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In vitro biocontrol activity of halotolerant *Streptomyces aureofaciens* K20: A potent antagonist against *Macrophomina phaseolina* (Tassi) Goid

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Abstract   A halotolerant actinobacterial strain isolated from salinity affected soil of Eastern Indo-Gangetic plains (IGP), Uttar Pradesh, India, was characterised for its antagonistic potential against *Macrophomina phaseolina* by dual-culture assay. It was shown to effectively inhibit the growth of *M. phaseolina* with an inhibition zone of 27 ± 1.33 mm. Further the actinobacterial strain was evaluated for its plant growth promoting (PGP) properties and its ability to produce biocontrol related extracellular enzymes *viz.* amylase, protease, cellulase, chitinase, gelatinase and urease. The results revealed that the actinobacterial strain had PGP potential along with positive assay for amylase, chitinase and urease. The interaction study between antagonist strain and fungal pathogen, performed by scanning electron microscopy technique revealed that the actinobacterium was able to damage fungal mycelia may be due to chitinase, establishing its role as a potential antagonist against *M. phaseolina*. The actinobacterial isolate was characterised by 16S rDNA gene sequencing, and was identified as *Streptomyces* genera. The identified gene sequence was deposited to NCBI GenBank with an accession number KP331758.

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1. Introduction

Fungal plant diseases are the major concern to worldwide agricultural production. The estimated yield loss due to plant diseases, range from 25% to 50% in the western countries and developing countries respectively (Gohel et al., 2006). Control of fungal diseases is imperative to ensure a constant food supply to an ever-increasing world population (Oskay, 2009; Evangelista-Martínez, 2014).

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The fungus *Macrophomina phaseolina* (Tassi) Goid is a soil-borne necrotrophic fungal pathogen that causes charcoal rot disease in over 500 different plant species, including various important crops *viz*. soybean, sorghum, maize, alfalfa and cotton. Charcoal rot has a wide geographical distribution, and the disease has been reported in tropical and subtropical regions of the world. Though, the fungus is primarily a soil-borne pathogen, in many crops it may be seed borne (Mah et al., 2012; Adhilakshmi et al., 2014). For decades, seed treatment with fungicides was recommended to control root rot of. *M. phaseolina*. It can be controlled effectively by fumigating the soil with methyl bromide; but the ever increasing cost of the application, restrictions on fungicide usage and concerns over environmental impact clearly indicate the need to search for effective control management strategies (Gopalakrishnan et al., 2011a). Research during the last two decades has led to the possibility of biological control as an increasingly realistic option for the management of plant pathogens. Biological control provides an environmentally safe and potentially stable alternative to chemical control. Several strains of actinomycetes have been found to protect plants against diseases through their potential to serve as: (a) source of agro-active compounds, (b) plant growth-promoting organisms and (c) biocontrol tools of plant diseases (Doumbou et al., 2001; Abdallah et al., 2013).

Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal agents that inhibit several pathogenic fungi (Gopalakrishnan et al., 2011b; Al-Askar et al., 2014) and antibacterial agents (Arasu et al., 2013; Zhang et al., 2013). The antimicrobial mechanisms may involve physical contact (hyperparasitism), synthesis of hydrolytic enzymes, toxic compounds or antibiotics as well as competition (Taechowisan et al., 2005; Dhanasekaran et al., 2008). However, there is still considerable interest in finding more efficient strains, which differ considerably with respect to their antimicrobial effectiveness. The need for new, safe and more effective antifungal agents are a major challenge to the plant protection industry today since saline stressed habitats are potential reservoirs for bioactive actinobacteria (Jose and Jebakumar, 2014). The success of a good biocontrol agent requires more than one parameter *viz*. it should be effective for biocontrol over a long duration and survive under adverse conditions, and if the biocontrol agent can improve plant growth, it will be an added advantage. Therefore, the present study seeks to isolate halotolerant actinobacteria from saline habitat with antagonistic activity against the phytopathogenic fungus *M. phaseolina*, to identify the actinobacterium by 16S rDNA gene sequence analysis and to investigate for plant growth promotory attributes *in vitro*.

