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Biological Control of Pythium Damping –off in Seedlings with Streptomyces Sp.

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Abstract: ***Damping-off is one of the severe diseases caused by soil-borne pathogens notably Pythium sp. the causative agent of this infection in raising tree saplings in forest nurseries. Biological control is an eco-friendly approach in disease management compared to chemical fungicides which in turn affects the soil environment. Biocontrol of Pythium sp. has been emphasized in vegetable nurseries than forest nurseries. The present research work is focused on identification of effective antagonistic organism from forest nursery soils against Pythium aphanidermatum. Bacteria were isolated from various forest soils collected from Boluvampati, Sirumugai and Mettupalayam forest nurseries in Coimbatore district and soil samples were screened for antifungal activity against Pythium aphanidermatum by dual culture technique. Among 245 bacterial isolates, one isolate KUMB1.1 exhibited clear zone of inhibition of 1cm and it was identified by 16S rDNA sequencing as Streptomyces sp. Solvent extraction was performed to isolate an active compound using ethyl acetate, dichloromethane, n-butanol, hexane and chloroform in the ratio 1:1. The antifungal activity of compound was performed by well plate method against Pythium sp. and n-butanol extract exhibited zone of inhibition. The antifungal activity of Streptomyces sp. was tested in a model plant Solanum lycopersicum (Tomato) seeds raised in Pythium aphanidermatum infested soils in seed trays under in vitro conditions. Pre-emergence and post-emergence disease incidences were observed, and the results exhibited promising efficacy of Streptomyces sp. against the fungal pathogen Pythium aphanidermatum. Seedbed study was carried out in Gmelina arborea seeds, where the seeds are treated with Streptomyces culture broth. In which seed treatment shows 43% increase in germination compared with control.***

Key words: Pythium, antifungal,Streptomyces, biocontrol, damping-off.

I. INTRODUCTION

The total value of the harvested wood is a sign of the contribution of forest to national economy. The prime objective of forest department is to develop and protect the forest for their maximum productivity. But the incidences of diseases caused by *Pythium* sp. causes heavy loss of economically important tree seedlings in the forest nurseries. The disease survey in nurseries of forest tree species in Kerala states that 50-60% mortality of seedlings [1].

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The damping-off disease survey in nurseries and main field in Coimbatore district causes 56.7% mortality of seedlings [2] and in forest trees causes 60% mortality of seedlings [3]
Pythium spp. belongs to the class oomycetes which mostly infects the plants in the seedling stage and also after the germination [5]. During the seed germination and when the roots grow, an ample amount of exudates are released and this attracts the zoospores which develops and infects the roots and seedlings [4].
Even though pesticides and fungicides have effective control over diseases caused by fungal pathogen, it deteriorates the environment and human health [6].
As an alternative approach, biological control which is an eco-friendly method is used to manage the plant diseases caused by *Pythium* spp. In order to implement this method, the interaction mechanisms in the environment and also the selection of antagonistic microbial inoculants should be studied [7]. Biological control is complicated when compared to chemical methods, as it requires exact procedures for application of the Biological Control Agents (BCA's) to specific fungal pathogen and different species of plant and also understanding of both target organisms and factors involved in biological control [8].
Few studies have been dedicated to monitor the feasibility of biological control and to evaluate the activity of bacterial and fungal BCA's which could control *Pythium* spp. in forest nurseries, forest stands and natural ecosystems [9].
Streptomyces sp. has been used as a biocontrol agent against different species of *Pythium* sp.. The current research is an attempt to explore the biocontrol efficacy of *Streptomyces* sp. against root rot pathogen *Pythium* sp. in forest nurseries.

II. MATERIALS AND METHODS

A. The Pathogen

Pythium aphanidermatum is a fungal pathogen which causes diseases in forest nurseries and vegetables and it is procured from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu. *Pythium aphanidermatum* was inoculated on Potato dextrose agar plates and incubated at 28± 4^{o} C for 3 days

B. Isolation of Bacteria from forest nursery soil samples

Six soil samples were collected from Boluvampatti forest nursery and ten soil samples were collected from Sirumugai and Mettupalayam forest nurseries of Coimbatore district, Tamil Nadu. Isolation of bacteria is done by serial dilution method. Mixture of soil was diluted with sterile distilled water up to 10^{-7}. 100 µl from 10^{-4} to 10^{-7} was spread gently on nutrient agar plates. After a 2-3-day incubation period at 37°C, single colony per propagation was selected for observation [10]

C. *In vitro* screening for antagonistic activity of bacterial isolates

In vitro screening of bacterial isolates for antagonistic activity was done by dual culture method on a Potato dextrose agar.

