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In vitro Screening of *Streptomyces* spp., against Necrotrophic Pathogen *Pythium aphanidermatum* Causing Damping-off in Tomato and Chilli

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A B S T R A C T

Damping-off in tomato and chilli caused by *Pythium aphanidermatum* is an opportunistic pathogen more prevalent on young or weak plants causing extensive damage in nurseries and mainfield. Control of this disease by biological method is gaining a momentum because of its high efficiency and environmental friendliness. In the present study, an attempt has been made to explore the bioactive rhizosphere actinomycetes for suppressing the pathogen. Rhizosphere soil samples were collected from various locations in The Nilgiris and Coimbatore districts and twenty seven different actinomycetes were isolated. All the isolated microbes were characterized morphologically by colour series and growth pattern on artificial media which showed that they were belong to *Streptomyces* spp. All the 27 isolates were screened under *in vitro* condition which revealed that the isolate ACM 14showed a maximum inhibition of 26.6 percent over the control, hence the antagonistic actinomycetes may probably be used against the damping-off pathogen.

Introduction

Tomato **(***Lycopersicon esculentum* Mill.,) and Chilli (*Capsicum annum* L.,) are the two important versatile vegetable crops with wide usage in Indian culinary tradition. Besides their cultivation and usage worldwide, the productivity is slowed down due to various pests and diseases. Damping-off is one among the diseases and it causes 30% seedlings mortality (Muriungi *et al*., 2014). Pre-emergence damping-off caused decaying or shrivelling of seeds, whereas post emergence damping-off caused death and toppling of the seedlings. It gets aggravated due to high soil temperature, moisture, poor soil aeration, lack of drainage and thick stand of seedlings. Control of soil borne diseases are tiresome

due to their wide host range, prolonged survival of spores and other resting structures in soil and lack of resistant cultivars (Kilany*et al.*, 2015). *Pythium*a saprophytic oomycete fungal like organism also called as water mould, is the largest genus causing severe damages in many crop plants. It forms resting structure called sporangia releasing numerous zoospores which later develops into oospores by surviving in soil and greenhouses (Loliam *et al.*, 2013). *Pythium aphanidermatum* causes damage to the economically important crops and is the most pathogenic (Muthukumar *et al.*, 2016). Management using fungicides like metalaxyl, strobilurin results in phytotoxicity, environmental pollution, development of fungicide resistance in plants, detrimental to non-targeted and beneficial microorganisms (Bharathi *et al.*, 2004).

Exploring the beneficial rhizosphere microbiome will be an alternate strategy for combating damping-off disease. The antagonistic microbes act directly by attacking the resting spores or mycelium by interfering with germination, infection process or indirectly by inducing host resistance (Termorshuizen and Jeger, 2008). Actinomycetes are Gram positive saprophytic soil inhabitants, widely distributed microorganisms with antibiotic producing capacity and growth promoting activity used for controlling soil-borne pathogens (El-Tarabily *et al*., 2008; Palaniyandi *et al*., 2011). Actinomycetes present in soil mostly belong to *Streptomyces* and 60% of the bioactive molecules obtained from them are used for agricultural purposes (Ilic *et al*., 2007). *Streptomyces* present in plant rhizosphere protect roots by inhibiting the pathogen growth through production of antifungal compounds and enzymes that degrade fungal cell wall (El-Tarability *et al.*, 2008) besides, it also enhances plant growth through production of plant growth promoters like auxin and gibberellin (El-Tarability 2008). It plays a dual role by acting as a plant growth promoter and as a suppressor of plant disease through mechanisms like increasing the supply of nutrients namely phosphorus, sulphur, iron, copper, production of IAA, cytokinin and siderophore (Gowdar *et al.*, 2018). Microbial antagonists like *Streptomyces* spp., *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Trichoderma* spp. have been used for managing damping-off diseases. *Streptomyces* spp., like *S. griseoviridis* (Mycostop) and *S. lydicus* WYEC108 (Actino-Iron) are the potent producer of hydrolytic enzymes that degrades the cellwall of the fungi like *Pythium, Phytophthora* and *Fusarium*. Cellulolytic *S. rubrolavendulae* S4 cause lysing of hyphaltips and abnormal swelling of mycelium of *Pythium aphanidermatum* (Loliam *et al.*, 2013), *S. rochei* from tomato rhizosphere produce IAA which aids in increasing seed germination, root elongation thereby increasing the plant growth (El-Tarabily 2008).

The intention of this study is to isolate the novel *Streptomyces* spp., perform preliminary characterization and identify the effective antagonistic for managing *Pythium* under *in vitro* condition.

