Original Research Article

Isolation, Screening and Identification of Herbicidal Actinomycetes from Rhizosphere Soils

ABSTRACT

Aim: Current study investigates the activity of rhizosphere microflora against common weeds of *Coccinia grandis*. Soil actinomycetes known for their potential antimicrobial and other secondary metabolites were taken for the study.
Study design: CRD.
Place and Duration of Study: Department of Agricultural Microbiology, Tamil Nadu Agricultural University (TNAU), between November to December 2021.
Methodology: Actinomycetes were isolated from rhizosphere soils collected from the western zone of Tamil Nadu (Ooty, Western Ghats, Tirupur, Erode and Coimbatore) using different media (KKA, SCA, HV & LMSA). Their morphological characterization was done by following standard protocol. The isolates were screened based on their herbicidal activity against *Trianthema portulacastrum*.*In vitro* seed germination assay (pre-emergence) was followed to identify potential actinomycetes.
Results: Thirty actinomycetes isolates were obtained from different locations in Tamil Nadu using four different media and their morphological characterization was done. Most of them are creamy white, grey, white and some of them were producing pigments. All the isolates showed positive for Gram's reaction.
Conclusion: Six efficient isolates were identified in the preliminary screening of weed seed germination. Further, the isolates will be studied at the field level.

Keywords: Weeds-Actinomycetes- herbicide-germination

1. INTRODUCTION

Weeds are undesirable plants in the crop field and caused a significant economic loss in crop production. About 200 plant species in the world act as weeds, of which around 80 species are troublesome enough to humans (Holm, 1977). The composition of weed species, their intensity, and competition offered by them to the crop vary with the geographic regions, soil and weather conditions, and the field and crop management practices (Mashingaidze *et al*., 2012).

Different weed management practices were followed to control weeds. Conventional weed control methods have their merit and demerit *viz*., manual weeding is time and labour-consuming; control with herbicide has host-specific/broad range limitation along with the emergence of herbicide resistance. To reduce the reliance on synthetic herbicides in cropping systems bio herbicides were introduced to reduce the toxic ill effect. Therefore weed management has changed from conventional practices to environmentally friendly biological approaches [3].

Bioherbicides derived from the microbial source are effective substances for weed control provide ecological advantages and maintain sustainable agricultural production. Secondary metabolites of identified microbial species cause plant phytotoxic activities such as necrosis, chlorosis, deformation and stunting. These features are prerequisites to use as bio herbicides for weed control. Actinobacteria represent the most prominent group of microorganisms, which produce bioactive compounds. The Majority of these molecules originate from the Streptomyces genus [4].

Trianthema portulacastrum L. belongs to the *Aizoaceae* family and is a common dicotyledon weed found in various agricultural and vegetable crops, especially during the rainy seasons [5]. Control of *T. portulacastrum* with various pre and post-emergence herbicides in different agricultural crops has been attempted. Pre-emergence application of oxyfluorfen, isoproturon, oxadiazon, pendimethalin and fluchloralin effectively controlled horse purslane in various crops [6, 7, 8].

Although these measures can control the weed on a small scale, they are not feasible for large infestations or infestations in environmentally sensitive areas. Further, increased and indiscriminate use of herbicides has resulted in herbicide resistance [9] and environmental concerns. Worldwide efforts are underway to reduce the heavy reliance on chemical herbicides and finding alternative strategies for weed management [10] is the need of time. Allelopathy and mycoherbicides could be an appropriate potential technology for this purpose.
Keeping these facts in mind, the present study was undertaken to isolate and screen actinomycetes for their herbicidal activity against Trianthema weed at the pre-emergence stage by microbial secondary metabolites.

2. MATERIAL AND METHODS

2.1 Sample collection and Isolation of actinomycetes

2.1.1 Sampling sites:

The soil samples taken for this study were collected from Agricultural Fields of the western zone of Tamil Nadu (Ooty, Western ghats, Tirupur, Karur & Erode) from November to December 2021. All the laboratory experiments were conducted in the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, following Completely Randomized Design (CRD) with three replications.

