Isolation of Actinomycetes from Pine Litter Layers

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Methods for the selective isolation of actinomycetes from pine litter layers were evaluated. Pretreatment of source material with a 0.05% of sodium dodecyl sulfate solution and the addition of 20 µg/ml of nalidixic acid to the agar media were effective in reducing bacterial growth. The addition of 200 µg/ml of benlate to the agar medium was effective in reducing fungal growth.

By removing actinomycetes from the surface of pine needles with the surface sterilization method by 70% of ethanol and sodium hypochlorite solution containing 1% of chloride, we were able to isolate those actinomycetes which reside under the waxy layer of needles.

Comparative studies of isolates obtained from pine litter layers showed both a qualitative and quantitative difference by layers.

INTRODUCTION

New actinomycete genera such as *Actinosynnema*¹⁾, *Herbidospira*²⁾, *Planopolyspora*³⁾ have been isolated from plant material as well as from soil, both places being the usual habitat for actinomycetes.

The selective isolation of actinomycetes from plants is important for obtaining various strains as a source for a microbial screening program and for studying the ecology. Although many reviews⁴⁻⁹⁾ have detailed the procedures for the selective isolation of a wide variety of actinomycetes from soil, there are a few reports which address the selective isolation of actinomycetes from plants.

For the isolation of actinomycetes we used the serial dilution method of Hayakawa⁴⁾ in which a sodium dodecyl sulfate solution was used to wash the plants. Then, the washing solution was spread onto agar media supplemented with nalidixic acid. It is effective in reducing bacterial growth during the isolation of actinomycetes.

For directly isolating actinomycetes from plants on agar medium by needles under stereo microscope, actinomycetes were often covered

with the rapidly growing common fungi. Rose bengal, dichloran and benlate are generally used for isolating fungi to inhibit the spreading common fungi^{10,11)}. Surface sterilization method by ethanol and sodium hypochlorite solution is a well-known method to isolate plant pathogenic fungi and endophytic fungi from plants¹²⁾. We applied these fungal inhibitors and surface sterilization method to the selective isolation of actinomycetes.

We also describe the comparison of actinomycetes from pine litter layers by our selective method thus developed.

MATERIALS AND METHODS

Susceptibility of actinomycetes towards fungal inhibitors: The susceptibility of actinomycetes to some fungal growth inhibitors such as rose bengal (Wako Pure Chemical Ind.), dichloran (2,6-dichloro-4-nitroaniline, Wako Pure Chemical Ind.) and benlate (50% benomyl, Dupont Japan Ltd.) were examined as follows. Rose bengal was added to the basal medium at concentrations of 500, 250, 125, 62.5, 31.2, 15.6, 7.8 µg/ml, respectively. Dichloran was added at concentrations of 10, 5, 2.5, 1.2, 0.6

µg/ml, respectively. Benlate was added at concentrations of 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 µg/ml, respectively. 0.02 % yeast extract agar (YEA) and humic acid-vitamin agar⁴⁾ (HV agar, except for nystatin) were used as the basal medium. Antibiotics such as nystatin (50 µg/ml), cycloheximide (50 µg/ml), polymixin B (8 µg/ml) and penicillin G (0.8 µg/ml), that are generally used in the isolation of actinomycetes, were added to the basal medium. For examining the fungal growth, these antibiotics were not supplemented the media.

Ten µl of spore suspensions in sterile saline of actinomycete and fungal strains were placed onto the test medium. The fungal strains were cultured for 7 days at 27°C and the actinomycete strains, for 14 days at 27°C. Diameters of fungal colonies of three plates were measured from the reverse side, and percentages of reduction of colony diameters were calculated.

Pine litter samples: Samples of intact pine needles and litter layers were collected from different locations (Table 1). Collected pine litters were divided into the following categories based on their appearance and degree of

decomposition as described by C. S. Millar¹³⁾; freshly fallen, brown needles (L type needles), dark brown to black needles (F1 type needles), fragmented but recognizable needles (F2 type needles), and black humus-like amorphous mass of needle fragments (H layer). L, F (F1), and H layers were used for our studies.

