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Isolation and Evaluation of an Antiviral Producing *Serratia* spp. Strain Gsm01 against *Cucumber mosaic virus* in Korea

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Abstract : An Antiviral producing bacterial strain was isolated from ginseng root environment in Hongcheon, Kangwon province of Republic of Korea. Identification of this bacterial strain was performed by physiological and biochemical tests along with 16S rRNA analyses. The results revealed that the bacterium was closer to genus *Serratia*, which was named as Gsm01. The strain was grown in Mannitol-Glutamate-Yeast (MGY) broth for 48 h. The culture was centrifuged and the filtrate obtained was tested for its ability to control *Cucumber mosaic virus* strain Y (CMV-Y) in greenhouse and field experiments. In the green house experiments, CF was evaluated for its ability to protect local host, *Chenopodium amaranticolor* and systemic host of CMV, *Nicotiana tabacum* cv. Xanthi-nc. It was found that, CF treatment reduced viral infection by 98% in local host; *C. amaranticolor*. The *N. tabacum* cv. Xanthi-nc plants treated with CF did not show visible viral symptoms 15 days post inoculation (dpi) and remained symptomless throughout the periods of the study. To evaluate effectiveness of CF under field conditions, experiment was carried out in a polyvinyl house. It was observed that, 52% plants were protected from viral diseases compared to non-treated plants, increasing the crop yield. This is the first report showing antiviral activity of a *Serratia* spp. against CMV. (Received October 23, 2006; accepted December 23, 2006)

Key words : *Cucumber mosaic virus* (CMV-Y), *Serratia* spp, culture filtrate (CF), *Chenopodium amaranticolor*.

Introduction

Viral diseases are major constraints in the production of many agricultural crops. Among them, CMV occurs in most countries of the world, and causes disease epidemics in many economically important crops (Gooding, 1991). It is transmitted primarily by aphids acquiring and transmitting CMV in less than one minute, which makes it a difficult pathogen to control by conventional methods. The conventional methods employed to reduce losses caused by CMV included, avoidance of source of infection, modified cultural practices, use of CMV resistant cultivars and use of transgenic plants. Besides these, there have been substantial efforts directed towards developing antiphytoviral compounds from various sources. A huge number of these compounds from plant sources have been known since long time, like Mirabilis antiviral protein (Kubo *et al.*, 1990), and Pokweek antiviral protein (Lee *et al.*, 1992). The issues related to isolation of these compounds on commercial scale however make them less attractive to use under field conditions. The studies on antiphytoviral substances of microbial sources date back since 1926, when first report on use of various bacteria inactivating tobacco mosaic virus was published (Mulvania, 1926). The antiviral substances of microbial

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origin are easy to produce and formulate compared to the plant sources. Recently a few studies showed systemic antiviral activity against CMV by bacteria or their culture filtrates. Raupach *et al.* (1996) showed the systemic control of CMV in cucumber and tomato by rhizosphere colonization of some bacteria by ISR mechanism. In Korea, Kim *et al.* (2004), used culture filtrates from *Acenetobacter* species KTB3, to control some viruses including CMV. Our group has previously reported use of a *Pseudomonas fluorescens* strain Gpf01 to control CMV-Y (Ipper *et al.*, 2005).

This paper describes the identification of Gsm01 strain isolated in Korea, based on biochemical characteristics and 16S rRNA analyses. In addition to that, it also elaborates on the antiviral activity from the culture filtrate (CF) of Gsm01 strain against CMV-Y under green house and field conditions.

Materials and Methods

Bacterial isolation and identification

Ginseng roots from a field at Hongcheon, Kangwon province, Republic of Korea, were collected and homogenized in demineralized water. The homogenate was serially diluted and plated onto Mannitol-Glutamate-Yeast (MGY) agar media (Keane *et al.*, 1970) followed by incubation at 28℃ overnight. Numerous colonies with different morphologies were picked from dilution plates. Each of them was assayed for antiviral activity. One colony, showing strong antiviral activity was selected and named as Gsm01. It was stored by three different preservation methods, 1) Storage of streaked MGY plates at 4~10℃ for short-term preservation and routine work; 2) Preservation at 70℃ using nutrient broth containing 20% glycerol; and 3) Freeze drying method using 10% skimmed milk for long-term preservation as described by Perry (1995).