2.1.2 Isolation of actinomycetes from soil:

Ten grams of the collected soil samples were pre-treated in Hot Air Oven. They were heated to 60-65°C for 3 hrs. This helps in decreasing the population of gram-positive bacteria. Actinomycetes were isolated from soil samples by Serial-dilution and plating technique. Four different media *viz*., Ken Knights Agar (Glucose -1; KH_2PO_4-0.1; $NaNO_3$ -0.1; KCl-0.1; $MgSO_4.7H_2O$ -0.1; Agar- 20 g/L), Humic acid Agar (Humic acid -1; KCl-1.7; Na_2HPO_4-0.5; $MgSO_4.7H_2O$-0.5; $CaCO_3$-0.02, $FeSO_4$-0.01; Agar -20 g/L), Low Nutrient mineral salt Agar (NaCl- 0.2; $MgSO_4$ -0.05; $CaCO_3$ -0.02; $FeSO_4$ -0.01; Soluble starch- 0.1; Yeast extract-0.1; K_2HPO_4- 2; Agar- 20) and SCA (Starch Casein Agar- Starch -10; Casein-0.3; KNO_3-2; $MgSO_4.7H_2O$- 0.05; NaCl- 2; $CaCO_3$-0.02; $FeSO_4.7H_2O$- 0.01; KH_2PO_4 -2; Agar- 20 g/L) were employed for isolation of actinomycetes with dilutions 10^{-2} and 10^{-3}. The culture medium was prepared and sterilized at 121°C at 15 lbs pressure for 15 min and supplemented with cycloheximide antifungal agent (50µ/ml) and tetracycline antibacterial agent (20µ/ml) to prevent bacterial growth and fungal growth respectively. The plates were incubated at 28±2°C for 5 to 7 days. After incubation, the actinomycetes were observed, purified using the subculture method and maintained in an ISP-4 medium for further investigation.

2.2 Identification of actinomycetes isolates:

The isolated actinomycetes colonies were studied by the following methods:

2.2.1 Cultural characterization

The cultural properties and growth characteristics of isolates were studied on the 7th day in ISP-4 media, the plates were incubated at 30°C and observations were recorded on the 7 th day.

2.2.2 Morphological Characterization:

Morphological Characterization was performed with a magnifying lens and observed on actinomycetes grown for 3 to 14 days on an ISP-4 agar media plate. Colony morphology was recorded with respect to aerial color, aerial mycelium, size, nature of colony, reverse side color and pigmentation and the isolated were observed under the microscope.

2.2.3 Grams staining:

A thin smear of each isolate was made on a clean glass slide and heat fixed. Then the smear was stained with crystal violet for 1 min and fixed by staining with Gram's iodine. After 30 sec the slide was washed again in tap water and decolorized with alcohol. After decolourization, the smear was counterstained with safranin for 1 min. Then the slide was washed, air dried and examined under the microscope.

2.3 Screening of actinomycetes isolates:

2.3.1 Weed seed germination assay (pre-emergence)

All the thirty isolates were purified and maintained in ISP-4 broth. Seeds of *Trianthema portucalastrum* were obtained from the Weed Management division, Department of Agronomy, Tamil Nadu Agricultural University and used for the screening of actinobacterial isolates as pre-emergent control. Before that culture filtrate was prepared. 24 hrs old cultures were centrifuged at 8000 rpm, 4^0C for 10 minutes. The supernatant containing secondary metabolites of the actinomycetes isolate was collected and the pellet was discarded.

For this experiment, a Petri dish was taken with suitable size filter paper and was moistened with culture filtrate. Seeds were soaked in 3 ml of extracted culture filtrate for 12 hours. After that seeds were placed in a Petri dish and observed after 4 days for germination. A Similar procedure without culture filtrate served as control. The experiment was repeated 3 times as replication to confirm the herbicidal activity of all isolates. From this efficient isolates were selected and taken for further investigation.

2.4 Identification of actinomycetes isolates:

2.4.1 Biochemical characterization of actinomycetes isolates

Biochemical characterization of selected six actinomycetes isolates after initial screening (Citrate utilization, Indole production, Methyl Red (MR) Vogues Proskauer (VP) test, Starch Utilization, Nitrate Reduction and Hydrogen sulphide production) was done by following standard protocol.

2.4.2 Identification of actinomycetes isolates by 16S rRNA method

Identification of actinomycetes isolates was done by the following 16S rRNA method by using specific primers i.e., Sm6F- forward (GGTGGCGAAGGCGGA) and Sm5R - reverse (GAACTGAGACCGGCTTTTTGA). Sequencing was done by Syngenome (OPC) private limited (Coimbatore, Tamil Nadu) following Sanger's sequencing method and nucleotide sequences were submitted in NCBI.

3. RESULTS

3.1 Collection of soil samples

3.1.1 Sampling sites:

Soil samples were collected from different locations in the western zone of Tamil Nadu i.e. Ooty, Western Ghats, Tirupur, Erode and Coimbatore. Their geographical coordinates were presented in table 1.