Materials and Methods

Isolation and phenotypic characterization of rhizospheric actinomycetes

The soil samples were collected from rhizosphere region of different plants from different locations in The Nilgiris (Muththorai 11°24'36" N, 76°41'59.99" E, Lovedale 11°22’54" N, 76°42’6" E, Nanjanad 11°36’68" N, 76°64’56" E) and Coimbatore districts (Eastern farm, TNAU 11°07'3.36" N, 76°59'39.91" E).Sampling was done in 40 days old crops to a depth of 10cm by

removing the top soil for about approximately 3cm and mixture of rhizosphere soils collected randomly from three plants in each location and stored in sterile polythene bags. Isolation was done by serial dilution technique using Kenknights agar medium amended with ampicillin (5 µg/ml) and cycloheximide (20 mg/l) to reduce bacterial and fungal contamination, respectively (Trabelsi *et al.*, 2016) and the plates were incubated at 28±2^0 C for 3-5 days. After incubation small white pinhead size powdery colonies appear which are purified and maintained by streaking on starch caesin agar medium (Kumar *et al.*, 2010). The morphological characters like aerial spore mass colour, substrate mycelium colour, colony texture and pigment production were recorded and compared with the observations made in International *Streptomyces* Project (ISP) medium containing data of 450 species of *Streptomyces* and *Streptoverticillum* (Shirling and Gottlieb, 1966).

Source of pathogen

Pythium aphanidermatum Udumalpet strain (NCBI accession no. MK817574) isolated from the tomato was obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore.

In vitro screening of antagonistic actinomycetes against *P. aphanidermatum*

The antifungal activity of the isolates against *P. aphanidermatum* was performed using the dual culture technique as described by (Dennis and Webster 1971). A quantity of 20 ml of PDA medium was poured into Petriplates after solidification, the actinomycetes isolates were streaked at one end of the plate and incubated for 3 days at 28±2^0 C. Later, mycelial disc of pathogen (9 mm dia) was placed opposite to actinomycetes and incubated at 28±2^0 C for 2 days. Control was maintained by placing pathogen alone. Efficacy of the isolates was determined by measuring the mycelial growth of pathogen over control. Percent inhibition over control was calculated using the formula.

$$PI = \frac{C - T}{C} \times 100$$

C - growth of pathogen (mm) alone in control plate

T - growth of pathogen (mm) in presence of antagonist isolate.

Statistical analysis

All the experiments were analysed independently and the treatment means were compared using the Duncan's Multiple Range Test (DMRT). SPSS version 16.0 developed by IBM Corporation was used for analysing the experiments.

Results and Discussion

Isolation of actinomycetes

A significant loss in the yield of many crops are mainly due to soilborne diseases. *Pythium aphanidermatum* (Edson) Fitz causing damping-off of chilli and other solanaceous vegetable crops is a severe threat to vegetable production and various methods like chemical and biological control measures are available for managing the disease (Ghosh, 2002). Actinomycetes have been extracted from many unexplored environments and extreme habitats for the past few years. Many of them could be considered as a unique or novel species with the potential of producing metabolites and enzymes with antagonistic activity (Martinez, 2012). *Streptomyces* occupies nearly 10% of soil microflora having the ability to colonize plant root surfaces under varied environmental conditions and soil types and the antibiotics produced are of

biodegradable used for making pathogen-specific fungicides with less side-effects to ecosystem. Using Kenknightsagar medium actinomycetes were isolated by serial dilutions of rhizosphere soil from 10^{-2} to $10^{-6.}$ After incubation for 5 days, small pinhead size white powdery colonies started to appear which were further streaked and maintained on starch casein agar medium. Consideration of using antibiotics as a precautionary measure has been suggested by many authors while isolating *Streptomyces* (Kitouni *et al.*, 2005; Errakhi *et al.*, 2009). Hence, for inhibiting the bacterial and fungal contamination ampicillin (5 µg/ml), either cycloheximide (50 µg/ml) or nystatin (50 µg/ml) were used (Fguira *et al.*, 2012). These revealed the importance of constituents added during isolation. Based on morphology, totally 27 different actinomycetes isolates were obtained from the samples collected and labelled from 1 to 27.

Table.1 Phenotypic characterization of isolated actinomycetes

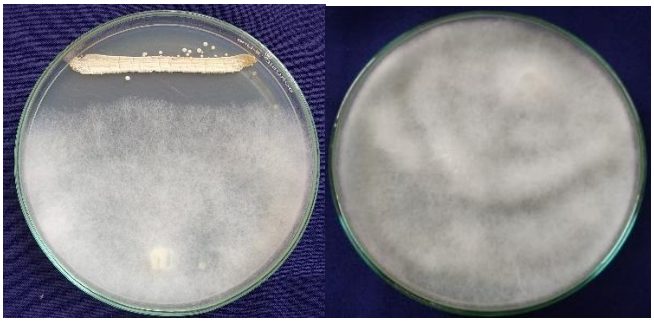
Isolate	Substrate Colony	Reverse Colony	Colony texture
AOR1	Grey	Light orange	Powdery
AOR2	White	Light yellow	Powdery
AOR3	White	Ash	Powdery
AOW4	White	Cream	Powdery
AOW5	White	White	Powdery
AOW6	White	White	Powdery
AOT7	Grey	Cream	Powdery
AOT8	Cream	Cream	Powdery
AOT9	grey	Light orange	Powdery
AOP10	White	White	Powdery
AOP11	Light orange	Light orange	Powdery
ACM12	White	Yellow-Orange	Powdery
ACM13	Grey	Light orange	Powdery
ACM14	Grey	Yellow-orange	Cottony
ACS15	White- grey	Cream	Cottony
ACS16	White	Orange	Powdery
ACS17	White	Pink	Powdery
ACS18	Dark brown	Orange	Powdery
ACS19	Grey	Light orange	Powdery
ACS20	Grey	Brown- yellow	Powdery
ACS21	Grey	Yellow	Cottony
ACS22	White	White	Powdery
ACD23	Brown	White	Powdery
ACD24	Grey	Light brown	Powdery
ACSO25	White	Light yellow	Powdery
ACPM26	Cream	Light yellow	Powdery
ACPM27	White	Cream	Powdery

