

トマト葉面から分離されたSerratia marcescens B2株によるBotrytis属菌の生育阻害

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Growth Inhibition of *Botrytis* spp. by *Serratia marcescens* B2 Isolated from Tomato Phylloplane

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Abstract

Serratia marcescens (isolate B2 from tomato phylloplane) inhibited markedly the growth of *Botrytis allii*, *B. byssoides*, *B. cinerea*, *B. fabae* and *B. tulipae* on LB agar medium containing colloidal chitin. The conidial germination and hyphal growth of *B. cinerea* and *B. fabae* were also suppressed by culture filtrate of the bacterium. The inhibitory effect of the bacterium correlated with its chitinolytic activity. On leaf disks of broad bean, the bacterium could control chocolate spot disease caused by *B. fabae*. These results indicate the possibility of using *S. marcescens* as a biocontrol agent for *Botrytis* spp.

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Key words : *Serratia marcescens*, chitinolytic enzyme, *Botrytis* spp., biocontrol.

INTRODUCTION

Botrytis species affect the vegetative and flowering structures of many crop plants throughout the growing season, often causing losses in crop quality and yield. The diseases by *Botrytis* spp. have been mainly controlled by chemical methods, because no resistant cultivars are available. Since 1971, many isolates of *Botrytis* spp. resistant to systemic fungicides have developed, which has made it difficult to control these diseases²⁾. Therefore alternative control measures for these diseases are urgently needed.

Biological control of many plant diseases has been attempted by a wide variety of antagonistic microbes^{1,4,5,9,10,14,23)}. For *Botrytis* spp., *Trichoderma harzianum* is well-known as a biocontrol agent against several fruit diseases caused by *B. cinerea*, e.g. grapevine grey-mold, strawberry grey-mold and apple dry eye rot^{6,21,22)}. This fungus is a mycoparasite and known to produce extracellular β -1,3-glucanase and chitinase which play an important role in degrading the host fungal cell wall⁸⁾. Recently, *Serratia marcescens*, a bacterial habitant in water, soil and phylloplane and an efficient producer of extracellular chitinase^{7,13)}, was shown to control effectively *Phytophthora capsici*, *Sclerotium rolfsii* and *Rhizoctonia solani*, all soil-borne fungal pathogens^{15,16,17)}. Transformed *Pseudomonas fluorescens* or *Escherichia coli* expressing *S. marcescens* chitinase gene also suppressed the growth of *Fusarium oxysporum* f. sp. *redolens*, *S. rolfsii* or *R. solani*^{18,20)}. Therefore it seemed worth while testing the possibility of using *S. marcescens* for the control of *Botrytis* spp.

We report here that a highly chitinolytic *S. marcescens* isolate B2 from tomato phylloplane effectively inhibited the growth of several *Botrytis* spp. *in vitro* and of *B. fabae* on leaf disks of broad bean.

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MATERIALS AND METHODS

Bacteria and fungi. One highly chitinolytic isolate of a bacterium producing red pigment was obtained from tomato phylloplane, identified as *Serratia marcescens* by the standard bacteriological tests using its type strain ATCC 13880 for comparison, and designated as isolate B2. This isolate was used for antifungal assay. The bacterium was routinely cultured at 28°C on LB chitin agar (LBCA) medium, composed of 10 g tryptone, 5 g NaCl, 5 g yeast extract, 1.8 g colloidal chitin and 15 g Bacto-agar (Difco) in 1 liter of distilled water. The colloidal chitin was made by the HCl-method from crab shells (Sigma C-3387) as in Shimahara and Takiguchi¹⁹.

For antifungal assay, *Botrytis allii* Munn (IFO No. 9430), *B. byssoidea* Walker (IFO No. 9431), *B. fabae* Sardina (IFO No. 5895) and *B. tulipae* (Libert) Hopkins (IFO No. 5896) were used (supplied by the Institute for Fermentation, Osaka (IFO)), as well as three strains of *B. cinerea* Persoon, a dicarboximide-resistant strain (CAES-2, from Chiba Prefectural Agricultural Experiment Station), a benomyl-resistant strain (IHES-3, from Ibaraki Horticultural Experimental Station) and a fungicide-sensitive strain (IPCR-1, from Institute of Physical and Chemical Research, Saitama). All fungi were preserved on potato sucrose agar (PSA) medium at -40°C.

