STUDIES ON VALIDAMYCINS, NEW ANTIBIOTICS. III

BIOASSAY METHODS FOR THE DETERMINATION OF VALIDAMYCIN

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Validamycin was found to cause an abnormal branching at the tips of the hyphae of *Pellicularia sasakii* under specific conditions. By application of this phenomenon, an agar dilution method, "dendroid-test method", was established. In another method called the "reversed layer method", agar plates inoculated with *P. sasakii* are incubated for 40 to 45 hours, then covered with more agar medium and paper disks dipped in the sample solution are applied on the surface. After overnight incubation the diameters of the inhibition zones are measured.

Although validamycin is effective against the sheath blight disease of rice plants caused by *Pellicularia sasakii* both in the KOSAKA's test[1] and green house test, and it is also effective against the damping off disease of cucumber seedlings caused by *Rhizoctonia solani* in green house, it does not inhibit *in vitro* growth of bacteria and fungi including *P. sasakii* and *R. solani*[4]. Therefore, it was necessary to establish suitable assay methods. We have developed two bioassay methods: "dendroid-test method", an agar dilution method, and "reversed layer method", a diffusion method.

Results and Discussion

I. Dendroid-test Method

An abnormal branching at the tips of the hyphae of *P. sasakii* described in the previous paper[4] was also observed with the diluted filtered broth or aqueous solution of the crude validamycin, and the minimum concentration in which the abnormal branching occurs could be calculated. Therefore, it was considered that the validamycin content of a sample could be estimated by recognizing the highest dilution at which the abnormal branching occurred.

To simplify the procedure, 10 ml of water agar was poured into a 9-cm Petri dish, a 7-mm glass disk was placed on the water agar plate, and the agar disk* of *P. sasakii* was put on the glass disk (Fig. 1). Investigations were carried out on the composition of media both for the plate and for the agar disk in this modified procedure. Samples were assayed by this method, and compared with results from rice plants tests. As shown in Table 1, a close correlation was found between the control-

* *P. sasakii* was inoculated on the modified PFEFFER's agar plate and cultivated at 27°C for 48 hours. Agar disks were cut from the periphery of the mycelium with a 5 mm cork-borer.

ling effect against the disease in a green house test and the validamycin titer assayed by this method.

The procedure of this method is summarized as follows:

(1) Test organism: *Pellicularia sasakii* IFO-9253

(2) Preparation of inoculum: *P. sasakii* from a potato sucrose agar slant is inoculated at the center of a modified PFEFFER's agar* in a 9-cm Petri dish, and incubated at 27°C for 40~45 hours. Agar disks for the inoculum are cut from the periphery of the mycelium with a 5-mm cork-borer.

(3) Assay procedure: Water agar plates with serial dilutions of validamycin are prepared. Two glass disks, 7-mm in diameter and 0.2-mm thick, are placed on the plate and an agar disk described above is put on each glass disk. After incubation at 27°C for 40~45 hours the highest dilution at which the abnormal branching occurs is taken as the end point (Fig. 2). Activity is expressed in dilution units. The titers of validamycins A and B assayed by this method are 100,000 units/mg and 2,000 units/mg respectively.

Table 1. Activities of some samples in simplified dish technique of dendroid test method and their controlling effects in a green house test.

Samples tested	Controlling effect (Expanding rate*, %)	Maximum dilution at which abnormal branching occurs
Untreated	100	
Filtered broth**	9	10,000
Aqueous residue after *n*-BuOH extraction**	13	10,000
Aqueous residue after carbon adsorption	100	100
Crude validamycin***	16	5,000
Monzet wettable powder****	10	

* Expanding rate $= \frac{\text{Average length of lesion per stem treated with drugs}}{\text{Average length of lesion per stem untreated}} \times 100$
The expanding rates in the above table were calculated 14 days after application of drugs.

** These samples were tested in 1/10 concentration in the green house test.

*** 15% purity, applied in 200 μg/ml solution in the green house test.

**** Containing 20% of methylarsine-bis-dimethyldithiocarbamate, 20% of zinc-dimethyl-dithiocarbamate and 40% of bis-(dimethyl-dithiocarbamoyl)-disulfide. Applied in 0.05% solution.

Fig. 1. Modified dish technique and its simplification (dendroid test method).

