Nepal Journal of Science and Technology 12 (2011) 97-102

Antifungal Activity of Actinomycetes from Vermicompost and Their Morphological and Biochemical Characterization

Rishi Baniya and Gita S. Vaidya
Nepal Academy of Science and Technology (NAST) Khumaltar, Lalipur
e-mail: rishi1baniya2@yahoo.com

Abstract

Thirty eight actinomycetes were isolated from saw dust and husk containing vermicompost samples. Of them, four (10.5%) were active against at least one of the tested phytopathogenic fungi; *Fusarium oxysporum, F.moniliforme, F. proliferatum, Stemphylium botryosum, Exserohilum turcicum* and human pathogenic fungi; *Candida albicans* and *Aspergillus* spp. during dual culture method. Of them, VAH3 showed broad spectrum activity against both pathogenic fungi with maximum inhibition of 24.53 ± 0.20% against *F. oxysporum* and minimum inhibition of 12.37 ± 0.24% against *Stemphylium botryosum* on solid agar surface. In agar well diffusion method, only VAH3 showed broad spectrum activity with maximum inhibition diameter of 14 mm against *F. proliferatum* and minimum inhibition diameter of six mm against *Sclerotium rolfsii*. Among them, one promising strain *Streptomyces* VAH3 was selected for further study. The minimum inhibitory concentrations of crude antifungal agent against *F. oxysporum, Stemphylium botryosum*, and *Exserohilum turcicum*, *Candida albicans* and *Aspergillus* spp. were found to be 560 µg/ml, 1120 µg/ml, 1120 µg/ml, 560 µg/ml and 1120 µg/ml, respectively. Morphological, biochemical and physiological tests confirmed that all four potent strains were from Streptomyces genus.

Key words: actinomycete strains, *F. oxysporum*, vermicompost, *Streptomyces* spp.

Introduction

Root rot disease of lentil, caused by *Fusarium oxysporum* f. sp *lentis* is one of the important diseases of the world. In Nepal, this disease and *Stemphylium* blight disease, caused by *Stemphylium botryosum*, at the early stage of plant growth cause heavy loss in lentil productivity (Joshi 2010). Similarly, *Exserohilum turcicum* cause heavy loss of maize productivity due to northern leaf blight disease.

The management of *Fusarium* root rot and wilting disease of lentil is very difficult because no single treatment is fully effective. Currently, solarization of soil, advance sowing date, use of disease free seeds and fungicide-treated seeds are in practice but with limited success (Jalali &Chand 1992; Haware &Nene 1982). Although the use of resistant cultivar is the most economical and efficient control measure, the evolution of variability in pathogenicity of these pathogens has caused difficulty in controlling diseases (Haware &Nene 1982, Jimenez-Gasco &Jimenez-Diaz 2003). The coordinated varietal trial experiment carried out in 45 lines of lentil crop at

Rampur, Chitwan and Khajura (Banke) showed 30 lines resistant, 5 moderately resistant and 10 susceptible to *Fusarium* root rot (Joshi 2010).

Biological treatments of these soil borne fungal phytopathogens by using different bacterial antagonists such as *Pseudomonas* spp., *Bacillus* spp. and fungal antagonists such as *Trichoderma* spp. were effective in different parts of the world. However, *Trichoderma koningi* was not effective in controlling root rot of lentil in Nepal (Joshi 2010).

Vermicomposts are rich source of beneficial microorganisms which not only degrade organic macromolecules into simpler forms but also help control phytopathogenic and human pathogenic diseases by producing secondary metabolities such as antibiotics, chitinase enzymes, siderophores and Indole acetic acid and other phenol containing compounds. In this study, actinomycetes from vermicompost were screened for antifungal activity against phytopathogenic fungi and human pathogenic fungi, *Aspergillus* sps and *Candida albicans*, and potent strains were characterized morphologically, biochemically and physiologically.

Methodology

Preparation of vermicompost

Two combinations of vermicomposts: a) Sawdust (100g): Chopped straw (100g): Mixed vegetable and fruit wastes (100g), and b) Husk (100g): Chopped Straw (100g): Mixed vegetable and fruit wastes (100g) were prepared in 10 L, 5-6 holed plastic buckets at room temperature in Nepal Academy of Science and Technology premises. The bedding materials were moistened with water and placed inside the bottom of bucket. Then, the crushed mixed vegetable and fruit wastes were composted on the top of bedding materials using 100 earthworms in each bucket covered with lid. Hundred grams mixture of vegetable and fruit wastes was added once a week as feed for earthworms. The whole set up was kept for 3 months until all the wastes were completely digested.