Pythium aphanidermatum disc was innoculated in the centre of the petriplates and incubated for 12 hrs, after which each bacterial isolate was streaked 3 cm away from the fungal disc and incubated for 3-5 days at 30 ± 2°C.The growth inhibition of fungal mycelia towards the bacterial isolate was the clear indication of antagonistic activity [11].

D. Molecular identification of isolate KUBPMB 1.1

Genomic DNA from the isolate KUBPMB1.1 was isolated using phenol: chloroform: iso-amyl alcohol method. 16S rDNA primer was used to perform PCR and the amplified PCR product was sequenced and it was assembled. The sequences were compared with already present sequences in the gene bank using BLASTn in NCBI GenBank and construct a phylogenetic tree with the higher similarity sequences.

E. Production of active compound

The composition of seed medium are 0.5% casein enzyme hydrolysate, 0.4% calcium carbonate, 2% starch, 0.5% ammonium sulphate, 0.5% yeast extract and 1% dextrose. Seed medium of 50mL is prepared and it is inoculated with *Streptomyces* sp. incubated in a rotary shaker at 150 rpm for 48 hrs at 27 °C. After incubation, 5 mL of the culture from seed medium was aseptically transferred to 95 mL of production media composed of 0.5% casein enzyme hydrolysate, 0.4% calcium carbonate, 2% starch, 0.5% ammonium sulphate, 0.5% yeast extract and 1% dextrose and water incubated on rotary shaker at 150 rpm for 10 days at 30 °C [17]

F. Extraction of active compound

After culture broth fermentation, the medium was centrifuged at 10,000 rpm at 4 °C for 10 min inorder to get rid of cell debris. Since the antibiotics are produced extracellularly, the supernatant of the culture is taken for antifungal activity and for solvent extraction. Solvents like Dichloromethane, Ethyl acetate, Chloroform, hexane and n-butanol were used for the solvent extraction. Solvent extraction is done using culture in the ratio of 1:1. And in a separating funnel, it is shaken actively for 15 min and kept inactive for another 15 min to separate the organic phase from the aqueous phase. The organic phase was collected and concentrated in a rotary vacuum evaporator [17]

G. Biocontrol efficacy of *Streptomyces* sp. in *Solanum lycopersicum* seeds against *Pythium* sp.

i) Seed procurement

Tomato seeds were obtained from the Department of vegetable crops, Horticultural College and Research Institute, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu. *Gmelina arborea* seeds were obtained from Institute of Forest genetics and tree breeding (IFGTB) nursery, Coimbatore, Tamil Nadu.

ii) Seed Treatment

Germination trays were used for the experiment. Soil was filled in the trays and the pathogen *Pythium aphanidermatum* was inoculated in the soil (10^4 spores/ml) and allowed to proliferate in the soil for a week. Tomato seeds were surface sterilized using teepol for 1 min and with 5% Sodium Hypochlorite for 2 mins and rinsed in sterile distilled water for 5 mins. The seeds were soaked in a bacterial suspension at a concentration of 10^7 CFU/ml for 1 hr and 3 hrs. The seeds soaked in sterile distilled water for 1 hr and 3 hrs served as control. Now, the treated seeds were sown onto the seed germination trays which were previously infested with *Pythium aphanidermatum* [18].

iii) Soil Infestation

In this treatment, Soil was filled in the germination trays and pathogen *Pythium aphanidermatum* was inoculated in the soil (10^4 spores/ml) and allowed to propagate in the soil for a week. The bacterial culture at concentration of 10^7 CFU/ml was added to the pathogen infested soil. After 16hrs, tomato seeds were surface sterilized and sown in the germination trays [18].

