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Antiviral Effects of the Culture Filtrate from *Serratia marcescens* Gsm01, against *Cucumber mosaic virus* (CMV)

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The potential antiviral effects of the culture filtrates (CF) from *Serratia marcescens* strain Gsm01 against yellow strain of *Cucumber mosaic virus* (CMV-Y) were investigated. The culture filtrate of *S. marcescens* strain Gsm01 applied on *Chenopodium amaranticolor* showed high inhibitory activity, likewise no necrosis appeared when applied on the tobacco plants 2 days before CMV-Y inoculation. When plants were challenge inoculated with CMV-Y for eighteen days, the disease incidence in plants with culture filtrate of *S. marcescens* Gsm01 did not exceed 59%, whereas 100% of control plants were severely infected. The results of double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA), reverse transcriptase polymerase chain reaction (RT-PCR), dot blotting, and western blotting showed that culture filtrate treatment highly affected the accumulation of CMV-Y or its CP protein gene in the treated plant leaves. It was also observed that the culture filtrate had no RNase activity on genomic RNAs of CMV-Y, suggesting that culture filtrate may not contain ribosome inactivating proteins (RIPs) or proteins with RNase activity. These data shows that culture filtrate of *S. marcescens* strain Gsm01 seems to be a promising source of antiviral substance for the practical use.

Keywords : culture filtrate, *Cucumber mosaic virus*, *Serratia marcescens*

Diseases caused by *Cucumber mosaic virus* (CMV) (genus: *Cucumovirus*, family: *Bromoviridae*) are the most wide spread plant virus in the world with extensive host range affecting many important crops. It is difficult to control because of its extremely broad natural host range and the ability to be transmitted in a non-persistent manner (Palukaitis et al., 1992; Zitter, 1991).

Historically, cultural practices, introduction of natural resistance genes through breeding have been used as effective means to control viral disease, alternatively transgenic plants containing viral coat protein genes and replicated association have been developed and implemented (Fitchen and Beachy, 1993). Systemic resistance for viral infection can be induced in plants inoculated with certain bacteria or bacterial products, various plant derived materials, and also by some chemicals (Cho et al., 2009; Doubrava et al., 1988; Kessmann et al., 1994).

Number of plant species contains potent inhibitory substances to protect them against virus infection, and few of potent inhibitors in the plants have been isolated and characterized which includes ribosome inactivating proteins (RIPs), PAP from *Phytolacca americana*, MAP from *Mirabilis jalapa* and dianthins from *Dianthus caryophullus* (Barbieri et al., 1982; Kubo et al., 1990; Verma et al., 1995).

Recently, nonpathogenic microbes such as plant growth-pormoting rhizobacteria (PGPR) have been reported to induce local and systemic resistance. Bergstorm et al. (1982) showed that *Colletrotrichum orbiculare* or *Pseudomonas syringae* treated in cucumber plants inhibits CMV. Maurhofer et al. (1994) successfully studied the resistance effect of *P. fluorescens* on tobacco against Tobacco necrotic virus (TNV) and Cho et al. (2009) has demonstrated the antiviral activity of *P. flourescens* strain Gpf01 against CMV and identified the genetic locus related to antivirus production.

Serratia marcescens strain B2 has been used effectively to inhibit the growth of several phytopathogenic fungi not limited to *Pyricularia oryzae*, *Rhizoctonia solani* AG-4, *Fusarium oxysporum* f. sp. *cyclaminis* (Someya et al., 2000). The PGPR strains of *Serratia* spp., has been reported to mediate induced systemic resistance (ISR) in protection against *C. orbiculare*, *P. s.* pv. *lachrymans* and CMV (Liu et al., 1995; Raupach et al., 1996). It has also been used to protect *Arabidopsis thaliana* against CMV infection (Ryu et al., 2004).

The objective of this research was to determine whether the culture filtrate of *S. marcences* strain Gsm01 active against CMV-Y in both local and systematic host, extended to protection against challenge inoculation with a systemic

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virus, cucumber mosaic virus (CMV-Y). Here we demonstrate that *S. marcences* strain Gsm01 were highly effective, and the culture filtrate may be used to induce resistance in plants against yellow strain of CMV-Y. A preliminary account of a portion of this work has been published (Ipper et al., 2006).