The direct method and surfacesterilization method described below were used for the isolation from L, F (F1) layers and intact needles, whereas the serial dilution method according to the method by Hayakawa⁴⁾ was used for the isolation from the H layer.

Intact leaves were used immediately after their collection, while fallen leaves were air-dried for 7 days in a chamber.

Serial dilution method: A 0.5 g of sample was treated with a 0.05% of sodium dodecyl sulfate (SDS) solution containing 6% yeast extract at 40°C for 20 min., according to the method of Hayakawa⁴⁾, and spread onto HV agar medium supplemented with 50 µg/ml of nystatin, 50 µg/ml of cycloheximide, 20 µg/ml of nalidixic acid and 0.8 µg/ml of penicillin G after a serial dilution with sterile saline. After incubation for 2 to 4 weeks at 27°C, the colo-

Table 1. Locations collected pine litters.

location	date	litter layers of
Kamakura, Kanagawa	Feb. 1992	<i>Pinus thunbergii</i>
Manazuru, Kanagawa	Mar. 1993	<i>P. thunbergii</i>
Sumiyoshi, Iriomote island, Okinawa	Sep. 1993	<i>P. luchuensis</i>
Komi, Iriomote island, Okinawa	Sep. 1993	<i>P. luchuensis</i>
Miyazaki, Miyazaki	Mar. 1994	<i>P. thunbergii</i>
Kanoya, Kagoshima	Mar. 1994	<i>P. thunbergii</i>
Sata, Kagoshima	Mar. 1994	<i>P. thunbergii</i>

nies grown on the agar medium were isolated.

Direct isolation method: Samples were cut into several pieces and placed onto the agar medium. After incubation for 2 to 4 weeks at 27°C, the colonies grown on plants and medium were picked up with a needle as observed through a stereo microscope and transferred to new agar medium.

Surface sterilization method: The samples were cut into several pieces, which were first soaked in 70% ethanol for 30 seconds, then soaked in a sodium hypochlorite solution (antiformin, Wako Pure Chemical Ind.) containing 1% of chloride for 30 seconds and washed twice with sterile water, and then finally dried on sterile filter paper overnight. After incuba-

tion for 2 to 4 weeks at 27°C, the colonies grown on plants and medium were picked up with needle as seen through the stereo microscope, and transferred to new agar medium.

Microorganism counts in pine litter layers: Dried needles (0.5 g) treated as described in serial dilution method were serially diluted with sterile saline and spread onto HV agar. For counting bacteria and fungi, 0.5 g of needles, which had not been dried, were suspended in sterile saline.

Nutrient agar supplemented with 50 µg/ml each of nystatin and cycloheximide was used for bacteria. Malt extract agar containing 50 µg/ml each of streptomycin and tetracycline, and 30 µg/ml of rose bengal was used for fungi.

Table 2. Minimum inhibitory concentrations (µg/ml) of rose bengal, dichloran and benlate in 0.02% yeast extract agar (YEA) and HV agar.

organism	rose bengal		dichloran		benlate	
	YEA	HV	YEA	HV	YEA	HV
<i>Streptomyces galbus</i> IFO 12864	63	63	>10	10	500	>1000
<i>S. griseus</i> IFO 12875	63	31	>10	>10	1000	1000
<i>S. toxytricini</i> IFO 12823	16	16	>10	>10	1000	1000
<i>S. gannmycicus</i> IFO 13467	63	63	>10	>10	>1000	>1000
<i>S. coeruleofuscus</i> IFO 12757	125	125	>10	>10	>1000	>1000
<i>Streptosporangium roseum</i> IFO 3776	16	<8	>10	>10	1000	500
<i>Streptosporangium</i> sp. NR 0721	<8	<8	>10	>10	500	500
<i>Promicromonospora sukumoe</i> IFO 14650	16	16	>10	10	>1000	>1000
<i>Nocardiopsis dassonvillei</i> subsp. <i>dassonvillei</i> IFO 13908	<8	<8	10	10	1000	1000
<i>Nocardia brasiliensis</i> IFM0236	16	<8	>10	10	>1000	500
<i>N. farcinica</i> IFM 0284	<8	<8	>10	>10	1000	500
<i>Actinokinespora riparia</i> IFO 14541	<8	<8	>10	>10	500	500
<i>Micromonospora</i> sp. NR 0708	<8	<8	>10	10	1000	1000
<i>Micromonospora chalcea</i> IFO 12135	<8	<8	>10	10	500	500
<i>Dactylosporangium aurantiacum</i> IFO 12592	<8	<8	10	>10	1000	1000
<i>Rhizopus stonifer</i> *	250		2.5		50	
<i>Aspergillus flavus</i> , <i>Penicillium expansum</i> & <i>P. islandicum</i> *	500		12.5		<2.5	