2. Materials and methodology

2.1. Isolation of actinobacteria and selection of halotolerant strains

Actinobacteria were isolated from composite soil samples collected from salinity affected regions of Eastern IGP, Uttar Pradesh, India (coordinates ranging from 25°93′ to 27°23′ N latitude and 79°02′ to 80°13′ E longitude). The pH of soil samples ranged from 7.4 to 10.8 and electrical conductivity (EC) from 2.24 to 13.26 dsm^{-1}. Isolation was done on starch casein agar (SCA) and actinomycetes isolation agar (AIA) media amended with cyclohexamide (50 mg L^{-1}) to reduce fungal contamination (Labeda and Shearer, 1990) by standard serial dilution spread plate technique. Sample dilution plates were incubated at 32 ± 2 °C for 7–10 days until sporulated or non-sporulated actinomycete colonies were observed. Selected colonies were then inoculated onto yeast extract malt extract agar (ISP-2) for purification and stored at 4 °C in slant agar and in 20% glycerol at −20 °C. Screening of halotolerance capacity of isolated actinobacteria was evaluated by observing the growth in starch casein agar amended with various concentrations of NaCl (2–10%; w/v) along with control plates maintained with 0.5% NaCl (w/v) followed by incubation at 32 ± 2 °C for 5–7 days.

2.2. In vitro antagonistic bioassay

The twenty eight halotolerant actinobacterial isolates were evaluated for their activity towards pathogenic fungi *M. phaseolina* by dual-culture *in vitro* assay following the protocol of Khamna et al., (2009) with a slight modification. The fungal pathogen *M. phaseolina* used in the present study was isolated from root rot infected plants on potato dextrose agar (PDA; Himedia) medium and was maintained as pure cultures on PDA slants at 4 °C. The test isolates were streaked at one corner (1 cm from the edge) of the modified potato dextrose agar (PDA) (50% PDA + 50% SCA) plates (90 mm diameter) 48 h before pathogen inoculation. 5 day old fungal discs (8 mm diameter), grown on potato dextrose agar (PDA) at 28 °C were placed at another corner (1 cm from the edge) of modified PDA plates opposite to actinobacterial streak. Plates without the test isolate served as control. All plates were incubated at 30 ± 2 °C for 5 days. After incubation, the zone of inhibition (in mm) was measured and colony growth inhibition (%) was calculated by using the formula: PI = $(C - T)/C \times 100$, where PI is the percent inhibition, C is the colony growth of pathogen in control, and T is the colony growth of pathogen in dual culture. All isolates were tested in triplicate.

2.3. Scanning electron microscopy to study interaction between promising isolate and fungal pathogen

The interaction of the test fungi with most promising antagonistic isolate (Actinobacterial strain K20) based on dual culture assay, was studied by scanning electron microscopy (SEM). The hyphae from the interaction zone was transferred on glass cover slips, then fixed with 1.5% glutaraldehyde and dehydrated with graded series of ethanol washes followed by drying in desiccator (Yuan and Crawford, 1995). Samples were affixed to SEM stubs using carbon tape followed by thin coating with gold: palladium (60:40) and examined by SEM (Hitachi model S3400).

2.4. Physiological and genotypic characterisation of the potent antagonist

Morphological and physiological characterisation of the potential antagonists was done on ISP-2 medium (10 g of malt extract powder, 4 g of yeast extract powder, 4 g of glucose and 20 g of agar in 1 l of distilled water) after 1 week of incubation. Sugar utilisation pattern was studied on basal medium [2.64 g

of $(NH_4)_2SO_4$, 2.38 g of KH_2PO_4, 5.65 g of $K_2HPO_4 \cdot 3H_2O$, 1.0 g of $MgSO_4 \cdot 7H_2O$, 6.40 mg of $CuSO_4 \cdot 5H_2O$, 1.10 mg of $FeSO_4 \cdot 7H_2O$, 7.90 mg of $MnCl_2 \cdot 4H_2O$ and 15 g of agar in 1 l of distilled water and pH was adjusted to 6.8–7.0] (Promnuan et al., 2009) amended with various carbon sources (Table 1). For molecular identification, genomic DNA of isolate K20 was extracted through enzymatic lysis (Pospiech and Neumann, 1995) with little modification in lysozyme and proteinase K concentration. PCR (Peltier Thermal Cycler, BIORAD) amplification was performed using actinomycete specific 16S universal primers sets, ACT283F (5′-GGG TAG CCG GCC UGA GAG GG-3′) and 1360R (5′-CTG ATC TGC GAT TAC TAG CGA CTC C-3′) (McVeigh et al., 1996). The final volume of the 50 μl reaction mixture contained: 1X PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 9.0 at 25 °C), 1.5 mM $MgCl_2$, 100 μM of each dNTP, 1 pM of each primer, 0.25U of Taq polymerase and 50 ng of template DNA under the amplification condition as follows: initial denaturation at 98 °C for 3 min, followed by 30 amplification cycles of 94 °C for 60 s, 54 °C for 60 s, and 72 °C for 90 s. The actinobacterial strain K20 was directly using Taq-mediated dideoxy chain terminator cycle in ABI 3130xl automated genetic analyser (Applied Biosystems, UK) with same primer.

