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Isolation and characterization of Actinobacteria Habiting Rhizosphere of Acid Lime Crop

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ABSTRACT: Citrus greening disease is one of the severe diseases causing major threat to Citrus industry across the world. It is caused by an unculturable phloem limited proteobacteria, Candidatus *Liberibacter asiaticus* (C*Las*). The available management practices are incapable of limiting *CLas* multiplication and proliferation in Citrus tissues. Concurrently, exploring new management options for this disease is also a highly complicated task due to its unculturable nature, prolonged latency and uneven distribution within the crop canopy. Exploitation of microbiome of Citrus crops is being considered as the best option to manage this systemic pathogen. Hence, this study was carried out with an aim to isolate actinobacteria residing in rhizosphere of healthy acid lime (*Citrus aurantifolia*) trees to manage the proliferation of C*Las* in phloem tissues and improve growth promotion in acid lime crops. Isolations were done with the 10 rhizosphere soil samples collected from acid lime cultivating areas of Tamilnadu. Characteristics of these isolates on Starch Casein Agar medium were observed, which revealed the differences in colony colour, surface, texture and consistency. Initially colony showed a slight colour variation, but after maturation, the colony colour at the reverse side of the Petri plates showed the considerable variations with different hues of yellow, pink, brown and violet colour. Colony surfaces of the isolates were granular and powdery. Colony texture also expressed variations such as discrete, lichenoid and butyrous consistency. Soluble pigment was found to seep into the medium of the isolates. Biochemical characterization studies also added substantial evidences to make a conclusion that five out of 10 isolates were *Streptomyces* spp. Further exploration on the plant growth promoters and antibiotics production by these *Streptomyces* spp. will definitely give a lead for improving the Citrus crop health.

Keywords: Acid lime, Rhizosphere soil, Actinobacteria, Cultural characteristics, Biochemical charactersistics.

INTRODUCTION

Actinobacteria are a group of prokaryotic microorganisms and are Gram-positive with high G+C content in their DNA (Lo *et al.,* 2002). They are widely distributed in soil, water and plants. They are aerobic organisms and are well known for the production of secondary metabolites. The most important and dominant genus within Actinobacteria is *Streptomyces* (Ceylan *et al.,* 2008). Streptomyces provides more than half of the naturally occurring antibiotics (Bérdy, 2005) with high commercial value and continue to be routinely screened for interesting bioactive substances (Takahashi, 2004; Meena *et al.,* 2013). The actinomycines represent an important class of natural products that continue to be a focus of many research areas (Kurosawa *et al.,* 2006; Praveen *et al.,* 2008).

Actinobacteria isolated from soil samples have the potential to inhibit the growth of plant pathogens and improve the plant health (Jeffrey *et al.,* 2007; Kekuda *et al.,* 2010). Few examples of Actinobacteria used to manage Citrus diseases are *Streptomyces chumphonensis* against Citrus green mould caused

by *Penicillium digitatum*, *Streptomyces* sp. against Citrus canker, Citrus foot rot and Citrus nematode *etc*. Xu *et al.* (2018) reported the structure and function of the global Citrus microbiome from bulk and rhizosphere soils of *Citrus* spp. in six continents, accordingly dominant prokaryotic phyla reported were Proteobacteria, Actinobacteria, Acidobacteria, and Bacteroidetes. Li *et al.* (2021) reported that the microbial therapy using Actinobacteria, Yeast, Rhizobium, along with other beneficial bacteria, and plant growth regulators is promising in managing greening disease in Citrus.

In accordance with these reports, the present study was carried out to explore the rhizosphere of healthy acid lime trees for isolation of actinobacteria and to choose the potential candidates among them for plant growth promotion and disease management in acid lime crop.

MATERIALS AND METHODS

Collection and pretreatment of soil samples. Rhizosphere soil samples from healthy acid lime trees were collected during the survey conducted in various districts of North Tamil Nadu such as Vellore, Tiruppattur, Ranipet, Kanchipuram, Tiruvallur, Dharmapuri, Krishnagiri, Perambalur, Ariyalur and Namakkal. The survey was taken up during March to April, 2021.

Collected soil samples were air dried separately for one week to reduce the population of Gram negative bacteria and were sieved separately. Heat treatment of the samples was done by placing them in hot air oven at 121°C for 1 hr to prevent the growth of other bacteria (Gurung *et al.,* 2020).