In vitro inhibition assay for *Botrytis* spp. After incubation on PSA medium at 20°C for 4 days, a 5 mm-diameter mycelial disk of each *Botrytis* sp. was placed singly on the center of a LBCA or potato sucrose chitin agar (PSCA) medium in a plastic Petri dish (90 mm diam., 15 mm high). After incubation at 20°C for 24 hr in the dark, four paper disks (7 mm in diam.) inoculated with 50 µl suspension (ca. 10⁸ cfu/ml) of *S. marcescens* isolate B2 were placed on the medium 4 cm distant from the growing front of the each fungus. Three replicate dishes were used for each assay. Mycelial growth was assessed after incubation at 20°C in the dark for 7 days.

For observing the inhibitory effects of the bacterium on fungal germination and growth, four 10 µl droplets of each conidial suspension (ca. 5×10⁴ cfu/ml) of *B. cinerea* IPCR-1, CAES-4 and IHES-3, and of *B. fabae* were placed on clear zones formed around the bacterial colonies and incubated at 20°C in the dark. After 24 and 48 hr of incubation, the conidial germination and the hyphal growth were observed under a light microscope.

Chitinolytic and antifungal assays of the culture filtrate. Chitinolytic activity of the filtrate of *S. marcescens* isolate B2 was assayed as follows. The bacterium was incubated in liquid LB chitin medium at 28°C on a reciprocal shaker (125 strokes/min) for 24 hr. The culture was centrifuged at 10,000 rpm for 10 min and the supernatant was passed first through a glassfiber filter (GF75, Advantec) and then through a sterilized nitrocellulose membrane filter (Advantec; pore size, 0.45 µm). Aliquots were assayed as such, after heating for 2 hr at 50°C and after autoclaving for 20 min at 121°C. The chitinolytic activity was assayed using *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (pNP-NAG1), *p*-nitrophenyl-β-D-*N,N'*-diacetylchitobioside (pNP-NAG2) and *p*-nitrophenyl-β-D-*N,N',N''*-triacetylchitobioside (pNP-NAG3) as substrates. The reaction mixtures containing 100 µl of the culture filtrate diluted 10 times and 10 µg substrate in 100 µl MacIlvaine buffer, pH 6.0, were incubated at 37°C for 60 min. The reaction was stopped with 100 µl 1 M Na₂CO₃ and absorbance of released *p*-nitrophenol (pNP) at 405 nm was measured by a spectrophotometer.

The antifungal activity of the culture filtrates was assayed as follows. Three 10 µl droplets of each conidial suspension of *B. cinerea* and *B. fabae* (ca. 1×10⁴ cfu/ml) in 2.5% glucose solution were aseptically placed on a glass slide in a Petri dish. After incubation for 24 hr at 20°C in the dark, 20 µl of the undiluted culture filtrate was added on the growing fungi. The hyphal growth of the fungi was observed under a light microscope after 24 hr of incubation.

Leaf disk inhibition assay for *B. fabae*. *S. marcescens* isolate B2 was tested for the ability to suppress *B. fabae* on leaf disks of broad bean plant (*Vicia faba* cv. Sosei). The seeds were sown in sterile soil in pots and grown for about one month at 20°C and 60% relative humidity. Disks of 15 mm diameter were cut with a cork borer from the young leaves and placed on moistened filter paper in sterile Petri dishes. Inocula were prepared by the following methods: 1) one ml of conidial suspension

(1×10^5 cfu/ml) of *B. fabae* in distilled water was mixed with 3 ml of bacterial suspension (ca. 10^8 cfu/ml) in liquid LB chitin medium, 2) with 3 ml of undiluted culture filtrate of the bacterium, and 3) with 3 ml of distilled water. The broad bean leaf disks were inoculated with one $10 \mu\text{l}$ droplet per leaf disk. After inoculation, the disks were incubated at $20 \pm 2^\circ\text{C}$ in the dark. Symptoms of the leaf disks were examined daily after inoculation and the disease incidence rate (%) was calculated by the method of Ko *et al.*¹²⁾

