

キュウリ灰色疫病菌の生物的防除用細菌 *Serratia marcescens* F-1-1株の抗菌性赤色色素の同定および同物質の各種植物病原菌に対する抗菌活性

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
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発行元	日本植物病理學會
巻/号	64巻4号
巻号補足	
掲載ページ	p. 294-298
発行年月	1998年8月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター

Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Identification of Antibiotic Red Pigments of *Serratia marcescens* F-1-1, a Biocontrol Agent of Damping-off of Cucumber, and Antimicrobial Activity against Other Plant Pathogens

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Abstract

The red pigmented strain F-1-1 of *Serratia marcescens* shows a strong antagonism against *Phytophthora capsici*, the pathogen of damping-off of cucumber seedlings, and its antagonistic activity is due to the production of antibiotic red pigments. In the present study, purification and identification of the red pigments produced by the strain F-1-1 were performed. The water-insoluble red pigments were extracted from cultures on agar plates with ethanol and then chloroform, and purified by silica gel column chromatography. An active substance was isolated using the antifungal activity assay and was identified as prodigiosin, a known compound based on the spectral data. Purified prodigiosin inhibited the germination of cystospores and the growth of the fungal hyphae of *P. capsici*. It also showed antimicrobial activity against some plant-pathogenic fungi including *Cochliobolus miyabeanus*, *Pythium spinosum*, *P. ultimum* and against plant pathogenic bacteria including, *Clavibacter michiganensis* subsp. *michiganensis* and *Erwinia carotovora* subsp. *carotovora*. These results suggest that *S. marcescens* F-1-1 could be used as a biocontrol agent against many plant diseases other than cucumber damping-off.

(Received March 6, 1998 ; Accepted May 11, 1998)

Key words : Prodigiosin, *Serratia marcescens*, *Phytophthora capsici*, antimicrobial spectrum, damping-off of cucumber.

INTRODUCTION

Damping-off of cucumber caused by *Phytophthora capsici* is one of the most destructive plant diseases in Japan. Since the chemical control of the disease is difficult, it is important to promote the biological control of the disease. Recently, we have isolated *S. marcescens* F-1-1 which was found to be a potential biocontrol agent against this disease⁹⁾. In addition, we suggested that the biocontrol mechanism is based on the inhibition of germination of cystospores and zoosporangia of *P. capsici* P-9-2 by some antibiotics contained in the red pigments produced by strain F-1-1⁹⁾. There have been no reports on antagonistic substances involved in the biocontrol mechanisms of *S. marcescens* against this plant disease. It was reported however that only the chitinase activity of this bacterium played an important role in

the biocontrol of some other plant pathogenic fungi such as *Botrytis* spp.^{1,7)}, *Sclerotium rolfsii*¹⁰⁾ and so on.

In the present study, we isolated a red pigment with antibiotic activity against *P. capsici* from the mixture of the pigments produced by *S. marcescens* and identified it as prodigiosin based on spectral data. In addition, we analyzed the antimicrobial spectrum of the compound against many plant pathogens for application as a biocontrol agent in other plant diseases.

MATERIALS AND METHODS

Bacterial and fungal strains and culture conditions *S. marcescens* F-1-1 used in this study was isolated from the rhizosphere of scarlet sage (*Salvia splendens* F. Sellow ex Roem. et Schult.) in Fukui Prefecture⁹⁾. For an assay of the antimicrobial spectrum, stock cultures of the National Institute of Agro-

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Environmental Sciences and other institutes were used (Tables 3, 4).

For the extraction of red pigments, bacteria were incubated for 2 days at 25°C on modified LB medium (polypeptone 10 g, yeast extract 5 g, NaCl 10 g, agar 15 g, distilled water 1 liter) or PPGSA medium (bact-pepton 5 g, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 0.5 g, Na_2HPO_4 2 g, sucrose 20 g and agar 15 g in 1 liter of decoction from potato 300 g, pH 6.8–7.0).

