IDENTIFICATION OF AN ANTAGONISTIC ACTINOMYCETE AND ITS USE IN THE BIOCONTROL OF POTATO SCAB

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Abstract

Eleven strains of Actinomycetes isolated from soils and potato periderm were screened for their antagonistic activities against the potato common scab pathogen, *Streptomyces scabies*. Strain 337 demonstrated the most significant activity in both the Cross-Streak and Cup Diffusion Agar tests. Based on the morphological and physiological characteristics, the strain was identified as *Streptomyces violaceusniger*. WB medium containing wheat bran 50 g, swine feces 10 g, $Ca(OH)_2$ 1 g, and moisture 50% was found to be a promising substrate for making a bioactinocide by principles of solid substrate fermentation. Pot bioassay showed that the ratio of scab incidence area was decreased to 6.7% using the bioactinocide, compared to 18.2% in control. The viable count of *S. scabies* in the bioactinocide pot decreased to $4.4 \times 10^4$from 1.6×10^6 CFU per gram soil by 12 weeks. There was also evidence of tuber yield increase to 12.4% in pots supplemented with the bioactinocide.

Intisari

Sebelas strain Aktinomisetes yang diisolasi dari tanah dan kulit kentang telah diuji daya antagonistiknya terhadap patogen penyakit kudis pada umbi kentang, *Streptomyces scabies*. Pada uji antagonisme dengan metode *Cross-Streak* dan *Cup Diffusion Agar*, strain 337 menunjukkan aktivitas yang paling tinggi dibandingkan strain-strain yang lain. Berdasarkan ciri morfologi dan sifat fisiologinya, strain 337 di identifikasi sebagai *Streptomyces violaceusniger*. Medium WB yang tersusun atas sekam gandum 50 g, kotoran babi 10 g, $Ca(OH)_2$ 1 g, dan kelembaban 50% terbukti sebagai medium yang baik untuk memproduksi bioaktinosida secara fermentasi substrat padat. Uji hayati pada pot menunjukkan bahwa tingkat serangan penyakit kudis pada kontrol dan perlakuan bioaktinosida berturut-turut 18,2% dan 6,7%. Bioaktinosida menekan populasi *S. scabies* dari $1,6 \times 10^6$ menjadi $4,4 \times 10^4$ *CFU/g* tanah selama inkubasi 12 minggu. Perlakuan dengan bioaktinosida juga meningkatkan hasil umbi kentang sebesar 12,4% dibandingkan dengan kontrol.

Introduction

Scab disease of potato (*Solanum tuberosum*) are incited by several *Streptomyces* spp. among which *Streptomyces scabies* predominates. Scab diseases are characterized by corky tuber lesions which may be superficial or pitted (Lambert and Loria, 1989). Some controlling strategies such as the maintenance of a soil pH below 5.2

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(Bishop *et al.*, 1954), green manuring (Hayashida *et al.*, 1989), irrigation during tuber formation (Wellings and Rosser, 1968), application of chemical compounds (Burrell, 1981) are generally employed to reduce the scab infection.

Nowadays, biological control of plant diseases is receiving much attention and is gaining popularity owing to the concern of overuse of pesticides and its potential contribution in the development of sustainable agriculture.

Nevertheless little is known as to the effective biocontrol of soilborne pathogen causing potato common scab. The purposes of this study were to investigate and identify a newly isolated strain of antagonistic actinomycete, to produce a potato scab-antagonistic bioactinocide, and to assess the efficacy of bioactinocide in controlling the potato scab disease.

Materials and Methods

Isolation and Screening of Potato Scab-Antagonistic Actinomycete

For the isolation of the antagonistic strains, suspension of various samples was inoculated onto a plate containing YMA medium (Nanri *et al.*, 1992) that was previously poured with *S. scabies*. Separated colonies showed inhibition zones were transferred onto agar slopes. Following isolation, eleven strains of scab-antagonistic actinomycetes which obviously differed from one another in appearance, were selected. The selected strains were individually examined their antagonistic activity against *S. scabies* using Cross-Streak and Cup Diffusion Agar methods (Hayashida *et al.*, 1988 and Nanri *et al.*, 1992). The strongest inhibiting strain was chosen for further study.