Materials and Methods

Bacteria and virus. *S. marcescens* strain Gsm01 was isolated from ginseng rhizosphere, in a field at Kangwon Province, Republic of Korea. A single colony was inoculated into 100 ml Mannitol Glutamate Yeast (MGY) broth and grown at 28°C for 48 h in a shaking incubator. The culture supernatant was then filtered through a 0.45 μm filter. The filtrate obtained was used for the antiviral assay. CMV-Y virus used in all the experiments was originally obtained from the virus collection of college of Forestry Sciences, Kangwon National University. The virus was maintained in tobacco leaves. The CMV-Y inoculum used throughout the experiments consisted of leaf tissue ground in 0.01 M phosphate buffer, pH 7.0, and 2 mM EDTA (1 g tissue:10 ml buffer). All inoculation materials were chilled at 4°C prior to inoculation and maintained on ice during inoculation.

Antiviral bioassay. The virus was mechanically inoculated into *N. tabacum* cv. Xanthi-nc by using carborundum-600 mesh as abrasive and maintained in the same host through out the period of study. The supernatant of the *S. marcescens* strain Gsm01 was tested for antiviral bioassay on local host of CMV-Y, *Chenopodium amaranticolor* using half-leaf method as described by Kubo et al. (1990).

To define antiviral effect systemically, 10 plants of same size were selected. The leaves of each plant were marked in the same place and the culture filtrate of *S. marcescens* strain Gsm01 was sprayed with a manual atomizer on to the whole surface of basal leaves of five host plants, *N. tabacum* cv. Xanthi-nc and *N. benthamiana*, and crude viral extract of CMV-Y was inoculated after 2 h onto upper treated leaves. Crude viral extracts were prepared from individual leaf tissue of *N. tabacum* cv. Xanthi-nc and *N. tabacum* cv. Samsun by grinding with a mortar and pestle in 15 ml of 0.1 M sodium-phosphate buffer (pH 7.0) and inoculated mechanically with carborundum (600 mesh) onto the youngest fully expanded leaves of 7-10 weeks-old *N. tabacum* cv. Xanthi-nc and *N. benthamiana*. The leaves were collected after 5, 10, 15 and 20 days, grinded in 0.01 M phosphate buffer (W:V=1:10) and used for further test.

Double antibody sandwich (DAS)-ELISA. CMV-Y accumulation in foliar tissues was determined by DAS-ELISA. Tobacco leaves inoculated with CMV-Y were harvested every 5 days till 20 days post inoculation (dpi). Approximately 5 g of diseased tobacco leaves were homogenized with a mortar and pestle in 2 ml PBS-T buffer containing 0.1% bovine albumin (Sigma). Further procedure was followed as described by Clark and Adams (1977) with some modification. Absorbance values were measured at 405 nm by an ELISA reader (Power waveX, Bio-TEK instruments Inc., USA).

Challenge inoculation with purified CMV-Y. Virus was purified 2 weeks after post inoculation from systematically infected leaf materials of *N. benthamiana*. The viruses were purified as described by Takanami, (1981). The purified preparation of CMV-Y was used for the inhibitory effect of Gsm01 substrate in *C. amaranticolor* by local lesion assay. Ten week old, *C. amaranticolor* plants were selected for this experiment and culture filtrate from *S. marcescens* strain Gsm01 was applied to 5 plants, and remaining 5 plants were used as control. Different concentrations (50, 70, 90, 120 and 150 pmol) of purified CMV-Y were inoculated on leaves. The plants were incubated at room temperature for 7 days and the local lesions were counted.

Total RNA isolation. Total nucleic acids from the leaf parts of virus-infected plants were extracted by phenol:chloroform extraction and ethanol precipitation. Briefly, sample of leaf tissue (1 g) was homogenized with a cooled sterilized pestle and mortar in a extraction buffer (10 mM Tris-HCl (pH 8.5)), 50 mM KCl, 1% SDS, 14 mM β-mercaptoethanol). It was extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (24:24:1, w:v) and chloroform iso-amyl alcohol (24:1, w:v) at 37°C for 15 min. The RNA was precipitated with 2.5 vol. 95% ethanol followed by incubation at –20°C for 12 h and was collected by centrifugation at 15,000 rpm at 4°C for 20 min. The pellet was washed in 70% ethanol, vacuum-dried and resuspended in 50 μl of TE buffer.

