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Evaluation of the antagonistic and plant growth promoting properties of *Streptomyces* isolated from aubergine rhizosphere soil of Kanchipuram, Tamil Nadu, India

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Abstract The actinobacteria were isolated from the rhizosphere soil and characterized its plant growth promotion and antagonistic ability against *Ralstonia solanacearum*. Totally seven actinobacteria strains were isolated from aubergine rhizosphere soil and their cultural and morphological characterization showed all the seven strains belongs to *Streptomyces* spp. *In vitro* antagonistic screening of actinobacteria against *R. solanacearum* showed over 20 mm zone of inhibition by KTR3, KTR-4 and KTR6. Similarly, KTR-3 and KTR-4 produced the maximum level of IAA. In addition, the highest level of ammonia produced by KTR-6 and KTR-3. Among these seven strains, KTR-3 is alone able to produce IAA, siderophore, ammonia, cellulase, amylase and protease and solubilized phosphate. Also, *in planta* study showed 100% seed germination and highest shoot and root length in tomato, aubergine and chili which higher than other treatments. The strain KTR-3 was expressed the highest antimicrobial activity against *R. solanacearum* on utilization of glucose (24mm), peptone (24mm), ferrous sulfate (15mm) and pH 7 (23mm) as different sources. It exhibited the potential plant growth promoting and antagonistic activities, *Streptomyces* strain KTR-3 would be a promising candidate for agricultural use.

Keywords: Actinobacteria, *Ralstonia solanacearum*, Plant growth promotion, Solanaceae crops

Introduction

Phytopathogens are creating plenty of issues in the production of commercially significant agricultural crops across the world (Adedeji *et al*.,

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2020). *Ralstonia solanacearum* is a gram-negative, aerobic, non-sporulating phytopathogen (Umadevi *et al*., 2021; de Pedro-Jové *et al*., 2021) which causes bacterial wilt in several food crops, particularly solanceaeus crops like tomato, aubergine and chili. It is also a significant soil-borne pathogen that causes wilt to more than 200 species and 50 plant families (Paude *et al*., 2020; Landry *et al*., 2020). Even there are many different pesticides are widely employed in modern agriculture to combat this disease, their widespread use has harmed human health, polluted the environment, and also cause the emergence of resistance among the phytopathogens (Lee *et al*., 2012; Yuliar *et al*., 2015).

Biocontrol agents are produced by potential microorganisms have a great potential for controlling phytopathogens while having no/limited influence on the environment or other non-target species (Eljounaidi *et al*., 2016; Legein *et al*., 2020; Ngalimat *et al*., 2021). In general, biocontrol microorganisms are chosen based on their *invitro* antagonistic activity against target phytopathogens. Plant growth regulating microbes can be found majorly in rhizosphere soils that are linked with plant roots, leaves, and tissues (Xiong *et al*., 2021; de Souza *et al*., 2015). In general microbial plant growth promotion is mediated by means of nitrogen fixation, phosphate zinc solubilization, and production of extracellular enzymes, plant growth hormones, ammonia, siderophores and some other volatile compounds (Ngalimat *et al*., 2021).

Biological control of bacterial wilt has been explored using a variety of microorganisms (Mamphogoro *et al*., 2020). Effective microorganisms, such as actinobacteria, are useful because they may enhance plant growth while simultaneously protecting it from a variety of phytopathogens. Actinobacteria thrive in plant rhizosphere soils, where they assist plant growth by degrading soil organic materials (Insuk *et al*., 2020). The selection potentials utilized for biocontrol agents include most soil actinobacteria belonging to the genus *Streptomyces*. They produce antibiotics that are believed to be effective against a variety of phytopathogens (Selim *et al*., 2021; Grubbs *et al*., 2021). Although *Streptomyces* act as biocontrol agent, it may produce a variety of enzymes for protection against phytopathogens via secondary metabolites. With cell wall degrading enzymes such as cellulase, amylase, and protease, PGPR has antagonistic activity in actinobacteria against several phytopathogens, including *R. solanacearum* (Mishra *et al*., 2020). *Streptomyces* strains also offer a wide range of biocontrol mechanisms against phytopathogens, including the production of cell wall degrading enzymes and antibiotics (Hassanisaadi *et al*., 2021). Seeds bio-primed with plant growth-promoting *Streptomyces* are more likely to germinate and are resistant to a wide range of phytopathogens (Deshmukh *et al*., 2020). Hence, the current study was conducted to explore