Isolation

One gram mature vermicompost sample from each combination was suspended in 9 ml sterile distilled water and further serial dilution was performed up to 10^{-6}. Dilutions from 10^{-4}-10^{-6} were spread plated onto Starch Casein Agar (SCA) and incubated at 28°C for 7 days (Collins *et al.* 1989).

Screening of antifungal activity

Antifungal activity of actinomycetes against phytopathogenic fungi (*Fusarium oxysporum, F. proliferatum, F. moniliforme, F. eridiforme, Stemiphylium botryosum, Exserohilum turcicum* and *Sclerotium rolfsii*) and human pathogenic fungi (*Aspergillus* sps and *Candida albicans*) was performed on potato dextrose agar by dual culture method (Crawford *et al.* 1993). Secondary screening of potent strains was further performed by using agar well diffusion method as described elsewhere (Barry &Thornsberry 1985).

Characterization of active actinomycetes

Morphological, biochemical and physiological characterization of active actinomycete isolates were performed as described elsewhere (Williams *et al.* 1989).

Extraction of antifungal agent

The extraction of antifungal agents was performed as described elsewhere (Augustine *et al.* 2004).

Determination of Minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations of crude antifungal agent and standard antifungal antibiotic Nystatin were determined against *F. oxysporum, S. botryosum, E. turcicum, Candida albicans and Aspergillus* spp as described elsewhere (NCCLS 2002).

Results and discussion

Altogether 38 actinomycetes were isolated from sawdust and husk containing vermicomposts. Among

them, four (10.5%) isolates were found to be effective against at least one of the tested phytopathogenic fungi by dual culture method. Isolate VAH3 showed broad spectrum activity with maximum inhibition of 24.53 ± 0.20% against *F. oxysporum* and minimum inhibition of 12.37 ± 0.24% against *Stemphylium botryosum*. Isolates VAH1, VAH8 and VAS9 inhibited all tested Fusarium sps but did not show activity against *Stemphylium botryosum* and *Exserohilum turcicum.* (Table 1)

Table 1. Antifungal activity of actinomycetes (% inhibition)

Isolates Fungi	VAH1	VAH3	VAH8	VAS9
F. oxysporum	8.11 ± 0.11	24.53 ± 0.20	10.05 ± 1.0	12.40 ± 0.23
F. moniliforme	12.25 ± 0.14	21.10 ± 0.64	12.90 ± 0.33	12.33± 0.33
F. proliferatum	12.15 ± 0.15	23.80 ± 0.57	12.66 ± 0.38	12.66 ± 0.38
F. eridiforme	12.60 ± 0.35	23.75 ± 0.38	12.99 ± 0.33	12.20 ± 0.20
Stemphylium botryosum	-	12.37± 0.24	-	-
Exserohilum turcicum	-	12.66 ± 0.38	-	-

Morphological, biochemical and physiological tests of potent strains confirmed that all four isolates were from *Streptomyces* genus (Figure 1 & Table 2). All four isolates were found to possess gram positive recti-flexibilis type of sporophores originating from branching thin mycelium during microscopy. Biochemical tests like sugar utilization tests, Nitrate reduction and polysaccharide hydrolysis tests and physiological tests such as temperature and Sodium chloride tolerance tests showed high variability among these four isolates as well as those of reported species in Bergey's Manual of Systemic Bacteriology Vol. IV. *Streptomyces* VAH3 is more than 70% similar to *Streptomyces noursei.* Although it is difficult to ascertain unknown isolates to their species level based on biochemical and physiological tests only, these tests are still useful to know the diversity of particular genera in natural samples.

Of four active strains, only *Streptomyces* VAH3 showed broad spectrum activity against tested phytopathogenic and human pathogenic fungi during secondary screening. It showed more than 10 mm inhibition diameter against *F. oxysporum*, *F. moniliforme, F. eridiforme, Stemphylium botryosum, Apergillus* spp., and *Candida albicans* (human pathogenic yeast) and no inhibition against *Sclerotium rolfsii* (Table 3). These results showed that antibiotic production occurred better in solid agar surface culture than in shake flask culture without rigorous optimization. Earlier studies also showed that agar surface culture was favorable for screening antibiotic production because of gradual availability of a key nutrient at suboptimal level and slight change in pH (Martin and Aharonowitz, 1983; Nisbet, 1982).