The sequence was searched against BLASTn programme for its identity against the sequences deposited in public databases and the phylogenetic tree was constructed with the MEGA software version 4.1 (Saitou and Nei, 1987) with its related sequences. Gaps were treated by pairwise deletions and bootstrap analysis was done using 1000 pseudo replications.
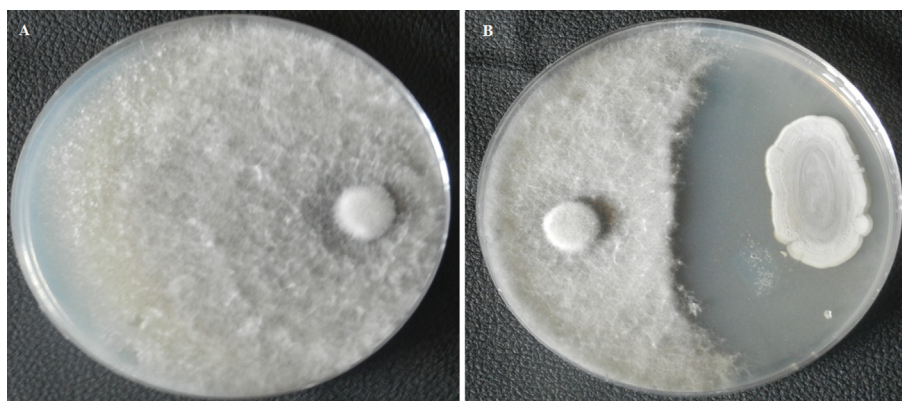
2.5. Accession number

The identified 16S rDNA partial gene sequence of antagonistic strain (K20) was deposited at NCBI GenBank database under accession number KP331758.

2.6. In vitro plant growth promotory (PGP) attributes of actinobacterial strain K20

Actinobacterial isolate K20 was assayed for various PGP properties in vitro viz., siderophore production was determined according to the methodology described by Schwyn and Neilands (1987). Antagonistic strain was streaked on Chrome Azurol S (CAS) agar media and incubated at 32 ± 2 °C for five days. When the actinomycetes consume the iron present

Table 1 Habitat, accession number, phenotypic characteristics, PGP traits and hydrolytic enzyme production by *S. aureofaciens* strain K20.

Characteristics		Observation
Habitat		Salinity affected soil of eastern IGP, Uttar Pradesh, India
GenBank accession number		KP331578
Morphological characteristics	Colour of substrate mycelia	Colourless to light cream
	Colour of spore mass	Greyish
	Diffusible pigment	–
	Motility	–
	Gram reaction	+
	Shape of spore chain	Rectiflexibilis
	Spore surface	
Cell wall amino acid analysis	Diaminopimelic acid (DAP)	L-DAP
Carbon source utilisation	Glucose	+
	Fructose	+
	Xylose	+
	Raffinose	+
	Cellobiose	+
	Arabinose	+
	Mannose	+
	Galactose	+
	Inositol	+
	Sucrose	+
Salt tolerance limit (% NaCl tolerance)	0–10%	+ (optimum at 8%)
Growth on different temperature	25–45 °C	+ (optimum at 35 °C)
Growth on different pH	7–8.5	+ (optimum at 7.5)
PGP traits and extracellular enzyme production		
	IAA production (μg mg^{-1} protein)	45.02
	Siderophore production	+
	Phosphate solubilisation	+
	Ammonia production	+
	HCN production	–
	Amylase production	+
	Protease production	–
	Cellulase production	–
	Chitinase production	+
	Gelatinase production	–
	Urease production	+

Figure 1   Micrographs for antifungal activity (dual culture assay) of *Streptomyces aureofaciens* strain K20 against *M. Phaseolina* (A) control, (B) dual culture plate.

