



Isolation, identification and antagonistic evaluation of *Streptomyces* spp. from rhizosphere soil against *Aspergillus flavus*

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Abstract

Groundnut (*Arachis hypogaea*, L.) is one of the important legume crops of tropical and semiarid tropical countries and known as “King of Oilseeds” it is third largest oilseed produced in the world and second largest in India. Preharvest and postharvest diseases are the major constraints in the groundnut among which aflatoxin producing *Aspergillus flavus* contamination is the major bottleneck. Biological control is one of the eco-friendly and reliable approach for managing those constraints. Therefore, in the present investigation rhizosphere soil was collected from the seven districts of central and northern Karnataka and twenty-one isolates of *Streptomyces* spp. isolates were obtained. Out of twenty-one, eleven isolates were confirmed as *Streptomyces* spp. through Gram staining, aerial mycelium characteristics and molecular characterization. Later these eleven isolates were tested for their antagonistic potential against *A. flavus* by dual culture assay under *in vitro*. The percentage inhibition of mycelial growth ranged from 11.11 per cent to 61.11 per cent. Isolate LIN15 showed highest (61.11 %) mycelium inhibition whereas, control showed zero per cent inhibition against the pathogen.

Keywords: Groundnut (*Arachis hypogaea* L.), oilseed crop, *Aspergillus flavus*, aflatoxin contamination, biological control, *Streptomyces* spp., rhizosphere soil

Introduction

Groundnut (*Arachis hypogaea*, L.) is one of the important legume crops of tropical and semiarid tropical countries, where it provides a major source of edible oil and vegetable protein (Prasad *et al.*, 2010) [11]. Groundnut is known as “King of Oilseeds”, third largest oilseed produced in the world and second largest in India (Bansal *et al.*, 2017) [2]. Preharvest and postharvest diseases are the major constraints in the groundnut among which aflatoxin producing *Aspergillus flavus* contamination is the major bottleneck (Waliyar and Bockiee, 1989) [18]. The naturally occurring toxins produced by the filamentous fungus *A. flavus* as an opportunistic and ubiquitous fungus. It produces aflatoxin B1 and B2 are commonly associated with food commodities grown in humid tropics and subtropics (Reddy *et al.*, 2010) [12]. In infected peanuts it can cause seed rot and reduce seed viability and germination (Kumar *et al.*, 2008) [9]. To avoid aflatoxin contamination from *A. flavus*, there are several possible control strategies employed in peanut such as treating peanuts with fungicides, fumigants, or biocontrol agents (Kong *et al.*, 2010) [8]. However, the indiscriminate and prolonged use of fungicides resulted in the development of fungicide resistance and environmental contaminations. Biological control of plant pathogens by antagonistic microorganisms is a potential nonchemical means and is known to be a cheap and effective eco-friendly method for the management of crop diseases (GE, 1991). *Streptomyces* isolated from soil have revealed their wide antifungal activity (Amaike and Keller, 2011) [1]. *Streptomyces* spp. are one of the important promising group of antagonists and root-colonizing microbes which survive in soils. Among them, the genera *Streptomyces*, with a high guanine and cytosine (G+C) content (69–78%), soil-dwelling Gram-positive bacterium, undergoes a complex cycle of morphological differentiation leading to

sporulation, production of diverged bioactive compounds including useful antibiotics, pigments, siderophores, chitinases and phytohormones with phosphate solubilizing abilities (Stackebrandt *et al.*, 1997) [15]. The major factor that goes into success of any biocontrol programme is the effectiveness with which the biocontrol agents are delivered. This requires identification of effective strains of biocontrol agents against various plant pathogens. Keeping in view, the present investigation was carried out in which collection, isolation, identification of *Streptomyces* spp. and evaluation of antagonistic potential of *Streptomyces* isolates against *A. flavus*.

