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SCREENING OF ACTINOMYCETES FOR ANTIMICROBIAL ACTIVITY AGAINST THE STRAINS OF *RALSTONIA SOLANACEARUM* (TOMATO BACTERIAL WILT DISEASE)

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Abstract–Actinomycetes constitute the most important group of bacteria, in which *Streptomyces* having the high potential in producing secondary metabolites e.g. antibiotics. Actinomycetes play an important role in the rhizosphere region where they influence plant growth and protect plant roots against invasion by pathogenic fungi. Actinomycetes are isolated by taking soil samples from 3 different habitats of Mahe Region of Puducherry Union Territory, India. These samples were serially diluted and plated on actinomycete isolation agar media. Potential colonies were screened and purified. Isolates were morphologically characterized. Antibacterial activity and Minimum Inhibitory Concentration (MIC) of the purified extract of isolates were evaluated. Six actinomycete isolates were observed according to the colour of aerial mycelium from different soils of Mahe region. They are characterized by their tough powdery, pigmented colonies. These were gray, cream, blue, pink, red and white. Among 6 actinomycete, antibiotics produced by one of the *Streptomyces* isolates had shown activity against *Ralstonia solanacearum*. Other isolates did not show any antibiosis against any of the test pathogens. The plant pathogen was inhibited by most of the *Streptomyces* isolates.

INTRODUCTION

Actinomycetes are the most economically valuable prokaryotes which are well known to produce chemically diverse metabolites with wide range of biological activity (Balagurunathan *et al*., 2007). Recent days the discovery of known metabolites and actinomycetes are increasing due to the exploitation of natural ecosystems. Exploitation of less and unexplored ecosystems for actinomycetes is highly necessary for the discovery of novel bioactive metabolites. Actinomycetes are important sources of new bioactive compounds such as antibiotics and enzymes (Vining *et al*., 1992) which have diverse clinical effects and are active against many organisms (Bacteria, Fungi, Parasites etc.). In fact more than 50% of the known natural antibiotics are produced from actinomycetes (Miyadoh *et al*., 1993). The most striking feature of the actinomycetes is their ability to produce a wide variety of secondary metabolites. These natural products have been extraordinary sources of lead structures in the development of newer drugs (Kutzner *et al*., 1986).

Actinomycetes are responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics (Berdy *et al*., 2005), anti-tumor agents (Crag *et al*., 2005), immunosuppressive agents (Mann, 2001) and enzymes. Because of the excellent track record of actinomycetes in this regard, a significant amount of effort has been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past fifty years. Recently, the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased (Fenical *et al*., 1999). Thus, it is crucial that new groups of actinomycetes from unexplored around exploited habitats be pursued as sources of novel bioactive secondary metabolites.

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A large number of actinomycetes have been isolated and screened from soil in the past decades, accounting 70-80% of relevant secondary metabolites available commercially. Actinomycetes which are profilic products of antibiotics and important supplier to the pharmaceutical industry. Actinomycetes have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. The researches have been remarkably successful and approximately two thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes. Goodfellow *et al.* (2000) reviewed the literature on isolation of actinomycetes and suggested that only 10% of the actinomycetes are isolated from nature. Most of the antibiotics in use today are derivatives of natural products of actinomycetes and fungi, (Butler *et al*., 2006). Actinomycetes can be isolated from soil and marine sediments. Although soils have been screened by the pharmaceutical industry for about 50 years, only a miniscule fraction of the surface of the globe has been sampled, and only a small fraction of actinomycetes taxa have been discovered (Baltz *et al.,* 2008).

The two major groups of soil actinomycetes that serve as important sources of antibiotics are *Streptomyces* and *Micromonospora*. It has been stated that *Streptomyces* account for about 80% of the total antibiotic products; while *Micromonospora* closely follows with less than one tenth as much as *Streptomyces*. Furthermore, previous experimental analysis, have also proven that secondary metabolites isolated from soil actinomycetes, are potent inhibitors of numerous plant pathogens. For example, it has been highlighted that actinomycetes from farming soils, have the capacity to inhibit *Erwina amylovora*; a bacterium that causes fire blight to apples.