Production and Preparation of Bioactinocide

A loopful of spores scrapped from slant culture was inoculated into 5 gram of solid medium in a 100 ml erlenmeyer, and then incubated at 30°C for 7 days. Eight solid media which have different composition are described as notes under the table 2. A culture which demonstrated abundant growth of a strain was added with 30 ml of distillated water, kept at 4°C overnight, and then filtered through the gauge. After centrifugation (12.000 rpm, 15 min), 0.325 ml of supernatant was tested against *S. scabies* on YMA medium by Cup Diffusion method. As the strong antagonistic activity was still demonstrated, it was used as the seed culture. The seed culture was inoculated at 10% (w/w) into the same medium for producing bioactinocide. The

bioactinocide was then used in pot test after being air-dried. The composition of the bioactinocide was determined to be as follows : total-N 1.72%, total organic-C 46.21%, C/N ratio 26.7, viable count of actinomycete 1.02×10^{10} CFU/g, pH (H_2O) 7.25, and moisture 12.5%.

Characterization and Identification of the Selected Strain

Cultural characteristics and physiological properties of selected strain were further studied using media as described by Shirling and Gottlieb (1981). The cultures were examined after incubation at 30°C for 7 days. Utilization of carbon sources was tested in PG medium (Pridham and Gottlieb, 1948) containing 1% of each carbon source. The morphology of the strain was examined under the light microscope (model Nikon Diaphot-TMD) as well as under the electron microscope (model JEM 100B, JEOL Co. Ltd.). Identification was confirmed according to Bergey's Manual of Determinative Bacteriology (Pridham and Tresner, 1974; William *et al.*, 1989).

Pot Test for Potato Scab Disease

To assess the effectiveness of the bioactinocide in controlling potato scab pathogen, pot experiment was carried out in triplicate. Humic volcanic ash soil that has been used successively for potato production was collected and passed through a 2 mm sieve, and 2250 g of soil sieved was weighed into a 4 l plastic pot. The properties of soil were pH(H_2O) 6.4, total-N 0.29%, total-C 4.24%, C/N ratio 14.62, moisture 31.3% and *S. scabies* population 2.39×10^6 CFU/g. Such population of *S. scabies* is enough to cause the scab disease on potato tuber (Hayashida *et al.*, 1988). The following trial protocol was adopted : (1) control pots were supplemented with only chemical fertilizer ($(NH_4)_2 SO_4$ 1.08 g, K_2SO_4 0.27 g, $Ca_3(PO_4)_2$ 0.9 g); (2) pots were supplemented with the same dosage of those chemical fertilizers and 0.825 g bioactinocide in order to bring 10^6 antagonist cells per gram soil. A sprouted tuber seedling of potato was planted in every pot. All pots were incubated at 25°C and humidity 75% in a thermohumid-stated glasshouse at Biotron Institute of Kyushu University, Japan. Water was daily added into the pot in order to maintain 60% WHC. Soil samples were monthly taken from each pot and were analyzed in the laboratory for moisture and *S. scabies* population. Viable count of *S. scabies* was performed by the dilution plate method on Tyrosine Casein Nitrate medium (Menzies and Dade, 1959). After 3 months cultivation, potato was harvested and severity of scab incidence on potato periderm was evaluated according to the formula as described by Nanri *et al.*, (1992).

Results and Discussion

Screening of Scab-Antagonistic Actinomycetes

As summarized in Table 1, among eleven strains screened, strain 337 showed the most significant antagonistic activity against *S. scabies* in either the Cross-Streak and Cup Diffusion Agar tests. The data also reveal that one strain indicated large inhibiton zone in the Cross-Streak test but lower, even zero, activity in the Cup Diffusion Agar, while another strain exhibited the opposite. It is apparent that the strain had different mode of antagonism, i.e.: antibiosis and/or nutrient competition. Evidence for the antibiosis was established in Cup Diffusion test, whereas Cross-Streak revealed those two antagonism modes.

Table 1. **Antagonistic Activity of Isolated Strains against *S. scabies*.**

Strain	Inhibition zone (mm)	
	Cross-Streak[a)]	Cup Diffusion[b)]
161	4	15
210	8.5	19
215	7	13
223	3	13.5
236	2	27.5
256	6.5	0
331	7	21
337	9.5	31
348	3.5	0
802	4.5	18
901	2.5	15

a) The zone was taken as the clear area from growth of antagonist periphery to the beginning of growth of test organism (*S. scabies*).
b) Inhibition zone denotes the diameter of clear area where no growth of *S. scabies* occuped.

