was prepared by mixing 1g of carbendazim in 1000 ml distilled water.

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#### 2.3 Dual culture:

The antifungal activity of isolated pathogen against pathogen was determined by dual culture. Briefly, an 8mm diameter mycelial disc was cut from the pathogen culture and *Trichoderma* pure culture had been cultured on PDA plates and *Pseudomonas* on Nutrient agar plates at 25°C for seven days. The *Trichoderma* disc was placed 1 cm from the edge of a fresh PDA plate, and *Pseudomonas* was streaked in the centre on nutrient agar media. The antagonistic effect was determined by measuring the size of the inhibition zone formed on plates after incubation at 24°C for 7 days. Zone of inhibition radius was recorded at 24, 48 and 96 hours of incubation and percentage of inhibition is calculated using the following formula

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$$\text{Percentage of inhibition (I)} = (C-T)/C \times 100$$

Where,

C - Diameter of fungal colony on PDA

T - Diameter of the fungal colony on treated plates

I - Percentage inhibition

#### 2.4 Poisoned food technique

The sterilized petri plates (90 mm) were poured with sterilized PDA agar medium and allowed to solidify. With the help of sterile pipette, 1000 ml of anti-fungal suspension was placed in the middle of the solidified plates and spread over the surface with the help of sterile spreader. one disc of the pathogen was placed at centre of petri plate. The inoculated plates were labelled and kept for incubation at room temperature (24°C). Zone of inhibition radius was recorded at 72, 120 and 144 hours of incubation.

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### 3. Results and Discussion

#### 3.1 Pathogen Isolation and Identification

*Fusculadium pongamiae* was isolated from fresh, infected leaves of *Millettia pinnata* collected from avenue plantations in selected villages of Mulugu Mandal. Infected leaf samples were incubated at 24°C, and mycelial growth with spores was observed after 5–7 days. The fungus was isolated from diseased leaves, transferred to Potato Dextrose Agar (PDA), and maintained as pure cultures following sufficient growth. Morphological characterization under a trinocular microscope confirmed the identity of *F. pongamiae*, with spores exhibiting long, one- to two-

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celled, septate, and capsule-shaped morphology, consistent with observations from dissected leaf samples (Fig. 1a–c).

### 3.2 Fungal Culture Purification

Pure cultures of *F. pongamiae* were established by excising an 8-mm disc from the initial culture using a sterilized cork borer and transferring it to PDA. Petri plates were incubated in a BOD chamber at 24°C for 3–4 days, allowing the development of mycelial blocks and spores. Purified cultures were stored at 4°C to prevent excessive fungal growth and used for subsequent experiments.

### 3.3 Pathogenicity Test on *Millettia pinnata*

Pathogenicity of *F. pongamiae* was assessed through spray inoculation on healthy *M. pinnata* leaves with a conidial spore suspension. Within 2 days, yellow spots appeared on the adaxial leaf surface, progressing to necrotic patches in 4–5 days. Brown lesions were observed on the abaxial surface on 7<sup>th</sup> day, associated by leaf curling and conidial accumulation on 8<sup>th</sup> day. Control leaves, inoculated with water and Tween 20, showed no symptoms, confirming the specificity of *F. pongamiae* induced symptoms. In a parallel experiment, conidial suspensions from infected leaves were sprayed onto healthy *M. pinnata* seedlings, resulting in small, scattered lesions on the abaxial surface after 2 weeks. These lesions expanded, turned brown, and indicated significant conidial production, consistent with previous reports (Dadwal and Soni, 1981).