Agrobacterium tumefaciens; the causative pathogen of crown gall disease in plants

The richness and diversity of actinomycetes present in any specific soil, is greatly influenced by the soil type, geographical location, cultivation and organic matter amongst other factors. Numerous studies have been done by scientists to isolate actinomycetes, as sources of antibiotics.

However, because actinomycetes occur widely in nature, only a small percentage of the globe and a small proportion of actinomycetes species have been screened. Actinomycetes have been intensively studied in several underexplored environments, niche, and extreme habitats in various parts of the world (including India) in the last few years. Yet, there is no report regarding isolation of actinomycetes from Mahe region of Puducherry Union Territory (India). Therefore, present study is aimed to isolate antimicrobial producing actinomycete which can resist the Tomato Bacterial Wilt Disease caused by *Ralstonia solanacearum* from this unexplored region in order to find novel species.

MATERIALS AND METHODS

Study of site and location

Mahe region is a tiny pocket of the Union Territory of Puducherry (Fig. 1) which is embedded on the west by the Arabian Sea, Moolakadavu River on the north and other two sides by stretch of hills of medium height. There are five villages viz. Pandakkal, Palloor, Chalakkara, Chembra and Cherukallayi besides the Mahe town. Total area of the region is 9 Sq.Kms.

Temperature of the region varies 19 to 36 degrees celcius depending upon the season and on an

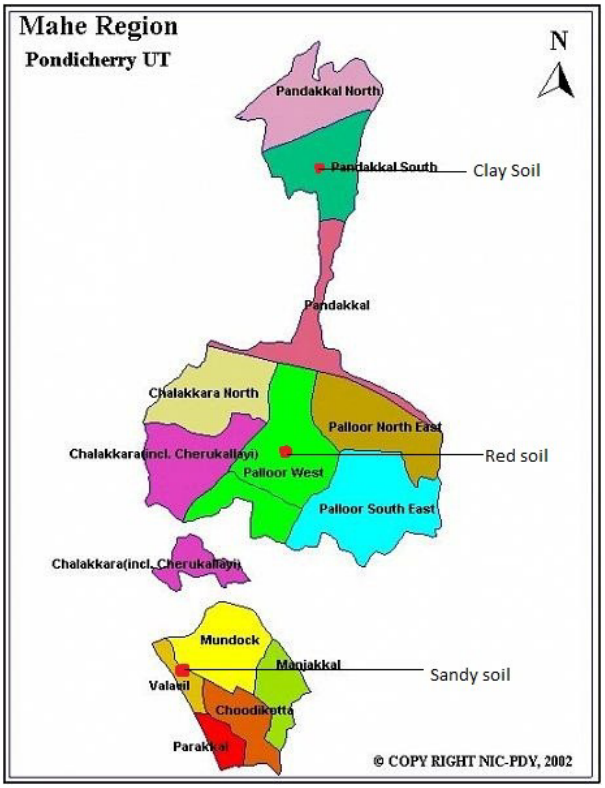

Fig. 1. Sample location

average of about 3000 mm of rainfall is received by this region. The region receives continuous rain between June and August mostly under the influence of South-West monsoon. During the month of September, moderate rains are received due to the influence of north-east monsoon. Soil is laterite in the inner areas and sandy towards the coastal areas. Patches of hils of moderate heights also is a specific nature in this region. Coconut, Arecanut, Pepper, Banana, Tapioca and Vegetables are cultivated in this region.

Sample collection

Soil samples were collected from three villages of Mahe Region which is of three soil types. Villages from where samples were collected type of soil have been depicted in Table 1.

Table 1. Sites for collection of soil samples

Sl.No	Revenue village	Type of soil
1	Pandakkal	Clay soil
2	Palloor	Red soil
3	Mahe	Sandy soil

Soil samples were collected from a depth of 0-5cm from surface of the site. Samples were collected in sterilized plastic bags, labelled properly and brought to lab. Soil samples were dried for appropriate time depending on their moisture content so that number of contaminant microbes decrease (Seong *et al.,* 2001).

Screening of Actinomycetes for Antimicrobial activity

The screening process were completed by two methods-

Primary and Secondary Screening

In primary screening the antimicrobial activity of pure isolates were determined by inoculation of isolated strains onto actinomycetes isolation agar by streak method. The plates were incubated at 28 °C for 3 days. The test organisms used was *Ralstonia solanacearum*. Secondary screening was performed by agar well diffusion method.