Isolation and characterization of actinobacteria. Isolation of actinobacteria from pretreated soil samples was carried out by the serial dilution and plating technique (Aparanji and Venkata 2013) using Starch Casein Agar (SCA) medium (Küster and Williams, 1964).

SCA Medium Composition

Starch - 10g	$CaCO_3$- 0.02g
K_2HPO_4- 2g	$FeSO_4.7H_2O$ - 0.01g
KNO_3- 2g	Agar agar- 15g
Casein - 0.3g	Distilled water -1000 ml
$MgSO_4.7H_2O$ - 0.05g	pH 7.0 ± 0.1

It was prepared by adding the required components in distilled water and boiled to dissolve the content completely. The prepared medium was sterilized by autoclaving at 15 lbs pressure (121°C) for 20 minutes.

From each preprocessed rhizophere soil samples, one gram of soil was taken and added to a test tube containing nine ml sterile water and shaken vigorously using an orbital shaker at 200rpm for 10 min at 25 ± 2°C and these test tubes were considered as stock culture for the soil samples. Then, one ml of aliquot from the stock solution was transferred aseptically to a test tube containing nine ml of sterile water and mixed well. From this test tube, one ml of aliquot was again transferred and mixed with another nine ml of distilled water to make 10^{-3} dilution. Similarly, serial dilutions up to 10^{-6} were made for all soil samples (Oskay *et al.,* 2004). One ml of aliquot from the aqueous dilutions of 10^{-2} and 10^{-3} was taken and spread onto the Petri plate containing SCA medium. The plates were incubated at 28°C, and observed from 5th day onwards to 20th day. After incubation, colonies with suspected actinobacteria morphology were sub-cultured on SCA medium and incubated at 28°C for 2 to 5 days.

Pure cultures were maintained in SCA slants and stored in glycerol broth at 4°C for further studies. Cultural and morphological characteristics of actinobacteria were recorded (Basavaraj *et al.,* 2010).

Biochemical characterization of actinobacteria. Reaction of actinobacteria isolates with Gram stain was assessed and biochemical tests such as citrate utilization test, starch hydrolysis test and catalase test (Sapkota *et al.,* 2020) were carried out. For Citrate utilization test, Simmons citrate agar medium was poured on sterile Petri plate and allowed to solidify. The isolates were inoculated and incubated for 5-7 days at 35°C to 37°C. Colour change in the reaction was observed.

Starch hydrolysis test was performed by streaking a loop ful of colony at the centre of the Petri plate containing Starch agar medium and incubated upto seven days at 37°C. After incubation 2-3 drops of 10 per cent iodine solution was added directly onto the edge of colonies. The observation was recorded after 10-15 minutes. Similarly, Catalase reaction was tested with a loop full of actinobacteria colony, which was transferred to the surface of a clean, dry glass slide. A drop of H_2O_2 (3%) was added on the culture and observed for the development of oxygen bubbles.

RESULTS AND DISCUSSION

Rhizosphere soil samples were collected from matured, healthy acid lime trees belonging to the age group starting from 6 yrs to15 yrs in the orchards located in North Tamilnadu, India. Ten actinobacteria species *viz.,* ACT- VEL-1, ACT- TPT-2, ACT- RAP-3, ACT- TRV-4, ACT- KAP-5, ACT- PER-6, ACT- DAM-7, ACT-KRG-8, ACT- NAM-9 and ACT- ARL-10 were isolated and maintained for further studies in Starch Casein Agar (SCA) medium (Table 1). In agreement with these findings, Xu *et al.* (2007) isolated various species of actinobacteria and reported that rhizosphere soils were the rich source of beneficial microbes, where prokaryotes dominated and actinobacteria played important role in symbiotic association with crop roots. Tindall *et al.* (2010) described the polyphasic approach for characterization of actinobacteria that depicted the variety of phenotypic, chemotaxonomic, and genotypic data and most actinobacteria were characterized and classified on the basis of their morphology.