RT-PCR analysis. The primer pairs, (P1-5' TAGTTTTGAGGTTCAATTCC 3'; P2-5' GACTGACCATTTTAGCCG 3') prepared from complete nucleotide sequences of RNA3 of the cucumoviruses from gene bank were used to amplify a DNA fragment of approximately 950-bp covering entire viral coat protein gene. Reverse transcription (RT) reaction was done in a reaction volume of 40 μl. RT was carried out at 42°C for 45 min with 8 μl viral RNA, 10 μl of 2.5 mM dNTPs, 2 μl of 0.5 pmol primer P2, 1 unit of RNase inhibitor and 50 units M-MLV reverse transcriptase in 12 μl distilled water and RT reaction was inactivated by incubation for 3 min at 94°C. Then, 20 μl of PCR cocktail premixture containing 2 μl of 0.5 pmol primers (P1 and P2), 10

mM dNTPs and 1.25 units of *Taq* DNA polymerase (Promega Co., USA) were added to the RT-finished tube. The cDNA was amplified by PCR in a programmable DNA thermal cycler (Perkin Elmer Cetus, model 480) for 35 cycles (94°C for 90 s, 48°C for 30 s and 72°C for 45 s) after an initial denaturation for 5 min at 94°C with final extension for 10 min at 72°C. A 10 μl aliquot of the RT-PCR reaction was analyzed by electrophoresis in a 1.2% agarose gel in 0.5× TAE (45 mM Tris-borate, 1 mM EDTA (pH 8.0)) containing 100 ng/ml ethidium bromide and visualized by UV transilumination.

Dot-blot hybridization. For dot blotting, viral RNA was denatured by boiling for 3 min, snap-chilled on ice for 10 min, and applied to nitrocellulose membrane (Bio-Rad, USA) using the 96-well Bio-Dot microfiltration unit (Bio-Rad, USA). The nylon membrane was baked at 80°C in a vacuum oven for 2 h. Pre-hybridization was performed at 48°C for 4 h. Hybridization was carried out overnight with DIG-labeled probe at 48°C. After hybridization, the membrane was washed two times for 5 minutes with 2×SSC, 0.1% SDS at room temperature, and twice with 0.2×SSC, 0.1% SDS at 65°C for 15 min.

Polyacrylamide gel electrophoresis (PAGE) and Western blotting. Protein extracts from the infiltrated leaves were centrifuged at 10,000 g for 10 min at 4°C and heated for 5 min at 100°C. The aliquots were electrophorased in SDS-PAGE with Penguin Dual Gel Vertical unit (OWL Co.) using the discontinuous polyacrylamide gel system (Laemmli, 1970). Proteins separated by SDS-PAGE were electron blotted onto a hybond-N^+ membrane (Ammersham, Buckinghamshire, UK) by electro-blotting with Trans-Blot SD Semi-Dry Transfer cell (Bio-Rad, USA) in transfer buffer. The membrane was blocked in TBS-T containing 1% bovine serum albumin (BSA) for 30 min at room temperature and immunoprobed with CMV antibody (Kisan Biotech Co. Ltd, Korea), and alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega, USA) antibody. The membrane was washed and then subjected to a color development reaction with the Proto Blot Western Blot AP System (Promega, USA).

RNase effect. To observe the RNase effect of the culture filtrate of *S. marcescens* strain Gsm01 on viral RNA, purified viral RNAs (5 μl) were added in Gsm01 culture filtrate (10 μl) and incubated at room temperature for 10, 20, 30, 40 and 50 min. The solution was than electrophorased in 1.75% agarose gel (TBE buffer, 1% SDS) at 50V for 45 min and stained on 100 ng/ml ethidium bromide and visualized by UV transilumination.

Results

Antiviral activity of culture filtrate of *S. marcescens* strain Gsm01 against CMV-Y. The antiviral effect of culture filtrate of strain Gsm01 on symptoms development by yellow strain of CMV-Y was tested in *C. amaranticolor*. The local lesions on the treated leaves were very low compared to lesions on untreated leaves (Fig. 1A). Furthermore, the effect of concentration of the culture filtrates was tested. The filtrate was diluted 2, 10, and 100 times and coated on the leaves of the *C. amaranticolor* by brush, and CMV-Y were applied after 2 h and incubated at room temperature for 7 days. The result showed that higher concentration of crude culture filtrates has stronger inhibitory effect against CMV-Y infection (Fig. 1B).