Table.2 *In vitro* screening of *Streptomyces* spp. against *P. aphanidermatum*

Antagonists	Mean mycelial growth (mm) *	Percent inhibition over control **
AOR1	70	22.20[c] (28.11)
AOR2	70	22.20[c] (28.11)
AOR3	70	22.20[c] (28.11)
AOW4	90	0.00[h] (1.62)
AOW5	86	4.44[e] (12.16)
AOW6	88	2.22[g] (8.57)
AOT7	68	24.40[b] (29.60)
AOT8	90	0.00[h] (1.62)
AOT9	90	0.00[h] (1.62)
AOP10	84	6.60[d] (14.89)
AOP11	86	4.44[e] (12.16)
ACM12	70	22.2[c] (28.11)
ACM13	70	22.20[c] (28.11)
ACM14	66	26.60[a] (31.05)
ACS15	70	22.20[c] (28.11)
ACS16	70	22.20[c] (28.11)
ACS17	68	24.40[b] (29.60)
ACS18	70	22.20[c] (28.11)
ACS19	70	22.20[c] (28.11)
ACS20	70	22.20[c] (28.11)
ACS21	68	24.40[b] (29.60)
ACS22	87	3.3[f] (10.47)
ACD23	87	3.3[f] (10.47)
ACD24	70	22.20[c] (28.11)
ACSO25	90	0.00[h] (1.62)
ACPM26	90	0.00[h] (1.62)
ACPM27	90	0.00[h] (1.62)
Control	90	0.00[h] (1.62)

*Values are means of three replications. In a column, means followed by a common letter are not significantly different at the 5% level by DMRT; **Values in parentheses are arcsine transformed values.

Figure.1 (1) Interaction of *P. aphanidermatum* with ACM 14; (2) Control *P. aphanidermatum* alone



Phenotypic characterization of actinomycetes

Phenotypic characterization of the isolates was done by observing the colour of matured aerial mycelium, colour of substrate mycelium and powdery or cottony textured colony characters. The isolates showed typical morphology of *Streptomyces* by growing on agar medium with earthy odour. Most of the isolates exhibited the colour series as white, brown, grey, cream and yellow with powdery growth colonies (Table 1). Phenotypic characterization notably the aerial mycelial colour as white, brown, grey and cream with substrate mycelial colours like light orange, yellow, cream, pink with powdery and cottony textured colonies indicated that they were confederated to novel *Streptomyces* genus which are considered as preliminary identification (Taddei *et al.*, 2006). Several authors *viz*., Nanjwade *et al.*, (2010), Kumar *et al.*, (2010) and Sharma *et al.*, (2014) have also followed the same phenotyping method for their isolates. The difference in colour series of the isolates may be due to the diversity of isolates in the sites chosen.

In vitro screening of actinomycetes

Actinomycetes which are abundant in rhizosphere colonize plant roots and play a role in plant growth promotion. It is well known that most of the *Streptomyces* spp., exhibit antimicrobial activity. Interaction of *Streptomyces* with fungal pathogens leads to the production of cell wall-degrading enzymes such as cellulases, hemicellulases, chitinases, amylases, and glucanases. In order to find the effective one, all the 27 isolates were screened against *P. aphanidermatum* by dual plate method. Isolate ACM 14 showed the maximum percent inhibition of about 26.6 % over control (Table 2; Fig. 1) followed by ACS 17 and 21 which showed 24.40 per cent inhibition over the control while, few isolates namely AOW4, AOT8, ACSO25, ACPM26 and ACPM27 had not inhibited the mycelial growth of the pathogen. *Streptomyces* spp., from rhizosphere regions showed antimicrobial activity against *Pythium* similarly *Streptomyces* sp. CA-2 against tomato damping-off with improved seedling vigour as reported by (Goudjal *et al.*, 2014), *S. griseoviridis* against cucumber damping-off and *S. rochei* ERY1 against damping-off of cabbage as reported by (Suwitchayanon *et al*., 2018) which showed the potentiality of using them as a biocontrol agent for damping-off disease.

Chemical fungicides are unsuitable for damping-off management because of residual effect. Hence biological control might be a better alternative. Thus, it was concluded that

Streptomyces sp., which possess growth promoting activities directly or indirectly benefit the plant growth and are rhizosphere competent, utilizing all plant sugars available in rhizosphere. *Streptomyces* sp., with its unique antifungal activity isolated from this study may be exploited to combat damping-off disease of tomato and chilli after field experiments.

Abbreviation

IAA – Indole Acetic Acid, ISP – International *Streptomyces* Project

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Conflict of Interest: None declared

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