Antibacterial Activity of Validamycin A against *Pseudomonas solanacearum* and Its Efficacy against Tomato Bacterial Wilt

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Abstract

Validamycin A (VM-A), the active ingredient of Validacin®, inhibits fungal growth by inhibiting trehalase activity. Therefore, VM-A was tested against *Pseudomonas solanacearum* for its efficacy in controlling tomato bacterial wilt. In media containing trehalose as the sole carbohydrate, VM-A at 50 µg/ml inhibited growth of *P. solanacearum* to rates similar to that of the bacteria in media without carbohydrate for seven days after inoculation. VM-A also gave excellent control of tomato bacterial wilt in greenhouse pot tests, when directly injected into plant stems. In field tests, foliar sprays of VM-A at 250 µg/ml five days before and two days after inoculation had reduced disease by 47.4% by four weeks after inoculation. In the tomato stem between 0 and 5 cm above the soil line, the bacteria population in the non-treated plot reached 3.84×10^{10} cfu/g fresh weight, whereas that in the VM-A (500 µg/ml)-treated plot reached 2.13×10^9 cfu/g fresh weight. Inhibition of bacteria growth by VM-A may delay the appearance of disease symptoms of tomato bacterial wilt.

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Key words : validamycin A, *Pseudomonas solanacearum*, control of tomato bacterial wilt.

INTRODUCTION

Bacterial wilt caused by *Pseudomonas solanacearum* is one of the most serious diseases affecting several hundreds species of crops in tropical, subtropical and temperate regions^{2,4)} by producing a slime which clogs the xylem and results in wilting. *Pseudomonas solanacearum* is soil-borne or carried on debris of diseased roots and stems. In order to control the disease, the soil must be sterilized by solarization or with chemicals such as methyl bromide or chlorpicrin, either independently or in combination. However, soil sterilization does not completely control the disease because chemicals are absorbed by soil particles and degraded by microorganisms. Moreover, under conditions of incomplete vaporization in the soil, methyl bromide and chlorpicrin fumigation can be irritating and phytotoxic. Therefore, they can not be used after the disease occurs in the fields. Furthermore, because methyl bromide affects the ozone layer, its application is controlled. No chemical controls bacterial wilt in culture.

Validamycin A (VM-A), the active ingredient of Validacin®, gives excellent control against rice sheath blight caused by *Rhizoctonia solani* by inhibiting the hyphal growth of the pathogen on rice leaves. VM-A does not exhibit antifungal activity on potato sucrose agar or potato dextrose agar medium unless the medium contains trehalose, a transport sugar in *R. solani*, as the

sole carbohydrate. Moreover, it inhibits *R. solani* trehalase activity *in vitro*^{1,6)}. The penetration hyphae of *R. solani* in rice sheath absorb carbohydrates and change them into trehalose⁷⁾, which is transported to hyphal tips, where it is metabolized into two molecules of glucose by trehalase. By inhibiting trehalase activity in the hyphal tips of *R. solani*, a shortage of utilizable carbohydrate results in the inhibition of hyphal elongation.

In this report antibacterial activity of VM-A against *P. solanacearum* and its control of tomato bacterial wilt after various methods of applications were examined. The distribution and population growth of *P. solanacearum* in tomato stems after VM-A treatments was also studied.

MATERIALS AND METHODS

Materials. *Pseudomonas solanacearum* E.F. Smith, strain OE1-1 was obtained from Dr. H. Date of Okayama Prefecture Agricultural Experiment Station. Tomato (*Lycopersicon esculentum* Mill. cv. Oogatafukuju) seeds were purchased from Takii Seed Corporation. Trehalose and thyrothricin were purchased from Sigma Chemical Company, triphenyl tetrazolium chloride (TTC), polymyxin B sulfate, chloromycetin and polypeptone were purchased from Wako Pure Chemical Ind. Ltd., and casamino acid was purchased from DIFCO Laboratories. Crystal violet was purchased from Merck

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& Co., Inc. VM-A used throughout this study was 97% in purity.