in the blue-coloured CAS media, orange halos are produced around the colonies, which indicate the presence of sidero-phores. Indole acetic acid (IAA) production was assayed as per the protocol of Patten and Glick (1996). The strain was grown in Starch casein broth supplemented with L-tryptophan (1 μg ml^{-1}) for five days at $32 \pm 2\,°C$. At the end of the incubation, the culture was centrifuged at 10,000 rpm for 10 min, and the supernatant was collected. One ml of this supernatant was allowed to react with 2 ml of the Salkowski reagent (1 ml of 0.5 M FeCl$_3$ in 50 ml of 35% HClO$_4$) for 30 min. At the end of the incubation, development of pink colour indicates the presence of IAA. Quantification of IAA was done by measuring the absorbance in a spectropho-tometer (Schimadzu, Japan) at 530 nm. For determining the

phosphate solubilisation capacity, the actinomycete isolate was inoculated on the Pikovskaya agar containing tricalcium phosphate and incubated at optimum temperature for 5–7 days for the determination of a halo or clear zone forma-tion around the colony, indicating the solubilisation of trical-cium phosphate (Mehta and Nautiyal, 2001). HCN was estimated qualitatively by sulphocyanate colorimetric method (Lorck, 1948). The isolate was grown on the Bennett agar amended with glycine (4.4 g l^{-1}). One sheet of Whatman filter paper no. 1 (9 cm diameter) was soaked in 1% picric acid (in 10% sodium carbonate; filter paper and picric acid were ster-ilised separately) for a minute and stuck underneath the Petri dish lids. The plate was sealed with parafilm and incubated at $32 \pm 2\,°C$ for five days. Development of reddish brown
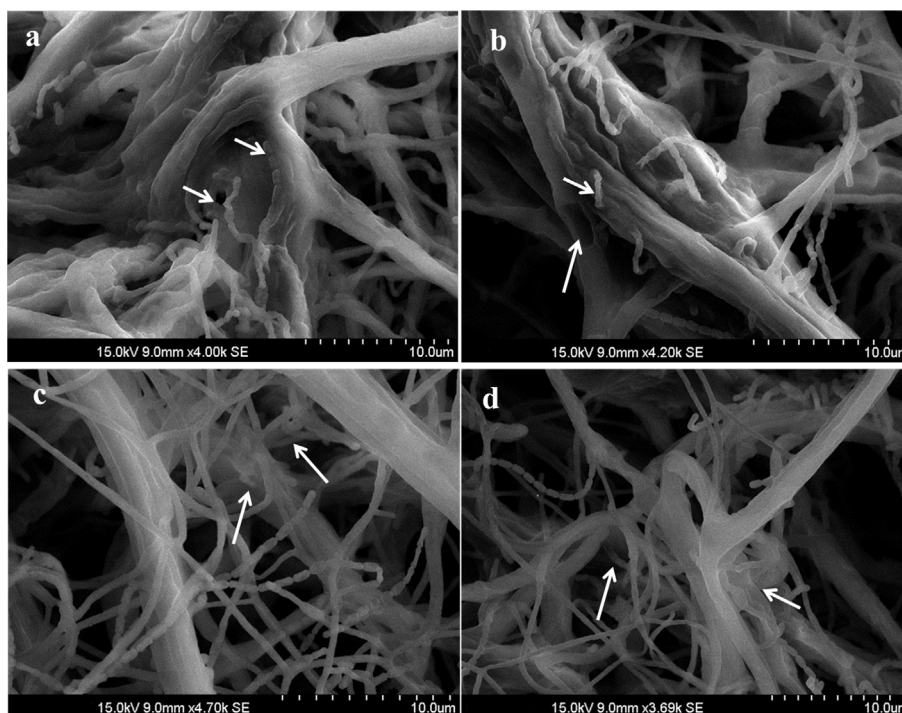
Figure 2   Scanning Electron Micrograph of *S. aureofaciens* strain K20 and the fungal pathogen *M. Phaseolina* after 5 days of interaction, (a–d) clustering of antagonist near fungal mycelia and causing destruction of the hyphae.

colour on the filter paper is an indication for HCN production. Nitrate reduction test was done by inoculating freshly grown cultures in 10 mL peptone water broth in test tube and incubated for 48–72 h at 32 °C. Nessler's reagent (0.5 mL) was added to the test tube and the development of brown to yellow colour was treated as a positive test for ammonia production (Cappuccino and Sherman, 1992).