RESULTS

Growth inhibition of Botrytis spp. in vitro

Chitinolytic enzyme production of *S. marcescens* B2 was detected by the formation of clear zones around the colony in LBCA medium (Fig. 1a, arrows). The clear zone indicated where the colloidal chitin had been hydrolyzed by the secreted chitinolytic enzymes. The clear zone of isolate B2 was markedly larger than that of ATCC 13880 (data not shown). Such a clear zone was not formed when the

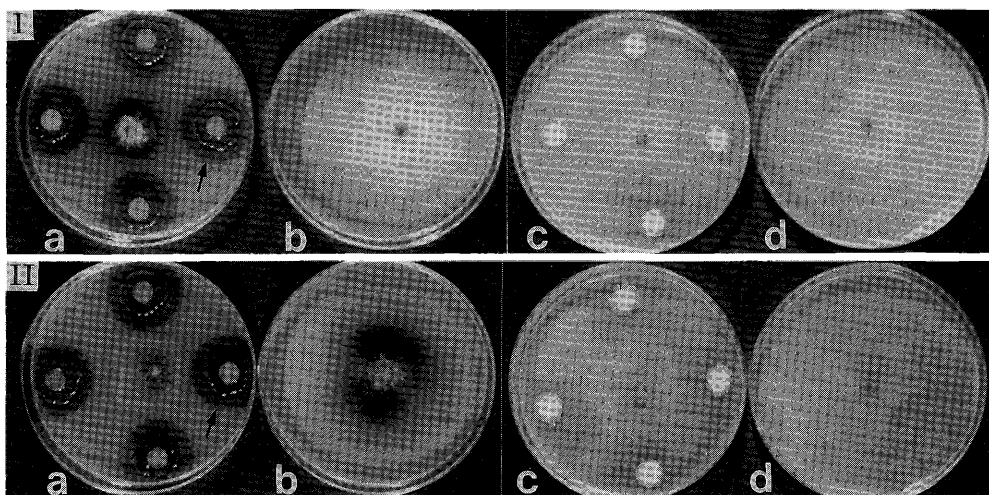


Fig. 1. Radial growth of *Botrytis cinerea* IPCR-1 (I) and *Botrytis fabae* (II) cultured with *Serratia marcescens* B2 (a and c) or without the bacterium (b and d) after 5 days of incubation on LBCA (a and b) and PSCA (c and d) media. The bacterium produced red pigment and formed clear zones (arrows) on LBCA (a), but not on PSCA (c).

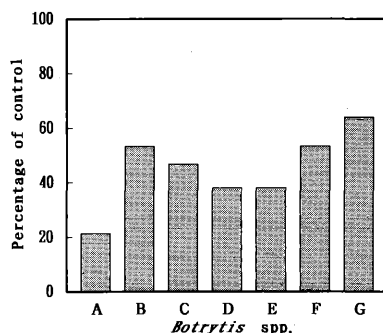


Fig. 2. Radial growth of *Botrytis* spp. co-cultured with *Serratia marcescens* B2 on LBCA media. A, *B. cinerea* IPCR-1; B, *B. cinerea* CAES-4; C, *B. cinerea* IHES-3; D, *B. fabae*; E, *B. byssoides*; F, *B. allii*; G, *B. tulipae*.

Table 1. Conidial germination and swelling of *Botrytis cinerea* isolates and *Botrytis fabae* on clear zones formed around *Serratia marcescens* B2 colonies on LB chitin agar medium^{a)}

<i>Botrytis</i> spp.	Clear zone		Control	
	Germination (%)	Swelling (%)	Germination (%)	Swelling (%)
<i>B. cinerea</i>				
IPCR-1	0.2	14.3	94.5	0
CAES-4	0	21.8	89.5	0
IHES-3	0	14.2	25.2	0
<i>B. fabae</i>	0	15.7	88.7	0

a) The conidia were inoculated on clear zones formed around 5 days-old colonies of *Serratia marcescens* B2 on the LB chitin agar plates. For the control, the conidia were inoculated on the same plates without the bacterium. Germination and swelling rates of 500 conidia were examined after 24 hr and 48 hr of incubation at 20°C, respectively. Data are means of three replicates.

