African Crop Science Conference Proceedings Vol. 8. pp. 2103-2107
Printed in El-Minia, Egypt
ISSN 1023-070X/2007$ 4.00

Characterization of antibiotic metabolites from actinomycete isolates

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Abstract: Metabolites from three actinomycetes isolates coded CS35, 28P and 14P antagonistic to *Pythium* spp were produced in shaken liquid media and centrifugation done to obtain cell free culture filtrates. The antibiotic metabolites from each of these isolates was characterized by performing chromatography & bioautography, partial purification, determination of minimum inhibitory concentration (MIC) and determining the effect of temperature, pH and shelve life on the stability of these metabolites. Rf values from the chromatography work and bioautography showed that, metabolites from CS35 and 14P had only one active compound whereas metabolite from isolate 28P had 2 active compounds. The antibiotic metabolites from isolate 28P and CS35 were stable up to a temperature of 70^0C but were sparingly stable at 80^0C. The stability of antibiotic metabolite from isolate 14P was drastically reduced from a temperature of 60^0C and above. High pH value (above 10) and low pH values (below 4.0) negatively affected the stability of the metabolites. Metabolites from isolate 14P lost stability with time whereas metabolites from isolates CS35 and 28P maintained their activity for the entire duration (5 months) tested. These metabolites were found to be sufficiently stable under different conditions and they possess a high potential to control the disease problems under field conditions.
Key words: Antagonistic, culture filtrates, growth rates, inhibition zones, stability

Introduction

Resistance development among the plant pathogens due to usage of chemical pesticides coupled with the environmental pollution and pesticide residues in the treated crops has been an issue of great concern necessitating the need to investigate other strategies such as use of antimicrobial metabolites. Metabolites from antagonistic microorganisms have shown promising results in the management of plant diseases and their rapid breakdown renders them environmentally friendly and safe to use. To fully utilize these metabolites, there is a need to characterize them and determine their stability which is an important factor in their formulation and application. Actinomycetes are a group of organisms that morphologically resemble fungi and physiologically resemble bacteria (Goodfellow *et al.,* 1984) and production of antibiotics is their most important contribution to mankind with the group producing half of all the known antibiotics (Misato and Yamuguchi 1977; Hongjuan *et al*., 2006). Application of antibiotics in plant disease control has been expanding rapidly and hundreds of antibiotics are already commercially available for plant disease control (Misato and Yamuguchi, 1977; Mustafa *et al*., 2004).

Antagonistic metabolites from the shaken liquid cultures are normally crude and dissimilar components (Porter, 1971). The mixture contains antibiotics and other secondary metabolites and the antibiotics are a very heterogeneous group of biologically active compounds (Betina, 1964). A simple and rapid method of separation and identification of the active metabolites is needed (Aszalos & Frost, 1968; Porter, 1971). The initial step involves separation in specific solvent systems followed by physical and chemical tests such as stability at different temperatures, pH ranges, storage duration, colour reactions and fluorescence, light absorption, paper chromatography of the whole antibiotic and of decomposition products, electrophoresis, counter current distribution, elementary analysis of physical constant and mass spectrometry (Porter, 1971; Augustine *et al*., 2005). Characterization of antibiotics is important since the ultimate usefulness of the antibiotic in plant disease control and the methods of purification are determined by the stability of the antibiotic (Porter, 1971; Mustafa *et al*., 2004).

Although the *in vitro* activity of antibiotics is not perfectly reflected in the *in vivo* conditions, factors such as stability of antibiotics to different pH ranges, temperature and different storage durations are good indicators of stability of the antibiotic in the environment. It is therefore, important to obtain a full characterization of an antibiotic metabolite before it can be put into use since its behavior under these different conditions will determine its application and ultimate usefulness (Betina, 1964; Porter, 1971; Simone *et al*., 1998). The objective of this study was to characterize the antibiotic metabolites from actinomycete isolates and to determine their composition and stability under different conditions.

Materials and Methods

Soils were collected from University of Nairobi farm and isolation of actinomycetes carried out using the dilution plate method as described by Herr (1959). The actinomycete isolates were screened for the production of antagonistic metabolites using *Pythium* spp as the test pathogen following the method of Henis & Baker (1979).

Testing for the production of antagonistic metabolites was done empirically using a range of modified media and incubation conditions as outlined by Pridham *et al*., (1956). The paper disc method as desribed by Loo *et al*., (1945) was used to test for the presence of antagonistic metabolites from the shaken liquid media where the actinomycetes had been cultured for defined durations of time.