Materials and Methods

1. Collection and isolation of *Streptomyces* spp. from healthy rhizosphere soil

Collection of rhizospheric soil sampling was done by scraping the top 2-3 cm soil with the kurpie at a depth of 20-30 cm and distance of 25-30 cm from the base of the plant. Soil samples were collected from 3-4 places in a field randomly. About 250 gm of rhizospheric soil was collected from the districts of northern and central regions of Karnataka viz Raichur, Ballari, Koppal, Yadgir, Vijayanagara, Chitradurga and Tumkur in polythene bags with proper labelling. Later, these rhizospheric soil samples were air dried and packed, then carried to laboratory for further work. Later isolation of *Streptomyces* spp. was carried out.

Rhizosphere soil samples were cleaned to remove the stones and debris. The standard serial dilution technique (Waksman, 1922) [17] was followed for the isolation of *Streptomyces* spp. from rhizosphere soil. The soil sample (10g) was collected and suspended in 90 ml of saline solution. Serial dilution was carried up to 10⁻⁶ dilution to avoid over-crowding. 1 ml aliquot of sample was taken from

the respective dilutions from 10^{-4} to 10^{-6} and was spread on petri dishes containing solidified actinomycetes isolation agar media (Actinomycetes isolation agar-21.8 g, Agar-Agar-20.00g and Distilled water-1000 ml) by using glass spreader (spread plate method of isolation). The inoculated plates were kept for incubation at $25 \pm 2^\circ\text{C}$ for 7-8 days. Later the obtained *Streptomyces* spp. isolates were confirmed as genus *Streptomyces* by Gram staining, aerial mycelium characteristics and molecular characterization.

2. Identification of *Streptomyces* spp.

Staining reaction was carried out by Gram staining technique according to the standard procedure as mentioned in Laboratory Guide for Identification of Plant Pathogenic Bacteria published by the American Phytopathological Society (Schaad, 1992) [13]. A loopful of colony of 24-hour old bacterial culture was taken and smeared on to the glass slide and passed over flame for two minutes to heat fix and then stained with crystal violet (primary stain). The slide was washed with distilled water after one minute and further rinsed with iodine solution for one minute. Thereafter, slide was washed with gram's decolourizer and subsequently drained with distilled water. Later, stained with counter stain safranin for one minute and washed with distilled water, dried with tissue paper and observed the bacterial cells under microscope for Grams reaction.

The observations of aerial mycelium were also carried out to identify the genus *Streptomyces*. The isolates were observed for colour and surface texture of aerial mycelium of *Streptomyces* spp. isolates for the further confirmation.

DNA was extracted from the pure cultures of *Streptomyces* spp. multiplied on starch casein broth at $25 \pm 1^\circ\text{C}$ for 7 days by using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980) [10] by inoculating 7 days old culture of eleven isolates in starch casein broth (Starch -2.0 g, casein-0.3 g, KNO_3 -2.0 g, K_2HPO_4 -2.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.05 g, CaCO_3 -0.02 g and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ -0.01 g), later The analysis was carried out using actinomycetes universal primers F27 and R1492^b (5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-TACGGYTACCTTGTTACGACTT-3') (Frank *et al.*, 2008) [4] with 35 cycles using the following PCR conditions of initial denaturation 94°C (4 min), denaturation 94°C (1 min), annealing 57°C (1 min), extension 72°C (1.30 min) and final extension 72°C (10 min). Later the PCR products are subjected to gel electrophoresis.

3. In vitro evaluation of antagonistic potential of *Streptomyces* spp. against *Aspergillus flavus*

The efficacy of obtained *Streptomyces* spp. isolates were tested using dual culture assay against *A. flavus*. *A. flavus* 11-4 strain which is already proved as aflatoxigenic in Groundnut Pathology lab of ICRISAT, Patancheru, Hyderabad was collected and maintained on Potato dextrose agar (PDA). The test culture of isolate of *Streptomyces* spp. was streaked at one side of the plate and on the opposite side the mycelial disc measuring six mm diameter from 4-5 days old culture of test pathogen was placed on sterile petri plate containing PDA medium. The petri plates with pathogen kept alone without streaking of test isolate which served as served as control. The petri plates were then incubated at $28 \pm 2^\circ\text{C}$. Three replications were maintained in each treatment. Growth of antagonists, pathogen and zone

of inhibition was measured after recording full growth of the pathogen in control plate. Per cent inhibition of mycelial growth of test pathogen was calculated by the formula given by Vincent (1947) [16].