Systematic antiviral activity of culture filtrate of *S. marcescens* strain Gsm01 against CMV-Y. The local inhibitory effect of culture filtrate of strain Gsm01 to CMV-Y, made us further experiment the systematic inhibitory effect. For this experiment, the *N. tabacum* cv. Xanthi-nc leaves were coated with culture filtrates of *S. marcescens* strain Gsm01 by brush and CMV-Y was inoculated after 2 h of incubation. As shown in Fig. 2A, the bacterial culture filtrate inhibited the viral infection significantly than those of the control (water). In addition, the spray treatment of the culture filtrate showed decreasing symptom development on *N. tabacum* cv. Xanthi-nc (Fig. 2B). When the systematic inhibitory activity was observed according to different host on *N. tabacum* cv. Xanthi-nc and *N. benthamaiana*, it was found to inhibit CMV-Y infection by 73.7% and 44.5% respectively (Table 1).

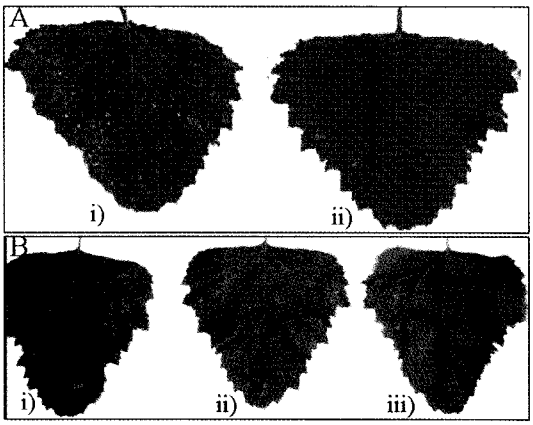

Fig. 1. Local lesions on the leaves of *C. amaranticolor* by CMV-Y against culture filtrate of *S. marcescens* strain Gsm01. (A) The leaves were coated with; i) distilled water ii) culture filtrate. (B) Inhibitory effects of different concentration, i) diluted 100 times, ii) 10 times and iii) 2 times and coated on the leaves of *C. amaranticolor* by brush before inoculation of CMV-Y.

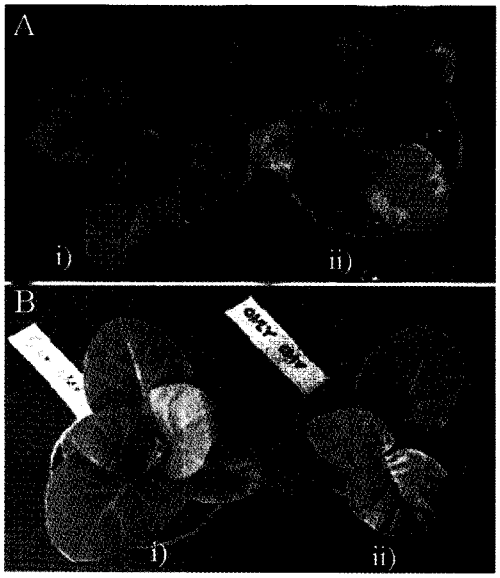

Fig. 2. Effects of bacterial culture filtrate of *S. marcescens* strain Gsm01 against CMV-Y inoculation on symptom expression of *N. tabacum* cv. Xanthi-nc. (A) The leaves were coated by brush with culture filtrates of i) Gsm01 and ii) distilled water. (B) The leaves were sprayed with culture filtrates of i) Gsm01 and ii) control plant before CMV-Y inoculation.

Effect of culture filtrate on accumulation of CMV-Y. To quantify the virus in the plant, the amount of CMV-Y accumulation in the leaves was analyzed at 5, 10, 15, and 20 dai by DAS-ELISA. The CMV-Y accumulation was slightly lower in culture filtrate treated leaves than in those with healthy plants until 20 dai, whereas only CMV-Y treated plants showed high values, however, after 15 and 20 dai accumulation of CMV-Y decreased (Table 2).

Effect of the time of treatment. To understand, whether mode of action of antiviral activity is protection or remedy, plants were treated with the culture filtrate before or after CMV-Y inoculation. The leaves of each plant were marked in the same place and the culture filtrate of Gsm01 was

Table 1. Spray effects with culture filtrates of *S. marcescens* Gsm01 on different host by CMV-Y

Culture filtrate	*N. tobacum* cv. Xanthi- nc			Inhibitory activity (%)[a]	*N. benthamiana*			Inhibitory activity (%)
Gsm01	6[b]	10	3	73.7	14	14	12	44.5
Control[c]	24	24	24	–	24	24	24	–

[a] {1–(No. of symptoms on the plants treated with the culture filtrates/ No. of symptoms on control plants)}×100=%.
[b] average no. of symptoms on the plants treated with the culture filtrates.
[c] The plants were inoculated with CMV-Y without treatment of the culture filtrates.