* quoted from King et al., the tested basal medium was glucose-peptone agar.

HV agar supplemented with the antibiotics as described in serial dilution method was used for the actinomycetes.

Plates were incubated at 27°C for 3 days for the bacteria, 7 days for the fungi and 10 days for the actinomycetes. CFU per gram (dry weight) were calculated based on the dry weight after dehydration for 1 week at room temperature.

pH measurement: Samples (0.5 g of L, F and H) were suspended in 10 ml of deionized water, and sonicated for 30 seconds. pH was measured after standing for 30 minutes at room temperature.

Taxonomic properties: Isolates were compared by aerial mass colors, pigmentation of colonies and microscopic characteristics. Types of diaminopimelic acid were determined by the method of Hasegawa¹⁴.

RESULTS

Establishment of a method for the selective isolation of actinomycetes from plants

1. Susceptibility of actinomycetes towards fungal inhibitors: Table 2 shows the minimum inhibitory concentrations of rose bengal, dichlo-

ran and benlate. Most of the actinomycetes tested showed susceptibilities to rose bengal at concentrations of less than 63 µg/ml. It seemed likely that they were significantly less sensitive to benlate than the fungi were but their sensitivities to dichloran were about the same. (Data shown in Table 2 were quoted from the data described by King *et al.*¹¹).

Table 3 shows percentages of reduction of colony diameters of fungal strains in 0.02% yeast extract agar (YEA) and HV agar supplemented with rose bengal, dichloran and benlate after 7 days. Rose bengal had no inhibitory activity, and dichloran showed a weak inhibitory activity to fungi.

When the antibiotics, such as nystatin, cycloheximide, polymixin B, and penicillin G, were added at the same concentration as that in the tests for actinomycetes to the medium containing rose bengal or dichloran, fungal growth was not inhibited as the same as the test without these antibiotics (data not shown). However, benlate showed a strong inhibitory activity to fungi except for *Mucor* sp. in the both YEA and HV agar media with and without these antibiotics.

Therefore, we used 200 µg/ml of benlate as antifungal agent together with nystatin and

Table 3. Percentages of reduction of colony diameters of fungal strains by fungal inhibitors in 0.02% yeast extract agar (YEA) and HV agar.

fungal strain	rose bengal									dichloran						benlate					
	YEA			HV			YEA			HV			YEA			HV					
	(µg/ml)	63	31	16	63	31	16	10	5	2.5	10	5	2.5	500	250	125	500	250	125		
<i>Trichoderma</i> sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100	100	100	100	100		
<i>Trichoderma</i> sp. 2	14	0	0	49	16	0	39	27	0	23	0	0	100	100	100	100	100	100	100		
<i>Fusarium</i> sp.	19	19	10	20	13	10	39	26	10	20	17	10	100	100	100	100	100	100	100		
<i>Penicillium</i> sp.	0	0	0	0	0	0	100	71	65	66	55	17	100	100	100	100	100	100	100		
<i>Mucor</i> sp.	34	20	0	44	29	21	57	43	39	66	57	57	55	42	40	82	69	48			

Table 4. Microorganism counts in pine litter layers collected at Manazuru.

layer	pH	CFU / g dry weight			
		bacteria	fungi	actinomycetes	
				UT ^a	SS ^b
I ^c	ND ^d	ND	ND	ND	ND
L	4.7	1.0×10^4	8.0×10^2	1.0×10^3	4.6×10^1
F	5.4	3.5×10^4	1.6×10^3	1.7×10^4	2.0×10^2
H	5.5	4.1×10^5	9.3×10^3	2.8×10^4	ND

^a untreated sample, ^b the surface-sterilized sample, ^c intact needles, ^d not determined.