Table 1. Geocoordinates of sampling sites

Location	Soil	Geocoordinates
Ooty	Belmounte Terrace	11.4046° N, 76.7230° E
Western ghats	Anaimalai	10.5821° N, 76.9343° E
Tirupur	Uttukuli	11.1559° N, 77.4433° E
Erode	Kodumudi	11.0788° N, 77.8867° E
Coimbatore	Pollachi	10.6609° N, 77.0048° E
	Orchard, TNAU	11.0122° N, 76.9354 E

3.2 Isolation and characterization of actinomycetes

3.2.1 Isolation of actinomycetes:

Thirty isolates were obtained from five different locations (table 2). Soil samples from Ooty register maximum isolates (8 No.) of which 3 isolates were obtained from normal soil and 5 isolates were obtained from preheated soils. Seven isolates were obtained from Coimbatore as well as Erode soils, where normal soils recorded 4 isolates from Erode and 3 isolates from Coimbatore. Pre-heated soils recorded 3 isolates from Erode and 4 from Coimbatore. Tirupur soils recorded the minimum number of isolates (3 Nos.) compared to other locations (table 2). Here the results denote the identically different isolates obtained from each soil not the total population of a particular soil.

Table 2. Isolation of actinomycetes from different rhizosphere soil

Particulars of sample	Normal soil sample				Soil heated @ 60-62°C for 3 hrs			
	KKA	SCA	HV	LMSA	KKA	SCA	HV	LMSA
Ooty	-	-	3	-	2	2	1	-
Western Ghats	1	-	1	-	-	-	3	-
Tirupur	-	-	2	-	-	-	-	1
Erode	-	4	-	-	-	3	-	-
Coimbatore	3	-	-	-	2	-	2	-

Values are the mean of three replications

3.2.2 Characterization of actinomycetes:

All thirty isolates were morphologically characterized based on the colony size, texture, color and pigmentation. Color of the isolates was Creamy white, white, Grey, Light yellow and Light grey. Out of 30 isolates, 15 isolates had a Creamy white colored colony, 8 had a white color, 4 recorded grey, 1 had Light yellow, 1 had Dull white and 1 had a Light grey color colony. All the isolates show pigmentation which was observed by the coloration on the reverse side of the colony. Pigmentation such as Yellowish white, Brown, Yellow, Pink, Dark greenish brown and pink diffusible pigments. Yellowish white pigmentation was predominant among the isolates (17 isolates) followed by brown and yellow. The texture of the colony was recorded as hard for most of the isolates and few of the isolates had a smooth surface. The

size of the colony ranged from 0.2 to 0.3 cm. All the isolates were positive for Gram's reaction (Table 3). All the isolates had characteristics earthy odour of actinomycetes.

Table 3. Morphological characterization of actinomycetes isolates

S.No.	Strains	Color Surface	Color reverse Side	Colony texture (cm)	Colony size (cm)	Gram staining
1.	A1	Creamy white	Yellowish white	Hard	0.3	+
2.	A2	white	Yellowish white	Hard	0.2	+
3.	A3	Creamy white	Yellowish white	Hard	0.3	+
4.	A4	Creamy white	Yellowish white	Hard	0.3	+
5.	A5	white	Brown	Hard	0.2	+
6.	A6	white	Yellow	Hard	0.3	+
7.	A7	white	Yellowish white	Hard	0.3	+
8.	A8	Creamy white	Yellowish white	Hard	0.2	+
9.	A9	Creamy white	Yellowish white	Hard	0.3	+
10.	A10	Creamy white	Yellowish white	Smooth	0.3	+
11.	A11	Creamy white	Yellowish white	Smooth	0.2	+
12.	A12	white	Pink	Smooth	0.3	+
13.	A13	Grey	Brown	Hard	0.3	+
14.	A14	Grey	Yellow	Hard	0.2	+
15.	A15	Creamy white	Dark greenish Brown	Hard	0.4	+
16.	A16	Creamy white	Yellowish white	Smooth	0.2	+
17.	A17	Creamy white	Yellowish white	Hard	0.3	+
18.	A18	Dull white	Yellowish white	Hard	0.4	+
19.	A19	Grey	Yellowish white	Hard	0.3	+
20.	A20	Light yellow	Yellowish white	Hard	0.2	+
21.	A21	Creamy white	Pink diffusible Pigment	Hard	0.3	+
22.	A22	Creamy white	Yellow	Smooth	0.3	+
23.	A23	Creamy white	Brown	Smooth	0.2	+
24.	A24	White	Yellow	Hard	0.3	+
25.	A25	White	Yellowish white	Hard	0.2	+
26.	A26	Grey	Yellowish white	Hard	0.3	+
27.	A27	Light grey	Yellowish white	Hard	0.2	+
28.	A28	White	White	Smooth	0.3	+
29.	A29	Creamy white	Brown	Smooth	0.2	+
30.	A30	Creamy white	Yellow	Hard	0.3	+