Since, the morphological characteristics were one of the most basic indexes providing in-depth information on a taxon, cultural characteristics of isolated actinobacteria were documented in this study. The isolated 10 actinobacteria isolates were observed over their growth on SCA medium. Initially, they produced the colonies with smooth surface and on maturation their colony morphology and colour expressed variations. Colony colour of the ten isolates was found to be in different shades of grey and white. After maturation, the colony colour at the reverse side of the Petri plates showed sharp variation. The isolates *viz.,* ACT- VEL-1, ACT-RAP-3, ACT-KAP-5, ACT-PER-6 and ACT-KRG-8 were produced different shades of yellow colour colonies, ACT-TPT-2 and ACT-TRV-4 produced pale pink and cinnamon brown colour colonies respectively. Isolates, ACT-DAM-7 and ACT-ARL-10 produced strikingly different colour colonies with violet and pale violet colour. Isolate ACT-NAM-9 alone had grey colour colonies. Similarly, Van Thanh *et al.,* (2019) isolated 26 endophytic actinobacteria from Horsetail plant and were classified into five color groups as White, Grey, Pink and Brown and Blue based on the color of sporulating aerial mycelium. Among them, Grey group accounted for the biggest portion with 12 strains followed by White group and Brown group. This study also reported that the colony colour of the isolated ten actinobacteria were grouped into two as grey and white.

Colony surface showed two different characters such as granular and powdery colonies; out of ten isolates, the following six isolates *viz.,* ACT- VEL-1, ACT-RAP-3, ACT-KAP-5, ACT-PER-6, ACT-DAM-7 and ACT-KRG-8 had granulated colonies and the remaining four isolates, ACT-TPT-2, ACT-TRV-4,ACT-NAM-9 and ACT-ARL-10 produced powdery colonies.

Likewise differences were also observed in colony texture. Colonies of the isolates like, ACT-TPT-2, ACT-PER-6 and ACT-DAM-7 were discrete, while ACT- VEL-1, ACT-RAP-3, ACT-KAP-5 and ACT-KRG-8 produced lichenoid colonies. The remaining three isolates such as ACT-TRV-4, ACT-NAM-9 and ACT-ARL-10 were observed to be with butyrous consistency. The observed characters were comparable with the findings of Basavaraj *et al.* (2010). They documented the antibiotic producing potential of many actinobacteria and studied their cultural characters using crowded technique. Accordingly the isolates exhibited the variations in colony colour from grey to white. Two types of colony texture *viz.,* lichenoid and butyrous were reported among them.

William Whitman and Aidan Parte (2012) also documented related findings in Streptomycetales members of actinobacteria and they explained that most of the actinobacteria colonies were discrete and lichenoid and few of them were leathery and butyrous. These colonies initially had a smooth surface, but later develop a weft of aerial mycelium, which appeared floccose and granular. Phan Thi Hong-Thao *et al.* (2016) also isolated endophytic actinobacteria from orange tissues and reported the similar observations on the colony characteristics. Accordingly the colony surface was powdery and curled. Aerial and substrate mycelia were grey to light brown or light yellow to brownish yellow on medium.

Soluble pigments were found to seep into the medium of the isolates *viz.,* ACT-TPT-2, ACT-DAM-7and ACT-ARL-10,but non soluble pigment production was observed with the other seven colonies as they retain their pigments in their colonies itself (Table 2; Plate1). Similarly, William Whitman and Aidan Parte, (2012) explained that *Microtetraspora* and *Streptomyces* strains produced diffusible melanoid pigments with diverse molecular structures that typically appear black or brown, red, yellow, orange, pink, brownish, distinct brown, greenish brown, blue, or black, depending on the strain, the medium used, and the age of the culture. Soluble yellow pigment production was observed on media *viz.,* ISP2 and ISP3 by actinobacteria isolatedfrom orange crop (Phan Thi Hong-Thao *et al.,* 2016).

Biochemical characteristics of isolated actinobacteria.

Biochemical characteristics were analyzed using Gram staining technique. The colonies of the 10 isolates were observed to retain the purple colour after Gram staining under light microscope which indicated that the isolates were Gram positive. It was well established by many studies that Actinobacteria species like Streptomyces, Micromonospora, Rhodococcus, and Salinisporas are Gram-positive bacteria with high G+C DNA content that constitute one of the largest bacterial phyla, and are ubiquitously distributed in both aquatic and terrestrial ecosystems (Muthu *et al.,* 2013; Van Thanh *et al.,* 2019).