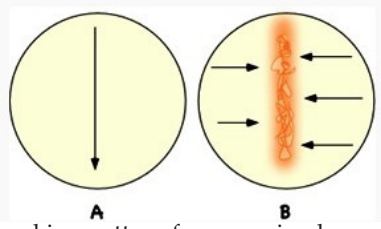

Fig. 2. Streaking pattern for screening by perpendicular streak method. (A) The placement of the Actinomycetes Colonies. B. The placement of the test culture of *Ralstonia solanacearum*

Cross streak method (Selvin *et al.,* 2009)

The actinomycete strains were streaked (Fig. 2) on one half of the medium plate. Plates were incubated at 28 °C for 7-10 days. 10 µl of the overnight grown culture broth of the sensitive strain was inoculated at right angles to the actinomycete tract. Plates were incubated at 28 °C for 48 hrs. If the isolate has antibacterial activity, it causes a zone of growth inhibition of the sensitive strain around the actinomycete culture.

Morphological Characterization of Actinomycetes Strains

Microbes are largely characterized on the basis of their morphological characters. The microscopic and macroscopic studies of an actinomycetes growing on agar can provide useful and rapid clues for identification of their respective genus. Macroscopic characters include colony characteristics such as size, shape, color, consistency on different media, the absence or presence of aerial mycelium and extent of spore formation. Cultures are observed for microscopic feature including fragmentation of substrate and arial mycelium, presence of sclerotic, spore chain morphology and spore surface ornamentation. On the basis of spore chains, the strains can be placed into groups.

For example, the species belonging to the genus *Streptomyces* are divided into three groups broadly, i.e. rectiflexibiles, ratinaculiberti and spirals. Characteristics of the spore bearing hyphae and spore chains can be determined by light microscopy using cover slip culture and slide culture techniques. Actinomycetes are also observed by the phase-contrast microscopy for the study of spore surface ornamentation. Genera of purified isolate can be identified based on morphological comparisons to the existing description of known genera as given in Bergey' s Manual Determinative Bacteriology. Then, selected and identified colonies of actinomycetes were transferred from the plate to starch casein agar slant and incubated at 37 °C for growth.

Testing of antimicrobial activity of Actinomycetes on Tomato Wilt

The bacterial culture was isolated from bacterial wilt

infected tomato plant.

Isolation of Bacterial Wilt Pathogen

The pathogen causing wilt in tomato was isolated from naturally infected plants. The infected plants were uprooted and washed in running tap water to remove adhering soil particles. The samples were then air dried. The stem portion of the infected plant was cut into small pieces and surface sterilized with 1% sodium hypochlorite for five minutes followed by rinsing in three changes of sterile water. The sample bits were then gently crushed on a sterile glass slide with a drop of sterile water and the suspension was streaked on Triphenyl Tetrazolium Chloride (TZC) medium in petri dish.

Plates were incubated at room temperature for 48 hours. Typical colonies of Bacterial Wilt pathogens were selected, purified by repeated streaking and sub cultured on nutrient agar slants.

Determination of the Antibacterial Activity by Agar well diffusion method (Okudoh and Wallis, 2001)

The antimicrobial activities of those extracts were tested against *Ralstonia solanacearum* by using agar well diffusion method. Sensitive strain seeded plates were prepared by mixing appropriate volume of fresh grown culture with 50 ml of soft agar, so as to have a final concentration of cells equal to 105cfu/ml and over-layering 3 ml of this suspension on to growth medium plates. The top layer was allowed to solidify for half an hour in the laminar flow hood. Wells of 7mm diameter were bored into the sensitive strain seeded agar plate. Different concentrations of ethyl acetate extract were added into the wells. Plates were incubated at 28 ºC for 24-48 hours and diameters of the zones of inhibition produced by bioactive compounds were measured.

Minimum Inhibitory Concentration (MIC)

Quantitative assay was done by agar dilution method which was used to determine MIC of extract against test bacteria. The minimum inhibitory concentration is the minimum concentration of the antibacterial agent in a given culture medium below which bacterial growth is not inhibited. MIC provides an idea of effectiveness an active extract or compound against a microorganism.

Statistical analysis

The data generated during this investigation for various characters were statistically analysed.