The culture filtrates from the three actinomycetes coded 14P, 28P and CS35 were then subjected to a series of procedures to characterize them. These procedures were separation of the different fractions using paper chromatography, bioautography to determine the active fractions, testing the effect of different temperature levels, pH ranges on the stability of these metabolites and partial purification. Shelve life of the metabolites at different temperatures, storage durations and the minimum inhibitory concentrations was also evaluated.

Chromatography and bioautography.

Using a micropipette, 5 µl of each culture filtrate was applied 3 cm from the lower edge of (1x 5mm) sterile chromatography paper strips (Whatman No. 1) and dried. These paper strips were immersed to a depth of 1 cm in 50 ml solvents put in chromatography tanks and attached to the inner surface of the lids using adhesive tapes. The different solvents tested for elution were methanol, butanol, water, acetic acid and n-hexane. The ascending development of chromatograms was stopped when the solvent fronts reached a distance of 15 cm from the origin. The solvents which moved and separated the antibiotics were used to make solvent systems. The solvent systems used were hexane:methanol:water [40:30:30 (v/v)], hexane:butanol:water [65:25:4 (v/v)], methanol:n-hexane [60:40 (v/v)] and butanol:acetic acid:water [50:25:25 (v/v)]. These solvent systems were used to make ascending developments of chromatograms as explained above. The solvent fronts were marked, measured and the paper strips used in bioautography.

Effect of temperature on the activity of the antibiotic metabolites

The culture filtrates were adjusted to pH 6.0 using 0.1 N HCl or 0.1 N Na0H and then 5ml of each culture filtrate put in sterile universal bottles and then subjected to different temperatures in a water bath for 10 minutes. The temperatures employed in these studies were 40^{o}C, 50^{o}C, 60^{o}C, 70^{o}C, 80^{o}C, 90^{o}C and 121^{o}C. For 121^{o}C, autoclaving was done at a temperature of 121^{o}C at one bar pressure for 15 minutes. The stability of the various culture filtrates to these temperatures was determined by measuring the size of the inhibition zones using the paper disc method described earlier. For each temperature, three plates each containing 3 paper discs were used and the plates were incubated at 25^{o}C in a completely randomized design.

Effect of different pH levels on the activity of the antibiotic metabolites

Five milliliters of culture filtrates were put in vials and using 0.1 N HCl or 0.1 N NaOH adjusted to the pH levels 2, 4, 6, 8, 10, and 12. These antibiotics were held for 3-4 hours at room temperature (20-24^{o}C) and then readjusted to pH 6.0. The experiment was replicated three times and arranged in a completely randomized design as outlined above. The activity of these treated antibiotic culture filtrates was determined by the sizes of the inhibition zones using the paper disc method described earlier.

Determination of the shelve life of the metabolites at different storage temperatures

The culture filtrates were adjusted to pH 6.0 using 0.1 N HCl or 0.1 N NaOH and 5ml of each filtrate from each isolate put in universal bottles and stored at the following temperatures; 4^{o}C, room temperature (22 $\pm$ 2^{0}C), and 40^{o}C for a period of 5 months. The treatments were replicated three times and arranged in a completely randomized design. The activity of the antibiotics in the culture filtrates was determined after every ten days. The paper disc method described earlier was used and the presence of inhibition zones around the paper discs that had been soaked in the culture filtrates was measured and used as a measure of antibiotic activity.

Partial purification of the antibiotic metabolites in culture filtrates

For partial purification of antibiotics, a modification of the method by Vanittankonn *et al*., (1986) was adopted. Charcoal (500gm) was dried and ground coarsely and put in a canister and activated by heating at 200^{o}C for 1 hour in the oven, and then cooled to room temperature 22 $\pm$2 ^{O}C. The whole fermentation broths prepared as explained above were mixed with 10% of the powdered charcoal (w/v) and stirred for 30 minutes to allow adsorption of the antibiotics onto the charcoal particles. Whatman No. 1 filter paper was used to filter the mixture and the antibiotic containing charcoal left after filtration was eluted with 60ml absolute methanol. This eluate which contained antibiotics dissolved in methanol was then concentrated *in vacuo* by the use of a rotary vacuum evaporator at 70^{o}C to about 2mls. This partially purified antibiotic was then used to determine the minimum inhibitory concentration.

Determination of the minimum inhibitory concentration (MIC)

Serial dilutions were made by dispensing 2mls of sterile distilled water in a series of vials. One ml of the culture filtrate was pipetted into the 2ml water blank and shaken well and successive serial dilutions were done up to the 10th dilution. Using sterile pipette and bent glass rods, 0.5ml of each of these dilutions was evenly spread on the surface of *Pythium* seeded Czapeks Dox Agar medium and incubation done at room temperature for 5-7 days and growth of *Pythium* monitored. The experiment was replicated three times and the treatments arranged in a completely randomized design.