Tomato plants were transplanted into 6 cm-diameter pots three weeks after seeding, then grown for four weeks in a greenhouse. The pathogen was maintained in sterile water at room temperature. Bacterial suspensions for the experiments were incubated in casamino acid-polypeptone-sucrose medium (CPS; 0.1, 1.0, 0.5%) at 34°C for seven days. For colony counts, 0.005% TTC was added to CPS agar medium⁵⁾.

Antibacterial activity in vitro. Czapek medium (0.3% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.001% FeSO₄·7H₂O), containing 0.8% carbohydrate, used for testing antibacterial activity against *P. solanacearum*. An 8 mm-diameter paper disk, containing about 30 µl VM-A solution, was placed on 10 ml of Czapek-trehalose agar medium in which *P. solanacearum* was embedded. After four days of incubation at 34°C, the diameter of the inhibitory zone was measured.

To 10 ml of Czapek liquid medium containing either 0.5% mannitol, glucose, mannose, inositol, sucrose, pyruvic acid, glycerol, galactose or trehalose as the sole carbohydrate, VM-A in 70% ethanol was added to make a final concentration of 50 µg/ml. The medium was inoculated with 100 µl of bacterial cells at a concentration of about 10⁴ cfu/ml, then incubated at 30°C for eight days in an incubator. Using a dilution plate technique on CPS-TTC agar medium, the number of bacteria in the medium containing trehalose was counted 0, 1, 3, 5 and 8 days after inoculation, but only after eight days for the media containing other carbohydrates.

Pot test in a greenhouse. Tomato plants were transplanted into 1/5000 a Wagner's pots three weeks after seeding, then grown for six weeks in the greenhouse. A paste of 5% VM-A was made with 70% CaCO₃ and 25% water. Tomato stems were pierced with a mini-drill to make a hole 10 cm above the soil line or just below the first inflorescence (at 30 cm above the soil line), which was filled with 0.2 g of VM-A paste using a syringe. VM-A paste was applied to nine tomato stems with two replicates. Seven days later, tomato plants were inoculated with 10⁸ cfu/ml of *P. solanacearum* by pouring 500 ml of the bacterial suspension on the soil in each pot. The number of wilted plants was counted daily until 20 days after inoculation.

Field test. A field test using VM-A foliar sprays against tomato bacterial wilt was carried out in 1994 at Ibaraki Prefecture. The tomato cv. Oogatafukuju was seeded on May 20, and transplanted to the field on June 29. Foliar spray of VM-A solution at 250 or 500 µg/ml was applied to 20 tomato plants in a plot on July 29 and August 5. The soil in each pot was drenched with 100 ml of 1.8×10⁸ cfu/ml *P. solanacearum* on August 3. A daily disease index for each plant was assigned according to the following scale: 0: no symptom, 1: slight wilt on the top part of the plant, 2: slight wilt in leaves, 3: severe wilt of the whole plant, 4: death of the plant.

Hydroponics culture test. Hydroponics water contained 0.033% KNO₃, 0.0167% Ca(NO₃)₂·4H₂O, 0.0098% KH₂PO₄, 0.015% MgSO₄·7H₂O, 0.0075% NH₄NO₃ and 0.0025% Greenplus® (Kyodo Hiryo) in deionized water. Roots of five-week-old tomato plants were washed well to remove soil and transplanted to hydroponics culture in trays. VM-A was mixed with the hydroponics culture water to obtain a final concentration of 100 µg/ml seven days after transplanting. Three hours later, a suspension of bacteria in distilled water was added to the hydroponics culture water to reach a final concentration of 2.4×10⁷ cfu/ml. The culture water was replaced seven days after inoculation.

Bacterial distribution in tomato stems. Six-week-old tomato plants, cv. Oogatafukuju, were transplanted to 1/20,000a Wagner's pots in a greenhouse. Their leaves were then sprayed to run-off with VM-A solution at 500 µg/ml. One week later, each plant was inoculated with 50 ml of a bacterial suspension (5×10⁸ cfu/ml) by soil drench. Tomato stems were excised 5 cm above the soil, then cut into fragments of several millimeters 10 days after inoculation. The pieces were immersed in 100 ml of distilled water to exude the bacteria and kept overnight at 6°C to avoid contamination by other bacteria and inhibit growth of *P. solanacearum*. The number of bacteria was counted by dilution plating after three days of incubation at 30°C on a selective medium containing CPS-TTC, crystal violet (50 µg/ml), polymyxin B sulfate (100 µg/ml), thyrothricin (20 µg/ml) and chloromycetin (5 µg/ml)³⁾.