Table 2. Cucumber mosaic virus (CMV-Y) accumulation in inoculated and non-inoculated leaves of *N. tabacum* cv. Xanthi-nc treated with culture filtrates of *S. marcescens* strain Gsm01 by DAS-ELISA

Treatment	DAS-ELISA[y]			
	5DAI[x]	10DAI	15DAI	20DAI
Gsm01 (CF)	0.583	0.763	0.104	0.105
CMV-Y-untreated	1.317	1.651	1.568	1.302
Healthy-untreated	0.128	0.109	0.099	0.124

[x] Days after inoculation.
[y] ELISA results are presented as absorbance values (405 nm) of means of three leaves of each plant.

sprayed with a manual atomizer on to the whole surface of basal leaves of *C. amaranticolor*. We observed marked difference in inhibition activity of culture filtrate on *C. amaranticolor* before and after inoculation. When the continuous effect of the culture filtrate of *S. marcescens* strain Gsm01 against CMV-Y in *C. amaranticolor* was observed, the antiviral activity was significantly higher after treatment of culture filtrate first than inoculation of CMV-Y or simultaneously after CMV-Y inoculation (Fig. 3).

Inhibition effects of accumulation of CMV-Y with culture filtrate of *S. marcescens* strain Gsm01. *C. amaranticolor* leaves were treated with culture filtrate of *S. marcescens* strain Gsm01, 5-10 min before inoculation of different concentration of purified CMV-Y by local lesion assay using *C. amaranticolor* with water treated controls. The average time of mosaic appearance was earlier in the untreated leaves compared to that of corresponding Gsm01 treated leaves. The culture filtrate inhibited the virus accumulation by 100% at 50 pmol inoculum and above 50% at 50-120 pmol inoculum, when compared to no inhibitory activity on control leaves (Fig. 4).

The accumulation of CP gene or coat protein by CMV-Y

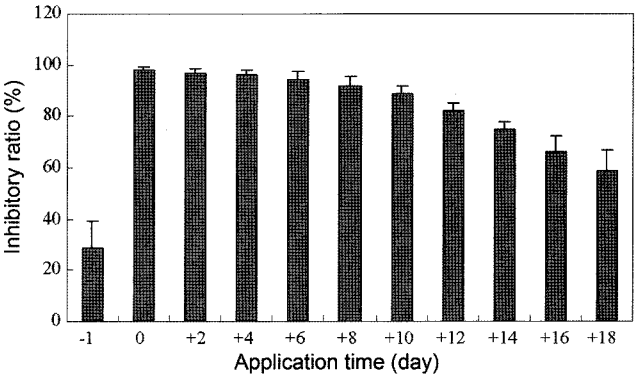

Fig. 3. Inhibitory ratio of local lesion formation on *C. amaranticolor* leaves on the time of treatment of *S. marcescens* strain Gsm01 before (–) or after (+) inoculation with CMV-Y. The control leaves were treated with distilled water.

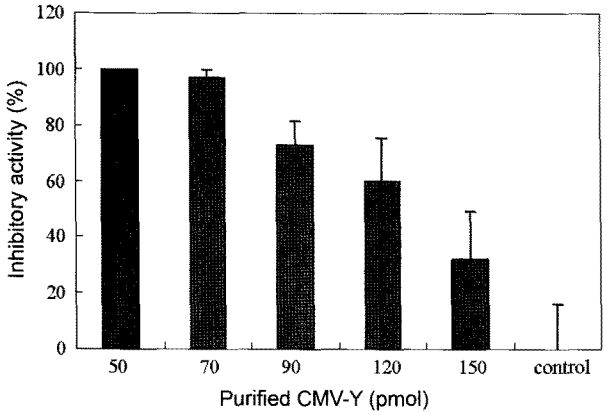

Fig. 4. Symptom expression by purified CMV-Y in *C. amaranticolor* challenge inoculation with culture filtrates of *S. marcescens* strain Gsm01. 1). Inhibition % of culture filtrates with different concentration of CMV-Y and control.