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent inhibition in mycelial growth.

C = Radial growth of fungus in control (mm).

T = Radial growth of fungus in treatment (mm).

Results and discussions

1. Collection and isolation of *Streptomyces* spp. from healthy rhizosphere soil

Total twenty-one isolates such as RAI1, GOL2, GAB3, BAL4, BAL5, GEN6, HIR7, DYA8, CHI9, KAL10, BLI11, KAN12, KOP13, TAD14, LIN15, MUN16, BAL17, SHA18, ANE19, DAS20 and BAN21 were isolated successfully on actinomycetes isolation agar medium from the samples collected.

2. Identification of *Streptomyces* spp.

The obtained cultures were subjected for the Gram staining to confirm the isolates are as *Streptomyces* spp. and the results showed that, out of the twenty-one isolates, only eleven exhibited distinctive staining features corresponding to *Streptomyces*. Hence, they are identified as Gram-positive bacteria with purple-coloured filamentous structures (Fig. 1 and Table 1). Further the confirmed isolates were studied for aerial mycelium characters and the results showed that, aerial mycelium colour ranged from white to grey, cream, and yellowish shades, while colony surface appeared powdery, rough, or smooth depending on the isolate. The isolate GOL2 from Raichur exhibited white, powdery growth, whereas GEN6 from Ballari produced creamy, smooth colonies. Similarly, isolates from Chitradurga (HIR7 and CHI9) displayed creamy powdery and creamy rough textures, respectively. Isolate KAL10 from Tumkur produced white, smooth colonies, while isolates from Koppal (KOP13 and LIN15) showed grey rough and white powdery growth, respectively. The isolates from Yadgir (MUN16 and BAL17) exhibited grey rough and creamy smooth growth patterns, while those from Vijayanagara (ANE19 and BAN21) displayed yellowish rough and grey rough colonies (Fig. 2 and Table 2). In the present study based on the colour of aerial mycelium of all isolates classified into four classes such as white, grey, cream and yellow. Therefore, the preliminary identification confirmed that eleven isolates GOL2, GEN6, HIR7, CHI9, KAL10, KOP13, LIN15, MUN16, BAL17, ANE19, and BAN21 belonged to the genus *Streptomyces* and later the total genomic DNA of *Streptomyces* spp. of eleven isolates obtained from seven districts were subjected to molecular characterization by using actinomycetes universal primers F27 and R1492^b. The genomic DNA was isolated by CTAB method, amplified using PCR and further confirmed by agarose gel electrophoresis. The amplification of PCR products of eleven isolates using actinomycetes universal primers resulted in a range of 1400 bp (Fig. 3).