Physiological and biochemical tests were performed to identify the bacterial strain. Carbon source utilization tests, enzyme production, and organic substrate utilization tests were carried out based on Bergey's manual of determinative bacteriology. 16S rRNA gene analysis was carried by PCR, performed with a DNA thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Total genomic DNA was isolated using a lysozyme dodecyl sulfate lysis procedure (Owen *et al.*, 1987), modified as described previously (Leach, *et al.*, 1990). The 16S rRNA gene was amplified using fD1 and rP2 primers (Weisburg *et al.*, 1991). The PCR product of the 16S rRNA was analyzed by electrophoretic separation in 1% (w/v) QA-agarose (Qbiogene, Irvine, CA), containing 0.5 $\mu g\ L^{-1}$ ethidium bromide. It was excised from the gel and purified using a QIAquick gel extraction kit (Qiagen Inc., Hilden, Germany). Purified DNA was ligated into the pGEM-T easy vector (Promega Co., USA). Plasmid containing the 16S rRNA region was then directly sequenced using an ALFred autocycle sequencing kit, with M13 forward and reverses primers. DNA homology searches were carried out with the NCBI databases, using the BLAST network service (Altschul *et al.*, 1990).

Antiviral bioassay

The *cucumber mosaic virus* strain Y was obtained from the virus culture collection of College of Forestry Sciences, Kangwon National University, Chuncheon, Korea. To prepare virus inoculum, 1 g systematically infected *N. tabacum* cv. Xanthi-nc leaves were finely ground in 40 ml phosphate buffer (0.01 M, pH 7.0). The virus was mechanically inoculated into *N. tabacum cv.* Xanthi-nc by using carborundum-600 mesh as abrasive. It was maintained on the same host throughout the period of this study.

A single colony of Gsm01 strain was inoculated into 100 mL MGY broth and grown at 28℃ for 48 h in a shaking incubator at 175 rpm. It was centrifuged at 12,000 rpm for 10 min. The supernatant obtained was filtered through 0.45 μm bacterial proof filter. CF thus obtained was used for antiviral bioassay using half leaf method on local host of CMV, *C. amaranticolor* as previously described by Kubo *et al.* (1990). The upper right halves of the leaves were treated with the CF using brush and the upper left halves were treated with autoclaved MGY broth as control. After one hour, virus preparation was inoculated onto both the halves of the leaves. The plants were allowed to grow in a green house with 12~14 h daylight and 30℃ temperature. The local lesion numbers were counted after seven days.

Table 1. Physiological and biochemical characteristics of Gsm01 strain

Characteristics	Gsm01	*Serratia* spp.	*Erwinia* spp.	*Pantoea* spp.	*Pseudomonas* spp.	*Rhizobium* spp.
Gram Stain (24 h)						
Indole production						nd
Voges Proskauer	+	+	+	+	nd	nd
Hydrogen sulfide production	nd				nd	nd
Urea hydrolysis					nd	nd
Phenylalanine deaminase (24 h)				+	nd	
Lysin decarboxylase	+	+	nd		nd	
Arginine dihydrolase	+	+	nd		+	V
Motility	+	+	+	+	+	+
Gelatin hydrolysis, 22℃	+	+	+		+	nd
D-Glucose, acid production	+	+	+	+	+	nd
D-Glucose, gas production	+	V				nd
Acid production:						
L-Arabinose		+	V	+	nd	+
Cellobiose	nd	+		V	nd	+
Dulcitol					nd	+
myo-Inositol					+	+
Lactose	nd	+			nd	+
Maltose	+	+		+	nd	+
D-Mannitol	+	+		+	nd	
D-Mannose	+	+		+	nd	+
Melibiose		+			nd	
Raffinose	+	+			nd	+
L-Rhamnose				+	nd	nd
D-Sorbitol			V		nd	nd
Trehalose	+	+	+	+	nd	nd
D-Xylose		+		+	nd	+
Tartrate	+	V	nd		nd	
Nitrate reduction	+	+		+	+	nd
Deoxyribonuclease, 25℃	+	+			nd	nd
Catalase	+	+	+	+	nd	nd

+, 80% or more strains positive; -, 80% or more strains negative; nd, not determined; V, variable.

The percent control effect was calculated using the formula: (1-T/C) × 100, where, C is the number of local lesions on the control half leaves and T is the number of local lesions on treated half leaves. The experiment was repeated three times with six replicates.

Systemic inhibitory activity of the CF was elucidated by using *N. tabacum* cv. Xanthi-nc plants at 6~7 leaf stage. CF from Gsm01 was applied on the lower three leaves of the plants, followed by CMV-Y inoculation on the upper untreated leaf after 24 h.

Similarly, MGY broth was applied as control treatment. The experiment was repeated three times, with five replicates.