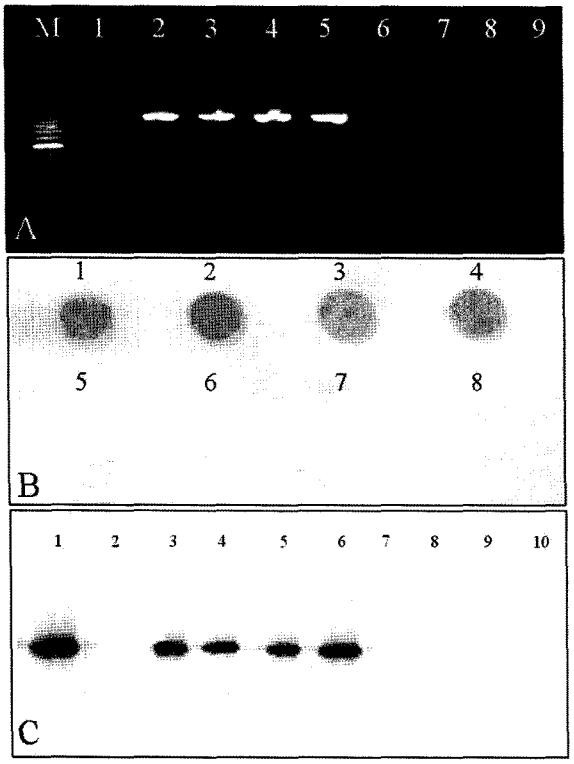

Fig. 5. Detection of CMV CP gene or coat protein in tobacco plants treated with culture filtrates of *S. marcescens* strain Gsm01. (A) RT-PCR analysis, Lane 1: Healthy tobacco plant. Lane 2-5: non treated plants (CMV-Y inoculated), Lane 6-9: Culture filtrate treated plants (culture filtrate treated before CMV-Y inoculation) M: 100 bp ladder (Promega). (B) Dot-blot hybridization, Lane 1-4: non-treated plants, Lane 5-8: treated plants. (C) Western blot analysis, Lane 1: purified CMV-Y, Lane 2: healthy tobacco plant. Lane 3-6: non-treated plants. Lane 7-10: treated plants. Tobacco plants were collected at 5, 10, 15, and 20 days after inoculation of purified CMV-Y.

in the plant leaves was determined using RT-PCR, dot blotting and western blotting analysis. The CP genes were not detected on treated leaves throughout the experiment days (5-20 day) by RT-PCR and dot blotting (Fig. 5A, B), and also coat proteins were not detected by western blotting (Fig. 5C).

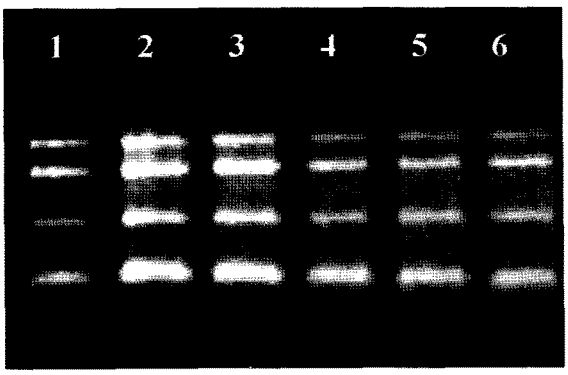

Fig. 6. RNase effects of culture filtrates of *S. marcescens* stain Gsm01 on CMV genomic RNAs (lane 2-6). CMV RNAs suspended in the culture filtrates were incubated at room temperature for 10 (lane 2), 20 (lane 3), 30 (lane 4), 40 (lane 5), 50 minutes (lane 6), and analyzed in agarose gel. Lane 1 is non-treated CMV RNAs.

RNase effects of cultrate filtrates on genomic RNAs. We investigated the influence of culture filtrate of *S. marcescens* strain Gsm01 on CMV-Y genomic RNA. When suspension of CMV-Y RNAs with culture filtrate was incubated at room temperature for different time intervals (10, 20, 30, 40 and 50 min) and analyzed in agarose gel, no change in the CMV-Y RNA was observed (Fig. 6). This indicates that the Gsm01 culture filtrate did not act as an RNase and thus it might contain components other than RNase.