rhizosphere soil actinobacteria plant growth promoting and biocontrol properties against *R. solanacearum*.

Materials and methods

Sample collection and isolation of actinobacteria

The rhizosphere soil samples were collected from the aubergine plantation field in Kanchipuram district, Tamil Nadu, India. After drying at room temperature for 2-3 days, the soil samples was further heat treated at 55-60 ℃ for 5 minutes so as to enhance the recovery of actinobacterial population and to suppress the growth of other microbes (Selvamohan *et al*., 2016). One gram of pretreated soil sample was serially diluted using 9 ml water blanks from 10^{-1} to 10^{-5} dilutions. Hundred micro litres (µl) of aliquot from each dilution was taken and spread on the starch casein agar plate using sterile L-rod. The isolation plates were incubated for 23-4 weeks at 28 ℃. Morphologically distinguished actinobacterial colonies were recovered and inoculated on the ISP2 agar media plates. The recovered cultures were incubated for seven days at 28 ℃ and stored in the ISP2 agar slants and glycerol stock (20%) for further studies.

Morphological characterization

The actinobacteria strains were identified by studying their microscopic morphology, and cultural characteristics. For the cultural morphology, the isolates were cultured on ISP2 agar medium at 28 ℃. The rate of growth, colonial texture, sporulation, color of aerial and substrate mycelium and pigment production were noted. The microscopic morphology was studied by adopting slide culture technique (Balagurunathan *et al.*, 2020).

In vitro antimicrobial activity against R. solanacearum

Ralstonia solanacearum is cultured in a *Pseudomonas solanacearum* agar medium whereas the actinobacterial isolates were cultured in ISP2 agar medium at 28^{o}C for 7-10 days. The antimicrobial activity of actinobacterial cultures was evaluated by agar plug method. Agar plug with 5mm diameter were cut from the ISP2 agar grown with actinobacterial cultures and placed over the nutrient agar plate which was already inoculated with *R. solanacearum.* After 24-48 hrs of incubation, all the plates were observed for the clear zone of inhibition around the actinobacterial plugs.

In vitro screening for plant growth promoting (PGP) properties

Indole acetic acid (IAA)

The actinobacteria cells were inoculated into 250-ml Erlenmeyer flasks containing 50 ml of Yeast extract-Tryptone (YT) broth amended with L-tryptophan (5 mg ml^{-1}) and incubated at 28 ℃ for 7 days in rotary shaker at 150 rpm. Later the actinobacteria supernatant (2ml) was collected by centrifugation at 15,000rpm for 15 min and mixed with 2ml of Salkowasky's reagent. After incubation for 20 min in dark, the appearance of pink color indicates the formation IAA. Optical density was measured at 530nm by UV-Vis spectrophotometer and compared with the standard curve of IAA.

Siderophore production

Actinobacterial strains were assayed for the siderophore production on the Chrome Azurol S agar according to the method of Alexander and Zuberer (1991). CAS agar plates were prepared and spot inoculated with actinobacterial strains and incubated at 28 ℃ for 7 days. The appearance of yellow to orange color zone around the actinobacterial growth was considered positive for siderophore production.