2.7. Extracellular enzymes production

The antagonistic halotolerant actinobacteria was screened qualitatively for the production of six important enzymes such as protease, chitinase, amylase, cellulase, gelatinase and urease. The qualitative assay for chitinase was performed on colloidal chitin agar (0.7 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.001 g of $ZnSO_4 \cdot 7H_2O$, 0.001 g of $MnCl_2 \cdot 7H_2O$, 1% glycerol, 5 ml of colloidal chitin in 1 l of distilled water and pH was adjusted 8.0 with 5 N NaOH). Colloidal chitin was prepared from crab shell chitin using concentrated HCl as described by Hsu and Lockwood (1975). Isolates were spot inoculated, then plates were incubated at 32 °C and clear halos around and beneath the growth, indicative of enzymatic degradation were observed after 5–7 days. Standard methods were followed for the assay of enzymes such as amylase (Mishra and Behera, 2008) protease (Manachini et al., 1988), cellulase (Farkas et al., 1985), gelatinase and urease (Cappuccino and Sherman, 1992).

3. Results

3.1. Isolation and selection of halotolerant actinobacterial isolates

In this study, fifty five actinobacterial isolates were isolated from the collected saline soil samples. The obtained isolates were screened for their salt tolerance potential. Among the fifty five actinobacterial isolates, twenty eight isolates were found to be halotolerant with salt tolerance capability of up to 10% NaCl (w/v). Out of these twenty eight halotolerant isolates screened for their antagonistic potential against *M. phaseolina* under a dual plate assay, only one isolate K20 efficiently inhibited the fungal growth, forming a large zone of inhibition and was therefore selected for further studies.

3.2. In vitro antagonism of isolate K20 and interaction studies

The actinobacterial isolate K20 strongly inhibited the growth of phytopathogen under dual culture assay (Fig. 1) with an inhibition zone of 27 ± 1.33 mm (Mean ± standard deviation) and percent inhibition of 64.52 ± 1.42%.

The *M. phaseolina* mycelia were obtained from the periphery of the inhibition zone of a 5 day old dual culture plate. Observations made under the scanning electron microscope revealed that the mycelia sample taken from dual culture assay plate was deformed, disintegrating and ruptured. As the antagonist entered the mycelial growth of fungal pathogen, it surrounded the mycelia growth completely and damaged it, reducing apical growth and irregular distortions in the fungal hyphae with reduction in the thickness of mycelia were observed (Fig. 2).

3.3. Morphological, physiological and genotypic characterisation of the isolate

The investigated antagonistic strain K20 formed an extensively branched substrate mycelium, aerial hyphae which carried smooth-surfaced spores in rectiflexibilis spore chains. It contained LL-diaminopimelic acid in the wall peptidoglycan, lacked characteristic major sugars and formed a greyish aerial spore mass on the starch casein agar (SCA) media and ISP-2 media with no soluble pigment formation which is a characteristic of the genus *Streptomyces*. Based on the physiological characterisation, the isolate was characterised as aerobic, mesophilic (growth between 25 and 45 °C with optimum at 35 °C), and moderately haloalkalitolerant (Salt tolerance up to 10% and pH tolerance range 7–8.5). Glucose, fructose, xylose, raffinose, cellobiose, arabinose, mannose, galactose, inositol and sucrose were used as sole carbon sources for energy and growth (Table 1). Based on the morphological and physiological characteristics, the isolate K20 was tentatively identified as belonging to genus *Streptomyces*. Assignment of the strain to the genus *Streptomyces* was confirmed
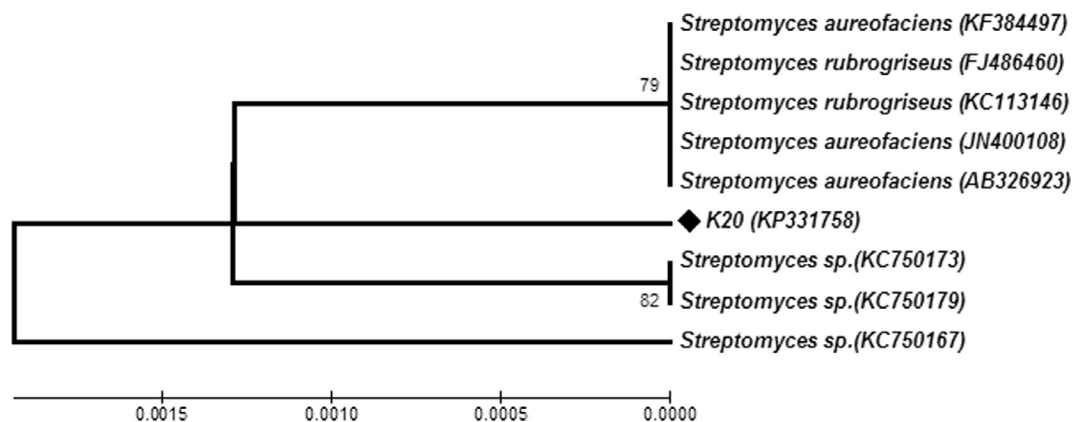
Figure 3 Phylogenetic tree based on the 16S rRNA gene sequences of halotolerant *Streptomyces* strain K20 and their closest phylogenetic relatives using UPGMA method. The bootstrap values from 1000 pseudo-replications are shown at each of the nodes. Bar indicates % similarity.