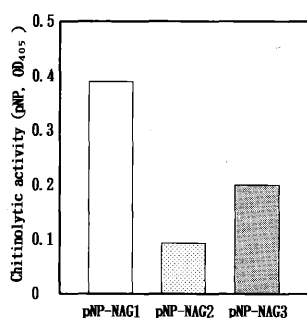


Fig. 3. Chitinolytic activity of the culture filtrate of *Serratia marcescens* B2. The activity was measured by optical density (405 nm) of *p*-nitrophenol (pNP) released from pNP-NAG1 (□), pNP-NAG2 (▨) and pNP-NAG3 (▩) after 1 hr of incubation at 37°C.

Table 2. Swelling of the hyphal tips of *Botrytis cinerea* isolates and *Botrytis fabae* by the culture filtrates of *Serratia marcescens* B2^{a)}

Filtrate	Chitinolytic activity (pNP, OD ₄₀₅)	Swelling of the hyphal tips (%)			
		<i>B. cinerea</i>			<i>B. fabae</i>
		IPCR-1	CAES-4	IHES-3	
CF	1.540	41.0	17.5	66.7	32.5
H-CF	0.335	9.0	11.7	10.1	2.1
A-CF	0.148	11.7	4.1	14.2	7.0
Control	0.040	9.7	0.3	7.8	2.5

a) Eighteen hr-old mycelia of the fungi were treated with *S. marcescens* B2 culture filtrate (CF), heated CF (H-CF; 50°C, 2 hr), autoclaved CF (A-CF), or LB chitin liquid medium (control). Swelling rates of 200 hyphal tips were examined under a microscope after 24 hr of incubation at 20°C. The chitinolytic activities of the filtrates were measured by the optical densities (405 nm) of *p*-nitrophenol (pNP) released from pNP-NAG1 after 1 hr of incubation at 37°C. Data are means of three replicates.

bacterium was cultured on PSCA medium (Fig. 1c). In the presence of 24 hr-old colonies of isolate B2 on LBCA, radial growth of all the tested *Botrytis* spp., especially *B. cinerea* IPCR-1, was much less than in the absence of bacterial colonies (Figs. 1 and 2). The growth inhibition by isolate B2 was not observed on PSCA (Fig. 1c).

Light microscopy showed that conidial germination of *B. cinerea* IPCR-1, CAES-4 and IHES-3, and of *B. fabae* was completely inhibited on the clear zones around isolate B2 colonies in LBCA (Table 1). Swelling of these fungal conidia within 48 hr was observed frequently (Table 1). On PSCA, no such

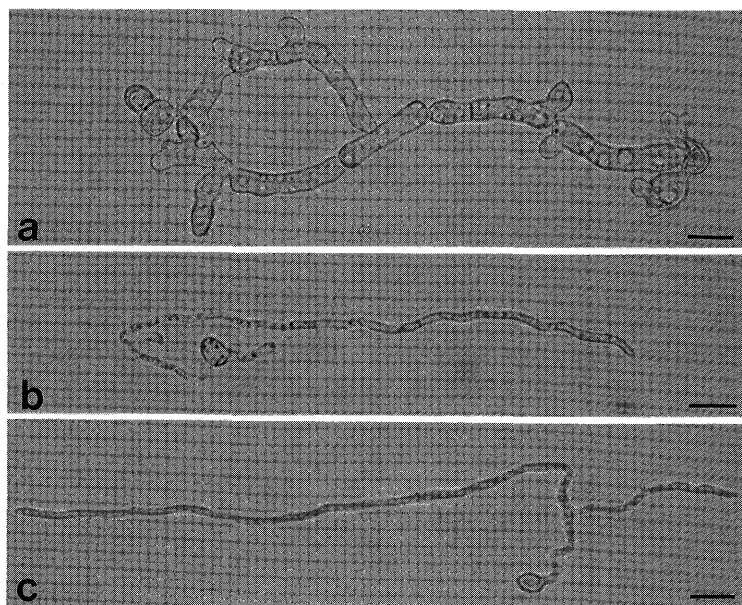


Fig. 4. Effects of the culture filtrate of *Serratia marcescens* B2 on the growing mycelia of *Botrytis cinerea* IPCR-1. Light micrographs showing swelling of whole hyphae caused by the crude filtrate (a), no swelling by the heated filtrate (b) and by the autoclaved filtrate (c). Scales: 20 μ m.