Production of Bioactinocide

For making bioactinocide, several media consisted of different materials of agricultural waste were used. As summarized in table 2, strain 337 showed a vigorous growth in CC-I, WB-I, WB-II, and WB-III media; but did not grow well in other media. Based upon the antagonistic activity of the extracted substances, medium WB-II gaved the strongest inhibition. It, therefore, was chosen in the production of larger amount of bioactinocide and tested in pot experiment. In addition to wheat bran as the main component, WB-II also contains a minor portion of $Ca(OH)_2$ and swine feces

Table 2. **Growth Response of Strain 337 in Various Solid Media and Their Antagonistic Activities against *S. scabies***

Medium	Growth Response	Inhibition Zone (mm)
CC	+ −	nd
CC-I	+ +	18
SF	+ −	nd
WB	+	nd
WB-I	+ +	25
WB-II	+ +	29
WB-III	+ +	22.5
WB-IV	+ −	nd

+ + : abundant + : fair + − : poor nd : not determined

CC : Cotton cake 50 g, Tap water 50 ml (pH: 6.1)
CC-I : Cotton cake 50 g, Ca(OH)2 1 g, Tap water 50 ml (pH: 7.2)
SF : Swine feces 50 g, Tap water 50 ml (pH: 6.1)
WB : Wheat bran 50 g, Tap water 50 ml (pH: 5.7)
WB-I : Wheat bran 50 g,Ca(OH)2 1 g, Tap water 50 ml (pH: 8.1)
WB-II : Wheat bran 50 g, Swine feces 10 g, Ca(OH)2 1 g. Tap water 50 ml (pH: 7.6)
WB-III : Wheat bran 50 g, Swine feces biof. 10 g, Ca(OH)2 1 g, Tap water 50 ml (pH: 7.6).
WB-IV : Wheat bran 50 g, Swine feces 10 g, Tap water 50 ml (pH: 5.7)

which seemed to play an important role in growth stimulation of strain 337. In Addition to favouring the pH, calcium ion is an essential element. The ion must be present during the entire initiation of germination period of spores (Lloyd, 1969), while the swine feces is likely to supply the offensive volatile fatty acids as carbon sources in the medium (Hayashida *et al.*, 1988). Assimilation of the volatile fatty acids was demonstrated by the growth of the strain on swine feces agar (Table 3).

Table 3. **Cultural Characteristics of Strain 337**

Agar Medium	Growth Response	Colony Color	Reverse Color	Soluble Pigment
Yeast Maltose	Abundant	Brown-gray	Brown	Positive
Gluc. Yeast-Ext Malt-Ext.	Abundant	Brown-gray	Brown	Positive
Glycerol Asparagine	Poor	White	Colorless	None
Inorg. Salt-Starch	Fair	Brown-gray	Light-brown	None
Nutrient	Poor	Brown-gray	Light-brown	None
Waksman	Fair	Brown-gray	Brown	Positive
Czapek's	Abundant	Brown-gray	Light-brown	None
Swine Feces Extract	Abundant	White	Brown	None
Tyrosine Casein Nitrate	Poor	Colorless	Colorless	None
Melanin Test (ISP)	Abundant	Brown-Gray	Brown	None

Determination and Identification of Strain 337

Strain 337 produced a well-developed branching of vegetative mycelium, but less heavy branching of aerial mycelium on agar medium. Mature spore chains produced a short spiral with up to 3 turns. Sclerotic granules, synnemata, and sporangia were not observed. The spore surface was smooth and had ellipsoidal in shape (Fig. 1).

Figure 1. **Smooth Spores of Strain 337; Transmission Electron Micrograph (× 21.000), Bar : 1 um.**

On series of recommended media, mostly aerial mass color is brownish gray and reverse side of colony is colorless to light brown (Table 3). The physiological properties are listed in Table 4. Optimal temperature for growth was 30°C and tolerance of pH medium was in the range of 4-9. The strain was obligately aerobic since vigorous growth was observed under aerobic condition but no growth under anaerobic conditions (BBL Gas-Pak Anaerobic Systems). No melanoid pigment was found in tyrosine agar medium. L-Arabinose, D-Fructose, D-Galactose, D-Glucose, Lactose, Maltose, D-Mannitol, Rhamnose, D-Xylose were utilized for growth. Trace of growth occured on Inositol, Na-Acetate, Na-Citrate, Na-Succinate, Raffinose, Sucrose; and no growth was found on Dulcitol and Sorbitol. NaCl tolerance was in the range of 0 - 7%. On the basis of morphological and physiological characteristics, strain 337 was identified to belong to major cluster of *Streptomyces violaceusniger* (Pridham and Tresner, 1974; William *et al.*, 1989).