Table 2. Morphological, biochemical and physiological characterization of potent actionmycetes

Characteristics	VAH1	VAH3	VAH8	VAS9
Aerial mycelium color	CW	CW	G	CW
Substrate mycelium color	W	B	W	W
Spore chain	RF	RF	RF	RF
Soluble pigment	+	+	-	-
Catalase	+	+	+	+
Oxidase	-	-	-	-
Nitrate reduction	-	+	+	+
Sulphur	-	+	-	-
Urea degradation	-	+	-	+
D-Gulcose	+	+	+	+
D-Fructose	+	+	-	+
L-arabinose	-	+	+	+
Glycogen	-	-	-	-
D-xylose	-	+	+	+
Raffinose	-	+	+	-
Sucrose	-	+	-	+
Galactose	-	+	+	-
Lactose	-	+	+	-
D-Mannitol	-	+	-	-
D-Mannose	-	+	-	-
Esculin	+	+	+	+
Salicin	-	-	-	-
Starch hydrolysis	+	+	+	+
Gelatin hydrolysis	+	+	+	+
Casein hydrolysis	+	+	+	+
Tween 20 hydrolysis	+	+	+	+
Chitin hydrolysis	-	+	-	+
5% NaCl	+	+	-	-
7% NaCl	+	+	-	-
10% NaCl	-	-	-	-
4°C	-	-	-	-
28°C	+	+	+	+
37°C	+	+	-	-
45°C	-	-	-	-

CW, Creamy white; W, White; RF, Rectus-flexibilis, ;B, Brown; G, Grey

Morphological, biochemical and physiological tests of potent strains confirmed that all four isolates were from *Streptomyces* genus (Figure 1 & Table 2). All four isolates were found to possess gram positive recti-flexibilis type of sporophores originating from branching thin mycelium during microscopy. Biochemical tests like sugar utilization tests, Nitrate reduction and polysaccharide hydrolysis tests and physiological tests such as temperature and Sodium chloride tolerance tests showed high variability among these four isolates as well as those of reported species in Bergey's Manual of Systemic Bacteriology Vol. IV. *Streptomyces* VAH3 is more than 70% similar to *Streptomyces noursei*. Although it is difficult to ascertain unknown isolates to their species level based on biochemical and physiological tests only, these tests are still useful to know the diversity of particular genera in natural samples.

Of four active strains, only *Streptomyces* VAH3 showed broad spectrum activity against tested phytopathogenic and human pathogenic fungi during secondary screening. It showed more than 10 mm inhibition diameter against *F. oxysporum*, *F. moniliforme*, *F. eridiforme*, *Stemphylium botryosum*, *Apergillus* spp., and *Candida albicans* (human pathogenic yeast) and no inhibition against *Sclerotium rolfsii* (Table 3). These results showed that antibiotic production occurred better in solid agar surface culture than in shake flask culture without rigorous optimization. Earlier studies also showed that agar surface culture was favorable for screening antibiotic production because of gradual availability of a key nutrient at suboptimal level and slight change in pH (Martin and Aharonowitz, 1983; Nisbet, 1982).

Table 3. Antifungal activity of *Streptomyces* VAH3 against phytopathogenic fungi

Isolate	Phytopathogenic fungi	Zone of inhibition diameter (mm)
Streptomyces VAH3	*F. oxysporum*	11(5)
	F. moniliforme	11 (5)
	F. proliferatum	14 (5)
	F. eridiforme	10 (5)
	Sclerotium rolfsii	6 (5)
	Stemphylium botryosum	10 (5)
	Candida albicans	10 (5)
	Aspergillus spp.	12 (5)
	Exserohilum turticum	10 (5)

( ), negative control

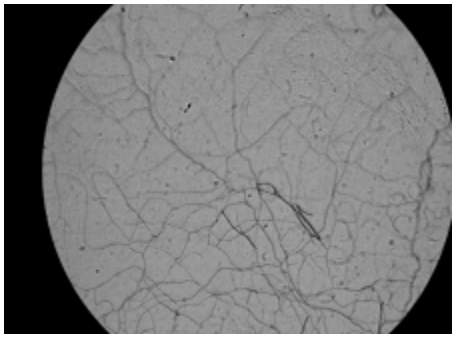

Fig. 1. Recti- flexibilis sporophore of *Streptomyces* VAH3 originating from thin mycelium under 100X oil immersion

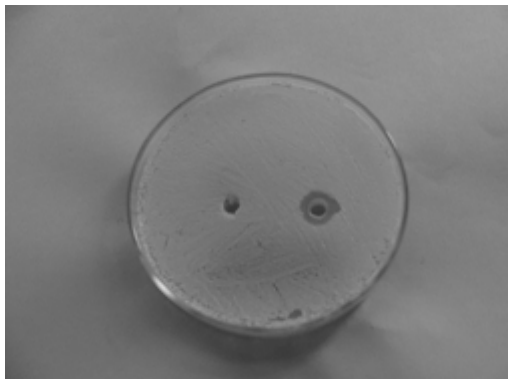

Fig. 2. Antifungal activity of *Streptomyces* VAH3 broth (162 µg /100µl) against *Candida albicans*