RESULTS

Antibacterial activity of VM-A in vitro

After seven days of incubation at 30°C, VM-A inhibited bacterial multiplication only in media containing

Fig. 1. Antibacterial activity of validamycin A against *Pseudomonas solanacearum* OE1-1 embedded in Czapek agar medium containing trehalose as the sole carbohydrate. A paper disk contains about 30 µl of validamycin A solution. a, validamycin A 500 µg/ml; b, 250 µg/ml; c, 125 µg/ml; d, 62.5 µg/ml.

trehalose as the sole carbohydrate (Fig. 1). VM-A inhibited bacteria at $62.5 \mu\text{g/ml}$, the lowest concentration in this test. VM-A did not inhibit the multiplication of *P. solanacearum* in media containing mannitol, glucose, mannose, inositol, sucrose, pyruvic acid, glycerol or

Fig. 2. Antibacterial activity of validamycin A against *Pseudomonas solanacearum* OE1-1 in Czapek liquid medium containing trehalose as the sole carbohydrate. To 10 ml of Czapek liquid medium containing 0.5% trehalose as the sole carbohydrate, $100 \mu\text{l}$ of validamycin A in 70% ethanol was added. *P. solanacearum* was added to the media for a final concentration of $7-8 \times 10^2$ cfu/ml. The media were incubated at 30°C for eight days in an incubator. The number of bacteria was counted by dilution plate technique on casein-peptone-sucrose-TTC media. Bars are the means of three replicates.

Fig. 3. Effect of $50 \mu\text{g/ml}$ validamycin A (VM-A) on the growth of *Pseudomonas solanacearum* in 10 ml Czapek liquid medium containing 0.5% trehalose as the sole carbohydrate. The liquid media were incubated at 30°C for eight days in an incubator. After dilution plating on CPS-TTC agar medium, bacteria were counted at 0, 1, 3, 5 and 8 days after inoculation. Bars indicate standard errors of three replicates.

galactose as the sole carbohydrate (data not shown).

In liquid media containing trehalose as the sole carbohydrate, VM-A strongly inhibited the multiplication of bacteria at concentrations of $0.02 \mu\text{g/ml}$ and above (Fig. 2). VM-A at the concentration of $0.02 \mu\text{g/ml}$ reduced multiplication of *P. solanacearum* to 10% of the growth attained when VM-A was not present. Through the entire eight days of the test period, the number of bacteria increased exponentially to 1.46×10^9 cfu/ml (Fig. 3). In the Czapek-trehalose medium with $50 \mu\text{g/ml}$ of VM-A, however, the number of bacteria increased exponentially for three days, reaching 6.94×10^6 cfu/ml. Thereafter, they remained in a stationary phase at 1.59×10^7 cfu/ml. Without a carbohydrate present, *P. solanacearum* also multiplied exponentially for three days, but only reached a population of 1.32×10^6 cfu/ml. Thereafter, little increase was observed. Growth rates in the media containing trehalose and VM-A were six times higher than that in the media without a carbohydrate and in the media with trehalose during the first day of incubation. Thereafter, populations in media containing trehalose and VM-A were almost the same as those in the medium without a carbohydrate.

Pot test in a greenhouse

Wilted tomato plants were first observed six days after inoculation in the non-treated plot (Fig. 4). Direct injection of the VM-A paste into stems 10 cm above the soil (AS) seven days before inoculation inhibited wilting symptoms until 12 days after inoculation. Injecting the paste below the first inflorescence (BI) seven days before inoculation inhibited disease development throughout

Fig. 4. Effect of validamycin A (VM-A) against tomato bacterial wilt in greenhouse pot tests. A tomato stem was pierced with a mini-drill to make a hole into which 0.2 g validamycin A paste (5% VM-A, 70% CaCO_3 , 25% water) was syringed. VM-A paste was applied to nine tomato stems with two replicates either 10 cm above the soil or just below the first inflorescence (at 30 cm above the soil line) seven days before the soil was drenched with a 500 ml bacterial suspension (1×10^8 cfu/ml).

the course of the experiment. After 20 days, 91.7% of the control plants had wilted, as opposed to 33.3% of those treated AS and only 25.0% of those treated BI. Both VM-A treatments by direct injection into the stem, either near (at 10 cm above the soil line) or far from the soil line (below the first inflorescence) were effective in delaying and reducing tomato bacterial wilt.