RESULTS AND DISCUSSION

Sampling

The samples were collected from the 3 villages with 3 different type of soil namely clay, red and sandy soil of Mahe region.

Isolation of Actinomycetes

Among the total actinomycetes isolation, six isolates were isolated from soil samples collected from the villages of Mahe Region (Table 2). Of these, the red soil shown more actinomycete population than other two samples.

Screening of isolated actinomycetes for their antimicrobial activities

Primary screening: As the results of primary screening, only one actinomycete isolate showed antimicrobial activity against *Ralstonia solanacearum*. The total isolates was designated as C-1, C-2, C-3, C-4, C-5 and C-6. Only C-3 isolate have shown the second antagonistic activity against *Ralstonia solanacearum.*

The present study of primary screening using single streak methods indicated that, only one out of 6 actinomycete isolates showed potential antimicrobial activity against *Ralstonia solanacearum*.

Inhibition of Indicator Bacteria

Table 2. Morphological characteristics observed on microscopic examination

Colony type	Morphological characteristic
Colony type 1 (C 1)	Appeared as large bright white filaments, with net-like mycelia.
Colony type 2 (C 2)	Appeared as pale white branching filaments and had powdery appearance.
Colony type 3 (C 3)	Had a dark brown uniformity and crumb-like appearance.
Colony type 4 (C 4)	Had a light brown appearance with cilia-like mycelia on its boundaries.
Colony type 5 (C 5)	Showed a dark brown appearance, embedded with concentric circular patterns. Each pattern was separated by regions of clear zones
Colony type 6 (C 6)	Appeared as yellow colonies with beautiful mycelia and transparent boundaries

From the 6 actinomycetes isolates only one inhibited the indicator bacteria. Some of the zones of inhibition were present but not very obvious. The widest inhibition zone (diameter = 9.5 mm) was recorded for C- 3.Observation of clear inhibition zones around the wells on the inoculated plates is an indication of antimicrobial activities of antibiotics extracted from actinomycetes against test organisms. Gurung et al. reported 0-18 mm inhibition zone of crude extracts against selected test organisms.

The efficiency of actinomycete isolate against *Ralstonia solanacearum* was evaluated under invitro condition. Out of six isolate only one was recorded with inhibition. Based on the morphological result obtained the isolate showing inhibition was identified to be genus streptomyces.

Soil actinomycetes isolated from soil (soil type 3) in this study, were of six colony types. On initial microscopic examination, the clear zones observed in some grown actinomycetes colonies (A-3 and A-