In Citrate utilisation test, the positive reaction was indicated by the change of colour from green to blue, which was due to the alkylation process by actinobacteria. The isolates *viz.,* ACT- VEL-1, ACT-TPT-2, ACT- TRV-4, ACT- KAP-5, ACT- PER-6, ACT- KRG-8 and ACT- ARL-10 showed positive reaction whereas the isolates, ACT- RAP-3, ACT-DAM-7 and ACT- NAM-9 were negative for this test.

Formation of clear zone around the colonies was observed for the positive result of starch hydrolysis test. Among the 10 isolates, seven isolates exhibited the positive reaction .The remaining three isolates like, ACT- TPT-2, ACT- PER-6 and ACT- NAM-9 were found to express negative reaction. When the colonies of all isolates were treated with H_2O_2 (3%), oxygen bubbles were observed. It indicated the positive reaction for catalase test (Table 3; Plate 2). Agreeing with these findings Sapkota *et al.* (2020) reported that *Streptomyces* spp belongs to the family

Streptomycetaceae and the order Streptomycetales were aerobic, Gram-stain-positive, non-acid-fast bacteria that form extensively branched substrate and aerial mycelia. They were Catalase-positive and reduced nitrates to nitrites and degraded polymeric substrates such as adenine, gelatin and hypoxanthine.

Both the morphological and biochemical characteristics revealed that out of ten isolates, five isolates *viz.,* ACT-VEL-1, ACT-TRV-4, ACT-KAP-5, ACT-KRG-8and ACT-ARL-10 were *Streptomyces* spp. and further exploration on the plant growth promoters and antibiotics production by these *Streptomyces* spp. will definitely give a lead for improving the Citrus crop health.

Table 1: Isolation of actinobacteria from rhizosphere soils of acid lime trees.

Sr. No.	Isolate code	District	Place of collection	GPS Coordinates	Variety	Age of trees (in yrs)
1.	ACT- VEL-1	Vellore	Nandibenda	12.524°N 78.425°E	Local	10
2.	ACT-TPT-2	Tiruppattur	Ambur	12.748°N 78.704°E	Local	11
3.	ACT-RAP-3	Ranipet	Walajah	12.932°N 79.333°E	Tenkasi local	6
4.	ACT-TRV-4	Tiruvallur	Ponneri	13.392°N 80.184°E	Local	8
5.	ACT-KAP-5	Kanchipuram	Agaramudli	12.574°N 79.743°E	Local	15
6.	ACT-PER-6	Perambalur	Ayylur	11.186°N 78.877°E	Local	7
7.	ACT-DAM-7	Dharmapuri	Onnappagoundanahalli	12.208°N 78.056°E	Local	6
8.	ACT-KRG-8	Krishnagiri	Thalikothanur	12.569°N 77.689°E	Tenkasi local	9
9.	ACT-NAM-9	Namakkal	Keerambur	11.231°N 78.104°E	Local	7
10.	ACT-ARL-10	Ariyalur	Papanacheri	11.078°N 79.044°E	Local	6

Table 2: Morphological and Cultural characteristics of Actinobacteria isolates.

Sr. No.	Isolate Name	Colony colour	Colony colour on Reverse	Colony Surface	Colony texture	Soluble Pigment	Non soluble pigment
1.	ACT- VEL-1	Bright white	Yellow	Granular	Lichenoid	-	+
2	ACT-TPT-2	Grey	Pale Pink	Powdery	Discrete	+	-
3.	ACT-RAP-3	Bright White with pale brown center	Pale Yellow	Granular	Lichenoid	-	+
4.	ACT-TRV-4	Grey	Cinnamon brown	Powdery	Butyrous	-	+
5.	ACT-KAP-5	Bright white	Yellow	Granular	Lichenoid	-	+
6.	ACT-PER-6	Dark grey	Pale Yellow	Granular	Discrete	-	+
7.	ACT-DAM-7	White with grey center	Violet	Granular	Discrete	+	-
8.	ACT-KRG-8	Grey	Greyish yellow	Granular	Lichenoid	-	+
9.	ACT-NAM-9	Bright white	Grey	Powdery	Butyrous	-	+
10.	ACT-ARL-10	Grey	Pale Violet	Powdery	Butyrous	+	-

+= Positive; -= Negative

Table 3: Biochemical characteristics of isolated actinobacteria.