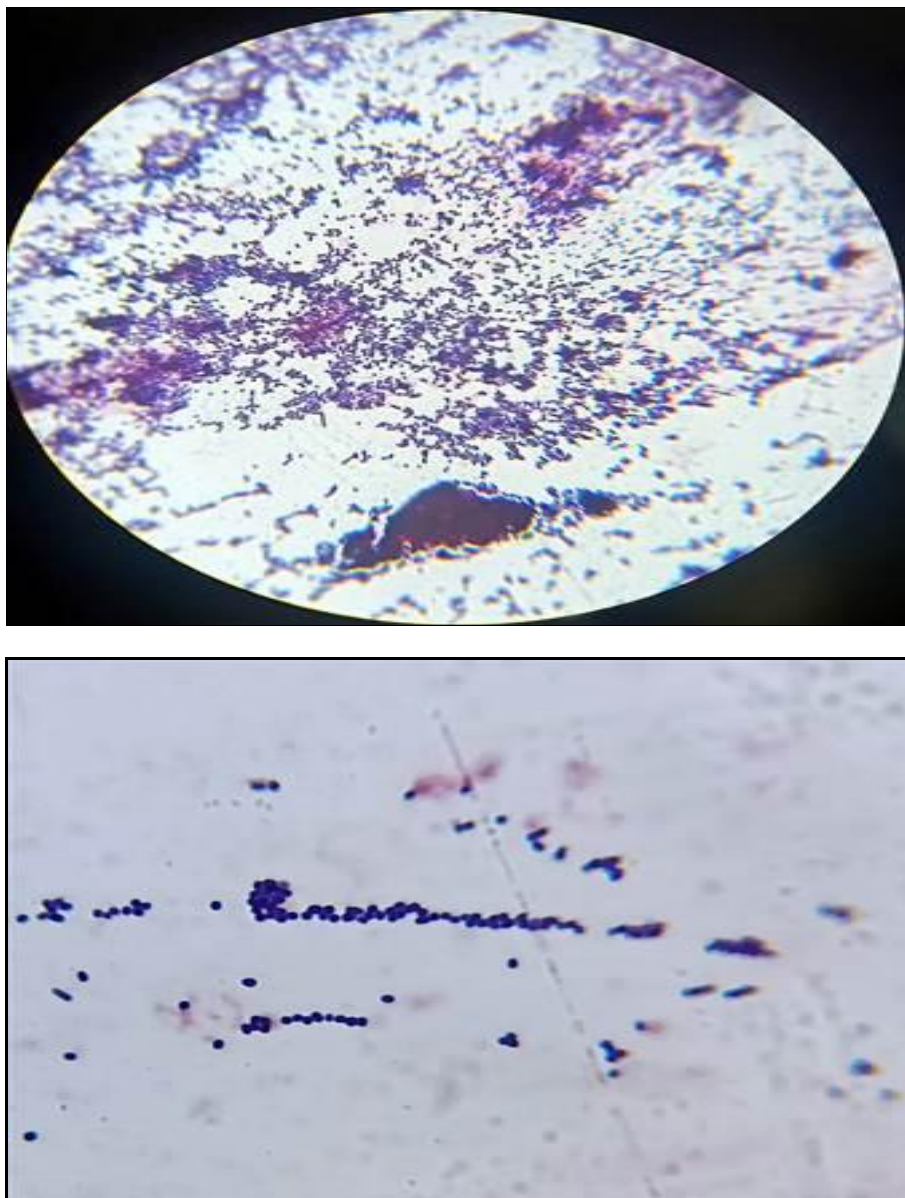


Fig 1: Gram staining reaction of *Streptomyces* spp. Isolate

Table 1: Gram staining reaction of *Streptomyces* spp.

SI. No.	Isolate code	Gram staining reaction
1	RAI1	-ve
2	GOL2	+ve
3	GAB3	-ve
4	BAL4	-ve
5	BAL5	-ve
6	GEN6	+ve
7	HIR7	+ve
8	DYA8	-ve
9	CHI9	+ve
10	KAL10	+ve
11	BLI11	-ve
12	KAN12	-ve
13	KOP13	+ve
14	TAD14	-ve
15	LIN15	+ve
16	MUN16	+ve
17	BAL17	+ve
18	SHA18	-ve
19	ANE19	+ve
20	DAS20	-ve
21	BAN21	+ve