Ammonia production

Freshly grown actinobacterial cultures were inoculated into 1 ml of peptone water and incubated at 28◦C for 7–12 days with shaking at 120 rpm. After incubation, 0.5 ml of Nessler's reagent was added in each culture tube. Development of yellow to brown color indicates ammonia production. The absorbance was measured at 530 nm using spectrophotometer, compared with the standard curve of ammonium sulphate $(NH_4)_2SO_4$ and expressed in mg/ml.

Phosphate solubilization

Seven days old actinobacterial cultures were inoculated onto Pikovskaya's agar plate supplemented with 0.5% tricalcium phosphate and incubated at 28 ℃ for 14 days. The colony which generated the halo zone around their growth was taken as phosphate solubilizer.

Cellulase activity

Actinobacterial cultures were inoculated into carboxymethyl cellulose (CMC) agar plates and incubated at 28 ℃ for 5-7 days. Later 1% Congo red solution was poured over the surface of congo red agar and incubated for 20min, at room temperature. Then the plates were stained with 1M sodium chloride solution. A clear zone formed around actinobacterial growth indicates the cellulase.

Amylase activity

Actinobacterial cultures were inoculated into agar media amended with 1% starch and incubated at 28℃ for 5-7 days. After incubation, the agar plates were mixed with 1% iodine solution and the amylase production was confirmed by clear zone around the actinobacterial growth.

Protease activity

Actinobacterial cultures were inoculated into skim milk agar plate and incubated for 28℃ for 5-7 days. The clear zones were detected around the actinobacteria colonies indicates the positive production of protease.

In planta evaluation of plant growth promotion

The actinobacterial cultures were further screened *in planta* for plant growth promoting properties in plain agar tubes. Briefly, the seeds of solanaceae plants such as tomato, aubergine and chili seeds were surface-sterilized for 3 min using 1% sodium hypochlorite and rinsed with sterile water for three times. All the seeds were treated individually with actinobacterial conidial suspensions (~10 conidia ml^{-1}) for 30 min under aseptic condition. Control seeds were treated with sterile distilled water. The experiment was conducted with ten seeds per treatment. After two weeks of incubation at 28±2℃, shoot and root lengths were measured. Percentage of germination was calculated as follows.

% Germination = Total number of seeds that germinated/total number of seeds used x100

Cultural and physiological characterization of potential strain KTR-3

Cultural characteristics were studied by inoculating the spores of potential actinobacterial strain KTR-3 into different ISP (International *Streptomyces* Project) media such as tryptone agar (ISP1), yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts-starch agar (ISP4), glycerol-asparagine agar (ISP5), peptone-yeast extract-iron agar (ISP6) and tyrosine agar (ISP7) (Shirling and Gottileb, 1966). Cultural characteristics recorded include the nature of growth, colony consistency, aerial mass color, presence of reverse side pigment and the production of soluble pigment, if any. The strain KTR-3 was studied for the utilization of carbon, nitrogen and mineral sources. Also, the effect of different pH levels on its growth was studied. The strain KTR-3 grown at all the above conditions was tested for its antimicrobial activity against *R. solanacearum* by agar plug method as described above.

Results

Isolation and characterization of actinobacteria

Totally 7 morphologically different actinobacterial cultures were isolated from aubergine rhizosphere soil. All the cultures showed good growth on ISP2 agar with leathery or powdery consistency of colonies (Table 1). Based on the observation of seven days old culture grown on ISP 2 agar, the aerial and substrate mycelium of actinobacterial strains were abundant and well developed. Based on the cultural and micromorphology, all the actinobacterial cultures were tentatively identified as members of the genus *Streptomyces* sp.

