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Diversity of Actinomycetes in Tomato Plants

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ABSTRACT

Background: Tomato plant holds great biodiversity of actinomycetes. This diversity can be explored for new potential actinomycetes strains.

Methods: Two different methods were followed for actinomycetes isolation (Serial dilution and direct streaking method). The direct streaking method was found to be the best method where it gave a higher number of actinomycetes compared to the plant extract method. All the actinomycetes were isolated by ISP2 medium supplemented with nystatin and cycloheximide (at 50 µg/ml). All the endophytic actinomycetes strains were isolated and purified based on the difference in appearances such as colony morphology, growth pattern, aerial hyphae growth, filamentous appearance and pigment production.

Result: The direct streak method, yielded 192 isolates and only 48 isolates by plant extract method. Thus, a total of 240 strains were isolated. Plants from Madurai yielded a maximum of 130 isolates (54%) and a minimum from Tuticorin 20 isolates (8%). The highest viable count of 80.01±14.24 (CFU g^{-1}) × 10^3 were recorded in the agro- fields of Dindugal followed by Madurai, Theni, Tirunelveli and Tuticorin. Out of 240 strains, 49.5% exhibited different aerial hyphae and 36.25% showed different filamentous appearances of growth and 5.8% produced pigments. The endophytic actinomycetes species isolated from tomato plants in different locations possess significant morphologically varied actinobacterial diversity. We can explore further on how these diverse actinomycetes are involved in plant growth promotion and protection against pathogens.

Key words: Colony morphology, Endophytic actinomycetes, ISP2 medium, Isolation, *Lycopersicon esculentum*.

INTRODUCTION

Tomato (*Lycopersicon esculentum*) belongs to the genus *Lycopersicon* under the *Solanaceae* family. Tomato is one of the most important “protective foods” because of its special nutritive value. It is one of the most versatile vegetables with wide usage in Indian culinary tradition (Helaly, 2021). Tomato is an important economic crop grown worldwide that is of commercial value and ecological importance. Tomato is the world’s largest vegetable crop after potato and sweet potato, and it tops the list of canned vegetables. The total global area under tomato was 46.16 lakh ha with global production of 1279.93 lakh tonnes. All India production estimate of Tomato in 2021 was estimated at 21 Metric Tonnes, estimated to have amounted to 852 thousand hectares. The cultivation area increased from the previous fiscal year. India ranked second on the list of nations producing tomatoes during the measured time period (Statista.com).

Endophytic microbes are a diverse group of bacteria, fungus, and actinomycetes that live inside plant tissues (Arini *et al*., 2021). Among these endophytes, actinomycetes are that associated with plants have played a crucial role in protecting the host from phytopathogenic invasions (Crawford *et al*., 1993). Several endophytic actinomycetes act as a plant growth promoter by producing plant growth hormones like Indole3-Acetic Acid (IAA) or iron-chelating molecules (Rungin *et al*. 2012). Endophytic microorganism has been in association for millions of years with their eukaryotic hosts, from lower crops to higher plants, representing an important increasing resource of new secondary metabolites (Firdous *et al*., 2019). In the last decade, approximately half of the newly discovered (5000 compounds) metabolites were isolated from endophytic strains. Antibiotic and vitamins-producing actinomycetes are beneficial for the plants physiological processes (Trajkovic *et al*., 2018). Plant endophytic microbes, often exhibit plant identities and plant second genes (Michelmore *et al*., 2017). These endophytes can live within cells, vascular system, or the capillary cells of the plant (Maggini *et al*., 2017). They are beneficial to the host plant, *e.g.*, biological control of plant diseases (Liu *et al*. 2017). Therefore, it is important to have a good understanding of the endophytic actinobacterial communities in the tomato plant. Endophytic *Streptomyces* sp. also provide advantages to the host plant by enhancing the physiological activity of the plant or through other modes of action and thus may serve as a source of agro-active compounds, biocontrol agents, or plant growth promoters (Fadiji and Babalola, 2020; Vurukonda *et al*., 2018). The possibilities of using these endophytes as biological control agents against tomato speck, wilt, fusarium crown, root rot

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are very high (Nandhakumar *et al*., 2020). Endophytic actinobacteria have attracted attention in recent years, with increasing reports of isolates from a range of plant types, including crop plants (cereals, such as wheat and rice, as well as potatoes, carrots, tomatoes and citrus (Singh and Dubey, 2018) and medicinal plants (Gos *et al*., 2017). Therefore, the present study aims to screen the diversity of endophytic actinomycetes in tomato plants of southern Tamil Nadu.