Field tests

Field study was conducted at Kangwon National University experimental field in Chuncheon, during spring, 2006. The pepper plants (*Capsicum annum* L.) 'Nok Kwang' were grown on raised beds, mulched by polyethylene cover with drip irrigation lines underneath reaching all plants in the polyvinyl house. The pepper plants were planted at 50 cm spacing. The experimental design was a completely randomized design with two treatments and three replications (seven plants per replication). The plants under treatment were sprayed with CF three times with an interval of 14 days in two successive treatments. The controls were kept untreated.

Table 2. 16S rRNA sequence similarity between Gsm01 strain and other bacteria

16S rRNA	Identity (%)
Serratia spp.	99
Pantoea spp.	97
Erwinia spp.	92
Pseudomonas spp.	83
Rhizobium spp.	76

ginseng roots in Hongcheon, Korea. The isolate was identified using physiological and biochemical tests and 16S rRNA gene analyses. The strain Gsm01 was Gram negative, motile and rod shaped. It showed, gelatin, lysine and arginine hydrolysis along with carbon source utilization, enzyme production and organic substances utilization pattern similar to the genus *Serratia* (Table 1). 16S rRNA gene sequence of Gsm01, obtained by PCR using fD1 and rP2 primers was compared with that available in the GenBank. It showed 99% similarity with *Serratia* spp., while for other bacteria; it ranged between 76 to 97% (Table 2). Based on biochemical and physiological characteristics and 16S rRNA gene analysis, it can be inferred that the Gsm01 strain belongs to genus *Serratia*.

Table 3. Effect of culture filtrate (CF) from Gsm01 on infectivity of CMV Y in local host, *C. amaranticolor*

Treatment	No. of local lesions / half leaf[a)]		Inhibition (%)
	Treated	Untreated	
CF[b)]	1	46	97.8
MGY media	99.6	103.2	3.4

[a)]Mean no. of local lesions from three experiments, with 6 leaves of C. amaranticolor under each treatment. Control effect was calculated using the formula: (1 T/C) × 100, where, C is the number of local lesions on the control half leaves and T is the number of local lesions on treated half leaves.
[b)]Gsm01 Culture was centrifuged and the supernatant obtained was filtered through 0.45 μm filter and used.

Table 4. Effect of spray treatment using CF, obtained from Gsm01, on disease incidence in pepper caused by mixed virus infection under field conditions

Treatment[a)]	Disease Incidence[b)] (%)				Inhibition[c)]
	I	II	III	Mean[d)]	(%)
CF	11.1	21.0	23.8	18.6	52.9
Control	42.9	29.6	46.0	39.5	-

[a)]Each treatment consisted of three replications (seven plants in each replicate).
[b)]Disease incidences were visually assessed on 15th day after the last application of CF. It was calculated as percentage of diseased plants in a replicate.
[c)]Mean of three replicates.
[d)]The inhibitory effect of CF treatment on viral infections.

The field trial started in mid May and ended in early July. No insecticides were sprayed throughout the field trials to allow insects to infect the plants with viral pathogens naturally. The disease incidences were recorded as number of plants survived the viral infection after fifteen days of the last treatment. The number and weight of the fruits were also recorded.

Results and Discussion

Identification of Gsm01

The Gsm01 strain was isolated from soil around

Antiviral effects of Gsm01 under greenhouse and field conditions

The antiviral CF from Gsm01 showed high inhibitory activity against CMV-Y. The CF treatment on local host of CMV, *C. amaranticolor* showed 98% inhibition of

Table 5. Effect of CF spray treatment on yield of pepper in the field experiment

Treatment[x]	Total no. of fruits (ea.)				Total weight of fruits (Kg)			
	I	II	III	Mean[y]	I	II	III	Mean[z]
CF	357	403	337	1097	4.52	4.14	3.48	12.14
Water	363	253	328	944	3.87	2.99	3.43	10.29

[x]Each treatment consisted of three replications (seven plants in each replicate).
[y]Total number of mature fruits were counted for each replicate and mean for three replicates was obtained on 15th day after the last application of CF.
[z]Total weight of mature fruits for each replicate and mean for three replicates was obtained on 15th day after the last application of CF.