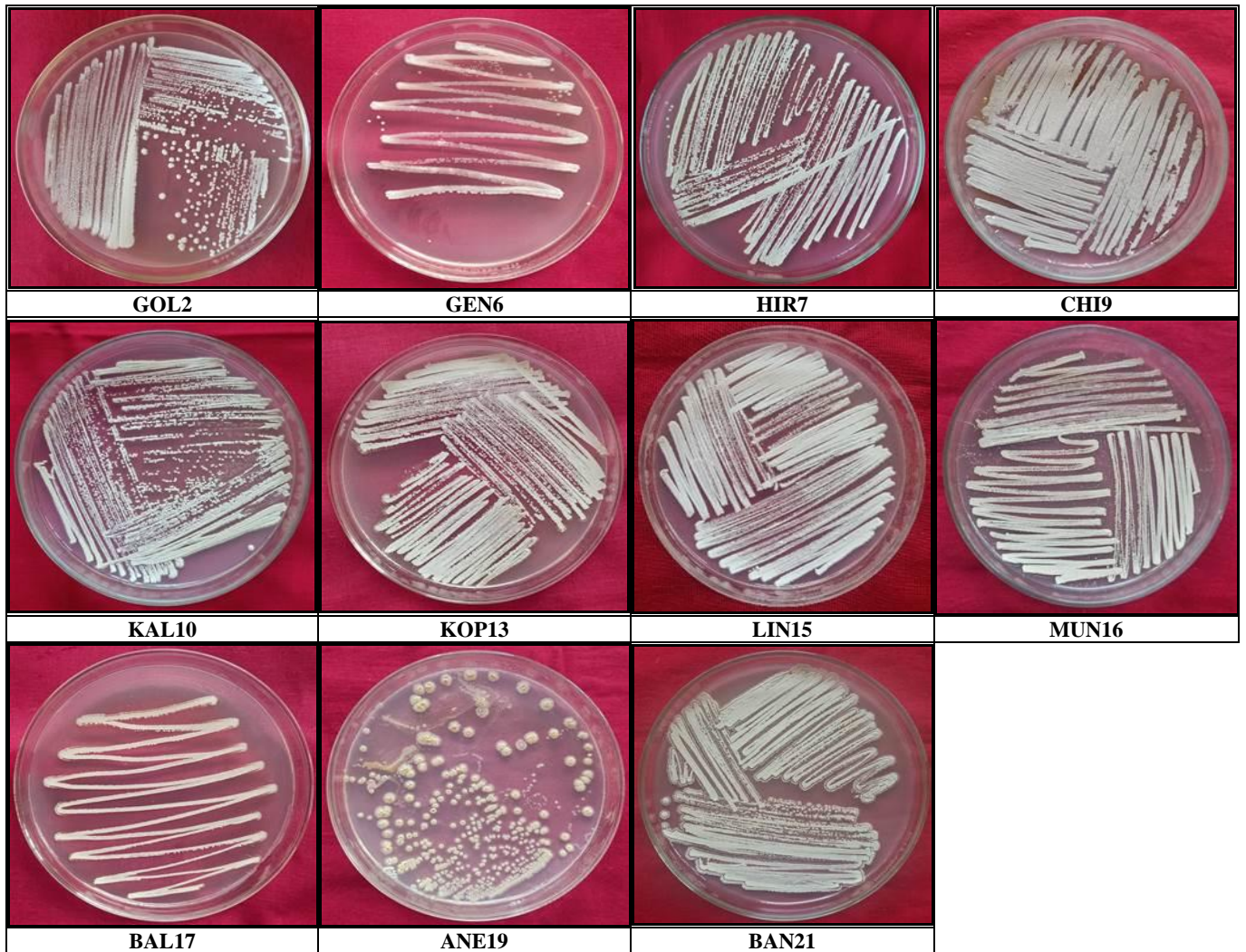


Fig 2: *Streptomyces* spp. isolates isolated on actinomycetes isolation agar medium