3.3 Screening of actinomycetes based on herbicidal activity

3.3.1 Germination of Trianthema seeds *in vitro*

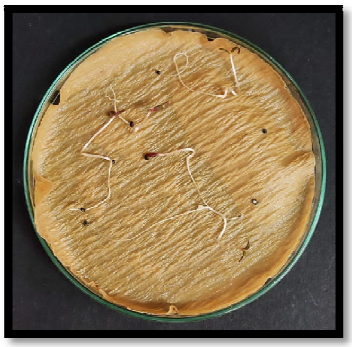
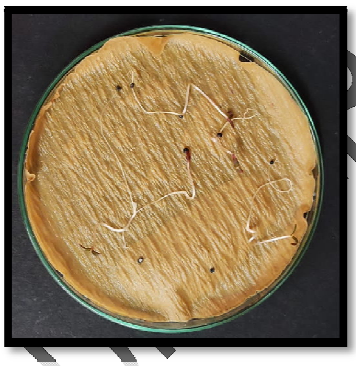
Common weed of garden soil crop (*Coccinia grandis*), *Trianathema portulacastrum* was taken for this study. Weed seeds were soaked overnight in the Culture filtrate of all thirty isolates and placed in a Petri plate containing moistened germination paper. Based on the germination behaviour of each isolate, their herbicidal activity was determined. This was the

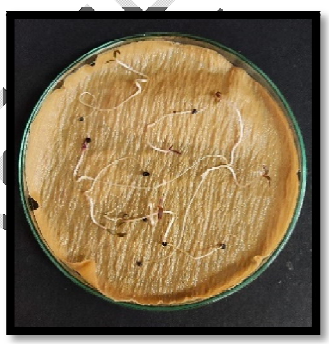
pre-emergence control of weeds by actinobacterial isolates. There is no germination/emergence of *Trianathema* seeds in A1, A 3, A8, A12, A13 and A17 isolates which denote the herbicidal activity of the isolates against the weed seed. Isolates A15, A19, A24, A25, A28, and A30 recorded 100 per cent germination, which indicates those isolates showed growth-promoting activity for weed seeds. Other isolates recorded a moderate growth rate for weed seeds. These results were tabulated in table 4 and plate 1.

Table 4. Effect of actinobacterial isolates on germination of weed seed

Isolates	Number of seeds germinated	Germination percent (%)
Control	8	100%
A1	0	0%
A2	6	75%
A3	0	0%
A4	5	62%
A5	7	87%
A6	4	50%
A7	6	75%
A8	0	0%
A9	7	87%
A10	4	50%
A11	3	37%
A12	0	0%
A13	0	0%
A14	7	87%
A15	8	100%
A16	4	50%
A17	0	0%
A18	6	75%
A19	8	100%
A20	4	50%
A21	5	62%
A22	6	75%
A23	4	50%
A24	8	100%
A25	8	100%
A26	6	75%
A27	7	87%
A28	8	100%
A29	7	87%
A30	8	100%

Values are the mean of three replications

		
Isolate A1 (0%)	Isolate A2 (75%)	Isolate A3 (0%)
		
Isolate A4 (62%)	Isolate A5 (87%)	Isolate A6 (50%)
		
Isolate A7 (75%)	Isolate A8 (0%)	Isolate A9 (87%)

		
Isolate A10 (50%)	Isolate A11 (37%)	Isolate A12 (0%)
		
Isolate A13 (0%)	Isolate A14 (87%)	Isolate A15 (100%)
		
Isolate A16(50%)	Isolate A17 (0%)	Isolate A18 (75%)

		
Isolate A19 (100%)	Isolate A20 (50%)	Isolate A21 (62%)
		
Isolate A22 (75%)	Isolate A23 (50%)	Isolate A24 (100%)
		
Isolate A25 (100%)	Isolate A26 (75%)	Isolate A27 (87%)

Isolate A28 (100%)	Isolate A29 (87%)	Isolate A30 (100%)

Control (100%)

Plate 1. Effect of actinomycetes isolates on germination of Trianthema
Values in () denotes germination per cent of weed seed

3.4 Biochemical characterization and molecular identification of selected actinomycetes isolates

Biochemical characterization of actinomycetes isolates was done following standard protocol. Selected isolates showed positive for Citrate utilization, Methyl red, production, Catalase test, Starch utilization and Nitrate reduction test and but negative for Indole production, Vogues Proskauer and H_2S production (table 5). Similar results were reported earlier by Reddy et al. (2011) for streptomyces isolates.