Extraction and purification of red pigments

Red pigmented bacterial cultures on plates were scraped and suspended in 10 ml of sterile distilled water per plate. Bacterial cells were collected by centrifugation (15,000 rpm, 5 min), extracted with ethanol by weak shaking overnight and centrifuged (15,000 rpm, 5 min). Then the supernatant fluid was filtered using a Millipore filter (0.2 μm) to exclude bacteria and concentrated under reduced pressure at 48°C. Next, the ethanol extracts were extracted with chloroform. The chloroform extracts were fractionated by silica gel column chromatography with elution by chloroform. Each fraction was analyzed by silica gel thin layer chromatography (TLC) and bioassayed for the inhibition of germination of cystospores as described later. The bioactive red pigment was further purified by high performance liquid chromatography (HPLC) and identified as prodigiosin by comparison of IR, ^1H -NMR, ^{13}C -NMR and mass spectral data with those previously reported^{2,4)}.

Antibiotic activity of prodigiosin against *P. capsici* The suspension of cystospores or zoosporangia of *P. capsici* P-9-2 was inoculated to a 5% ethanol solution containing various concentrations of prodigiosin in a micro-test tube (1.5 ml). The germination rate of spores was determined 3 days after shaking culture (90 reciprocations/min).

Antimicrobial spectrum of prodigiosin Antimicrobial activity against various plant pathogenic fungi was evaluated as follows. Hyphal disk of each strain was placed on PDA medium containing 0.1, 1 and 10 μg

of prodigiosin in a petri dish. Growth inhibition induced by prodigiosin was estimated after 4 to 10 days of incubation at 25°C when fungal hyphae in a control dish (without prodigiosin) grew and covered the plate completely.

A bioassay with the plant pathogenic bacteria was performed as follows. Bacterial suspension was inoculated to a PD broth supplemented with 5% of an ethanol solution containing various concentration of purified prodigiosin in a micro test tube (1.5 ml). Maximal allowable concentration (MAC) was estimated 3 days after shaking culture (90 reciprocations/min) at 25°C.

RESULTS

Identification of prodigiosin

Red pigments of *S. marcescens* F-1-1 were extracted with ethanol and then chloroform. The chloroform extracts were fractionated by silica gel column chromatography and four fractions, Fr.1 (light purple), Fr.2 (red), Fr.3 (whitish pink) and Fr.4 (dark purple), were obtained. They were assayed for the inhibition of cystospore germination of *P. capsici* P-9-2. As shown in Table 1, a strong antibiotic activity was detected in the red pigmented fractions, Fr.2 and Fr.0 (mixed with Fr.1, 2, 3 and 4) but not in other fractions.

Fr.2 was purified by HPLC and monitored by ultraviolet and visible absorption spectra. It showed absorption bands at 275, 336 and 467 nm (Fig. 1). IR, NMR and mass spectral data were identical with those of prodigiosin

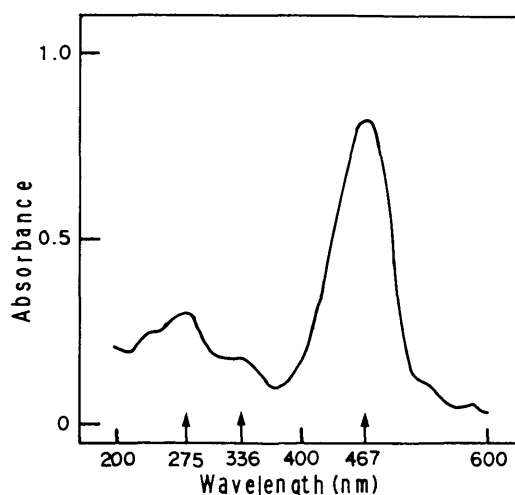


Fig. 1. Ultraviolet and visible absorption spectra of the purified red pigment which was extracted from strain F-1-1 and dissolved in isopropanol.

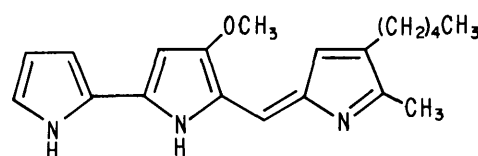


Fig. 2. Structure of prodigiosin.