Table 1. Morphological characteristics of actinobacterial cultures isolated from aubergine rhizosphere soil

Actinobacterial strains	Growth	Consistency	AMC	RSP	AM	SM
KTR-1	Good	Leathery	White	-	+	+
KTR-2	Good	Powdery	Yellowish white	-	+	+
KTR-3	Good	Cottony	White	-	+	+
KTR-4	Good	Powdery	Pale yellow	-	+	-
KTR-5	Good	Cottony	White	-	+	+
KTR-6	Good	Leathery	White	-	+	+
KTR-7	Good	Cottony	White	-	+	+

In vitro antimicrobial screening

Out of seven actinobacterial cultures tested, three strains viz, KTR3, KTR-4 and KTR6 were found to inhibit *R. solanacearum* with more than 20mm zone of inhibition. Similarly, many researchers have studied the potential of several *Streptomyces* species to inhibit or suppress the growth of *R. solanacearum*. *Streptomyces* isolated from solanaceae crops such as tomato, aubergine and chili rhizosphere soil showed biocontrol potential against *R. solanacearum in vitro.*

Plant growth promoting (PGP) properties

Among seven actinobacterial cultures strains four isolates were able to produce IAA with the range from 15.45 to 86.57 µg/ml. Maximum of 86.57

µg/ml of IAA was produced by KTR-3 followed by 50.24 µg/ml and 25.78 µg/ml by the strains KTR-4 and KTR-7, respectively. The least IAA production was observed by KTR-6 (15.45 µg/ml) and remaining three strains KTR-1, KTR-2 and KTR-5 failed to produce IAA.

The findings found that the isolated *Streptomyces* sp. can be able to produce IAA in the range of 22 - 82.36 µg/ml in the presence of L-tryptophan. Out of seven actinobacteria strains, KTR-3 and KTR-7 were able to solublize phosphate. Similarly, the actinobacteria strains KTR-2, KTR-3 and KTR-4 were able to produce siderophore. The actinobacteria strain KTR-5 produced maximum amount of ammonia (83.5 mg/ml), followed by KTR-3 (70.8 mg/ml) and KTR-1 (46.2 mg/ml) (Table 2). The least amount of ammonia was observed by KTR-6 and KTR-7 showing 14.5 and 16.2 mg/ml respectively. The actinobacterial strains KTR-3 and KTR-7 were able to produce cellulase, amylase and protease (Table 2). Interestingly, the strain KTR-3 was able to produce IAA, siderophore, ammonia, cellulase, amylase and protease and also solubilize the PO_4.

Table 2. *Invitro* plant growth and enzymatic properties of actinobacteria strains

Strain name	IAA (µg/ml)	Phosphate solubilization	Siderophore	Ammonia (mg/ml)	Cellulase	Amylase	Protease
KTR-1	-	-	-	46.2	-	+	+
KTR-2	-	-	+	-	+	-	+
KTR-3	86.57	+	+	70.8	+	+	+
KTR-4	50.24	-	+	-	+	+	-
KTR-5	-	-	-	83.5	-	-	+
KTR-6	15.45	-	-	14.5	+	-	-
KTR-7	25.78	+	-	16.2	+	+	+

In vitro plant growth promotion by paper towel method

Among seven actinobacterial cultures tested, KTR-3 and KTR-4 produced 100% germination in tomato, aubergine and chili (Figure 1a). Notably, the strain KTR-3 had induced highest shoot and root length in tomato, aubergine and chili seeds. It was observed that KTR-3 showed maximum shoot length (22mm) in tomato seeds followed by KTR-2 (15mm) and KTR-7 (13mm) (Figure 1b). Similarly, the maximum shoot length observed with the treatment KTR-3 in aubergine (16mm) and chili (13mm). In addition, KTR-3 showed maximum root length than other actinobacteria treated and control tomato (15mm), aubergine (10mm) and chili (9mm) seeds (Figure 1c). In this present study, it was observed that the maximum IAA-producing actinobacteria strain KTR-3 enhanced better shoot and root length of solanaceae plants as compared to other inoculated and un-inoculated control plants in *in planta*.