Table 5 Biochemical characterization of selected actinomycetes isolates

S. No.	Test Name	Isolates					
		A1	A3	A8	A12	A13	A17
1.	Citrate utilization	+	+	+	+	+	+
2.	Indole	-	-	-	-	-	-
3.	Methyl Red (MR)	+	+	+	+	+	+
4.	Vogues Proskauer (VP)	-	-	-	-	-	-
5.	Catalase	+	+	+	+	+	+
6.	Starch Utilization	+	+	+	+	+	+
7.	Nitrate Reduction	+	+	+	+	+	+

8.	Hydrogen sulphide production	-	-	-	-	-	-

Values are the mean values of three replications
(+ positive, – negative)

Selected six isolates were identified by the 16 S r RNA method and identified as *Streptomyces griseorubens* (A1), *Streptomyces lavendulocolor* (A3), *Streptomyces diastotochromogenes* (A8), *Streptomyces griseocarneus* (A12), *Streptomyces variabilis* (A13) and A17 (*Streptomyces althioticus*) (table 6).

Table 6. Identification of actinomycetes by 16S rRNA method

S.No.	Isolate No.	Isolate Name	Accession No.
1	A1	*Streptomyces griseorubens*	ON970374
2	A3	*Streptomyces lavendulocolor*	OP001737
3	A8	*Streptomyces diastotochromogenes*	OP001743
4	A12	*Streptomyces griseocarneus*	OP001744
5	A13	*Streptomyces variabilis*	OP001423
6	A17	*Streptomyces althioticus*	OP001424

3. DISCUSSION

There is no clear-cut and established method available at present for the control of this weed. However manual, chemical and biological methods are considered to control this weed. Herbicides have become one of the main environmental problems, causing long-term toxicity to water and soil resources, as well as mammals. Therefore, integrated approaches to biological weed control in arable crops along with other weed management techniques are being broadly studied. Biological control of Trianthema with fungal pathogens was studied earlier by many researchers and it has gained acceptance as a practical safe and environmentally beneficial method. Earlier studies [10, 11, 12] indicate the potential of biological control of trianthema using plant pathogens.

Actinomycetes are an important group of microorganisms producing many extracellular active compounds such as Anisomycin, Bialaphos, Herbicidin A & B [13]. Christy et al. [14] reported that a small number of microbial products combined with herbicides improved weed control e□cacy.

In the present investigation, selective isolation of Actinobacteria using specific media was done and 30 actinomycetes isolates were obtained from different rhizospheric soil samples taken from the western zone of Tamil Nadu. Isolates were characterized following methods prescribed by the International streptomyces project (ISP) and identified as actinomycetes. The results of the present study are comparable with the study on endophytes in which, the genus Streptomyces was dominant [15]. Similarly, Zhang et al. [16] reported mainly Streptomyces from medicinal plants.

In the present investigation out of 30 isolates, 6 isolates showed 0 per cent germination, which shows there is a 100 % inhibition of weed seed at the pre-emergent stage. These isolates were taken for further study. A study was conducted by Helly Singh et al. (2017) [17] on the herbicidal activity of endophytic actinomycetes against *Parthenium hysterophorus*, *Ageratum conyzoides* and *Bidens biternata*. The direct fermentation method was used for phytotoxin production in the submerged culture to study the herbicidal activity against different test weeds. Significant differences were observed in the production of phytotoxin in

SCN and GS medium. In the case of *Ageratum conyzoides* (billygoat weed), the culture filtrate of *Nocardiodes sp*., *Nocardiodes sp*. and *Actinomadura sp*. a showed a 60% reduction in seed germination. These findings suggest that endophytic actinomycetes are a rich source of herbicidal metabolites. Further studies are required to isolate, purify and structure elucidation of the metabolites.

4. CONCLUSION

In the present study, the phytotoxic effect of rhizospheric actinomycetes was tested against Trianthema weed and six effective isolates were identified with 100 % inhibition of weed seed *in vitro* compared to other isolates and control. These isolates will be further studied at the field level to find out their efficiency, and their secondary metabolites will be studied to find out the active ingredient responsible for phytotoxicity of weed.

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