H. Biocontrol efficacy of *Streptomyces* sp. against *Pythium* sp. in *Gmelina arborea* seeds

i) Seedbed Studies of *Gmelina arborea*

Seedbed preparation is an important step that can optimize seed germination and survival rate. Length and width of the bed is 1 meter. The beds are raised 15 to 20 cm high from the ground level. There is a space left between two beds which are of 30 - 40 cm that helps in weeding, draining out excess rainwater, prevention of insect pest and diseases in plants. Seeds were soaked in natural Gum which is an adhesive agent used for seed coating then the seeds were treated with *Streptomyces* sp. culture broth and tested in *Gmelina arborea* seeds under nursery conditions.

III. RESULTS AND DISCUSSION

A. Isolation of bacteria from forest nursery soil samples

Bacterial isolates were obtained in a total of 245 (Fig 2) from different Coimbatore forest nurseries by serial dilution method.

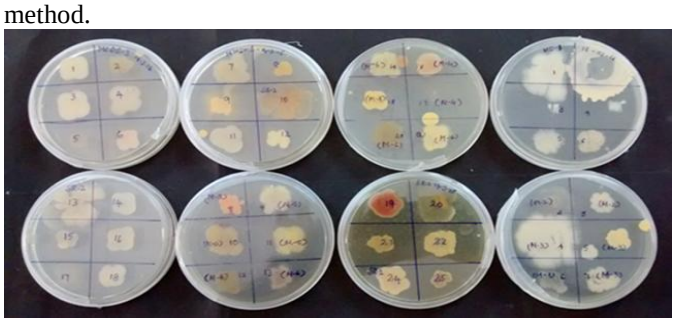


Figure 2: Bacterial isolates from forest nursery soils

B. Screening of bacterial isolates against *Pythium aphanidermatum*

Among 245 bacterial isolates, four isolates showed activity against *Pythium aphanidermatum*, of which one isolate KUBPMB1.1 exhibited zone of inhibition of diameter 1.5cm against *Pythium aphanidermatum* (Fig 3). Antagonistic activity is usually confirmed by the formation of zone of inhibition between bacterial isolate and the fungal isolate [12].

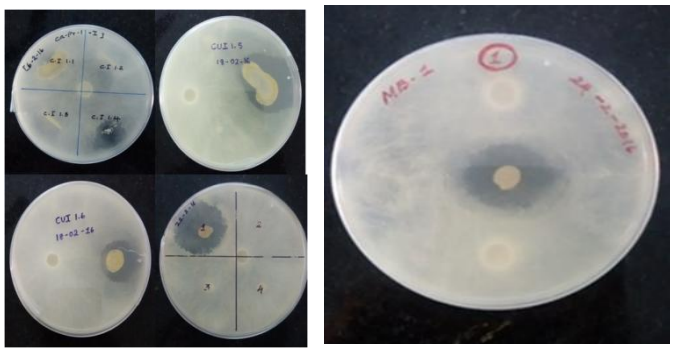


Figure 3: Antagonistic activity of KUBPMB1.1 against *Pythium aphanidermatum*

C. Molecular identification of KUBPMB1.1

The bacterial isolate KUBPMB1.1 showed 99% similarity with *Streptomyces* sp when subjected to BLAST. Phylogenetic analysis indicated that it formed a clade with the *Streptomyces* sp. (Fig 4). The 16S rDNA sequence was submitted to NCBI GenBank and the accession number (MK251462) was obtained

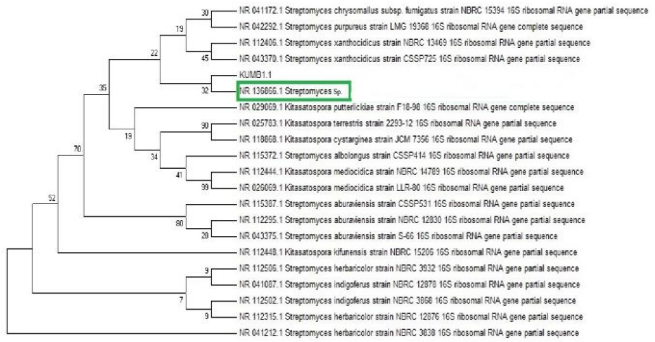


Figure 4:Phylogenetic tree of bacterial isolateKUBPMB1.1

D. Extraction and Isolation of active compound

The active metabolite (antibiotic) was extracted using ethyl acetate, dichloromethane, n-butanol, hexane and chloroform in the ratio 1:1.

