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Research Article

ANTAGONIST ACTIVITY OF STREPTOMYCES SAMPSONII MN 93/2 AND RESEARCH ON JASMONIC ACID

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A B S T R A C T

As a result of isolation of *Streptomyces* actinomycetes, which are antagonists of some plant fungal pathogensfrom the soil using Humic acid, vitamin agar and selective nutrient medium,a pure culture similar to *Streptomyces* in terms of morphology was isolated and it was the closest to *Streptomyces sampsonii* when determining species affinity through 16SrRNA sequential analysis.

Upon determination of antagonist activity of *Streptomyces sampsonii* MN93/2 culture by double culture method, it was determined to have antagonist activity, creating 15.5 mm inhibition zone for*Cladosporium fulvum* and 19 mm inhibition zone for *Alternaria alternata* pathogens.

Tests of *S.sampsonii MN93/2* under greenhouse conditions, neutralized the course of tomato alternariosis and showed 72.1% biological activity in 21 days. *Streptomyces sampsonii MN93/2* contains 0.00679 mg /kg of jasmonic acid in plants treated with 10^7cells/ml and 0.0267 mg / kg in plants treated with 10^8cells/ml.

INTRODUCTION

The main habitat of actinomycetes is soil, which belongs to the largest class of gram-positive bacteria, taxonomically classified as *Actinobacteria*, and consists of 6 groups, 39 families, 130 species, and various fibrous and hyphae-forming microorganisms. Most species are facultative anaerobes and some species grow under aerobic and anaerobic conditions (Bergey's Manual). Produces biologically active antibiotics, amino acids, enzymes and vitamins, plays an important role in soil formation and fertility. Actinomycetes carry out all forms of metabolism except photosynthesis and secrete large amounts of secondary metabolites (EssaidAitBarka, ParulVatsa *et al*, 2016). The genus *Streptomyces* was first identified by Waksman & Henrici in 1943 and is the largest genus in the genus Streptomycetaceae and includes more than 550 species conditions (Bergey's Manual). Actinomycetes of the genus *Streptomyces* are the major antibiotic-producing microorganisms and, under suitable conditions, synthesizes approximately 7,600 types of biologically active metabolites (BerdyJ, 2005.). Many species of actinomycetes, such as *Streptomyces scabies, Streptomyces lydicus, Streptomyces griseoviridis, Streptomyces hygroscopicus, Streptomyces viridochromogenes,* are used against plant diseases and weeds.

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(Yuan W M, *et al*, 1995, Andrea Minuto *et al*, 2006, Charles J. Thompson *et al*, 1987, Dirk Schwartz *et al*, 2004, B. E. Wiggins and L. L. Kinkel. 2007) Jasmonic acid - dependent signalling pathways are involved in the defence response against pathogens, pests and wound damage (Walling, 2000; Eric *et al*., 2003; Rojo *et al*., 2003)

Research materials and methodology

Soil sampling

Taken from 0-20 cm deep soil in potato field, Bornuur soum, Tuv aimag.

Methods of isolating pure actinomycetes culture from soil

Dilute the soil sample to 10^4 with physiological solution by Koch reduction method and after sterilization under humic acid-vitamin agar (HVA) in selective medium / Humic acid - 1.0 g, Na2HPO4 - 0.5 g, KCl - 1.7 g, FeSO4 * 7H2O - 0.01 g, MgSO4 * 7H2O - 0.05 g, CaCO3 - 0.02 g, agar - 16.0 g 900 ml of distilled water, pH 7.0 ± 0.2, incubated for 14 days at 28°C in vitamin B, biotin, nystatin, nalidixic acid, transferred from single cell colony to ISP2 /Yeast extract and malt extract agar/and a pure culture was isolated by culturing for 14 days at 28°C.[12].