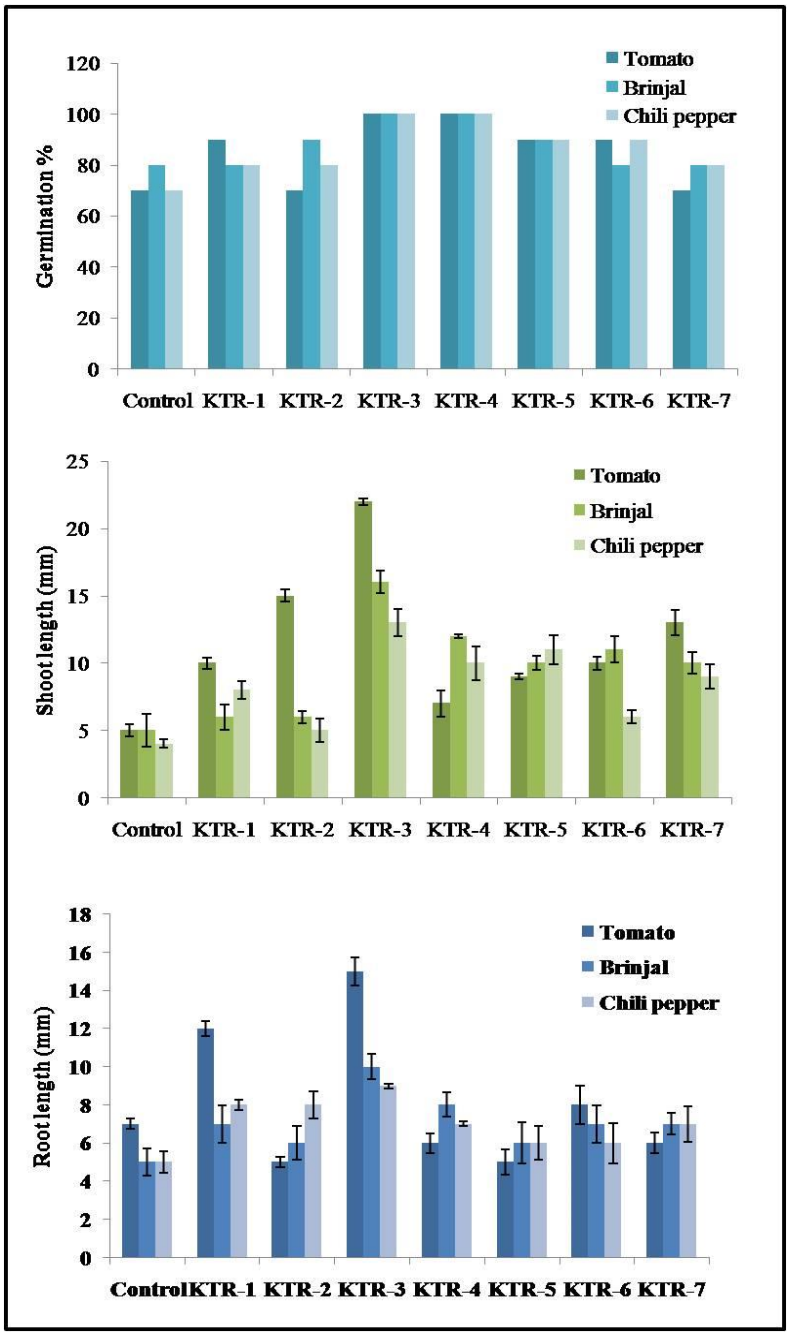

Figure 1. Germination percentage, shoot length and root length of tomato, aubergine and chili treated with potential strain KTR-3

Characterization and optimization of potential actinobacterial strain KTR-3

The cultural characteristics of the strain KTR-3 is shown in Table 3. It showed good growth on ISP2, ISP3, ISP4, ISP5 and ISP7 and moderate growth on ISP1 and ISP6. The potential strain KTR-3 was found to utilize wide range of carbon (glucose, starch, ionositol), nitrogen (Peptone, Malt extract and Soybean) and mineral sources (ferrous sulfate) for their growth and secondary metabolite production. Other factor (pH) also found to regulate the growth and secondary metabolite production of the strain KCA1 (Figure 2). Among all the parameters tested, the potential strain KTR-3 was found to show maximum antimicrobial activity against *R. solanacearum* on utilization of glucose as carbon source (24mm), peptone as nitrogen source (24mm), ferrous sulfate as mineral source (15mm) and pH 7 (23mm) as seen in Figure 2. *In vitro* and *in planta* studies revealed that *Streptomyces* from aubergine rhizosphere soil was found to be a promising candidate to develop as potential biocontrol agent and growth promoters for solanaceae crops.