Figure 5: Solvent extraction of active compound from *Streptomyces* sp. culture supernatant

The antifungal activity of compound was performed by well plate method against *Pythium* sp. and n-butanol extract exhibited zone of inhibition of diameter 0.8cm.

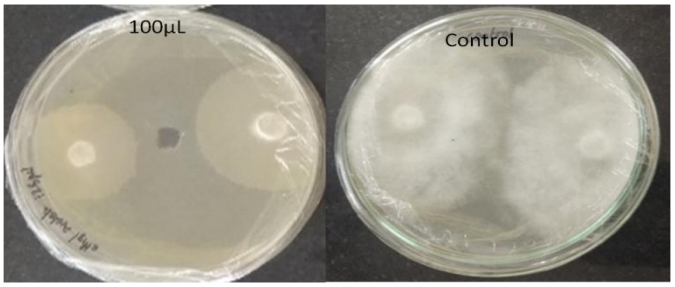


Figure 6: Zone of inhibition of n-butanol extract against *Pythium* sp.

Sodium phenylacetate and phenylacetic acid are the antifungal compounds isolated from n-butanol extract of *Streptomyces humidus* culture filtrate [19].

E. Biocontrol efficacy of *Streptomyces* sp. against *Pythium* sp. in *Solanum lycopersicum* seeds

Solanum lycopersicum seeds started to germinate on next day of sowing. The germination was monitored on day 4 and day 7 and germination percentage was calculated. On day 4, Germination percentage was found to be 37% for control and 40% in seed treatment for 1 hr incubation and 46% in seed treatment for 3 hrs incubation. On day 7, germination was found to be 69% in control and 70% in seed treatment for 1 hr incubation and 78% for seed treatment for 3 hrs incubation. The germination percentage has increased as the duration of the treatment increased. Disease incidences were observed on day 8 and day 14. On day 8, pre-emergence damping-off was monitored where the control showed disease incidence of 31% and seed treatment for 1 hr incubation showed 30% of disease incidence and seed treatment for 3 hrs incubation showed 22% of disease incidence. Pre-emergence damping-off cannot be detected easily because of non-visibility of seeds and often losses are pointed as “poor seed” [13]. After an extensive period, if the seed has not emerged then it has to be dig out and examine; the damping-off fungi is involved when seeds are decayed inside [14].

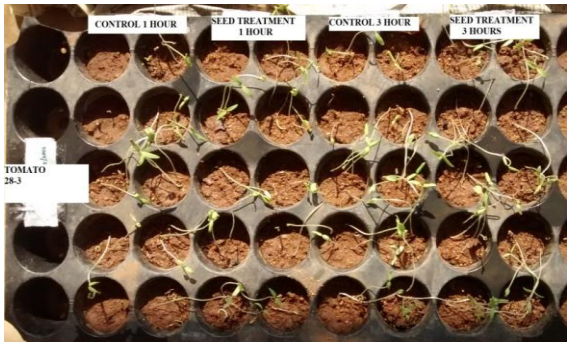
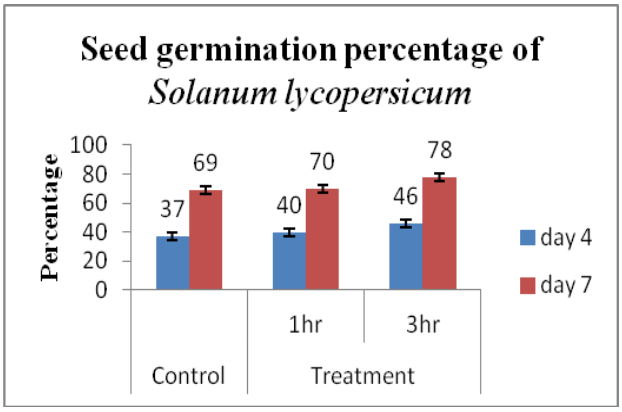