16S rRNA sequencing and phylogenetic analysis

Bacterial DNA was isolated using the lysis buffer (splitting solution) method. Polymerase chain reaction amplifies the DNA control portion of the genome. The polymerase chain reaction amplified the DNA control portion of the genome. To

identify the microorganism, primers 27F - 5`AGAGTTTGATCCTGGCTCAG and 338-5 'GCTGCCTCCGTAGGAGT were used in the gDNA control zone.The total PCR reagent is 50 µl, 5 µl of 10x Dream buffer, 1 µl of dNTP, 1 µl of each primer, 3 µl of sample, 1 µl of polymerase (Dream taq polymerase), 39 µl of ultra-sterile water (Thermofischer ultrapure) conditions for preddenaturization at 94 ° C for 5 min, 35 cycles: 30 sec at 94 ° C, 30 sec at 58 ° C, 30 sec at 72 ° C, and 7 min at 72 ° C for the final lengthening step (My Genie ™ 32 Thermal Block , Bioneer). The PCR product was tested by 1.5% agarose gel electrophoresis.

DNA sequencing and phylogenetic analysis

The PCR product was refined, and DNA sequencing was performed on an automatic sequencing machine (Macrogen, South Korea) and the result was searched, compared with sequence at Genbank (NCBI), the genetic relationship was established, and the species was identified.

Determination of antagonistic activity

1. *Pathogen culturing:* Two pathogens, *Alternaria alternata and Cladosporium fulvum,* were incubated in potato glucose agar medium at 25°C for 7-10 days.
2. *Actinomycete culturing*: Actinomycete culture was planted entirely on the surface of the ISP2 medium and incubated at 28°C for 7–10 days.
3. *Determining Antagonistic activity*: Antagonistic activity was determined by double culture method. The pathogen and the studied actinomycetes were incubated in potato glucose agar medium for 4 repetitions at 27°C for 14–21 days, and the diameter of the inhibition zone and pathogenic fungal colonies was measured against the diameter of the pathogen grown in the control cup.

Determining biological activity against tomato alternariosis

Culturing Alternaria alternata

The plant pathogen *Alternaria alternate* was cultured in PDA medium at 25 ° C for 10 days, washed with a 0.1% solution of Tween-80 and 10^4 spores were prepared.

Infection of Alternaria alternata in tomatoes

When the tomatoes had 5-6 leaves, the spore suspension / 10^4 / was sprayed with 30-40 ml per plant. To make the infection more effective, it was covered with a plastic mesh for 24-48 hours after infection, and symptoms appeared within a week.

Determining biological activity

When planting tomato seedlings in bucket, antagonistic cultures around the roots were irrigated with 10^7, 10^8 cells / ml to 50 ml. The antagonist culture was tested with 2 doses of 10^7 and 10^8 cells / ml and 3 repetitions when the degree of disease of artificially infected tomato leaves was 1 point. There should be at least 5 tomatoes per repetition and the culture per plant was sprayed by 30 – 40 ml twice at 7-day intervals. In determining biological activity, progress and severity of the disease were assessed on 7^{th}, 14^{th}, and 21^{st}days, and compared with control plants and it was determined by the Abbott method.

METHODOLOGY FOR PLANT DISEASE DETECTION

Detection of plant disease degree is completed by determining the percentage of diseased part (spot, stain etc) of the plant on the total surface area the plant. The severity of the disease is expressed in points. Usually a 5-point classification is used. A score of 1-2 indicates low morbidity, a score of 3 indicates an increase in morbidity, and a score close to 4 indicates epiphytotic. 0 point means no disease symptom on plant.
1 point – up to 10 percent of leaf surface of the plant is diseased

2 point - 25 percent of leaf surface of the plant is diseased
3 point - 50 percent of leaf surface of the plant is diseased
4 point - 75 percent of leaf surface of the plant is diseased
5 point - the leaves of the plant are completely diseased

The degree of plant disease is determined by a score of 0-5, and the progress or index of plant disease is determined by the average degree of plant disease.

$$Px = \frac{\sum(a \times b)}{n \times k} \times 100\%$$

Px – progress of plant disease, %
a – quantity of diseased plant
b – disease/sickness rate, score
n – quantity of plants taken for calculation
к –the highest score of plant disease rate
Biological activity of bio preparation has been calculated by using Abbott formula.

$$Э = \frac{(k - 0)x100}{k}$$

Э – biological activity, %
к –disease progress of plant under the control, %
0 – disease progress in version processed by biological preparation, %

Jasmonic acid research

After the above biological activity determination test, 2gr sample was taken from each plant treated with 10^7 and 10^8 cells/ml, diseased control, healthy control, *Streptomyces sampsonii MN93/2* culture, ground it, put in a tube with 10 ml solution (95% methanol: 5% ethyl acetate) and mixed with the vortex for 15 seconds. After that centrifuged at 13,000 rpm for 10 minutes, the supernatant was separated, filtered through a 0.45 µm nylon membrane, and placed in a glass jar.