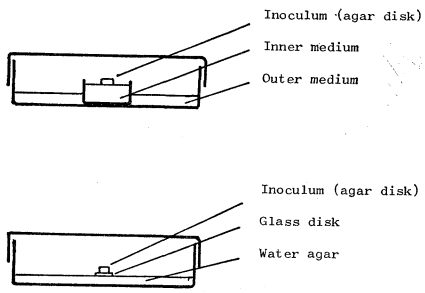
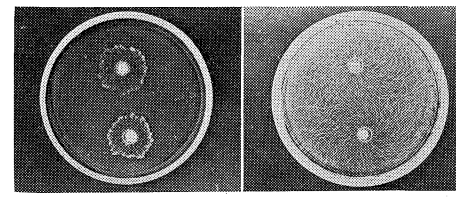


Fig. 2. Growth of *Pellicularia sasakii* in dendroid-test method.



Validamycin added Control

II. Reversed Layer Method

In searching for a diffusion assay method, the following was noted:

(1) In a green house test validamycin was more effective when rice plants were inoculated with *P. sasakii* a few days before the application of validamycin.

(2) In the dendroid-test method, *P. sasakii* developed with normal branching after overnight incubation, but after 40~45 hours abnormal branching was observed.

* This medium is composed of 3.0% sucrose, 0.2% L-asparagine, 0.3% NH_4NO_3, 0.1% KH_2PO_4, 0.1% $MgSO_4$, 0.001% versenol (iron sodium ethanolethylenediaminetriacetate 50%), 1.5% agar (pH 7). The following vitamins are added to 100 ml of the medium before use: 100 μg vitamin B_1 hydrochloride, 100 μg riboflavin, 100 μg calcium pantothenate, 100 μg niacin, 0.5 μg biotin, 50 μg folic acid, 200 μg vitamin B_6 hydrochloride, 50 μg PABA, 0.2 μg vitamin B_{12}.

It seemed that validamycin dose not inhibit initiation of growth, but it inhibits development of hyphae of partially grown fungus. Thus, following experiments were carried out. A modified PFEFFER's agar plate seeded with *P. sasakii* was incubated at 27°C for 1~2 days. Five ml of water agar was overlaid on it. A paper disk dipped in aqueous solution of validamycin was applied. The plate was incubated at 27°C for two days. When the seed layer was incubated for 1 day before the overlay procedure, no inhibition zone was observed, whereas when incubated for 2 days prior, an inhibition zone, though not clear, was observed. To utilize this inhibition zone for the assay of validamycin, further investigations were carried out.

Inhibition zones obtained with combinations of various media for the bottom and the upper layers were compared (Tables 2 and 3). The upper layer medium was overlaid after incubation of the bottom layer medium for 1 or 2 days, but better results were obtained with 2 days. A clear inhibition zone was observed with bouillon agar for the bottom layer and B-medium for the upper layer.

As a result of many investigations, a modified bouillon agar was selected as the bottom layer medium.

Various kinds and concentrations of carbon and nitrogen sources and inorganic salts to be contained in the upper layer medium were examined and the best one selected.

As the nature of the growth of *P. sasakii* on the bottom layer seemed to influence greatly the size and the appearance of the inhibition zone, the effect of the inoculation temperature upon the subsequent growth was examined. Melted bottom layer medium was kept at the temperatures of 40, 45, 50, 55 and 60°C, inoculated with the hyphal

Table 2. Media for the bottom layer and the upper layer

Medium	Composition
Media for the bottom layer	(1) 1.0 % sucrose, 0.2 % L-asparagine, 0.2 % NH_4NO_3, 0.1 % KH_2PO_4, 0.1 % $MgSO_4$, 1.5 % agar.
	(2) 1.0 % lactose, 0.2 % L-asparagine, 0.2 % NH_4NO_3, 0.1 % KH_2PO_4, 0.1 % $MgSO_4$, 1.5 % agar.
	(3) 1.0 % sucrose, 0.1 % KH_2PO_4, 0.1 % $MgSO_4$, 1.5 % agar.
	(4) 1.0 % lactose, 0.1 % KH_2PO_4, 0.1 % $MgSO_4$, 1.5 % agar.
	(5) 0.2 % L-asparagine, 0.2 % NH_4NO_3, 0.1 % KH_2PO_4, 0.1 % $MgSO_4$, 1.5 % agar.
	(6) Glucose bouillon agar.
	(7) Bouillon agar.
	(8) Potato sucrose agar.
	(9) Rice straw infusion agar.*
	(10) V-8 juice agar.**
Media for the upper layer	A : 0.2 % L-asparagine, 0.2 % NH_4NO_3, 0.1 % KH_2PO_4, 0.1 % $MgSO_4$, 1.5 % agar.
	B : 0.2 % sucrose, 0.2% L-asparagine, 0.2 % NH_4NO_3, 1.5 % agar.

* One hundred grams of rice straws were cut and autoclaved at 1.5 kg/cm² for 20 minutes in 1 liter of water, and solution obtained was added with 2 % sucrose and 1.5 % agar.