Table 2: Identification of *Streptomyces* spp. isolates by aerial mycelium

Sl. No.	District	Isolate code	Aerial mycelium
1	Raichur	GOL2	White and powdery
2	Ballari	GEN6	Cream and smooth
3	Chitradurga	HIR7	Cream and powdery
4		CHI9	Cream and rough
5	Tumkur	KAL10	White and smooth
6	Koppal	KOP13	Grey and rough
7		LIN15	White and powdery
8	Yadgir	MUN16	Grey and rough
9		BAL17	Cream and smooth
10	Vijayanagar	ANE19	Yellow and rough
11		BAN21	Grey and rough

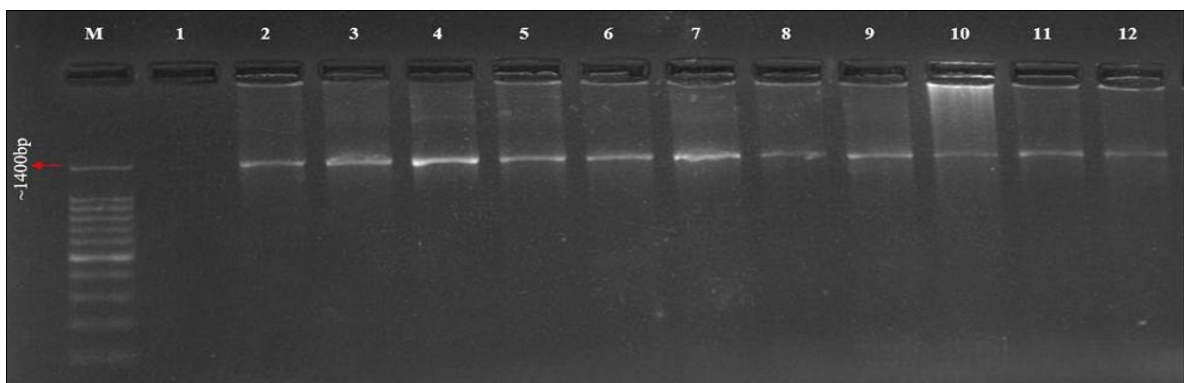


Fig 3: Agarose gel showing amplification of PCR products of *Streptomyces* spp. isolates using actinomycetes universal primers (F27 and R1492^b)

3. In vitro evaluation of antagonistic potential of Streptomyces spp. against Aspergillus flavus

The antagonistic potential of eleven species of *Streptomyces* isolates were studied against the toxigenic fungus *A. flavus* strain 11-4 strain under *in vitro* using the dual culture technique. All the tested species exhibited a clear inhibitory effect on the mycelial growth of *A. flavus*, though the degree of inhibition varied significantly among the isolates. The percentage inhibition of mycelial growth ranged between 11.11 per cent and 61.11 per cent. Among the eleven species, isolate LIN15 demonstrated the highest antagonistic activity with 61.11 per cent inhibition, thereby emerging as the most potent isolate and GOL2 (33.33%), GEN6 (18.88%), HIR7 (25.55%), CHI9 (11.11%), KAL10 (32.22%), KOP13 (33.33%), MUN16 (18.88%), BAL17 (27.77%), ANE19 (42.22%), BAN21(38.88%) and control showed zero per cent mycelial inhibition (Fig. 4, Fig. 5 and Table 3).

The present study is in accordance with Wongsariya *et al.* (2019) [19] who evaluated thirty soil actinobacterial isolates against *A. flavus* IMI 242684 and reported that six isolates inhibited fungal growth, with inhibition ranging between 21.42 and 42.85 per cent. Among them, isolate N39Wh exhibited the strongest activity with 42.85 per cent inhibition. Shakeel *et al.* (2018) [14] investigated *S.*