by 16S rRNA gene sequencing studies. The 16S rRNA gene sequence of strain K20 was compared to the corresponding *Streptomyces* sequences already deposited in NCBI GenBank database, using BLASTn (NCBI website). The strain displayed 99% similarity with *S. aureofaciens*. 16S rRNA sequence of the strain K20 was deposited in public database (GenBank) under accession number KP331758. The *S. aureofaciens* strain K20 was grouped with other known *Streptomyces* species by UPGMA clustering (Fig. 3).

3.4. Plant growth promotory attributes and hydrolytic enzyme production

The study of plant growth promoting potential of the strain K20 under *in vitro* conditions revealed that the strain was capable of producing plant hormone IAA, siderophore (the iron chelating compound), and ammonia. The *Streptomyces* strain K20 was also solubilising phosphate but lacking the capacity to produce hydrogen cyanide (HCN). The amount of IAA produced by strain was 45.02 μg mg^{-1} protein. Among the tests performed for six important hydrolytic enzymes, the strain was positive for three enzymes i.e. amylase, urease and chitinase production while it tested negative for protease, cellulase and gelatinase production as shown in Table 1.

4. Discussion

The search for new natural products derived from microorganisms, especially from actinomycetes, has been focused on the isolation of species from unexplored and stressed niches to isolate novel or endemic species. These natural environments are still either unexplored or under-explored with adverse environmental conditions such as salinity and thus, can be considered as prolific resource areas for the isolation of diverse microorganisms. In the present study, 28 halotolerant actinobacterial strains isolated from salinity stressed habitat were assayed for antagonism against the phytopathogen *M. phaseolina*. One of the halotolerant isolate, *Streptomyces aureofaciens* K20 exhibited strong antifungal activity against the fungal plant pathogen, mainly attributed to the production of mycolytic enzymes, hyperparasitism and production of iron chelating compound siderophore. An appropriate screening system is important for selecting biocontrol agents. The screening system should be established according to the characteristics of the pathogen and the properties of the actinomycetes. Dual-culture assay has commonly been used for evaluating the antagonistic activity of actinomycetes against plant pathogenic fungi *in vitro* (Khamna et al., 2009; Kaur et al., 2013; Adhilakshmi et al., 2014). In this study, scanning electron microscopy was performed to find out the mode of action and interaction of the antagonist isolate K20 with the pathogen. The observations revealed that the strain K20 was capable of surrounding the fungus and degrading its mycelia further leading to holes in mycelia with its disruption. In a study done by Patil et al. (2010) on antagonism of *Streptomyces toxytricini* against fungal pathogen *Rhizoctinia solani* similar findings were reported under SEM interaction studies with the exception of hyperparasitism of antagonistic strain.