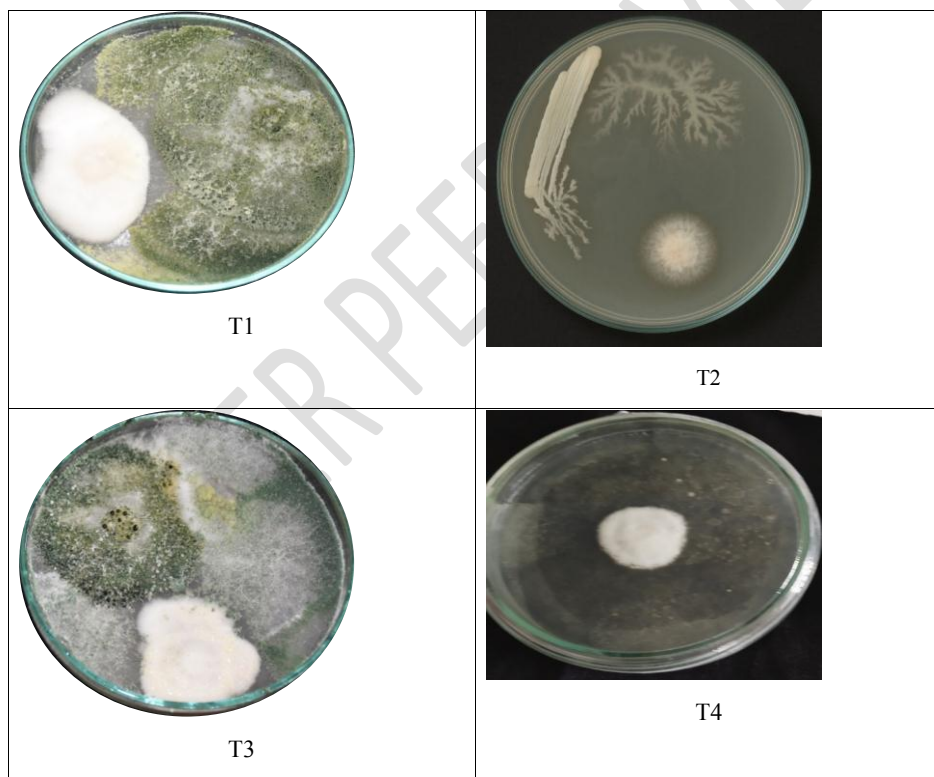
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### 3.4 Efficacy of Biocontrol Agents and Antifungal Compounds

The antagonistic activity of two biocontrol agents, *Trichoderma viride* and *Pseudomonas fluorescens*, against *F. pongamiae* was evaluated in vitro using the dual culture technique. Both agents were co-cultured with the pathogen on PDA for 7 days, and the zone of inhibition was measured relative to a pathogen-only control. *P. fluorescens* exhibited a higher inhibition of radial mycelial growth (68.46%) compared to *T. viride* (53.79%) ( $p < 0.05$ ). When both biocontrol agents were combined in a triangular arrangement with the pathogen, the inhibition rate was 61.46%. Antifungal efficacy was also assessed using the poison food technique, with PDA added with 5% Pongam oil and 0.1% Carbendazim. Carbendazim exhibited the highest inhibition (83.82%), followed by *P. fluorescens* alone, while Pongam oil resulted in 41.28% inhibition (Table 2 & Figure 3, 4). Control treatments showed no inhibition.

Table 2: *In vitro* evaluation of biocontrol agents and antifungal treatments against *Fusicladium pongamiae*

Treatment No.	Treatment Details	Mycelial Growth (mm)	Growth Inhibition (%)
T <sub>1</sub>	<i>Trichoderma viride</i>	33.70 ± 1.69	53.79 ± 1.20
T <sub>2</sub>	<i>Pseudomonas fluorescens</i>	23.00 ± 1.69	68.46 ± 1.20
T <sub>3</sub>	<i>T. viride</i> + <i>P. fluorescens</i>	28.10 ± 1.69	61.46 ± 1.20
T <sub>4</sub>	Pongam oil (5%)	42.82 ± 1.69	41.28 ± 1.20
T <sub>5</sub>	Carbendazim (0.1%)	11.80 ± 1.69	83.82 ± 1.20
T <sub>6</sub>	Control (Pathogen only)	72.94 ± 1.69	-



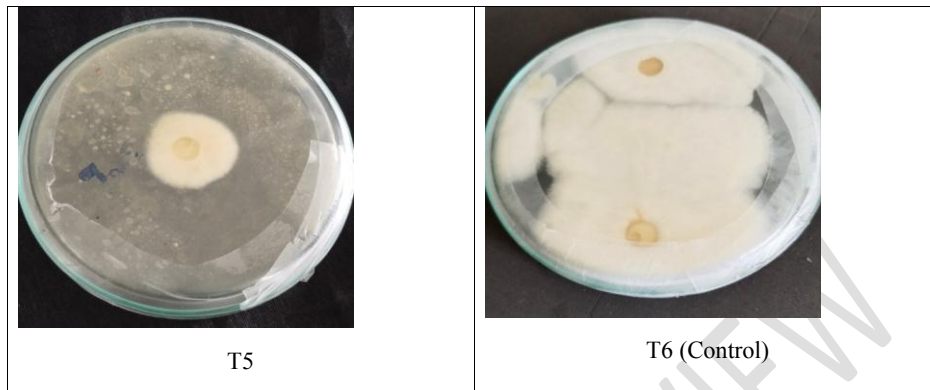


Figure 3: In vitro efficacy test of biocontrol and anti-fungal agents against *F. pongamiae*