MATERIALS METHODS

Tomato plant sample collection

Healthy tomato plants (*Lycopersicon esculetum*) were collected from five different districts (Tirunelveli - 8.7815° N, 77.3942°E; Tuticorin 9.1727°N, 77.8715°E; Madurai - 9.9420°N, 77.9724°E; Dindukal - 10.4489°N, 77.9360°E and Theni - 10.0015°N, 77.6164°E) of southern Tamil Nadu in India. Plants were collected from three different places for each district. And three samples from each farm at the fruiting stage. Three tomato plants were randomly collected from the corner and centre of the field. Thus, a total of fifteen plants were collected in a sterile polypropylene bag and brought directly to the laboratory for microbiological processing. Thus, 45 samples (3 from each site) were collected from different areas/locations of Tamil Nadu.

Sample preparation

The collected plants were washed in running water to remove all adherents. Each isolation procedure was done in triplicate for each plant sample. The plant samples were cut to 4-5 cm length using the sterile surgical cutter. The plant parts were shoot tip, stem (upper and lower regions), root-(upper and lower regions). The disinfection and isolation of actinomycetes were performed according to Araújo *et al*., (2000) with minor modifications. The plant parts were disinfected superficially disinfected as 70% alcohol for 1 min, 90% ethanol 1 min, sodium hypochlorite (0.9%) for 4 min, 70 ethanol for 30 seconds, 10% $NaHCO_3$ for 5 min and finally rinsed in sterile, distilled water. Sterile distilled water wash was done for 5 minutes for every change in the solution. To ensure the disinfection protocol, an aliquots of the sterile water used in the final rinse were plated in an ISP2 medium (Yeast malt extract agar- yeast extract - 4.0 g malt extract - 10.0 g dextrose - 4.0 g agar 20.0 g).

Actinomycetes isolation

Two different methods were followed to evaluate and to get the maximum number of actinomycetes from tomato plants.

Serial dilution method

Small pieces of shoot tip, stem, root were separately ground separatively with 6 mL of aqueous solution (0.9% NaCl) in a sterile mortar and pestle. The tissue extract was subsequently incubated at 28°C for 3 hours to allow the complete release of endophytic microorganisms from the host tissue. For the isolation of endophytic bacteria, the tissue extract was diluted in an aqueous solution and plated on five ISP2 agar plates supplemented with nystatin and cycloheximide (at 50 µg/ml) for each dilution (from 10^{-1} to -10^{-5}). The plates were incubated for 15 days at 30°C. Colonies were selected on 7th, 10th, 12th and 15th days of incubation. The isolated colonies are pure cultures of actinomycetes that were separated on the following methodology, time of appearance, growth rate, and morphology (color, shape, and size). All of the colonies were counted and expressed as CFU (Colony Forming Unit) per one gram of fresh tissue.

Direct streaking method

The washed and surface-sterilized stems were cut in a cross direction and streaked on the ISP2 agar supplemented with nystatin and cycloheximide (at 50 µg/ml) incubated at 30°C for 7th, 10th, 12th and 15th days. Thus, actinomycetes alone were allowed to grow and separated as specified in the previous serial dilution method.

Identification

Each petri dish was evaluated; the colonies were selected according to their time of growth and morphology (color, size and shape). Different strains were identified based on the morphological characteristics of colonies on the plate, areal hyphae on the agar plate, the morphology of spores, and pigment production. Isolates were labelled serially as VITGV. These separated strains were purified and maintained in glycerol suspensions (30% v/v) at -80°C.