Field test

In the non-treated plot, wilting occurred by seven days after inoculation. The disease severity of non-treated control tomato plants 15 and 23 days was 56% and 90%, respectively (Fig. 5). Almost all the plants died by 28 days.

Fig. 5. Control of tomato bacterial wilt by foliar sprays of validamycin A (VM-A) in field tests. Twenty tomato plants (cv. Oogatafukuju) in two replicates were sprayed to run-off with 100 ml per plant of VM-A solution on July 29 and August 5 in 1994. On August 3 each tomato plant was inoculated with a soil drench of 100 ml of a bacterial suspension (1.8×10^8 cfu/ml). Disease severity for each plant was rated daily until 28 days after inoculation according to the following: 0, no symptom; 1, slight wilt on the upper part of the plant; 2, slight wilt in leaves; 3, severe wilt of the whole plant; 4, death of the plant.

Foliar spraying with VM-A at 250 µg/ml and 500 µg/ml resulted in wilted tomato plants by 20 days after inoculation. By 28 days, disease severity in the VM-A treated plot at 250 µg/ml was 51% and at 500 µg/ml was 45%. VM-A delayed the appearance of disease symptoms for about 10 to 14 days.

Hydroponics culture test

In the hydroponics culture test, all the non-treated Oogatafukuju plants had wilted after nine days (Table 1). On the other hand, only 25% of the plants in the VM-A-treated plot had wilted after 12 days. By 12 days, 83% of the non-treated Patio cultivar had wilted. VM-A completely inhibited wilting until eight days when only 8% of the plants had wilted. No further increase in wilting was observed.

Fig. 6. Effect of foliar sprays of 500 µg/ml validamycin A (VM-A) on the distribution of *Pseudomonas solanacearum* in tomato stems. One week after application, each tomato plant was inoculated by soil drench with 50 ml of a bacterial suspension (5×10^8 cfu/ml). Ten days after inoculation tomato stems were excised at 5 cm intervals, then cut into fragments several millimeters thick, then immersed in 100 ml of distilled water. The number of bacteria from the tomato stem was counted by dilution plating on a selective medium for *P. solanacearum*. Bars indicate standard errors of three replicates.

Table 1. Efficacy of validamycin A (VM-A) against tomato bacterial wilt in hydroponics culture

Cultivar	VM-A treatment	% of wilted tomato plants				
		DAI ^{a)}	6	7	8	9
Oogatafukuju	+		12.5	12.5	12.5	12.5
	-		62.5	87.5	87.5	100.0
Patio	+		0.0	0.0	8.3	8.3
	-		16.6	41.6	58.3	66.7

a) DAI means days after inoculation. For cv. Oogatafukuju eight plants were tested and for cv. Patio, 12 plants. VM-A was dissolved in hydroponics water at a concentration of 100 µg/ml. Three hours after application, the bacterial suspension was poured into hydroponics water at a final concentration of 2.4×10^7 cfu/ml.

b) Numbers in columns followed by the same letter are not significantly different in Tukey HSD test at $p=0.05$ level.

Distribution of *Pseudomonas solanacearum* in tomato stems after VM-A foliar sprays

All three untreated plants wilted. Bacterial populations in stems from these plants, reached 3.84×10^{10} cfu/g fresh weight in stem fragments excised in the first 5 cm from the soil line (Fig. 6). None of the three tomato plants receiving the VM-A foliar spray at 500 μ g/ml wilted, even though 2.13×10^9 cfu/g fresh weight of bacteria were recovered from tomato stems excised from the basal 5 cm. From stems between 30 and 35 cm above the soil line, 1.54×10^6 cfu/g fresh weight of bacteria were isolated from VM-A-treated plants, as opposed to 1.93×10^7 cfu/g fresh weight of control tissue.