Table 1. Antifungal activity against cystospore germination of *Phytophthora capsici* P-9-2 of chloroform-extracts of fractions of red pigments

Fraction No. ^{a)}	Cystospore germination rate ^{b)}					
	Non-diluted	1:2 ^{c)}	1:4	1:8	1:16	1:32
Fr. 0	—	—	+	3+	4+	4+
Fr. 1	4+	4+	4+	4+	4+	4+
Fr. 2	—	—	—	2+	3+	4+
Fr. 3	2+	3+	4+	4+	4+	4+
Fr. 4	4+	4+	4+	4+	4+	4+
Water (control)	4+					

a) Fractions were obtained by silica gel column chromatography.

b) Germination rate: —, 0 (%); +, 1–10 (%); 2+, 11–49 (%); 3+, 50–79 (%); 4+, 80–100 (%).

c) Dilution of extracts.

Table 2. Antifungal activity of prodigiosin against *Phytophthora capsici* P-9-2

Concentration of prodigiosin ($\mu\text{g/ml}$)	Germination rate (%)	
	Cystospores	Zoosporangia
50.0	0.0 A ^{a)}	0.0 A
25.5	0.0 A	0.0 A
12.5	15.8 B	0.0 A
6.3	52.4 C	18.3 B
3.1	60.6 C	24.3 B
1.6	92.0 D	41.7 C
Water (control)	95.2 D	88.5 D

a) Values followed by the same letter are not significantly different ($p=0.01$), according to Duncan's multiple range test (inhibition grade; $A>B>C>D$).

Table 3. Growth inhibition of plant pathogenic fungi by various concentrations of prodigiosin produced by *Serratia marcescens* F-1-1

Fungal strain ^{a)}		Growth inhibition (%)		
		0.1 ^{b)}	1.0	10.0 ($\mu\text{g/ml}$)
<i>Cochliobolus miyabeanus</i>	H-1-1	16.7	57.8	83.3
<i>Fusarium moniliforme</i>	F-3-2	0	0	5.9
<i>F. oxysporum</i> f. sp. <i>allii</i>	F-1-5-1	11.8	12.5	17.6
<i>F. oxysporum</i> f. sp. <i>raphani</i>	F-1-6-3	0	8.8	16.3
<i>F. oxysporum</i> f. sp. <i>cucumerinum</i>	F-1-2-7	0	0	23.5
<i>F. oxysporum</i> f. sp. <i>spinaciae</i>	F-1-1	0	0	1.2
<i>F. oxysporum</i> f. sp. <i>cepae</i>	F-1-4	0	12.0	17.8
<i>F. roseum</i>	MS-1	0	0	5.8
<i>F. solani</i> var. <i>coeruleum</i>	WP-1-2	0	0	14.3
<i>F. ventricosum</i>	MS-5-3	0	0	11.1
<i>Phytophthora cactorum</i>	P-6-4	25.5	63.8	89.7
<i>P. capsici</i>	P-9-2	15.2	37.5	63.5
<i>P. castaneae</i>	P-2-2	12.0	40.6	74.5
<i>P. citrophthora</i>	P-4-7	23.4	48.5	85.1
<i>P. infestans</i>	MAFF 03-05586	15.5	58.8	80.5
<i>P. infestans</i>	MAFF 03-05587	11.1	29.4	83.2
<i>P. melonis</i>	P-11-2	16.8	50.0	93.0
<i>Pyricularia oryzae</i>	Pr-1-5	0	3.8	28.8
<i>Pythium spinosum</i>	Py-8-11	1.0	33.3	66.4
<i>P. ultimum</i>	Py-9-1	11.0	15.2	44.2
<i>Rhizoctonia solani</i>	MAFF 03-05250	0	8.5	52.9
<i>R. solani</i>	R-1-2	0	11.8	17.6
<i>R. solani</i>	R-1-4	0	9.4	24.2

a) MAFF: Ministry of Agriculture, Forestry and Fisheries of Japan.

b) Concentration of prodigiosin.

reported previously (Fig. 2)^{2,4)}.