RESULTS AND DISCUSSION

Actinomycetes isolation

Endophytic actinomycetes diversity in the Tomato plant was explored in this study. The surface sterilization process is the basic step to isolate and purify the endophytes. The addition nystatin and cycloheximide (at 50 µg/ml supplemented with ISP2 agar plate surface-sterilized sample showed no microbial contamination. In addition, ISP2 agar plates spread with the last washed water also did not have microbes. The results show the surface sterilization protocol is very effective. Similar validation of sterilization was done by Cao *et al*. (2004) for *Streptomyces* sp.

A total of forty-five plant samples were randomly collected from fifteen different agro sites. The sampling sites are shown in Fig 1. The highest number of actinomycetes were recorded in Madurai (54.16%) followed by Dindugal (16%) and the lowest count was recorded in Tuticorin (8.33%) (Fig 2).

Among the two methods tested, the direct streaking method was best with 80% (192) collection while serial dilution yielded 20% (48) (Table 1). Thus, a total of 240 isolates were obtained. Madurai recorded the highest number of isolates in serial dilution and direct streak method 20 and 110 of isolates, while the lowest no of isolates were recorded in Dindugal 4 by serial dilution method) and in Tuticorin 12 by direct streaking method (Fig 3). The results

were recorded in Table 2. The microbial colonies were segregated based on different morphological characters. They are separated and purified as separate strains (Fig 4). As for the isolation methods, sample streaking had the best result compared with grinding plant parts and spreading. As we directly streak plant parts and placed plant parts in the ISP2 media, the positive environment around the plant parts might favour the endophytes to come and colonize. While grinding plant parts could be stressful leading to sudden change in environment thus a low number of colonies. The serial dilution plate count method for actinomycetes isolation from crushed plant extract was cultured by serial dilution technique. From this method, only 48 different colonies were isolated and purified. Previously Costa *et al*. (2012) isolated 158 endophytic bacteria from the bean in a TSA (Tryptic Soy Agar) medium by serial dilution technique. Similar isolation of endophytic bacteria from the leaves of *Anredera cordifolia* CIX1 was reported by Nxumalo *et al*. (2020). By placing the cut plant parts, helps enlarged colonies attracting all actinomycetes to come out of plant parts and colonize in the ISP2 medium. This result coincided with Tan *et al*. (2006) reported as 619 actinomycetes were isolated from tomato plants by the plant streak method.

Based on the morphological analysis was performed for all 240 endophytic isolates, they are categorized as aerial hyphae, colony appearance and pigment producing strains. The total number of isolates and their category is given in Table 3. According to the preliminary morphological identification, the most abundant genus was *Streptomyces* sp. a similar finding was reported for different hosts plant wheat by Coombs and Franco (2003) and neem plant Verma *et al*. (2009). Accordingly, there were more aerial hyphae (49.5%) followed filamentous (45%) and less pigment producing strains (6%).

These isolates belong to the different genera of actinomycetes. Out of 240 isolates, 23.33% (n=56) are small-

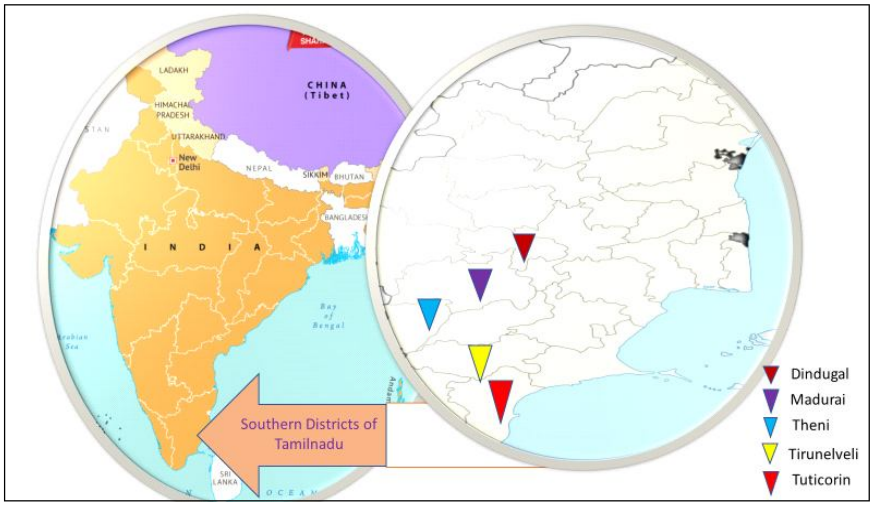

Fig 1: Showing the different locations from where tomato plants were collected for actinomycetes isolation.