GC-MS Conditions

CLARUS SQ 8 GC / MS equipment is adjusted as follows using RxiR-5ms type 30 m long, 0.25 mm diameter I.D. Using the x 0.25 µm column. Detector temperature is 280^0C, Source temperature is 240^0C, Injector temperature is 250^0C, Carrier gas helium is 1ml/min, Split total flow is 20ml/min, Injection volume is 0,2µл

Mass Spectrometer Adjustment

Detector mass range – 45gr – 480gr

Gas Chromatograph Adjustment

Keep the gas chromatograph oven temperature at 400^0C for 3 min. Then increase the temperature to 150^0C at 20^0C/min speed. Then increase the temperature to 280^0C at 10^0C / min

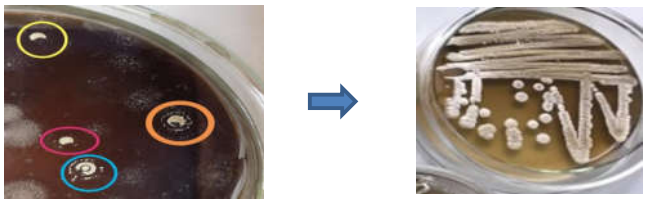
speed and finish the analysis by keeping the temperature at 280^{0}C for 6.5 min.

RESULTS AND DISCUSSION

Isolation for Streptomyces pure culture from the soil

Diluted the soil sample and incubated for 14 days at 28 ° C in a selective culture medium with humic acid, vitamin HVA and isolated pure actinomycetes culture by transferring culture morphologically similar to *Streptomyces* to ISP2 media.

Streptomyces sampsonii MN93/2 forms spherical, convex, white-gray colonies on the surface of the ISP2 medium with spherical, chained spores, and the substrate mycelium is brown. It does not create turbidity in liquid media (ISP2 broth) and can cause scale and sedimentation.



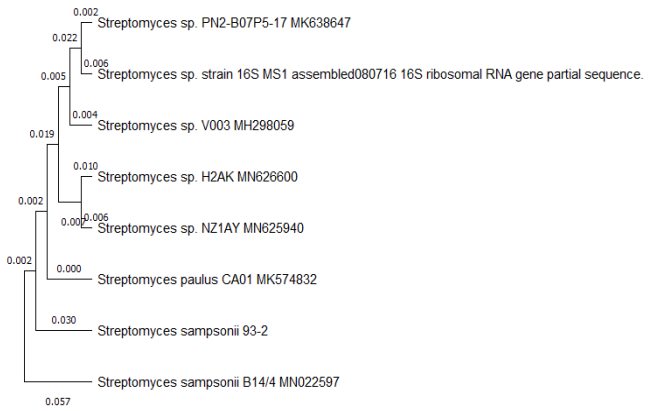
Picture 1 *Streptomyces* pure culture isolated from the soil by using HVA nutrient medium

The *Streptomyces sampsonii PM33* was isolated from the sediment collected from Vellar estuarine, Parangipettai, Tamil Nadu, India (Venugopal G, Manikkam R *et al*, 2019). The *Streptomyces sampsonii* GS1333 was isolated from garden soil (Praveen Kumar Jain and PC Jain, 2006).

16S rRNA sequence and phylogenetics analysis

The DNA of the culture was isolated by Lysis buffer method and the DNA was amplified using a primer of the 16S rRNA gene 27F – 5′AGAGTTTGATCCTGGCTCAG, 338–5 ’GCTGCCTCCCTAGGAGT, and the sequence was determined.

As a result of DNA sequences, processing was conducted using the BioEdit program and MEGA software version X and read into the BLAST (Basic Local Alignment Search Tool) of the NCBI (National Center for Biotechnology Information) *Streptomyces sampsonii* strain ATCC 25495 16S ribosomal RNA matched with complete sequence by 98.93%, *Streptomyces sampsonii* strain NRRL B12325 16S ribosomal RNA matched with partial sequence by 98.93%.