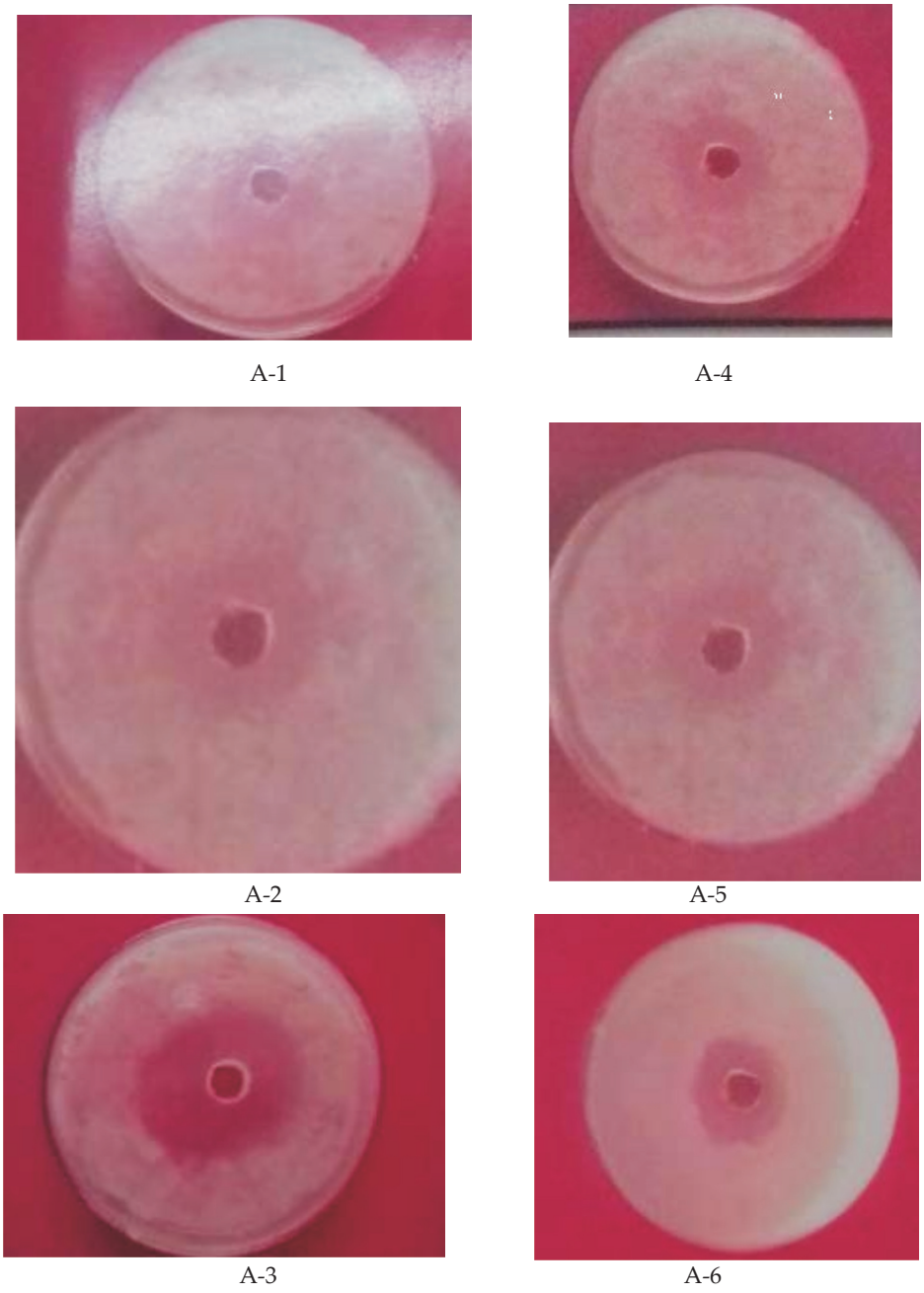

Fig. 3. Effect of isolated actinomycete against *Ralstonia solanacearum* under invitro condition

4), indicate that the organisms broke down the chitin for growth and development. Colony types (A-1, A-2, A-3 and A-4) that did not develop clear zones, may have utilized other nutrients in the medium such as nitrates, phosphates and sulphates, in order to grow.

Among six actinomycete isolates were obtained only two has potential used against bacterial wilt in tomato by the production of both\ primary and secondary metabolites. Members of this genus are now known to share many phenotypic characteristics and to continue a distinct phyletic line, and they are currently assigned to over 23 validity described species. It is clear that minimal standards for description of *Streptomyces* species need to be based on a judicious selection of genotypic and phenotypic properties.

Streptomyces, soil-dwelling filamentous bacteria are profile producers of wide range of antimicrobial agents and this continue to be a rich source of vitamins, carbon, nitrogen, amino acids utilization and enzymes.

It has been reported that 1g of soil when plated, harbours up to 10 billion microorganisms, of which about 4.2×10^6 CFU/g (dry weight) are accounted for by bacteria species. The number of actinomycetes (3.5×10^5 CFU/g dry soil) obtained in this study was however lower than expectation. This may be partly attributed to the geographical location of the soil. The soil was collected from an area that was relatively steep and not quite in the shade. The steepness of the terrain, exposure to the sun rays may have caused leaching of actinomycetes and therefore have partly contributed to the observed low actinomycetes CFU/g of dry soil.

Absence of clear zones found in three (A-1, A-2, A-3 and A-4) (Fig. 3) actinomycetes isolates during final examination of chitin agar plates, shows that no antibiotics were produced in such colonies. Although, the other two isolates (A-5 and A-6), were able to inhibit the growth of indicator bacteria, however the inhibition was not observed in all tested indicator bacteria. This shows that there was a low secretion of antibiotics by such actinomycetes isolates. A probable explanation for these results could be that the culture method used in this study, did not provide ideal conditions that should have enabled the growth of large numbers of actinomycetes and secretion of high amounts of antibiotics.

It is evident from the present study, actonomycetes have the potential to control tomato wilt disease and further in depth study is needed to assess the potential of antimicrobial activity of actinomycetes.

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