After studying the cultural and morphological characteristics of isolate K20 it was assigned under the genus *Streptomyces*. Concerning phenotypic characteristics, the isolate produced grey colour aerial and colourless to cream coloured substrate mycelia. According to microscopic morphological studies, the isolate formed rectiflexibilis type spore chain which bore smooth spores. The chemotaxonomic characteristics of the strain further validated that it belongs to genus Streptomyces as the cell wall contains L-diaminopimelic acid (cell wall type-I) and no characteristic sugar was detected in whole cell hydrolysate. Thus, this study is in accordance with the previous reports that Streptomycetes are known to be predominant among actinomycetes with antagonistic potential and produce antifungal compounds (Ramesh and Mathivanan, 2009; Khamna et al., 2009; Patil et al., 2010; Kaur et al., 2013; Rashad et al., 2015). The identity of the *Streptomyces* isolate was further confirmed by 16S rDNA sequence analysis, and isolate *Streptomyces* K20 revealed 99% sequence similarity with *Streptomyces aureofaciens*. The ribosomal gene (16S rDNA) sequencing has been used as a basic approach for the identification of microbial communities as well as for assessing microbial diversity in natural environments (Solanki et al., 2014).

It has been already established in several reports that microorganisms with stress tolerance capacity and plant growth promotion attributes are very helpful in growth and development of plants under stressed environments (Yandigeri et al., 2012; Shrivastava and Kumar, 2015). There are various mechanisms involved in the disease suppression, one of the primary mechanism of pathogen inhibition as used by plant growth promoting rhizobacteria (PGPR) includes the production of antibiotics, lytic enzymes, volatile compounds and siderophore (Harikrishnan et al., 2014). Our strain along with biocontrol capability exhibited plant growth promoting attributes (phosphate solubilisation, IAA, siderophore and ammonia production) and produced hydrolytic enzymes chitinase, amylase and urease. The halotolerant strain *S. aureofaciens* K20 was producing indole acetic acid (IAA) in a fairly good amount (45.05 μg mg^{-1} protein). There are many reports which demonstrate the ability of endophytic and rhizospheric soil streptomycetes to produce indole acetic acid and thus promote plant growth (Yandigeri et al., 2012; Harikrishnan et al., 2014). Khamna et al. (2009) and Gopalakrishnan et al. (2011a) have also reported the production of IAA by antagonistic actinobacteria isolated from medicinal plant rhizosphere and herbal vermicompost respectively. In agriculture, biological phosphate solubilisation as an alternative to natural phosphate utilisation plays an important role in efficient nutrient uptake and it was carried out by our strain in the present work and ammonia was also produced by *S. aureofaciens* K20. Similar results were reported by Kaur et al. (2013) in a study performed for evaluation of antagonistic and plant growth promoting activities of endophytic and soil actinobacteria. We reported the production of siderophore while HCN production was not seen in our antagonist strain K20. The data suggest that siderophore production may be involved in the inhibition of pathogen by the isolate and thus promote plant growth indirectly because *Streptomyces* species are known for the production of hydroxamate type siderophores, which inhibit phytopathogen growth by competing for iron in rhizospheric soils (Khamna et al., 2009; Gopalakrishnan et al., 2011a; Harikrishnan et al., 2014). *In vitro* evaluation showed that *S. aureofaciens* K20 produced lytic enzymes viz., chitinase and amylase which might be responsible for strong antagonistic activity against fungal pathogen observed in our study. Chiti-

nase production by antagonistic strain could be one of the possible mechanisms of biocontrol for fungal phytopathogens, because the cell wall of fungi consists of polysaccharides such as chitin and glucan (Patil et al., 2010; Kaur et al., 2013). Inhibition of *M. phaseolina* mycelial growth by K20 culture can be attributed to production of extracellular compounds such as cell wall degrading enzymes and antibiotics, which damaged and disintegrated the hyphae of pathogen as observed under SEM. The contact of strain K20 with pathogen resulted in the abnormalities such as distortion and hyphae destruction.

5. Conclusion

In the present scenario the search for new bioactive compounds has extended from medical field to agricultural field for crop protection and enhancing yields. The present study publicises that the saline stressed habitats are potential reservoirs for bioactive actinobacteria. *In vitro* antagonistic assay with our antagonist strain uncovers its potential to be utilised as biocontrol mechanism against phytopathogenic fungi *M. phaseolina*. The halotolerant Streptomyces strain K20 even possesses plant growth promotion potential by producing IAA, siderophore and ammonia and solubilises phosphate. Thus, directly as well as indirectly it can promote plant growth. So, it may be concluded that the strain *S. aureofaciens* K20 could be utilised for biocontrol management (*M. phaseolina*) programme in saline stressed soils.

Acknowledgements

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