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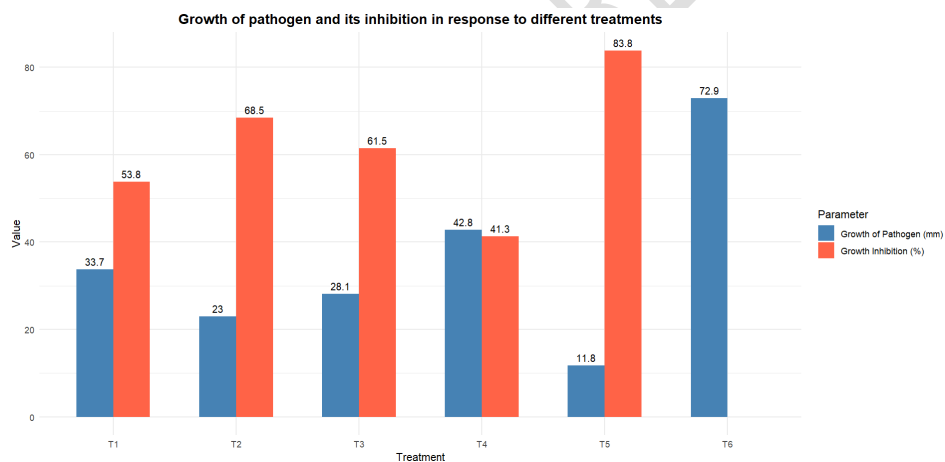


Figure 4: Growth of pathogen and its inhibition in response to different treatments.

The antagonistic activity of *Pseudomonas spp.* against phytopathogenic fungi is well documented (Whipps, 1997) and this property has often been used as means for *in vitro* screening of various biocontrol agents against novel phytopathogens (Elad *et al.*, 1980). It was reported that the combined role of both *Trichoderma viride* and *Pseudomonas fluorescens* (1+2%) in reducing mycelia growth of *Fusarium oxysporum* (Rajeswari and Kapoor, 2017) and also reported on cashew against *C. gloeosporioides* (Satapathy and Beura, 2020). Our *in vitro* data of antagonistic activity by *P. fluorescens* and *T. viride* reflect on their direct interaction with *F. pongamiae* via

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antibiosis or competition with the pathogen for nutrients. These interactions between the biocontrol agents and the phytopathogen are likely a result of highly coordinated and regulated cascade of metabolic events involving production of compounds and enzymes and other interfering metabolites in situ at low concentrations (Raaijmakers and Mazzola, 2012). The results of the present study were also in accordance with the findings of Bhanumathi and Rai, 2007 who reported in *Azadirachta indica* against the pathogens like *Colletotrichum dematium* and *Fusarium solani*. In which the spray of carbendazium at 50, 100, 150 ppm was found to reduce the mycelia growth of *Fusarium solani* in range of 88.54 - 86.32%, carbendazim (0.1%) has showed the 100 percent inhibition of fungal growth and also other fungicides like Propiconazole (0.1%), Mancozeb+Carbendazim (0.25%), tricyclazole (0.15%) were also showed 100 percent inhibition of fungal growth of *Colletotrichum gloeosporioides* on *Garcinia indica* and similar results were recorded by Sahoo, (2015), in terms of fungicidal role against the pathogen of *Colletotrichum gloeosporioides* in *Rauwolfia serpentina* and among, the biocontrol agents, *Pseudomonas fluorescens* reduced the 55.88 percent of mycelia growth and among the plant extracts, the Eucalyptus oil was able to reduce 39.8 percent of mycelia growth of *Colletotrichum gloeosporioides* and (Patil *et al.*, 2010) in sapota with leaf blight due to *C. gloeosporioides*. Dual culture and poison food technique in turmeric was reported by Kothikar (2017).

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#### 4. Conclusion

The current research confirmed *Fusicladium pongamiae* as the cause of leaf spot and blight of *Millettia pinnata*. Pathogenicity tests confirmed its disease-causing ability. Of the treatments under test, *Pseudomonas fluorescens* exhibited better in vitro antagonistic activity, followed by *Trichoderma viride*. The chemical fungicide Carbendazim (0.1%) inhibited greatest growth of fungi. These results demonstrate the potential of combining biocontrol agents with chemical management practices for successful disease suppression. These integrated strategies can decrease chemical dependency, encourage eco-friendly plant health management, and minimize environmental damage in Pongamia plantations infested by *F. pongamiae*.

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#### Conflict of interest:

No potential conflict of interest was reported by the authors

COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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