Discussion

The present study shows that the culture filtrate of *S. marcescens* strain Gsm01 is a potent inhibitor of virus infection. CMV-Y infection was inhibited when plants were treated with culture filtrate of *S. marcescens* strain Gsm01 at the time, before as well as after the inoculation of CMV-Y. The culture filtrate inhibited the production of local lesions on the leaves of *C. amaranticolor* effectively compared to the untreated leaves indicating that it is capable of preventing CMV-Y infection substantially. Although infection sites were present in Gsm01 substrate filtrate treated leaves, they were smaller and fewer than those in the water treated controls, which could be due to the blockage of viral invasion into the cell and restriction of cell to cell spread from initially infected cell by culture filtrate.

Treatment of Gsm01 culture filtrate in *N. tabacum* cv. Xanthi-nc plants reduced the symptom formation up to

100% as compared to the control plants. Challenge inoculation of CMV-Y in the tobacco leaves with Gsm01 culture filtrate, decreased the disease incidence by 73.7% and 44.5% in *N. tabacum* cv. Xanthi-nc and *N. benthamaiana*, respectively compare to 100% in control, which could be due to some form of induced systemic resistance. These results are in accordance to the previous studies, where induction of the cucumber and tomato plants reduced CMV disease incidence (Ghandi, 2000; Raupach et al., 1996). It was also observed that higher the concentration of culture filtrate treated on the leaves, higher the inhibitory activity.

Treatment of culture filtrate of *S. marcescens* strain Gsm01 on the leaves of *N. tabacum* and *N. benthamaiana* prevented the appearance of disease symptoms caused by CMV-Y. CMV-Y was not determined in the treated leaves of the tobacco plants till 20 dai by DAS-ELISA, confirming the inhibition of its growth by culture filtrate. These results are in harmony with those of Raupach et al. (1996) and Murphy et al. (2003), where they found tomato plants treated with PGPR (*P. fluorescens* and *S. marcescens*) strains could prevent initial symptoms for 14 days after CMV inoculation.

Challenged inoculation of different concentration of purified CMV-Y with the culture filtrate of *S. marcescens* strain Gsm01 showed that culture filtrate are capable of reducing the virus accumulation effectively. In our experiments, 100% of plants challenged with 50 pmol of purified CMV-Y escaped mosaic development completely, and more than 50% of the plants escaped mosaic development completely with 70-120 pmol of inoculum when compared to no activity in control plants. This shows that culture filtrate of *S. marcescens* strain Gsm01 is effective for even higher concentration of purified virus for their effective control.

The absence of CMV-Y or its CP gene in the systematically protected plant leaves clearly indicate that culture filtrate treatment has probably affected virus replication and/or movement, which are verified by RT-PCR analysis, dot blot and western blotting. However, further work has to be done to prove the mechanisms of resistance by culture filtrate to degrade virus.

RNases have been known to inhibit plant virus infection, although the mechanisms remain unclear (Ehara and Mink, 1986; Francki, 1968). Studies have implicated RNases in many key processes in plant development and in defense against pathogens (Green, 1994). S-RNases are presumed to play a key role in recognition and rejection of self pollen tubes (Rivers et al., 1993). PR-10 protein classified as a pathogenicity-related protein is also an RNase (Moiseyev et al., 1997). No effect of the culture filtrate of *S. marcescens* strain Gsm01 on CMV-Y RNAs indicates that the culture filtrate of Gsm01 in our study may not contain RNases or protein related to similar function. However further extended studies would give more detailed information about the RNases activity of the culture filtrate.

In our preliminary experiment in greenhouse, we observed higher number and heavier in weight of pepper fruits treated with culture filtrate of *S. marcescens* strain Gsm01, which indicates that culture filtrate not only protects plants from viral infection, but also helps to increase the yield (Ipper et al., 2006). In the above experiments, no damage to the host plant was observed due to culture filtrate of *S. marcescens* strain Gsm01 treatment. It, thus, can be characterized as a non-toxic antiviral agent, which could give necessary efficiency in combating CMV-Y and other virus without any harmful effect.

In conclusion, these results suggest that the culture filtrate of *S. marcescens* strain Gsm01 may be an effective and persistent bio-control agent for yellow strain of CMV-Y in different plants. Lytic enzymes, antibiotics, siderophores and salicylic acid have been characterized in *S. marcescens* having antifungal activity (Someya et al., 2000). Further studies on determining these or other products responsible for antiviral effect present in culture filtrate of *S. marcescens* strain Gsm01 and how it interferes with the infection process of CMV-Y are in progress.

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