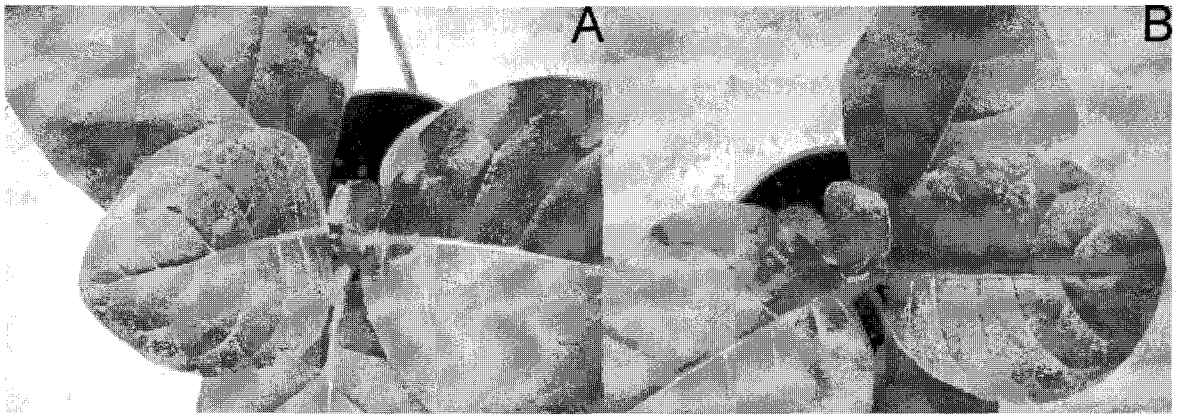

Fig. 1. Systemic control of CMV-Y in *N. tabacum* cv. Xanthi-nc 15 days post inoculation.
(A) Plants treated with CF obtained from *Serratia* spp. strain Gsm01 on the lower leaves of *N. tabacum* cv. Xanthi-nc. After 24 h, CMV-Y was inoculated onto the upper untreated leaves.
(B) MGY broth as control was treated on the lower leaves of *N. tabacum* cv. Xanthi-nc. After 24 h, CMV-Y was inoculated onto the upper untreated leaves.

the local lesions (Table 3). This indicates that the treatment of CF prevents CMV-Y infection substantially. When CF was used to elucidate the systemic effect of the antiviral component on *N. tabacum* cv. Xanthi-nc plants, it was observed that the MGY broth treated control plants showed visible CMV symptoms 15 dpi, where as the CF treated plants did not show viral symptoms (Fig. 1). This shows that the plants were protected systemically against infection by CMV-Y. The media used as control had no role in antiviral activity of the CF.

In the field, pepper plants were observed to have mixed viral infections. The treatment with CF provided effective protection against viral diseases with 52.9% plants protected (Table 4). The number of pepper fruits and their weight was higher in CF treated plants than that of non treated controls (Table 5). This indicates that the CF treatment protects plants from viral infections and help to increase the yield of the produce.

In all the above experiments, no damage to the host plants was observed due to CF treatment. Thus, CF can be characterized as non toxic antiviral agent efficient in combating CMV-Y infections and can be used as a biological control agent to combat viral diseases.

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한국에서 CMV에 항바이러스 효과를 나타내는 *Serratia* spp. GSm01 균주의 분리 동정 및 효과 검정

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요약 : 항바이러스 효과를 나타내는 세균은 강원도 홍천 인삼재배지의 수삼으로부터 분리하였다. 분리세균은 생리 생화학 테스트와 16s rRNA 유전자 분석을 통해 동정한 결과, *Serratia* 속으로 동정 되어져 본 세균을 Gsm01으로 명명하였다. Gsm01 균주를 MGY 액체배지에 증식시켜 배양 여액을 0.45 μm filter에 통과시킨 Culture filtrate (CF)를 명아주 (*Chenopodium amaranticolor*)에 반엽법으로 처리한 결과, 오이모자이크 바이러스 (CMV)에 대한 억제율이 98%로 매우 높은 것을 확인하였다. 또한 전신유도저항성을 확인하기 위하여 담배 (*Nicotiana tabacum* cv. Xanthi-nc)의 하엽에 CF와 CMV-Y를 처리하였을 때, CF를 처리한 식물에서 접종 15일 후까지 바이러스 증상이 관찰되지 않았다. 고추의 포장시험에서 무처리 식물과 비교해 보았을 때 바이러스 증상은 52.9% 감소한 것으로 보아 포장 시험에서 CF를 처리한 농작물의 산출량이 무처리 식물에 비해 14% 증가함을 확인 할 수 있었다. 한편, Gsm01 균주의 CF는 고추에 어떠한 약해도 나타내지 않아 매우 안전한 것으로 판단되었다.

색인어 : 생물학적 방제, 항식물바이러스, *Cucumber mosaic virus* (CMV-Y), *Serratia* spp, *Chenopodium amaranticolor*

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