** Composed of 20 % (v/v) V-8 juice, 2.0 % sucrose and 1.5 % agar.

Table 3. Size and characterization of inhibition zone* observed on various media.

Media for the bottom layer	Media for the upper layer			
	A		B	
	Size (mm)	Characterization	Size (mm)	Characterization
(1)	40	not clear	+	not clear
(2)	±		−	
(3)	−		−	
(4)	−		−	
(5)	±		±	
(6)	36	not clear	±	
(7)	+	not clear	21	clear
(8)	−		−	
(9)	−		−	
(10)	−		−	

* Obtained by applying the paper disk dipped in 500 μg/ml solution of purified validamycin A on the surface of the upper layer media.

− : Absence of inhibition zone.

± : Doubtful existence of inhibition zone.

+ : Large but irregular inhibition zone difficult in measuring the diameter.

Fig. 3. Inhibition zones in reversed layer method.

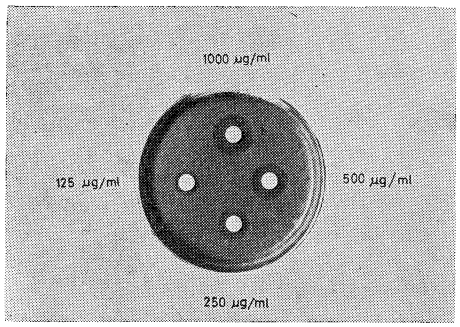
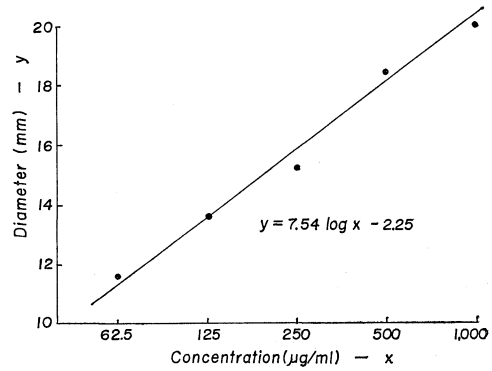


Fig. 4. Relation between the concentration of validamycin A and the diameter of the inhibition zone in reversed layer method.



suspension of the fungus, and kept at each temperature for 0, 10, 15 and 20 minutes. Then agar plates were prepared with each medium, incubated at 27°C for 24 hours and the growth of the fungus on the agar plates was observed. *P. sasakii* seemed to be fairly sensitive to temperature, and it was considered that the inoculation of the test organism to the bottom layer medium should be carried out below 45°C.

The effect of temperature of the upper layer medium during the overlay procedure was examined, and it was found that the upper layer medium should be overlaid at 55°C.

Incubation temperatures of 25, 27 and 30°C were compared, and 27°C was chosen.

As the present test organism seemed to be rather sensitive to temperature, less sensitive strains were looked for among 47 strains of *P. filamentosa* f. sp. *sasakii* (*P. sasakii*) deposited in the Institute for Fermentation, Osaka, but none were found.

Several inoculation media were examined to select the best.

Procedure of the reversed layer method

(1) Test organism: *Pellicularia sasakii* IFO-9253.

(2) Preparation of hyphal suspension (inoculum): The agar disk cut from a culture on modified PFEFFER's agar plate is inoculated to 50 ml of the sterilized medium in a 300-ml flask containing 3.0 % sucrose, 0.2 % L-asparagine, 0.3 % NH_4NO_3, 0.1 % KH_2PO_4 and 0.1 % $MgSO_4$, and incubated at 27°C for 4 days on the reciprocal shaker (10 cm, 120 spm). The whole culture obtained was homogenized with a blender, and used as a hyphal suspension of the test organism.

(3) Assay media:

Bottom layer medium: 0.1 % sucrose, 1.0 % beef extract, 1.0 % peptone, 0.8 % agar.
Upper layer medium: 0.25 % sucrose, 0.45 % peptone, 0.1 % NaCl, 1.2 % agar.

(4) Procedure: Melted bottom layer medium is kept at 45°C and to it is added 2.5 % of the hyphal suspension of the test organism. After mixing, 5 ml of this medium are poured into a 9-cm Petri dish. The plate is incubated at 27°C for 40~45 hours, and overlaid with 10 ml of the upper layer medium melted and kept at 55°C. After solidification, the paper disk dipped in a sample solution is placed on the surface. The inhibition zone is measured after incubation for 20~25 hours at 27°C (Fig. 3).

When validamycin B was assayed by this method using purified validamycin A as a standard, its potency was found to be 30 μg/mg.

Fig. 4 shows a dose-response curve for purified validamycin A in the reversed layer method. The response was observed to be linear over a range of 62.5~1,000 μg/ml.

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