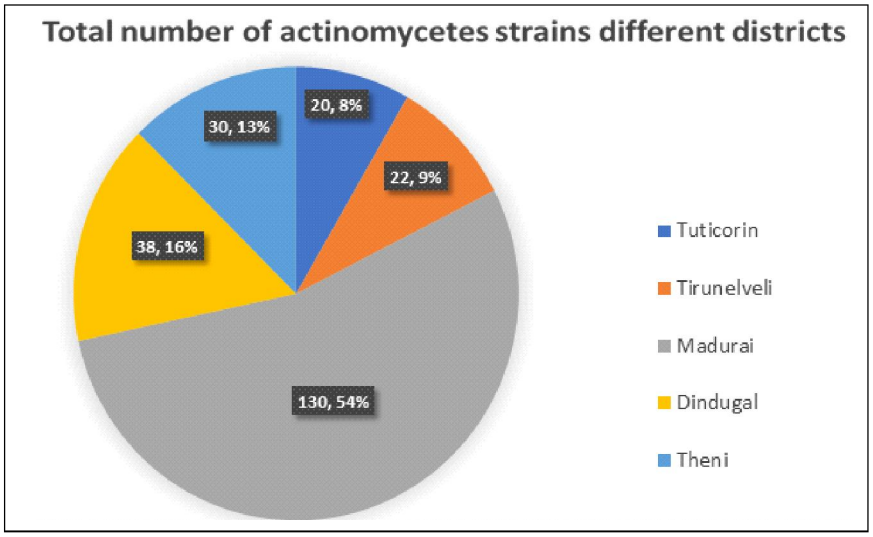

Fig 2: A pie chart illustrating the total number of actinomycetes strains isolated from different districts of Tamil Nadu.

sized colonies, 22.08% (n=53) are large-sized colonies, 22.08% (n=53) rough-surfaced colonies, 10.83% (n=26) smooth-surfaced colonies, 7.5% (n=18) dry textured colonies, 8.3% (n=20) viscid textured colonies and 5.8% (n=14) pigment-producing colonies appeared in ISP2 agar medium (Table 3).

Three major criteria were analysed in the growth pattern of actinomycetes in ISP2 and results were recorded in Table 3. The first condition for growth is aerial hyphae, which is recorded in all the districts 49.5% (n=119). Among these, the highest aerial hyphae colonies (n=80) were recorded in Madurai (33.3%) while the lowest number (n=8) was

Table 1: Showing samples that were collected from different districts, their code and number of strains.