yanglinensis and its antifungal metabolites against *A. flavus* under *in vitro*. The isolate 3-10 of *S. yanglinensis* reduced the mycelial growth of *A. flavus* by 41 per cent in dual culture assays, and also significantly suppressed aflatoxin B1 production. These findings agree with the present study, which also recorded substantial inhibition of *A. flavus* mycelial growth, though with a different isolate. Goredema *et al.* (2020) [6] reported that *Streptomyces* isolates exhibited broad-spectrum antagonistic activity against multiple plant pathogens, including *A. flavus*, *F. oxysporum*, and *P. italicum*. Although the majority of isolates did not exhibit significant inhibition, about 22 per cent of the tested strains suppressed fungal growth by more than 55 per cent. Isolates CUT-*Streptomyces* 4, 10, 11, 20, and 23 showed consistent antagonism across multiple pathogens. Jacob *et al.* (2016) [7] isolated actinomycetes from groundnut rhizospheric soils and screened for antagonism against *S. rolf sii* through a dual culture assay. Of the 108 actinomycete isolates screened, 26 isolates inhibited growth of *S. rolf sii* ranging from 21 to 69 per cent and highest (69 % inhibition) antagonism against *S. rolf sii* was shown in dual culture assay. Fifty-nine *Streptomyces* isolates and a *S. griseoviridis* strain from the commercial product Mycostop, evaluated against *A. flavus* growth. Fifty-eight isolates were able to inhibit fungal growth