Table 5. Number of actinomycete isolates from various locations.

sample location	number of isolates by litter layers			
	I ^a	L	F	H
Sumiyoshi, Iriomote island, Okinawa	1	3	10	19
Komi, Iriomote island, Okinawa	0	7	11	36
Miyazaki, Miyazaki	ND ^b	0	19	23
Sata, Kagoshima	ND	1	21	18
Kanoya, Kagoshima	ND	2	19	19
Manazuru, Kanagawa	12	19	24	17
Kamakura, Kanagawa	12	15	9	39

^a intact needles, ^b not determined.

cycloheximide.

2. Effect of pretreatment with SDS: By using the method by Hayakawa⁴⁾ (pretreatment with 0.05% SDS solution containing 6% yeast extract and addition of 20 µg/ml of nalidixic acid to agar plates), we obtained bacterial counts 0.6 to 5% lower than those obtained with those that were untreated.

Therefore, all isolation agar media were supplemented with 50 µg/ml of nystatin, 50 µg/ml of cycloheximide, 20 µg/ml of nalidixic acid, 0.8 µg/ml of penicillin G and 200 µg/ml of benlate.

Comparative studies of microorganisms in pine litter layers

1. Microorganism counts obtained from pine litter layers: Table 4 shows the bacterial, fungal and actinomycete counts obtained from pine needles in different litter layers. Actinomycetes in the needles were also counted after sterilization of the surface of the needles.

Microorganism counts was directly proportional to the degree of decomposition; namely, the more decomposed the layers was, the greater the counts. For all of litter layers, the counts were greater for the fungi, then for the actinomycetes, and finally for the bacteria. The acti-

Table 6. Number of actinomycete isolates in which the surface-sterilization method was applied and not applied to litter layers.

sample location	layer	number of isolates			
		UT ^b	SS ^c	total	duplicates from UT & SS
Manazuru, Kanagawa	I ^a	12	0	12	0
	L	19 (1)	2	19	2
	F	25	5 (1)	24	3
	H	17	ND ^d	17	
Sumiyoshi, Iriomote island, Okinawa	I	1 (1)	0	1	0
	L	3	0	3	0
	F	7 (1)	4 (2)	10	1
	H	19	ND	19	
Komi, Iriomote island, Okinawa	I	0	0	0	0
	L	6 (1)	1 (1)	7	1
	F	7 (1)	5 (2)	12	0
	H	36	ND	36	

^a intact needles, ^b untreated sample, ^c the surface-sterilized sample,

^d number of isolates obtained from both of untreated and the surface-sterilized samples, * not determined, Number in parentheses means number of isolates having meso type of diaminopimelic acid.

nomycete counts of the L and F layer samples were significantly lower (by 1~4% of the direct method) after a surfacesterilization procedure.

2. A comparison of the surfacesterilization method and the direct method: Table 5 shows the number of total actinomycete isolates obtained from intact needles (I) and litter layers collected from various locations. The direct method and the surfacesterilization method were for the I, L, F layers samples; the serial dilution method, for the H layer samples only. The number of isolates were the greatest in the highly decomposed needle samples compared with the lesser decomposed samples.

Table 6 shows the number of actinomycete

isolates obtained by applying the direct isolation method and the surface sterilization method. No strains were isolated from intact needles by the surface sterilization method; but if the needles were in the decomposing litter layers, the number of isolates would be greater in proportion to the degree of decomposition. There were a few duplicated isolates by such as two methods.