Picture 2 Phylogenetic tree stored in the GenBank and established in the order in which it was discovered.

This research identified the species with a 98% probability of being studied by other scientists, and according to the phylogenetic tree it is the closest to the *Streptomyces sampsonii* species registered with the Genbank in terms of genetic distance.

Streptomyces sampsonii MN93/2, a local strain with antagonist activity isolated from soil, was registered in the NCBI GenBank under MT256205.1.

Antagonist activity

The antagonistic activity ofactinomycetes pure culture isolated from soil was tested by dual/double culture method on plant pathogens such as *Alternaria alternata* and *Cladosporium fulvum*. The pathogen and the studied actinomycetes were incubated in potato glucose agar medium for 4 repetitions at 27°C for 14–21 days, and the diameter of the inhibition zone and pathogenic fungal colonies was measured against the diameter of the pathogen grown in the control cup.

If the organism under research releases an antibiotic-like compound, it will infiltrate the culture medium, inhibiting the growth of the pathogen and creating frame/circle. The size of the circle/range depends on the emission of antibiotic-like compounds and their activity.

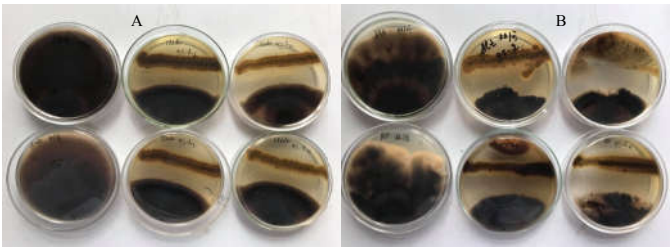
Table 1 Antagonist activity of *Streptomyces sampsonii MN93/2*

Variants	Repeat	*Cladosporium fulvum*			*Alternaria alternata*		
		Colony diameter (average, мм)		*Antagonist activity %*	Colony diameter (average, мм)		*Antagonist activity %*
		14thday	*21th day*		*14th day*	*21th day*	
Streptomyces sampsonii MN93/2	4	40	40	52.3	32.25	32.25	58.1
Pathogen	4	74	84		59	**77**	

Streptomyces sampsonii MN93/2 isolated from the soil delays the growth of pathogens such as *Cladosporium fulvum* and *Alternaria alternata* by 52.3-58.1%.

Table 2 Inhibition zone of *Streptomyces sampsonii MN93/2*

Strain	Inhibition zone (мм)	
	Cladosporium fulvum	*Alternaria alternata*
Streptomyces sampsonii MN93/2	15.5	19



Picture 3 Anatagonist activity *Streptomyces sampsonii MN93/2*. A. *Cladosporium fulvum, B. Alternaria alternata*

The study found that after 14 days, *Streptomyces sampsonii MN93/2* formed a 15.5 mm inhibition zone o for *Cladosporium fulvum* and 19 mm inhibition zone for *Alternaria alternata* and was antagonistically active. (Picture 3, Table 2).

Biological activity of S.sampsonii MN93/2against tomato alternariosis

Laboratory-grown tomato seedlings were transplanted into drum in a greenhouse, adapted to the environment for 10-14

days, and then sprayed with *Alternaria alternata* pathogen (10^5) and artificially infected. When infected tomatoes had 1-point disease, 10^8 and 10^7 spore cultures of *S.sampsonii* MN93/2 was sprayed twice at 10-day intervals, determined disease progress and disease development, compared with disease control, and calculated biological activity after 21 days.

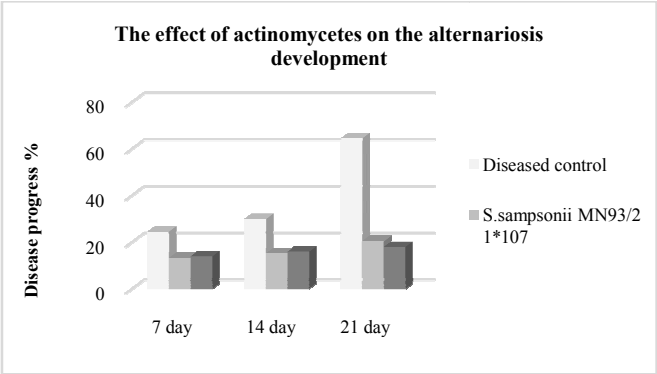
One week after spraying Alternariosis pathogen into tomato, all plants had 1 point disease.