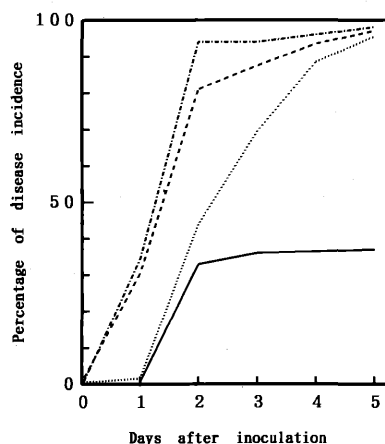


Fig. 5. Effects of *Serratia marcescens* B2 and its culture filtrate on *Botrytis fabae*-induced chocolate spot disease of broad bean. —, *S. marcescens* B2; ·····, filtrate; ----, LB chitin; -·-·-, distilled water. Thirty leaf disks per specimen were inoculated with 10 μ l of the conidial suspension (2.5×10^4 cfu/ml) containing the bacterium (ca. 10^8 cfu/ml) or the cultural filtrate. The sizes of lesions were measured, and disease incidence rates (%) were calculated by the method of Ko *et al.*¹¹⁾

abnormal conidia were seen around B2 colony (data not shown).

Chitinolytic and antifungal activities of the culture filtrate

After 24 hr of liquid shake culture, culture filtrate of *S. marcescens* B2 released pNP from pNP-NAG1, pNP-NAG2 and pNP-NAG3 (Fig. 3). The chitinolytic activity was highest with pNP-NAG1. Interestingly, activity with pNP-NAG3 was higher than with pNP-NAG2. These results indicate the presence of both exo-chitinase and endo-chitinase activities, since exo-chitinases (β -*N*-acetylglucosaminidases) can release *N*-acetylglucosamine from the non-reducing end of the chitin chain

quite slowly and that endo-chitinases can cut the chitin oligomers longer than trimer quite rapidly. After heating or autoclaving, the filtrate showed very low chitinolytic activity (Table 2).

When *S. marcescens* B2 culture filtrate was inoculated on 24 hr-old mycelia of *B. cinerea* IPCR-1, CAES-4 and IHES-3, and on mycelia of *B. fabae*, 20-70% of the fungal hyphae became swollen, especially at the hyphal tips, within 24 hr of incubation (Table 2 and Fig. 4). When the heated or autoclaved filtrates were added on the mycelia, the frequency of the hyphal swelling was much lower, corresponding to the lower chitinolytic activity of the filtrates (Table 2).

Suppression of chocolate spot disease on broad bean leaf disks

Inoculation of broad bean leaf disks with *B. fabae* conidia mixed with *S. marcescens* isolate B2 resulted in less than 40% disease incidence for up to 5 days after inoculation, compared to ca. 100% in control with *B. fabae* only (Fig. 5). When the conidia were inoculated together with bacterial culture filtrate, the disease incidence was also ca. 40% for 2 days after inoculation, but, later increased close to 100%.

DISCUSSION

Serratia marcescens B2 isolated from tomato phylloplane inhibited the growth of *Botrytis* spp. *in vitro* and the chocolate spot disease caused by *B. fabae* on leaf disks of broad bean, indicating a possibility for biocontrol. Chitinolytic activity of the bacterial culture filtrate and growth inhibition assay of the fungi with microscopy showed that the inhibitory effect of the bacterium was correlated with the chitinolytic activity. This agreed with the result that the inhibitory effect was observed on the clear zone of LBCA medium where the bacterium secreted the chitinase but not on PSCA medium where the bacterial chitinase was not produced.