Fig. 3. Antifungal activity of *Streptomyces* VAH3 broth (162 µg/100µl) against *Fusarium oxysporum*

Among four solvents; benzene, chloroform, ethyl acetate and n-butanol used for the extraction of antifungal agent, only ethyl acetate extract showed activity against both phytopathogenic and human pathogenic fungi. The ethyl acetate extract yield was found to be 162 mg per 100ml of fermented broth. The minimum inhibitory concentrations of crude antifungal agents against *F. oxysporum*, *E. turcicum*, *S. botryosum Candida albicans*, *Aspergillus* spp. were found to be 560 µg /ml, 112 µg /ml, 112 µg /ml, 560 µg / ml and 112 µg /ml respectively and that of Nystatin (positive control) was found to be 64 µg /ml for each of tested pathogenic fungi (Table 4).

Table 4. Minimum inhibitory concentrations of ethyl acetate extract of *Streptomyces* VAH3 against pathogenic fungi

Antifungal agents	**Minimum inhibitory concentrations (µg/ml)** *F. oxysporum* *E. turcicum* *S. botryosum* *C. albicans* *Aspergillus* **spp**				
Ethyl acetate extract	560	1120	1120	560	1120
Nystatin	64	128	128	64	128

Acknowledgements

We thank Nepal Academy of Science and Technology (NAST) for providing space and resources to conduct this research in Biotechnology Laboratory. We also thank Plant Pathology Division of Nepal Agricultural Research Council for providing phytopathogenic fungi and Microbiology Department, Institute of Medicine, Maharajgunj for providing human pathogenic fungi.

References

Augustine, S. K., S. P. Bhavsar, M. Baserisalehi and B. P. Kapandnis. 2004. Isolation, characterization and optimization of antifungal activity of an actinomycete of soil origin. *Indian Journal of Experimental Biology* **42**: 928-932.

Barry, A. L. and C. Thornsberry. 1985. Susceptibility tests: Diffusion test procedure. In: *Manual of Clinical Microbiology* (Ed. E. H. Lennette). 4th ed. American Society of Microbiology, Pp. 978-987.

Collins, C. H., P. M. Lyne and J. M. Grange. 1989. *Microbiological methods.* 6th ed. Butterworth-Heinemann Ltd., Pp. 495-410.

Crawford, D. L., J. M. Lynch, J. M. Whipps and M. A. Ousley. 1993. Isolation and characterization of actinomycetes antagonsists of a fungal root pathogen. *Applied and Environmental Microbiology* **59** (11): 3899-3905.

Haware, M. P. and Y. L. Nene. 1982. Races of *Fusarium oxysporum* f. sp. *ciceri*. *Plant Diseases* **66**: 809-810.

Jalali, B. L. and H. Chand. 1992. Chickpea wilt. In: *Plant diseases of international importance. Diseases of Cereals and Pulses* (Eds. U. S. Singh & K. A. Mukhopadhaya). Prentice Hall, Eaglewood cliff, Pp. 429-449.

Jimenez-Gasco, M. M. and R. M. Jimenez-Diaz. 2003. Development of a specific polymerase chain reaction-based assay for the identification of *Fusarium oxysporum* f. sp.*cicer* and its pathogenic races 0,1A, 5 and 6. *Phytopathology* **93**: 200-209.

Joshi, S. 2010. *Lentil*. Annual Report. Plant Pathology Division, National Agricultural Research Institute, Kumaltar, Lalitpur, Nepal, Pp. 45-49.

Martin, J. F. and Y. Aharonowitz. 1983. Regulation of biosynthesis of b-lactam antibiotics. In: *Antibiotics containing the beta lactam structure* (Eds. A. L. Demain & N. A. Solomon). Springer-Verlag, Berlin, Pp. 229-254.

NCCLS, 2002. *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi*. Approved standard. NCCLS document M38-A. Clinical and Laboratory Standards Institute Villanova, Pa.

Nisbet, L. J. 1982. Current strategies in the search for bioactive microbial metabolites. *Journal of Chemical Technology and Biotechnology* **32**: 251 -270.

Williams, S. T., M. Goodfellow and G. Alderson. 1989. Genus *Streptomyces* Waksman and Henrici 1943, 339AL. In: *Bergey's Manual of Systematic Bacteriology* (Eds. S.T. Williams, M.E. Sharpe & J. G. Holt). Williams and Wilkins, Baltimore **4**: 2452-2492.