Table 4. **Physiological Properties of Strain 337**

Property	Result
Temperature range for growth (°C)	20 – 40
Optimal temperature (°C)	30
pH range for growth	4 – 9
Optimal pH	7
Formation of melanin pigment	Negative
Growth under Anaerobic	Negative
NaCl tolerance (%)	0 – 7

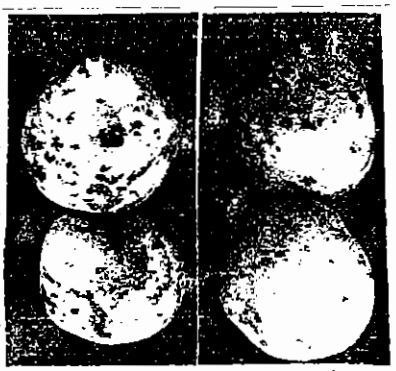
Test for Controlling the Scab Disease

Changes in *S. scabies* population in the pot soil is shown in Table 5. In control pots, the population was relatively unchanged throughout the period of pot test. Unlike the control pots, application of antagonistic bioactinocide caused a decline in the population of the pathogen to 4.4×10^4 CFU/g from 1.6×10^6 CFU/g.

Table 5. **Changes in *S. scabies* Population in the Pot Soil with Potato Cultivation**

Supplement	Population (CFU/g)			
	Days after Planting			
	1	30	60	85
Control	2.85×10^6	3.82×10^6	3.60×10^6	3.19×10^6
Bioactinocide	1.57×10^6	4.24×10^5	8.38×10^4	4.36×10^4

Observations regarding to the occurence of scab incidence and the yield of harvested potato are presented in Fig. 2 and Table 6. The effect of amendment of bioactinocides provided a suppression to potato scab disease. The antagonistic bioactinocide had repression index of 63.1% since the ratio of incidence area was only 6.7%, compared to 18.2% in control. The less suppression of the bioactinocide on potato scab might be due to the low amount of bioactinocide applied. It is also possible that the significant antagonistic activity observed in culture media did not reflect the effectiveness of disease suppresion by the strain 337 in the rhizosphere since many components may be limiting the efficacy of inhibitory substances. Rothrock and Gottlieb (1984) reported that the decrease of activity of inhibitory substances produced by soil microorganisms is due to the degradation in the soil. In addition, some microorganisms other than *S. scabies*, which also cause scab disease, might be more resistant to the antagonist action.



control supplemented with the bioactinocide.

Figure 2. **Comparison of scab lesions on the harvested potato**

Table 6. **Effect of Bioactinocide on the Scab Incidence and Yield of Harvested Potato**

Supplement	Incidence area[a] %					Ratio of[b] incidence area (%)	Ratio of Reppression (%)	Yield (g/pot)	Yield Increment (%)
	0–1 (A)	1–10 (B)	10–25 (C)	25–50 (D)	>50 (E)				
Control	0	3	1	2	0	18.2	–	61.7	–
Bioactinocide	1	3	1	0	0	6.7	63.1	69.3	12.4

a) Measured as percentage of tuber with scab coverage

b) Scab severity in term of percent coverage:

$$\frac{0.5(A) + 5(B) + 18(C) + 38(D) + 75(E)}{\text{Total number of tubers}}$$

A – E, the number of tuber with corresponding percent coverage.

Based upon the pot test results in Figure 2 and Table 6, we could propose the following mechanisms of scab control. In the control pots, the growth of *S. scabies* encountered less antagonism from soil microflora than what occured in the bioactinocides pots. The antagonistic bioactinocide which could counteract directly on the outbreak of the pathogen was due to the inhibitory substances produced by strain 337.

There was also evidence of the increase of potato yield to 12.4% in the pots supplemented with antagonistic bioactinocides. It seemed to supply some additional nutrients in the soil which then became

available for potato crop after being mineralized within the pot cultivation.

However, further investigations are necessary to find out the optimal amount of the bioactinocide application and to manipulate the soil environment to create conditions conducive to successful bio-control without any harmful effect in the soil ecosystem.

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