These results are compatible with the previous reports on the antifungal action of *S. marcescens* and its chitinase toward other fungi^{11,17,18,20}. Specifically, Ordentlich *et al.*¹⁷ demonstrated that culture filtrate of *S. marcescens* containing chitinase caused swift swelling of the hyphal tips of *Sclerotium rolfsii*. They concluded that the inducible extracellular chitinase produced by the bacterium attacked the hyphal cell wall, probably causing the release of fungal β -1,3-glucanase, which accelerated the complete degradation of hyphae. More direct evidences demonstrating the degradation activity of the *S. marcescens* chitinases have been obtained by using transformed *E. coli* and *P. fluorescens* expressing the cloned chitinase genes^{11,18,20}.

In preliminary tests the growth of *Phytophthora capsici* and *Pythium aphanidermatum*, both Oomycetes fungi containing little chitin³, was also suppressed by *S. marcescens* B2 *in vitro* (data not shown). Okamoto *et al.*¹⁵ also recently reported that *S. marcescens* inhibited *in vitro* cystospore germination of *P. capsici*. Therefore there may be other additional mechanisms involved in the inhibitory activity of *S. marcescens*, e.g. antibiotic pigment production, as shown by Okamoto *et al.*¹⁵ However, as far as *Botrytis* spp. are concerned, our data are compatible with the role of the bacterial chitinase as the main suppressing factor for growth in other fungi.

The phylloplane is a very unfavorable environment to introduce antagonistic bacteria into, so that the introduced bacteria are difficult to survive and multiply under the harsh and dry conditions in the field^{4,5,14}. However, in humid greenhouses, it may be possible to introduce *S. marcescens* successfully so as to maintain high numbers of cells sufficient for biocontrol. This is especially so for our isolate B2, because it was obtained from tomato phylloplane, and *Serratia* spp. are known to be often associated with many species of vegetable crops, including tomato, lettuce and broccoli, and to survive well on such plants⁷. In a modification of this strategy, another species of a transformed phylloplane bacterium expressing *S. marcescens* chitinase gene could be used for biocontrol of *Botrytis* spp., as already shown for some soil-borne fungal pathogens²⁰.

Pot tests in the greenhouse on the biocontrol ability of *S. marcescens* B2 against *Botrytis* spp. on the phylloplane are now in progress.

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和 文 摘 要

阿久津克己・平田暁子・山本美智子・平八重一之・奥山 哲・日比忠明：トマト葉面から分離された *Serratia marcescens* B2 株による *Botrytis* 属菌の生育阻害

トマト葉から分離されたキチナーゼ活性細菌・*Serratia marcescens* B2 株は、コロイダル・キチンを含む LB 平板

培地で高いキチナーゼ活性を示し、5種7系統の *Botrytis* 属菌 (*B. cinerea* IPCR-1, CAES-4, IHES-3, *B. allii* IFO No. 9430, *B. byssoidea* IFO No. 9431, *B. fabae* IFO No. 5895, *B. tulipae* IFO No. 5896) の生育を著しく阻害した。*S. marcescens* B2株の培養ろ液にはキチナーゼ活性が検出され、このろ液で処理した *B. cinerea* IPCR-1, CAES-4, IHES-3 および *B. fabae* の胞子は、100%発芽せず、膨潤した形状の異常な胞子が高頻度で観察された。また、菌糸に処理した場合には、短時間で菌糸の先端部が肥大し、処理24時間後には菌糸全体が膨潤するとともに、一部で細胞膜の突出が観察された。一方、熱処理した培養ろ液では、キチナーゼ活性が著しく低下し、供試菌に対する阻害効果はみられなかった。また、本細菌を *B. fabae* の胞子懸濁液と混合し、ソラマメ葉片に接種したところ、60%以上の発病阻止効果が認められた。以上から、*S. marcescens* B2株の *Botrytis* 属菌に対する生育阻害作用は、主として、本細菌の産生するキチナーゼによることが明らかになるとともに、本細菌を *Botrytis* 属菌のバイオコントロールに利用し得る可能性が示唆された。