Figure 7: Germination trays used for the *In vitro* study

On day 14, post-emergence damping-off was monitored where control showed 19% disease incidence and seed treatment for 1hr incubation showed disease incidence of 12% and the seed treatment of 3 hrs incubation showed 10% disease incidence. Disease incidence has decreased in 3hrs treatment compared to control on day 8 and day 14. But when comparing 1hr and 3hrs treatment on day 8, the disease incidence increased for 3hrs treatment and it found to be decreased when compared within day 8 and day 14 for 3hrs treatment. Postemergence damping-off which leads to seedling death after emergence or transplantation as the stem tissue near the soil line is weakened and decayed, causing plants to fall over and die [14]. Post-emergence damping off symptoms like root rot; chlorosis and wilting were noted on day 14. Comparison of the germination percentage within the treated seeds indicated that the percentage of germination increased (78%) in the seeds that received 3h treatment on day 7. The germination percentage and damping-off disease percentage was examined at 50th day after the seeds were sowed. The germination rate of the ginseng seeds was found to be 75% [15]. The germination percentage of tomato seeds was 91%, using biocontrol agent *Streptomyces* sp. DPTB13 to control damping-off disease [16]. The disease incidence decreased as the day progressed. This can be attributed to the induced defense responses in the growing plant triggered as a result of the treatment.



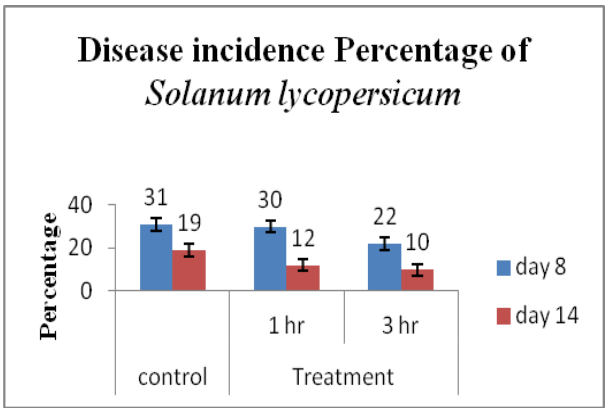


Figure 8: Germination and disease incidence percentage of *Solanum lycopersicum*

F. Biocontrol efficacy of *Streptomyces* sp. against *Pythium* sp. in *Gmelina arborea* seeds

i) Seedbed Studies of *Gmelina arborea*

Seeds were pretreated with natural gum and then treated with *Streptomyces* sp. culture broth. Seedbeds were infested with *Pythium aphanidermatum* and the treated seeds were sown in the seedbed. Seed treatment shows 26% increase in germination compared with control

Table 1: Germination percentage of *Gmelina arborea* seeds in seedbed study

Treatments	Germination Percentage of *Gmelina arborea*
Seed Treatment	31%
Control	5%

Figure 9: Details of seed bed germination of *Gmelina arborea* in pretreatment of natural Gum, Seed treatment + Soil application of *Streptomyces* sp. culture broth

Figure 9: Details of seed bed germination of *Gmelina arborea* seeds sown in the soil infested with *Pythium* sp. as a control setup

IV CONCLUSION

This present research work is a first attempt to isolate bacteria from forest nursery soils of Boluvampatti forest nursery and screened for its antifungal activity against *Pythium aphanidermatum*. Further the bacterial isolate exhibited zone of inhibition of diameter 1.5cm. The antifungal activity of compound was performed by different solvent extraction and confirmed by well plate method against *Pythium* sp. Out of which n-butanol extract showed zone of inhibition.In *Solanum lycopersicum*, the germination percentage was comparatively found to be higher about 78% in seed treatment of 3 hrs treated bacterial culture. The disease incidence was found to be very less of 22% in the same treatment. Further studies need to be performed to determine the antifungal metabolite of *Streptomyces* sp. KUBPMB1.1. and to elicit the production of the active compounds.

In seedbed studies, germination percentage of *Gmelina arborea* was found to be 43% increase in germination in Seed treatment compared with control.

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