DISCUSSION

Pot test showed that direct injection of VM-A into tomato stems gave excellent control of bacteria populations regardless of the distance of the injection site from the roots. Foliar sprays of VM-A in the field were also effective against tomato bacterial wilt. Judging from these results, VM-A is thought to be absorbed into the plant, and translocated to sites where *P. solanacearum* multiplies.

VM-A inhibited bacterial growth *in vitro* when trehalose was the sole carbohydrate. VM-A also inhibited bacterial growth *in vivo*, either directly or indirectly. Moreover, in tomato stems treated with VM-A, *P. solanacearum* populations were less than those in non-treated stems. This growth inhibition of bacteria by VM-A may cause the delay of disease symptoms.

Foliar sprays of VM-A also delayed and reduced disease symptoms of tomato bacterial wilt. Because of the soil-borne nature of the pathogen, however, no chemical can control tomato bacterial wilt completely. To effectively control tomato bacterial wilt, however, complete control of the pathogen in the soil may not be necessary. Inhibiting multiplication of the pathogen in the plant may be enough to control the disease. Therefore, direct injection of VM-A into stems was aimed at by-passing soil factors and inhibiting growth of *P. solanacearum* in the stem. Unfortunately, direct injections are not practical for large-scale applications.

VM-A was proven to be effective against tomato bacterial wilt when applied in foliar sprays, which are a more efficient, so far and more feasible method of eliminating soil complications in control.

Literature cited

1. Asano, N., Yamaguchi, T., Kameda, Y. and Matsui, K.

- (1987). Effect of validamycins on glycohydrolases of *Rhizoctonia solani*. J. Antibiot. 40: 526-532.
2. Buddenhagen, I. and Kelman, A. (1964). Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 2: 203-230.
3. Granada, G.A. and Sequeira, L. (1983). A new selective medium for *Pseudomonas solanacearum*. Plant Dis. 67: 1084-1088.
4. Hayward, A.C. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29: 65-87.
5. Kelman, A. (1954). The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44: 693-695.
6. Shigemoto, R., Okuno, T. and Matsuura, K. (1989). Effect of validamycin A on the activity of trehalase of *Rhizoctonia solani* and several sclerotial fungi. Ann. Phytopathol. Soc. Jpn. 55: 238-241.
7. Shigemoto, R., Okuno, T. and Matsuura, K. (1992). Effects of validamycin A on the growth of and trehalose content in mycelia of *Rhizoctonia solani* incubated in a medium containing several sugars as the sole carbohydrate. Ann. Phytopathol. Soc. Jpn. 58: 685-690.

和 文 摘 要

石川 亮・藤森健一・松浦一穂：バリダマイシン A の *Pseudomonas solanacearum* に対する抗菌活性とトマト青枯病防除効果

バリダシン®の有効成分であるバリダマイシン A (VM-A) は *Rhizoctonia solani* によるイネ紋枯病に卓効を示す薬剤である。VM-A は *R. solani* 由来のトレハラーゼを阻害することが報告されている。本報告では、*Pseudomonas solanacearum* に対する VM-A の抗菌活性と、トマト青枯病防除効果について調べた。VM-A はトレハロースを唯一の糖源としたときのみに、*P. solanacearum* に対して抗菌活性を示し、その活性は増殖抑制効果であった。トレハロース液体培地に 50 μ g/ml の VM-A を処理したときの *P. solanacearum* の増殖速度は、培養 1 から 8 日後までは、糖源を含まない培地のときと同程度に強く抑制された。VM-A は温室内でのポット試験、圃場試験で高い防除効果を示した。圃場試験で、VM-A は 250 μ g/ml の茎葉散布 (接種 5 日前および 2 日後) で、接種 4 週間後に 47.4% の防除効果を示した。無処理のトマト茎からは、地上部から 5 cm の範囲で *P. solanacearum* が 3.84×10^{10} cfu/ml 生重検出されたが、VM-A 処理のトマトの同部位からは病原菌は 2.13×10^9 cfu/ml 生重しか検出されなかった。以上の結果より、VM-A はトマト内での *P. solanacearum* の増殖を抑制することで、青枯病の発病遅延効果を発現するものと考えられた。