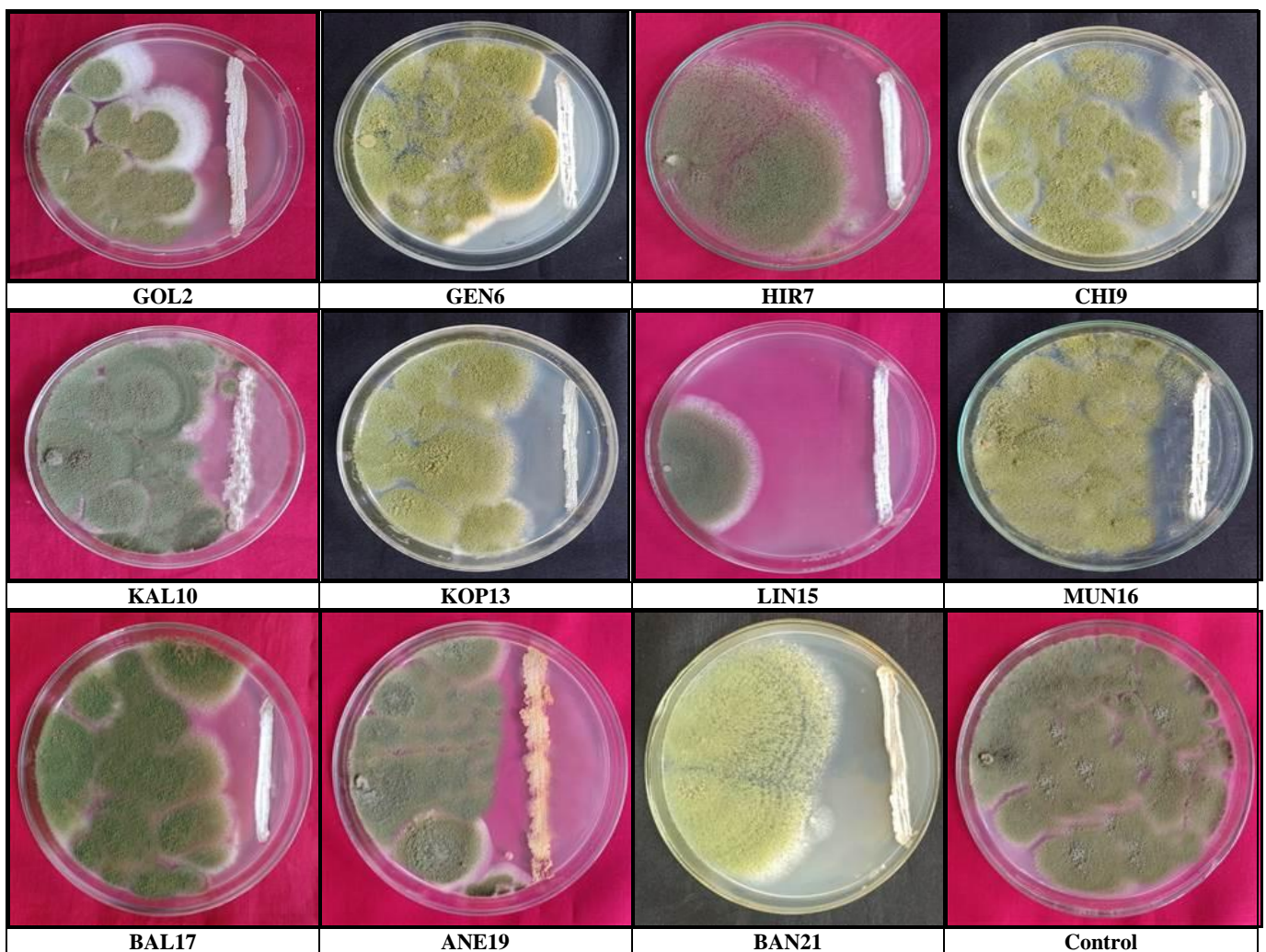


Fig 4: Antagonistic potential of *Streptomyces* spp. against *Aspergillus flavus* in dual culture assay

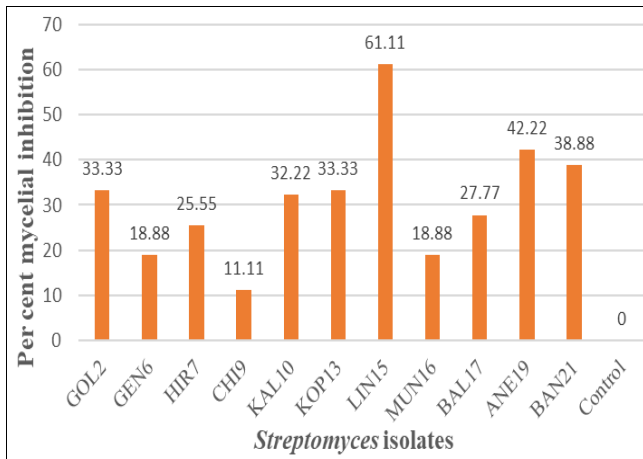


Fig 5: Antagonistic potential of *Streptomyces* spp. against *Aspergillus flavus*

Table 3: Antagonistic potential of *Streptomyces* spp. against *Aspergillus flavus* in dual culture assay

Sl. No.	<i>Streptomyces</i> spp. isolates	Colony growth* (mm)	Per cent mycelial Inhibition*
1	GOL2	60	33.33 (35.26)
2	GEN6	73	18.88 (25.76)
3	HIR7	67	25.55 (30.36)
4	CHI9	80	11.11 (19.47)
5	KAL10	61	32.22 (34.58)
6	KOP13	60	33.33 (35.26)
7	LIN15	35	61.11 (51.41)
8	MUN16	73	18.88 (25.76)
9	BAL17	65	27.77 (31.80)
10	ANE19	52	42.22 (40.52)
11	BAN21	55	38.88 (38.58)
	Control	90	00.00 (00.00)
	S.Em. ±		0.64
	CD @1%		2.56

*Mean of three replications, Figures in parentheses are arc sine transformed values

during dual culture assays, with a maximal reduction going down to 13 per cent of the control. Aflatoxin-specific production was decreased by all isolates to at least 54 per cent of the control (Campos-Avelar *et al.*, 2021)^[3].

Conclusion

In the present investigation, twenty-one actinomycete isolates were successfully obtained, out of which eleven were confirmed as *Streptomyces* spp. based on Gram staining, aerial mycelium characteristics and molecular characterization. The eleven *Streptomyces* isolates exhibited varying antagonistic potential against *A. flavus* in dual culture, with inhibition ranging from 11.11 per cent to 61.11 per cent. Thus, usage of *Streptomyces* spp. is one of the eco-friendly and reliable approach to manage aflatoxin contamination in groundnut.

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