Antibiotic activity of prodigiosin against *P. capsici*

The antibiotic activity of purified prodigiosin against *P. capsici* P-9-2 was examined. The germination of cystospores and zoosporangia of *P. capsici* P-9-2 was completely inhibited by 25.5 $\mu\text{g/ml}$ and 12.5 $\mu\text{g/ml}$ of prodigiosin, respectively, and even at concentrations as low as 3.1 $\mu\text{g/ml}$ and 1.6 $\mu\text{g/ml}$, prodigiosin partially inhibited the germination of cystospores and zoosporangia, respectively (Table 2). A potent antibiotic activity against *P. capsici* P-9-2 was thus confirmed.

Antimicrobial spectrum of prodigiosin

Antifungal activity of prodigiosin against plant pathogenic fungi including 20 species (species, f.sp., var.) in 6 genera was tested. As shown in Table 3, several fungi such as *C. miyabeanus*, *P. cactorum*, *P. infestans* (MAFF 03-05586) and *P. melonis* were most sensitive and their growth was inhibited by 50% at concentrations of 0.1 to 1.0 $\mu\text{g/ml}$. At the concentrations of 1.0 to 10 $\mu\text{g/ml}$, prodigiosin inhibited the growth of *P. capsici*, *P. cas-*

Table 4. Antibacterial activity of prodigiosin produced by *Serratia marcescens* F-1-1 against plant pathogenic bacteria

Bacterial strain ^{a)}	MAC ^{b)} of prodigiosin ($\mu\text{g/ml}$)
<i>Acidovorax avenae</i>	NIAES 1141 50.0
<i>Agrobacterium tumefaciens</i>	NIAES 1001 50.0
<i>Bacillus subtilis</i>	NIAES 1002 12.5
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	NIAES 1037 6.3
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	MAFF 03-01618 25.0
<i>E. carotovora</i> subsp. <i>carotovora</i>	BNS 2-2 25.0
<i>E. carotovora</i> subsp. <i>carotovora</i>	SR 79-33-3 12.5
<i>E. herbicola</i>	E1-3-1 50.0
<i>Pseudomonas cichorii</i>	NIAES 1368 100.0<
<i>P. fluorescens</i>	IAM 1201 100.0<
<i>P. gladioli</i> pv. <i>gladioli</i>	NIAES 1066 100.0<
<i>P. glumae</i>	H-8303 100.0<
<i>P. marginalis</i> pv. <i>marginalis</i>	NIAES 1078 100.0<
<i>P. syringae</i> pv. <i>lachrymans</i>	NIAES 1415 100.0<
<i>P. syringae</i> pv. <i>mori</i>	NIAES 1020 100.0<
<i>P. syringae</i> pv. <i>phaseolicola</i>	NIAES 1023 100.0<
<i>P. syringae</i> pv. <i>pisi</i>	NIAES 1208 100.0<
<i>Ralstonia solanacearum</i>	Ps 3-1 100.0<
<i>R. solanacearum</i>	MAFF 03-01556 100.0<
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	NIAES 1076 25.0
<i>X. campestris</i> pv. <i>carotae</i>	NIAES 1101 50.0
<i>X. campestris</i> pv. <i>oryzae</i>	NIAES 1086 25.5

a) NIAES: National Inst. of Agro-Environmental Sciences.

MAFF: Ministry of Agriculture, Forestry and Fisheries of Japan.

b) MAC: maximal allowable concentration.

taneae, *P. citrophthora*, *P. infestans* (MAFF 03-05587), *Pythium spinosum* and *Rhizoctonia solani* (MAFF 03-05250) by 50%. The growth of all the tested fungi was inhibited at a concentration of 10 µg/ml, among which some were inhibited by more than 80%.

Next, the antibacterial activity of prodigiosin against plant pathogenic bacteria including 19 species (sp., subsp., pv.) in 6 genera and *Bacillus subtilis* was investigated. As shown in Table 4, *C. michiganensis* subsp. *michiganensis* was most sensitive to prodigiosin with a value of 6.3 µg/ml for the maximal allowable concentration (MAC). *Erwinia carotovora* subsp. *carotovora*, *Xanthomonas campestris* pathovars and *Bacillus subtilis* were also sensitive, whereas, pseudomonads and *Ralstonia solanacearum* were resistant to this chemical.