Districts	Sampling sites	VIT GV strains	No of strains	Designation of series of endophytic actinomycetes strain
Tuticorin	TCSS1	VITGV 1-6	6	VITGV 1, VITGV 2, VITGV 3, VITGV 4, VITGV 5 and VITGV 6
	TCSS2	VITGV 7-13	7	VITGV 7 and VITGV 8
	TCSS3	VITGV 14-20	7	VITGV 15, VITGV 16, VITGV 17, VITGV 18, VITGV 19 and VITGV 20
Tirunelveli	TVSS1	VITGV 20-34	14	VITGV 22, VITGV 29, VITGV 30, VITGV 31, VITGV 32 and VITGV 34
	TVSS2	NIL	Nil	Nil
	TVSS3	VITGV 34-42	8	VITGV 35 and VITGV 36
Madurai	MSS1	VITGV 43-172	130	VITGV 59, VITGV 63, VITGV 65, VITGV 66, VITGV 68, VITGV 69, VITGV 72, VITGV 76, VITGV 77, VITGV 81, VITGV 87, VITGV 88, VITGV 83, VITGV 94, VITGV 95, VITGV 96, VITGV 98, VITGV 99, VITGV 100, VITGV 103, VITGV 104, VITGV 105, VITGV 106, VITGV 107, VITGV 110, VITGV 113, VITGV 114, VITGV 115, VITGV 117, VITGV 119, VITGV 121, VITGV 122, VITGV 125, VITGV 128, VITGV 137, VITGV 140, VITGV 156, VITGV 161, VITGV 162 and VITGV 164.
	MSS2	Nil	Nil	Nil
	MSS 3	Nil	Nil	Nil
Dindugal	DSS1	VITGV 173-202	30	VITGV 173, VITGV 176, VITGV 177, VITGV 178, VITGV 200, VITGV 201, VITGV 193 and VITGV 194
	DSS2	VITGV 203-210	8	VITGV 206, VITGV 205 and VITGV 209
	DSS3	Nil	Nil	Nil
Theni	TSS1	VITGV 211-240	30	VITGV 224, VITGV 225, VITGV 226 and VITGV 228
	TSS2	Nil	Nil	Nil
	TSS3	Nil	Nil	Nil

Table 2: Showing the number of endophytic actinomycetes count from serial dilution method and direct streaking method from different districts.

Sampling districts of Tamil Nadu	Serial dilution method		Different isolates from direct streaking method
	Plate count (CFU g^{-1}) × 10^3	Different isolates	
Tuticorin	18±34.94	8	12
Tirunelveli	45.3±15.9	6	16
Madurai	65 6±10.4	20	110
Dindugal	80.01±14.24	4	34
Theni	49.3±64.48	10	20
	Total	48 (20%)	192 (80%)

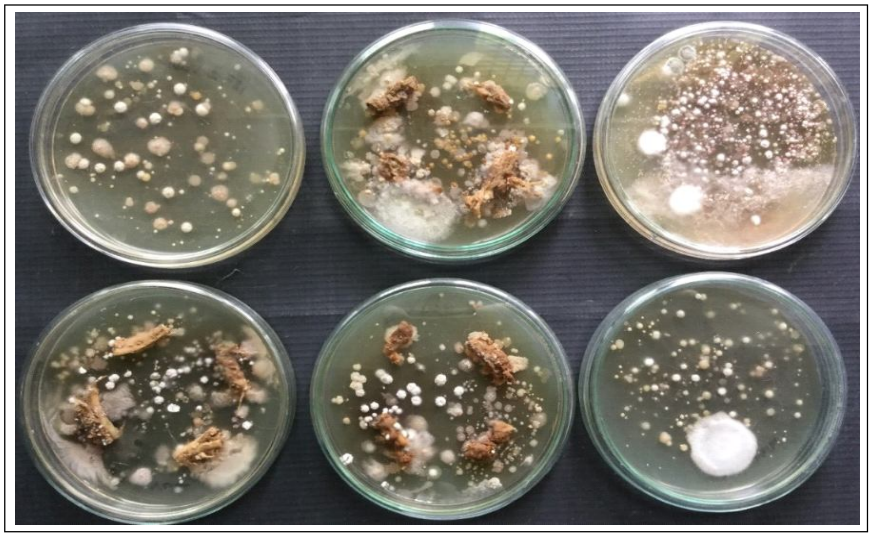

Fig 3: Growth of Actinobacterial colony for isolation by serial dilution method and streak plate method.