Table 3. Morphological and cultural characterization of potential strain KTR-3

Media	Growth	Colony texture	Aerial mass Color	Reverse side pigment	Soluble Pigment
ISP 1	Moderate	Leathery	Yellowish white	Pale yellow	No
ISP 2	Good	Powdery	White	Pale brown	No
ISP 3	Good	Cottony	White	Orange	No
ISP 4	Good	leathery	White	Yellow	No
ISP 5	Good	Cottony	White	Yellow	No
ISP 6	Moderate	Leathery	White	Yellow	No
ISP 7	Good	Cottony	White	Pale yellow	No

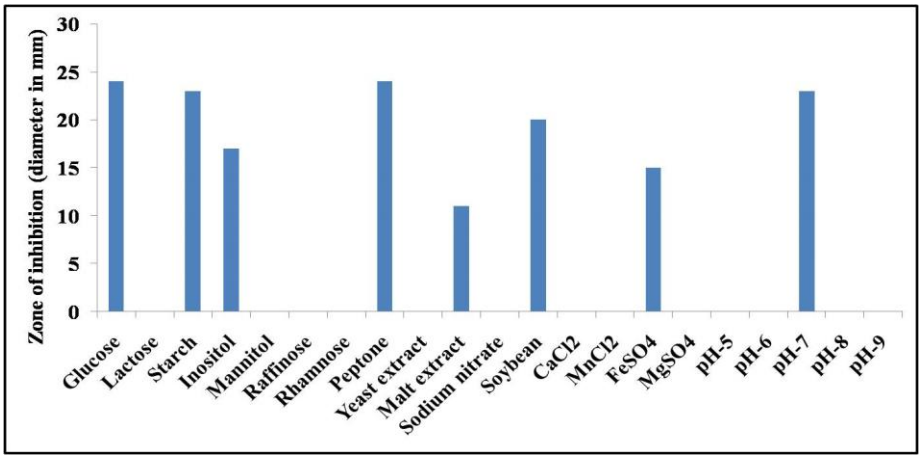

Figure 2. Antimicrobial activity of different physiological media parameter against *Ralstonia solanacearum*

Discussion

All the cultures actinobacterial cultures showed good growth on ISP2 agar with leathery or powdery consistency of colonies. Based on the cultural and micromorphology, all the actinobacterial cultures were tentatively identified as members of the genus *Streptomyces* according to Williams *et al*. (1989). Three strains actinobacterial cultures of KTR3, KTR-4 and KTR6 were inhibited *R. solanacearum*. Similarly, many researchers have studied the potential of several *Streptomyces* species to inhibit or suppress the growth of *R. solanacearum* (Manigundan *et al*., 2020; Tan *et al*., 2006). *Streptomyces* isolated from solanaceae crops such as tomato, aubergine and chili rhizosphere soil showed biocontrol potential against *R. solanacearum in vitro* and in field conditions which also reported by Manigundan *et al*. (2020) and Minuto *et al*. (2006).

Four isolates actinobacterial cultures, KTR-3, KTR-4, KTR-6, and KTR7 were produced IAA with the range from 15.45 to 86.57 µg/ml. Withthis, El-Tarabily and Sivasithamparam; and Tsavkelova *et al*. (2006) reported that actinobacteria isolated from rhizosphere soils of many vegetable crops have the promising ability to produce IAA and promote plant growth. IAA, a plant growth hormone which is believed to enhance plant growth.