DISCUSSION

In our previous study⁹⁾, it was suggested that the antagonism of *S. marcescens* F-1-1 might be due to the production of red pigments. In the present study, an antibiotic red pigment was purified and identified as prodigiosin, a known compound. Purified prodigiosin showed a strong antibiotic activity against *P. capsici* P-9-2 *in vitro*. Thus, it was suggested that the red pigment, prodigiosin plays an important role in biocontrol of damping-off of cucumber caused by *S. marcescens*.

Several antibiotics such as phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (Phl), pyoluteorin, and pyrrolnitrin have been reported to be involved in the mechanism of plant pathogen suppression^{6,8,11)}. A chitinase produced from *S. marcescens* has also been known to be involved in the biocontrol mechanism of several plant diseases as mentioned in another section. However, it had not been reported that prodigiosin was responsible for the biocontrol of plant pathogens.

Prodigiosin and homologue pigments (prodiginines) have been known to be produced by some prokaryotes, including *S. marcescens*, *Pseudomonas magnesiiorubra*, *Vibrio psychroerythrus*, *Actinomyces* (*Nocardia*) *pellestieri*, *Streptomyces longispororuber* and *Streptovorticillium* sp.¹²⁾. It had been reported that prodigiosin was an antimicrobial agent against clinical pathogenic microorganisms³⁾, but not against plant pathogens. In the present study, many species of plant pathogenic fungi in addition to *P. capsici* were found to be sensitive to prodigiosin. Especially several species of fungi such as *C. miyabeanus*, *P. cactorum*, *P. infestans* and *P. melonis* were more sensitive than *P. capsici* to prodigiosin. And also prodigiosin displayed a strong antibiotic activity against *C. michiganensis* subsp. *michiganensis*. The pigment has been reported to be more active against Gram-positive than Gram-negative pathogenic bacteria⁵⁾. In the present study, two Gram-positive bacteria, *C. michiganensis* subsp. *michiganensis* and *Bacillus subtilis*, were most sensitive among the tested bacteria. These results suggest that prodigiosin

might be effective for the suppression of these fungal or bacterial plant diseases, and that *S. marcescens* which produces prodigiosin might be used as a biocontrol agent in these diseases.

S. marcescens has been known to be a bacterium capable of inducing an opportunistic infection in human. Therefore, the introduction of a large quantity of the bacterium as a biocontrol agent into the natural environment may be hazardous. To alleviate this shortcoming, useful transgenic biocontrol agents could be developed by introducing prodigiosin-biosynthesis genes into harmless rhizobacteria with a fixative ability to plant roots.

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和 文 摘 要

岡本 博・佐藤善司・佐藤 守・小磯邦子・岩崎成夫・伊阪実人：キュウリ灰色疫病菌の生物的防除用細菌 *Serratia marcescens* F-1-1 株の抗菌性赤色色素の同定および同物質の各種植物病原菌に対する抗菌活性

赤色集落を形成する *Serratia marcescens* F-1-1 株は、キュウリ灰色疫病菌 *Phytophthora capsici* P-9-2 に対し強い拮抗作用を示すが、その拮抗性は、本菌株の赤色色素産生能と関連する。そこで、この拮抗性赤色色素の純化およびその同定を行った。水不溶性の赤色色素は、平板培養からかきとった菌体からエタノールで、ついでクロロフォルムで抽出され、シリカゲルカラムクロマトグラフィーにより純化した。*P. capsici* P-9-2 に対する抗菌活性を指標にして、抗菌活性を有する色素を単離した。この物質は、NMR、マススペクトル等の解析から、既知物質 prodigiosin と同定された。*Cochliobolus miyabeanus*, *Pythium spinosum*, *P. ultimum* 等の植物病原菌、および *Clavivacter michiganensis* subsp. *michiganensis*, *Erwinia carotovora* subsp. *carotovora* 等の植物病原細菌に対しても抗菌活性を示した。これらの結果から、*S. marcescens* F-1-1 株がキュウリ灰色疫病のみならず、他の多くの植物病害の生物防除に利用できる可能性が示唆された。