Sr. No.	Isolate Name	Gram staining	Citrate utilization test	Catalase test	Starch hydrolysis test
1.	ACT- VEL-1	+	+	+	+
2.	ACT-TPT-2	+	+	+	-
3.	ACT-RAP-3	+	-	+	+
4.	ACT-TRV-4	+	+	+	+
5.	ACT-KAP-5	+	+	+	+
6.	ACT-PER-6	+	+	+	-
7.	ACT-DAM-7	+	-	+	+
8.	ACT-KRG-8	+	+	+	+
9.	ACT-NAM-9	+	-	+	-
10.	ACT-ARL-10	+	+	+	+

+= Positive; -= Negative

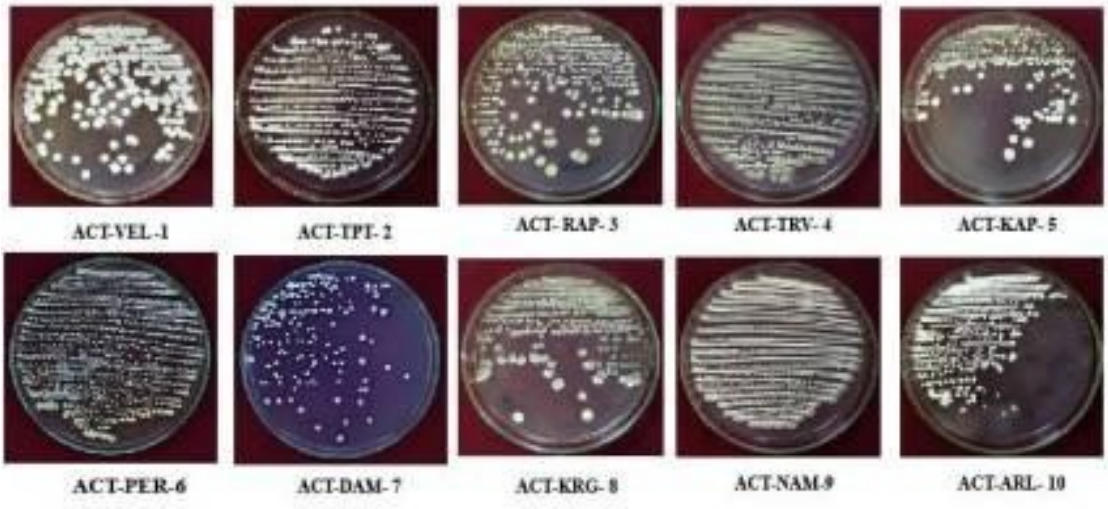

Plate 1a: Cultural characters of Actinobacteria isolates on SCA medium.

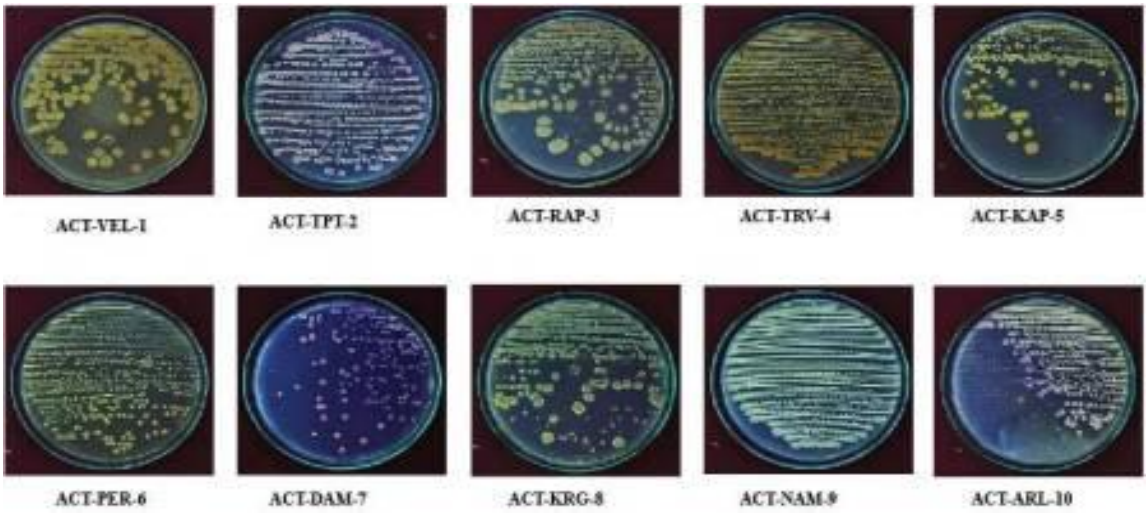

Plate 1b: Cultural characters of Actinobacteria isolates (Reverse).