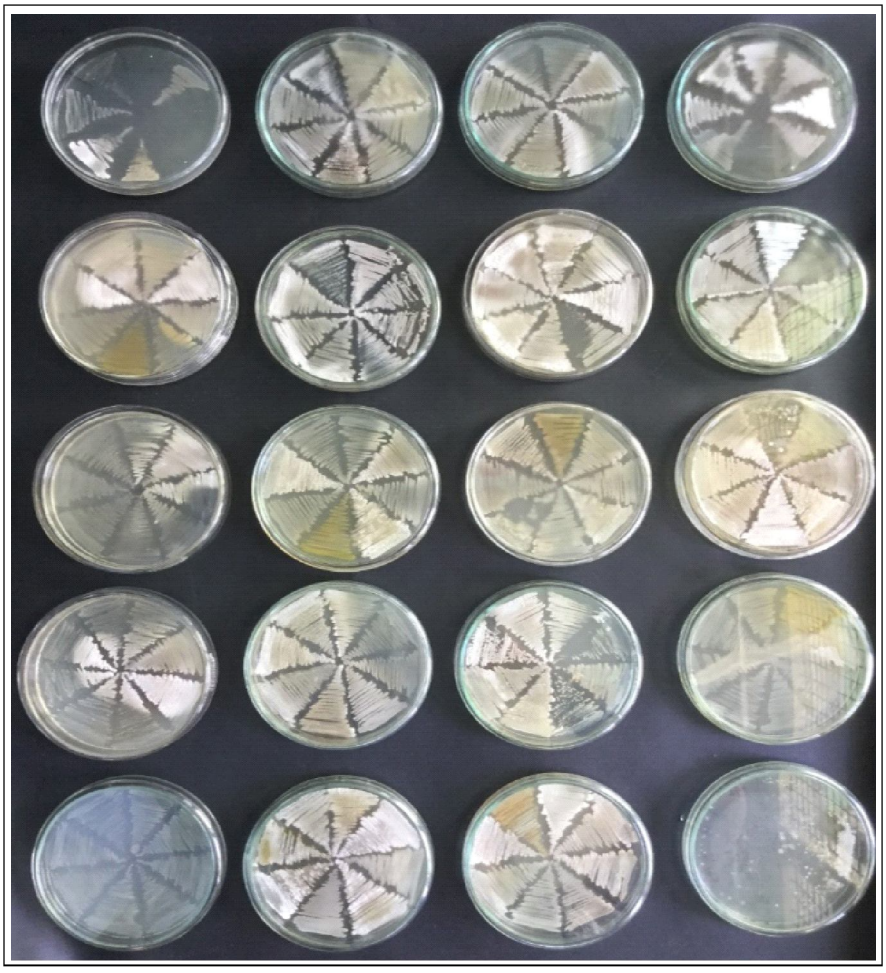

Fig 4: Purified endophytic actinomycetes isolate colonies in ISP2 medium.

Table 3: Different morphological characteristics of actinomycetes count from different sites of Tamil Nadu.

Analysis of the Structure of colony			No of colonies isolated from different districts of Tamil Nadu				
			Tuticorin	Tirunelveli	Madurai	Dindugal	Theni
Aerial hyphae	Size	Small	3	-	26	1	3
of growth		large	-	2	32	4	3
	Surface	Rough	2	1	10	2	1
		Smooth	1	3	6	2	1
	Texture	Dry	1	1	3	1	2
		Viscid	2	1	3	-	2
	Total-19 (49.5.%)		9	8	80 (33.3%)	10	12
Filamentous	Size	Small	2	-	14	2	5
appearance		large	-	2	5	2	3
of growth	Surface	Rough	3	4	16	14	-
		Smooth	1	1	5	6	-
	Texture	Dry	1	3	2	2	2
		Viscid	1	1	4	-	6
	Total-07 (44.58%)		8	11	46 (19.1%)	26	16
Pigment	Colour	Whitish	-	-	-	-	-
production		Grey	-	1		-	1
of growth		Blue	1	-	1	-	1
		Red	1	1	1	-	
	Diffusible		1	1	1	2	
	soluble		-	-	1	-	-
	Total-14 (5.83%)		3	3	4 (1.6%)	2	2
Total			20 (8.3%)	22 (9.1%)	130 (54.1%)	38 (15.8%)	30 (12.5%)