Particularly, *Streptomyces* isolated from rhizosphere soil showed significant amount of IAA production and could be useful for the plant growth promotion (Myo *et al*., 2019; Khamna *et al*., 2010). Our findings are correlated with others finding of *Streptomyces* sp. can produce IAA in the range of 22 - 82.36 µg/ml in the presence of L-tryptophan (Abd-Alla *et al*., 2013; Myo *et al*., 2019).

KTR-3 and KTR-7 strains were found to solublize phosphate and KTR-2, KTR-3 and KTR-4 strrains were recorded to produce siderophore. This observation is also agreement with others results showed actinobacteria particularly *Streptomyces* strains isolated from rhizosphere soil showed positive observation of phosphate solubilization would be used for plant growth promotion (Hamdali *et al*., 2008). Ammonia synthesis is an indirect mechanism for promoting plant growth that can also help to inhibit phytopathogens (Minaxi *et al*., 2012). The actinobacteria strain KTR-5 produced maximum amount of ammonia followed by KTR-3 and KTR-1. Similar findings were reported by Passari *et al*. (2017) who indicated that the isolate *Streptomyces* sp. produced 82.3 mg/ml of ammonia.

Cellulase, amylase and protease are not only support in the decomposition of organic materials and the stimulation of seed germination, but they also aid in disease prevention by suppressing phytopathogens (Kavamura *et al*., 2013). The actinobacterial strains KTR-3 and KTR-7 were found to

produce those enzymes. The strain KTR-3 recorded to produce IAA, siderophore, ammonia, cellulase, amylase and protease and solubilize the PO_4. The finding was agreed with others showing that the specific strains of *Streptomyces* sp. could produce cellulose, amylase and protease which also correlated with disease suppression against various phytopathogens (Passari *et al*., 2016; 2017; Sirisha *et al*., 2013).

Among seven actinobacterial cultures tested, KTR-3 and KTR-4 produced 100% germination in tomato, aubergine and chili (Figure 1a). Notably, the strain KTR-3 had induced highest shoot and root length in tomato, aubergine and chili seeds. It was observed that KTR-3 showed maximum shoot length (22mm) in tomato seeds followed by KTR-2 (15mm) and KTR-7 (13mm) (Figure 1b). Similarly, the maximum shoot length observed with the treatment KTR-3 in aubergine (16mm) and chili (13mm). In addition, KTR-3 showed maximum root length than other actinobacteria treated and control tomato (15mm), aubergine (10mm) and chili (9mm) seeds (Figure 1c). In this present study it was observed that the maximum IAA-producing actinobacteria strain KTR-3 enhanced better solanaceae plant shoot and root length as compared to other inoculated and un-inoculated control plants in *inplanta*. This finding is also correlated with previous studies reported by other authors (Wahyudi *et al*., 2019; Manigundan *et al*., 2020). The current *inplanta* seed germination finding is consistent with earlier results that *Streptomyces thermocarboxydus* BPSAC147 increased germination percentage to 100% as compared to 90% in untreated control seeds (Passari *et al*., 2019).

Our findings showed good growth performance on ISP2, ISP3, ISP4, ISP5 and ISP7 and moderate growth on ISP1 and ISP6. The strain KTR-3 was utilized a wide range of carbon), nitrogen and mineral sources for their growth and secondary metabolite production. The potential strain KTR-3 showed a maximum antimicrobial activity against *R. solanacearum* on utilization of glucose as carbon source, peptone as nitrogen source, ferrous sulfate as mineral source. This present study is correlated with others studies showing different carbon, nitrogen and mineral sources influenced the growth and antimicrobial activity against different pathogens (Manikkam *et al.*, 2015; Al-ghazali and Omran, 2017). Further investigation would be conducted in green house and field evaluation of potential actinobacterial cultures which needed to be undertaken to substantiate development goals (SDGs).

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