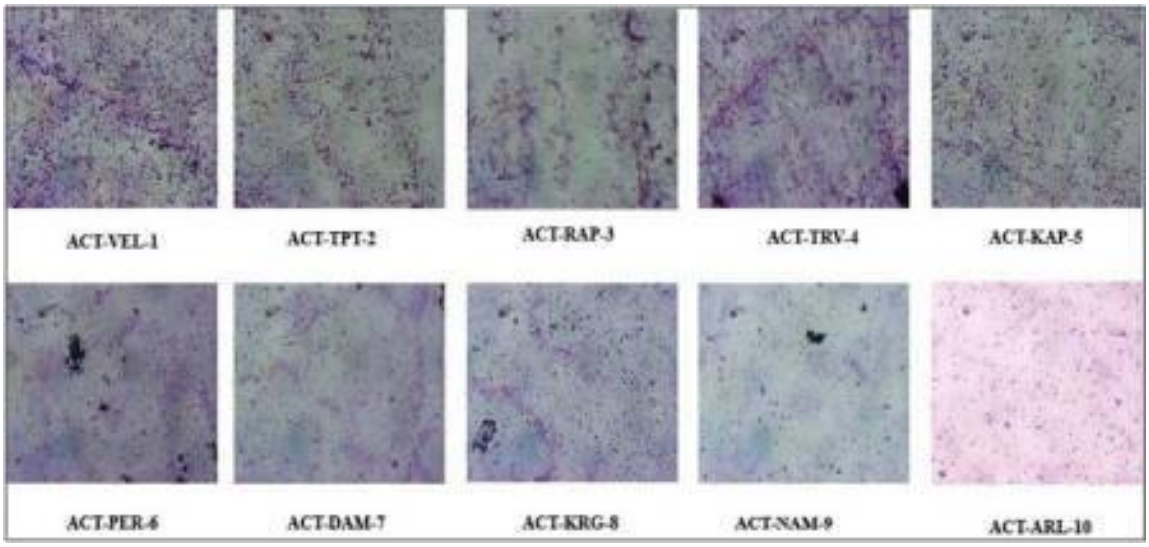

Plate 2a: Reaction of Actinobacteria isolates to Gram stain.

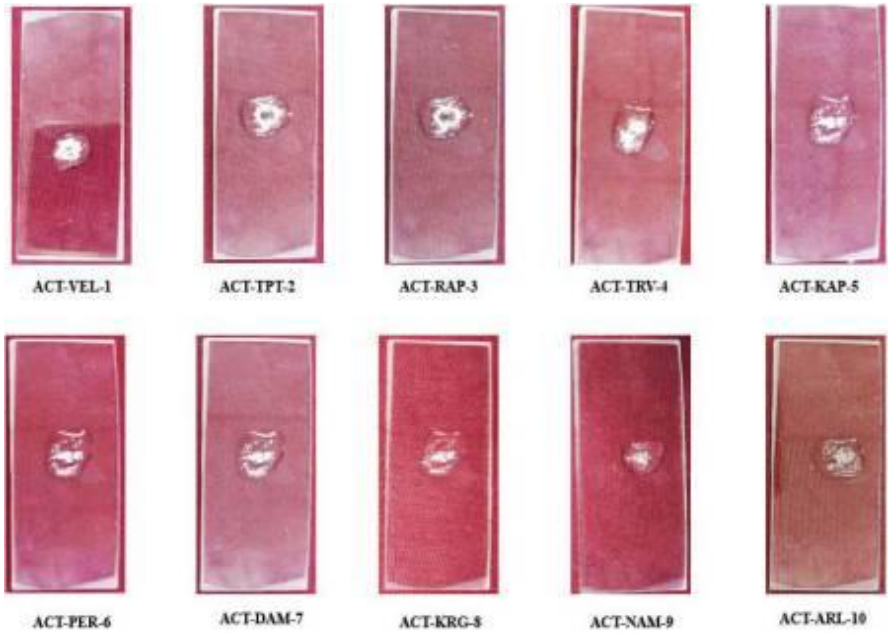

Plate 2b: Starch Hydrolysis Test: Clear zone around the culture indicated the positive reaction (+).