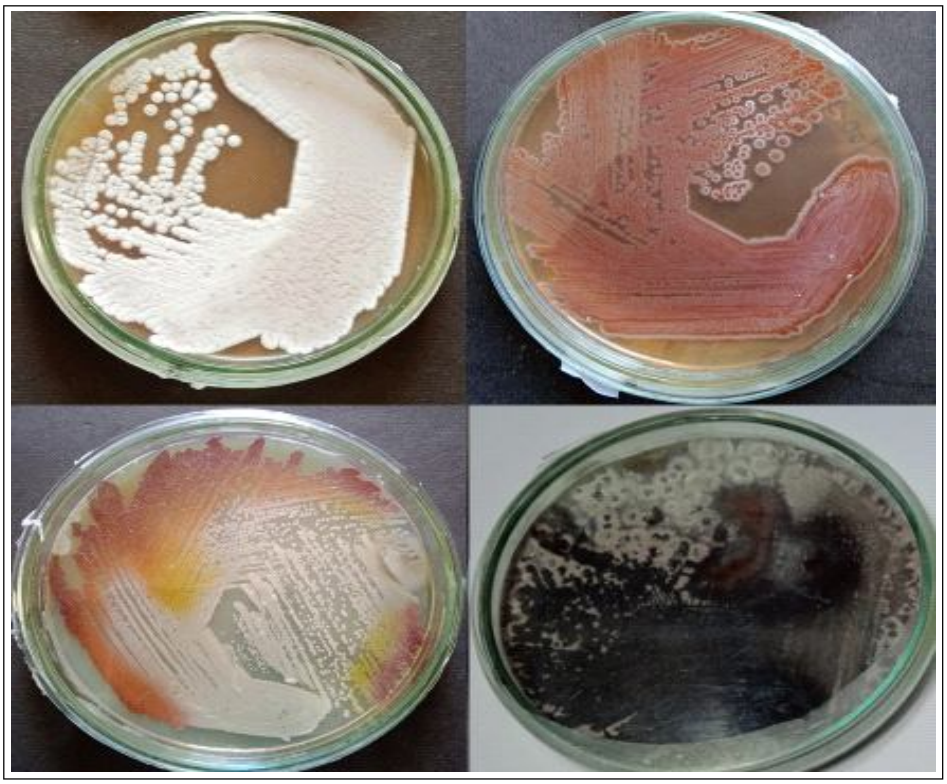

Fig 5: Sample plates showing colonies of pigment-producing different actinomycetes.

recorded in Tirunelveli (3.3%). This study coincides with (Basavaraj *et al*., 2010) reports such as morphological and cultural characteristics of the strain actinomycetes A-4 showed cellular and aerial growth as well as soluble pigment formation in various ISP media.

The second condition is filamentous appearance found in 44.58% (n=107) endophytic strains. Madurai recorded highest as 19.1% (n=46) and Tuticorin lowest with n=8. The filamentous appearance was continuous, and connecting producing aerial or substrate mycelium. Similar observation was reported in the diversity of filamentous soil actinomycetes by Chaudhary *et al*. (2013). Similar results have been reported by (Karkouri *et al*., 2019) from their studies associated with 22 different actinomycetes sp. using ISP2 agar medium. The entire actinomycetes diversity from all the collected plants in different districts sites where a major significant quantity of actinomycetes was reported in MSS (130).

A total of 14 (5.83%) pigment-producing strains were recorded in (Table 3). ISP2 medium supports the growth and pigment production of versatile growth types of actinomycetes (Fig 5). Actinomycetes are capable of producing coloured substances certain actinomycetes produce melanin pigments. This study covers 14 morphologically different actinomycetes producing different pigments (Fig 4, shows different shades). This study coincides with Srinivasan *et al*. (2016) in actinomycetes strain Ac 14 with grey color aerial mycelium, dark brown substrate mycelium and produces diffusible dark brown pigment.

CONCLUSION

Tomato plants collected from selected locations of Tamil Nadu contains a total of 240 different species of endophytic actinomycetes. Out of which some 5 to 10% are expected to be new species. On an average, a tomato plant harbours 25 to 30 different species of actinomycetes. As actinomycetes are well-known organisms for bioactive compounds, there could be a large symbiotic association ensuring plant protection. The presence of certain pigment-producing species further opens the way to explore their genetic relations.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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