When the sample was read on a GC/MS device, the standard substance was peak at 13.22 minutes, and the peak of the standard substance was compared with that of the sample, peak was detected at 13.03, 13.01 minutes on samples of plants treated with *Streptomyces sampsonii MN93/2*culture and it was determined to contain jasmonic acid. And jasmonic acid was not detected in diseased control and healthy control plants and 0.00679mg/kg jasmonic acid contained in plant treated with 10^7 cell/ml *Streptomyces sampsoniiMN93/2* culture and 0.0267mg/kg in plant treated with 10^8 cell/ml, respectively.

Table 3 Biologically activity of *S.sampsonii* MN93/2 (on tomato alternariosis)

Strain	Variants	Quantity of plants to be calculated	Quantity of plants diseased	Disease rate/degree /score/					Disease distribution%	Disease progress %	Biological activity %
				0	1	2	3	4			
Tomato											
	Diseased control	30	30	-	3	6	2	19	100	64.6	-
S.sampsonii MN93/2	$1*10^7$	30	24	6	21	1	-	2	80.0	20.6	68.1

Note: (-) – no pathogenic symptom on corresponding score.



Pigure 1 *S.sampsonii MN93/2*caused delay in alternariosis development

The graph above shows that the progression of the two variants affected by *S.sampsonii MN93/2* was reduced by 10.6-11.3% at 7thweek, 14-14.7% in 14 days, and 44-46.6% in 21 days compared to the control variant.

Experimental results showed that the progression of tomato disease treated with *S.sampsonii MN93/2* was neutralized from the 7th day, and the biological effect was 72.1% on the 21st day.

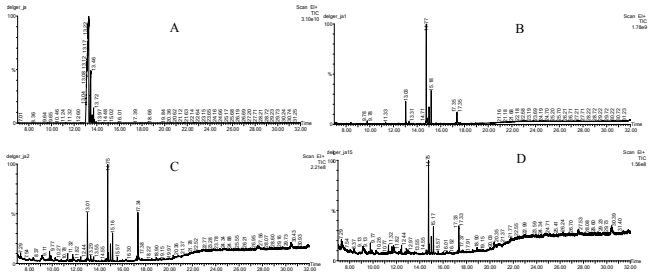
RESULT OF JASMONIC ACID DETECTION

After the above tomato experiment, 2 gr sample was takenfrom the plant and conducted jasmonic acid detection analysis.Jasmonic acid is a compound that represents the plant disease resistance.

Table 4 Sampling of the jasmonic acid content in the GC/MS device

GC/MS Sample number	Sample name	Jasmonic acid mg/kg
Ja 1	Plant treated with 10^7 cell/ml *Streptomyces sampsonii MN93/2*	0.00679
Ja 2	Plant treated with 10^8 cell/ml *Streptomyces sampsonii MN93/2*	0.0267
Ja 15	Diseased control	0
Ja 14	Healthy control	0
Ja	Jasmonic acid standart	100

Experiments have shown that when treating with the *Streptomyces sampsonii MN93/2* culture, it forms/creates alternariosis resistance in tomatoes.



Picture 4 A. Standard, B. Plant treated with 10^7 cell/ml *Streptomyces sampsonii MN93/2* culture, C. Plant treated with 10^8 cell/ml *Streptomyces sampsonii MN93/2* culture, D. Diseased control

CONCLUSION

Streptomyces sampsonii MN93/2 forms spherical, convex, white-gray colonies on the surface of the ISP2 medium with spherical, chained spores, and the substrate mycelium is brown. It does not create turbidity in liquid media (ISP2 broth) and can cause scale and sedimentation.

Streptomyces sampsonii MN93/2 was the closest to *Streptomyces sampsonii* when determining species affinity through16SrRNA sequential analysis.

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