The ratio of isolates having *meso*-diaminopimelic acid was greater for those isolates obtained from samples subjected to the surface sterilization method (2/4, F layer in Sumiyoshi; 2/5, F layer in Komi), than for those that had not been subjected to the treatment (1/7, F layer in Sumiyoshi; 1/7, F layer in Komi).

Table 7. Percentages of overlapping actinomycete isolates from pine litter layers.

sample location	layer	number of total actinomycete isolates	% of overlapping isolates		
			I ^a	L	F
Manazuru, Kanagawa	I	12			
	L	19	27		
	F	24	13	38	
	H	17	7	11	1
Kamakura, Kanagawa	I	12			
	L	15	15		
	F	9	4	27	
	H	39	5	0	0
Sumiyoshi, Iriomote island, Okinawa	I	1			
	L	3	0		
	F	10	0	65	
	H	19	0	19	7
Komi, Iriomote island, Okinawa	I	0			
	L	7	0		
	F	11	0	35	
	H	36	0	9	6

^a intact needles.

Thus, the number of isolates having *meso*-diaminopimelic acid detected by the surface-sterilization method was higher than that of non-treated method.

3. Comparison of overlapping actinomycete isolates in pine litter layers: Table 7 shows percentages of overlapping isolates among litter layers. A large number of isolates overlapped with L and F layer, and overlapping percentage was 27~65% in 4 locations, while the isolates obtained from the H layer did not highly overlapped with those of any of the other layers; the overlapping percentage was between 7 to 19%.

DISCUSSION

Rapidly growing bacteria and spreading fungi often mask the presence of actinomycetes; this is one of the main reasons why their isolation from plants is difficult. Therefore, pretreatment of the sample or the application of selective pressures are needed to inhibit their growth on the isolation plates.

The effective isolation methods of actinomycetes from plants were established, based on these results. The addition of benlate together with nystatin and cycloheximide to agar plates was effective in inhibiting the growth of fungi. To reduce the number of bacteria, pretreatment of samples with 0.05% of SDS solution containing 6% yeast extract and then growing the samples on agar plates supplemented with 20 µg/ml of nalidixic acid described by Hayakawa⁴⁾ was useful for isolating actinomycetes from plants by the serial dilution method.

In the surface sterilization method by 70% ethanol and sodium hypochlorite solution is commonly used for isolating plant pathogenic fungi and endophytic fungi from plants¹²⁾. We applied this surface sterilization method towards the isolation of actinomycetes.

We considered that isolates by the surface-sterilization method would penetrated inside

living plants in general, and might be called as "endophytic actinomycetes". Therefore we thought that these actinomycetes might also be present in living pine needles, but based on our present results, we were not able to find any "endophytic actinomycetes". Although "endophytic actinomycetes" were not isolated from living pine needles, many saprophytic actinomycetes were isolated from dead needles by the surface sterilization method.

Although the isolation frequency by the surface sterilization method was lower than by the direct method, the ratio of isolates having *meso*-diaminopimelic acid by the surface sterilization method was higher than that by the direct method. The percentage of the isolates having *meso*-diaminopimelic acid was 10% by the direct method, and 40% by the surface sterilization method. It seemed thus likely that the surface sterilization method was effective in isolation of rare actinomycetes.

We did not isolate "endophytic actinomycetes" from living pine needles. However, we have isolated a few "endophytic actinomycetes" from the live leaves of *Camellia japonica*, *Camellia sinensis* and *Citrus unshiu* (unpublished data). Sardi *et al.* have reported on the isolation of endophytic *Streptomyces* strains from surface-sterilized roots¹⁵⁾; however, there is few report from surface-sterilized leaves. Using surface-sterilized leaves will be probable to find unique actinomycetes.

Although some researchers have described the overall composition of actinomycetes in pine litter^{7,16-18)}, those which have described the taxonomies of organisms found in litter layers are few. We have obtained many isolates from different layers, and showed that the type and amounts of each are different for each layer. This suggests that the substratum succession of actinomycetes is similar to that of fungi.

Further studies, particularly the identification of isolates, are necessary for the clarification of actinomycete ecology in plants.

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