Results

Characterization of antibiotic metabolites in the culture filtrates

The solvents tested namely methanol, butanol, water, n-hexane and acetic acid eluted all the antibiotics in culture filtrates of isolates 28P and CS35 but n-hexane did not elute the antibiotics in the culture filtrate of actinomycete isolate 14P. The results of the bioautography showed that, Rf values were different for each of the metabolites except in cases where the clear zones were not observed. Two clear zones were observed in metabolites from isolate 28P indicating that this culture filtrate had two compounds which were active against *Pythium* spps. Only one clear zone was observed from culture filtrates from isolate 14P and CS35 indicating that these metabolites had one compound active against *Pythium* spps (Table 1).

Table 1: Rf. value of antibiotic metabolites from the various actinomycete isolates in the different solvent systems

		x	y	z	S. F.	Rf. Value
Hexane:butanol: water	14P	5	5.5	6	6	0.92
Hexane:butanol :water	28P	0.7	2.1	3.5	6.5	0.32
Butanol:acetic –acid:water	14P	3.25	3.5	3.7	6.1	0.57
Butanol:acetic –acid:water	28P	12.6	13.3	14	15	0.89
Butanol:acetic –acid:water	CS35	4.1	4.275	4.45	6.8	0.63
Methanol:hexane	28P	1	2	3	8.75	0.23
Methanol:hexane	CS35	3	3.675	4.35	6.1	0.6
Hexane:methanol:water	14P	0	0	0	4.3	0
Hexane:methanol:water	28Pzone 1	1.25	0	1.25	5.4	0
Hexane:methanol:water	28Pzone 2	2.4	3.2	4	5.4	0
Hexane:methanol:water	CS35	0	0	0	6.25	0

Key:
X: is the distance from the origin to the lower end of the clear zone.
Y: is the distance from the origin to the geometrical center of the clear zone.
Z : is the distance from the origin to the upper end of the clear zone.
S.F. is the distance from the origin to the solvent front
Rf.Value=Y/S.F

The antibiotic in the 4 metabolites responded differently when subjected to different temperatures. None of the antibiotic was stable at 90^{o}C and above. Antibiotics from culture filtrates 28P and CS35 were sparingly stable at 80^{o}C. Antibiotics from culture filtrates 14P were unstable from temperatures 40^{o}C and above. Temperature had a significant ($P \leq 0.05$) effect on the activity of the metabolites and raising the temperature decreased the inhibitory activity (Table 2).

Table 2: Mean inhibitory diameters (cm) produced by culture filtrates from the three actinomycete isolates subjected to different temperatures levels

Actinomycete isolates	40^{0}C	50^{0}C	60^{0}C	70^{0}C	80^{0}C
14P	1.81 bc	1.5 b	1.61 ab	1.41 a	0 b
28P	2.05 b	1.74 a	1.48 ab	1.46 a	0.14 b
CS35	2.53 a	1.38 b	1.89 a	1.41 a	0.85 a
L.S.D. (0.05)	0.3	0.15	0.41	0.32	0.32

Each value represents a mean of three replicates.
Values followed by the same letters are not significantly different.

The stability of antibiotic metabolites from the four actinomycetes isolates tested were affected by both very high and low pH. Low pH levels had a more detrimental effect on the stability of antibiotics than the high pH levels. The stability of antibiotics was best at pH 6.0 and 8.0 in all the culture filtrates (Table 3).

Table 3: Mean inhibitory diameters (cm) produced by culture filtrates from the three actinomycete isolates subjected to different pH levels.

Actinomycetes	pH 2	pH 4	pH 6	pH 8	pH10	pH12	pH14
14 P	0.83 a	1.42 a	2.02 a	1.85 a	1.77 a	1.58 a	1.5 a
28 P	0.8 a	1.55 a	1.8 b	1.75 a	1.75 a	1.52 ab	1.52 a
CS35	0.4 a	1.5 a	1.83 ab	1.72 a	1.57 a	1.38 b	1.47 a
L.S.D.(0.05)	1.15	0.18	0.19	0.17	0.22	0.16	0.19

Stability of antibiotics was significantly affected by the different pH levels at P≤0.05. Metabolites from isolates CS35 and 28P stored at fridge, room temperature and at 40^0C retained their activity for the entire duration (5 months) of testing but reduction in activity with time was observed. Metabolite from isolate 14P stored at room and at 4^0C retained the activity for the entire testing period but it lost activity after 130 days of storage at 40^0C (Table 4).