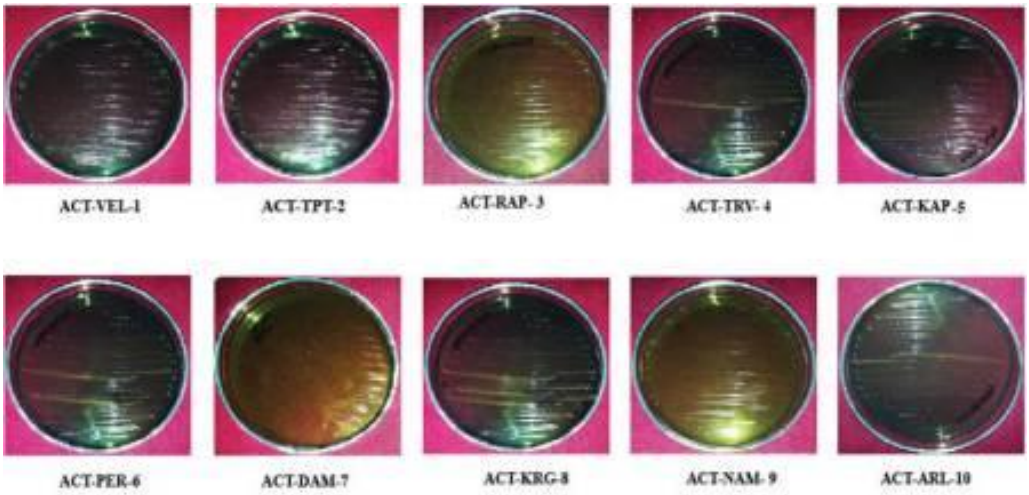

Plate 2c: Catalase test: Copious bubbles production.

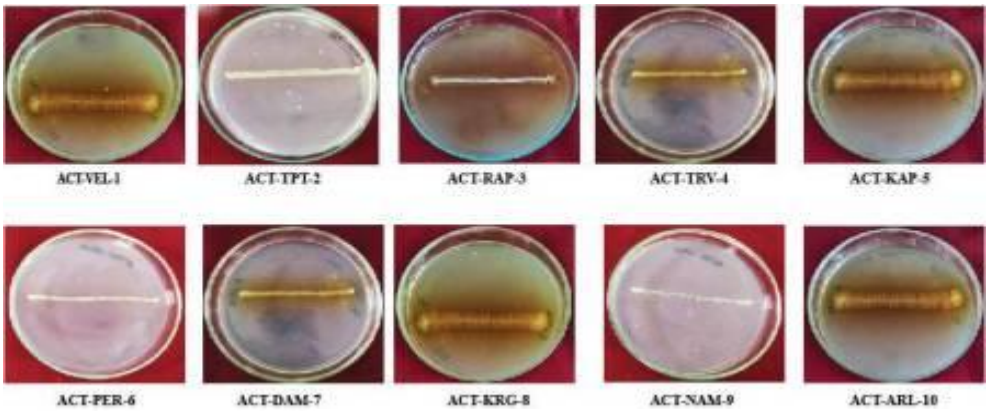

Plate 2d: Citrate utilization test: Growth on the medium changes from green to blue - positive reaction.

CONCLUSIONS

Studies conducted on actinobacteria highlighted the biochemical characteristics of the 10 isolated species and out of which, five isolates were confirmed at the genus level as *Streptomyces* spp. Based on the above work and the detailed discussions, it is clearly concluded that actinobacteria, and especially Streptomyces are the beneficial bacteria, and are the prospective agents for use as plant coinoculants in microbial consortium to improve plant–microbe symbiosis and thus it could help in managing greening disease in Citrus crop.

FUTURE SCOPE

Further extensive studies on the complex Streptomyces–rhizosphere environment and the mechanisms of plant growth promotion are needed. Revealing out the details of symbiotic association of Streptomyces with other plant growth promoting rhizobacteria might lead to the development of highly effective bioagent suitable for various soil types and environmental conditions. Still more focus is needed to produce effective formulations with *Streptomyces* spp. using different carriers, additives and with various delivery methods in field level.

Author Contributions: KM and RS conceived the idea and designed the work, RS carried out the experiment, PS helped to design the study. All the authors contributed equally to the manuscript and approved the final manuscript.

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Conflicts of Interest. None.

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