Table 4: Mean inhibitory diameters (cm) produced by culture filtrates stored at different temperatures levels

Actinomycete isolate	4^0C	Room temperature (22 ± 2^0C)	40^0C
CS35	1.71 a	1.72 a	1.39 ab
28P	1.83 a	1.74 a	1.76 a
14P	1.64a	1.55 a	1.23 b
L.S.D. (0.0 5)	0.29	0.27	0.41

Means followed by the same letters are not significantly different.

The stability of the antibiotic metabolites was significantly (P≤0.05) different for the 3 temperature levels tested. There was an increase in activity as evidenced by the increase in size of the colony diameters when partial purification of the metabolites was done. The activity of the isolate 14P had the highest increase in activity followed by isolate CS35 and 28P in that order. Partial purification had a significant (P≤0.05) effect on the enhancement of activity of the antibiotic metabolites. The dilution levels of the metabolites were shown to vary in their ability to inhibit the growth of the test organism. Actinomycete isolate coded 14P, had the highest dilution (3^{-5}) inhibiting the fungal growth followed by isolate CS35 at dilution 3^{-3}. Actinomycete isolate coded 28P, had the lowest dilution (3^{-2}) inhibiting the growth of the test organism (Table 5). Diluting the antibiotic metabolites significantly (P≤0.05) reduced their activity, but metabolites from some isolates were more affected than others.

Table 5: Mean inhibitory diameters (cm) produced by various dilutions of culture filtrates

D	14P	14P	14P	av	28P	28P	28P	av	CS35	CS35	CS35	av	C	C	C	av
	R_1	R_2	R_3		R_1	R_2	R_3		R_1	R_2	R_3		R_1	R_2	R_3	
3^{-1}	1.53	1.6	1.55	1.56	1.68	1.67	1.63	1.66	1.62	1.63	1.58	1.61	0	0	0	0
3^{-2}	1.6	1.5	1.43	1.51	1.3	1.37	1.28	1.32	1.33	1.33	1.41	1.36	0	0	0	0
3^{-3}	1.43	1.5	1.41	1.45	0	0	0	0	0.83	0.8	0	0.54	0	0	0	0
3^{-4}	1.33	1.33	1.27	1.31	0	0	0	0	0	0	0	0	0	0	0	0
3^{-5}	1.23	1.3	1.31	1.28	0	0	0	0	0	0	0	0	0	0	0	0
3^{-6}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Key:
R= replications, C= control, Av= average, D =Dilutions

Discussion

The antibiotic metabolite from isolates CS35 and 28P were eluted by all the solvents tested namely, n-hexane, methanol, water, butanol and acetic acid. This is an indication that, the antibiotics in these culture filtrates were soluble in these solvents. All these solvents, except the n-hexane eluted the antibiotic from isolate 14P so this antibiotic was soluble in all these solvents apart from the n-hexane. The highest solvent fronts were observed in water and this was expected since the antibiotics in all these culture filtrates were dissolved in water. Different Rf values were recorded for all the culture filtrates in the different solvent systems indicating that these antibiotics could all be different. Although other factors like purity of the metabolite could affect the Rf values, antibiotics with similar Rf values are suggested to be closely related (Mustafa *et al*., 2004). The different values are also indicative of the movement of antibiotics in the different solvent systems as also reported by Aszalos & Frost, (1968).

The different metabolites showed a varied response on subjection to different ranges of temperatures and all were thermolabile from a temperature of 90°C and above. The stability of antibiotics in all the isolates was highest in culture filtrates stored at 4°C followed by storage at room temperature and the least by storage at 40°C. High temperatures were found to destabilize the antibiotics and this has an important implication on the storage of the antibiotics from these culture filtrates. The duration of storage was found to affect the antibiotics and even where the activity of the antibiotics was recorded for the entire duration of testing, the activity reduced with time. Reduction of antimicrobial activity of antibiotic metabolites on subjection to different temperatures and pH levels has also been reported in other studies (Mustafa, *et al*., 2004; Augustine, *et al*., 2005).

Low and high pH levels were found to have a detrimental effect on stability of antibiotic metabolites and this agrees

with reports by Augustine *et al*., (2005). All the antibiotics in these culture filtrates could be partially purified by adsorption onto activated charcoal and then eluted from the charcoal by the use of solvents like methanol. This partial purification increased the activity of these metabolites. Partial purification of antibiotic metabolites using charcoal has also been reported by other researchers and other solvents such as n-butanol has been used to elute toximycin adsorbed on charcoal during partial purification (Mustafa *et al*., 2004). Minimum inhibitory concentration is important as it gives an indication as to what level of dilution can be achieved while still retaining the level of activity. The higher degree of activity from isolate 14P could be attributed to the presence of higher amounts and concentrations of antibiotics compared to metabolites from the other actinomycete isolates.

Acknowledgements

This work was funded by the University of Nairobi and the International Potato Centre (CIP).

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