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Involvement of Heat-stable and Proteinaceous Materials in the Culture of *Pseudomonas putida* JB-1 for the Inhibition of *Tobacco mosaic virus* Infection

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Out of various fungi and bacteria tested for inhibition of *Tobacco mosaic virus* (TMV) infection using *Nicotiana tabacum* cv. Xanthi-nc, a bacterial isolate JB-1, identified as *Pseudomonas putida* had a strong direct inhibitory activity against the TMV infection. Its systemic or indirect activity was also noted at more than a half level of the direct control efficacy. Disease severity was reduced significantly in the susceptible tobacco *N. tabacum* cv. NC 82 by the treatment of the bacterial culture filtrate, somewhat more by the pretreatment than by simultaneous treatment, probably by inhibiting the TMV transmission and translocation in the plants, showing negative serological, which responses in the viral detection by DAS-ELISA. TMV-inhibitory substances from *P. putida* JB-1 were water-soluble, stable to high temperature (even boiling), and to a wide range of pH. As proteinase K nullified their antiviral activity, the TMV inhibition activity of *P. putida* may be derived from proteinaceous materials. In electron microscopy, TMV particles treated with the JB-1 culture were shown to be shrunken with granule-like particles attached on them. All of these aspects suggest that *P. putida* JB-1 may be developed as a potential agent for the control of TMV.

Keywords : antiviral activity, heat-stable, proteinaceous materials, *Pseudomonas putida*, *Tobacco mosaic virus*

Tobacco mosaic virus (TMV) is one of the most important viruses, having a wide host range of about 119 plant species including tobacco and distributing worldwide (Zaitlin and Israel, 1975). It is very stable and easily transmitted mechanically by the direct transfer of sap through contact of a healthy plant with another infected plant in which enormous virus particles usually accumulate (Lucas, 1975). TMV transmission occurs easily by tools and hands during culture practices especially at the time of transplanting. In the control of TMV other than the use of resistant varieties, sanitation is one of the most important methods, which includes removing infected plants and then washing hands with soap (Agrios, 2005). However, this cannot prevent such mechanical transmission from taking place after various injuries on the leaves of adjacent diseased and healthy plants by winds or by cultivation during the growing season in field conditions.

No pesticide for plant virus control has been developed until now, although inhibitors of plant viruses have been investigated from various sources including higher plants, microorganisms, and some mushrooms (Aoki et al., 1993; Hudson, 1990; Ito et al., 1992; Kim et al., 2004; Klement et al., 1966; Stevens and Reynolds, 1992; Yeo et al., 1997, 1998). In their studies, a few of potent virus inhibitors have been isolated and characterized; however, they have been limited for practical uses because of their control efficacy, mass production and/or stability in field conditions inferior to commercial pesticide standards. Antiviral materials with all characters of high control efficacy, easy and economical mass production and high stability to severe outdoor environments are to be developed in order to meet the standards that can be practically used for the virus control.

Anti-phytoviral agents can be mainly divided into two action modes, prevention of virus infection and inhibition of virus multiplication, and they usually have either one or another action mode (Hirai, 1977). For example, milk, with which plants are sprayed or dipped, gives some control of TMV by inhibiting its infection (Agrios, 2005). Likewise, many macromolecules originated from plants and microorganisms are not systemically acting into plants, and thus mainly show a preventive activity against virus infection (Kim et al., 2000). These materials are little applicable to the control of TMV because a minimal uncontrolled TMV infection may cause severe disease due to its high infectivity and rapid multiplication capability. On the other hand, antiviral materials with small molecules that may have systemic activity have also been rarely developed as viral pesticides probably because of low control efficacy in an optimum concentration and short persistence (Yeo et al., 1997, 1998). Thus, it is much proper to consider antiviral materials with both action modes and together with persistence in harsh environments in the development of

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practical viral pesticides. This study was aimed to develop a potent biological agent for the control of TMV.

Materials and Methods

Screening of antiviral bacteria. In 1999 and 2000, storage root rots of Korean ginseng and cactus stem diseases were surveyed in the areas of Geumsan, Anseong, and Goyang. About 300 microbial isolates were obtained from surveyed areas, out of which approximately 50 bacterial isolates were tested for antiviral activity.

The antiviral activity was assayed by the local lesion assay of TMV infection using the supernatant of the bacterial cultures grown in nutrient broth (Difco, Becton, Dickson and Company, MD, USA) at 28°C shaking on a rotary shaker (200 rpm) for 4 days. The bacterial culture was centrifuged at 6,000× *g* for 10 min to remove bacterial cells and culture debris. The supernatant was adjusted to proper concentrations either concentrating using a rotary evaporator (Büchi Rotavapor R-200, Flawil, Switzerland) or adding with sterile distilled water. The bacterial culture filtrate was mixed with an equal volume of 200-fold diluted TMV-infected plant sap (systemically infected *Nicotiana tabacum* cv. NC 82). The mixture of an equal volume of the TMV-infected plant sap with an equal volume of distilled water was used as control. The mixture of culture filtrate and infected plant sap was inoculated on the half leaf of the local lesion host plant, *N. tabacum* cv. Xanthi-nc at 6-8 true leaf stages, and the other half leaf was inoculated with the infected plant sap alone. The inoculated plants were placed at 25-27°C in a greenhouse, and the number of lesions formed on each half leaf was measured and compared each other at 3-4 days after inoculation, which hereafter named the half-leaf local lesion assay. Three replicates were used for each bacterial isolate.

Identification of isolate JB-1. Screening experiments showed over 80% TMV-inhibitory efficacies in two bacterial isolates. One of which, isolate JB-1, from the surface of a cactus collected in Anseong showed more active and consistent antiviral effect in several antiviral efficacy trials. For the bacterial identification, the bacterial morphology was examined by electron microscopy. A two-day-old bacterial colony was picked with a spatula and placed in distilled water on a formvar-coated copper grid, dried and stained with 2% uranyl acetate for negative staining, and examined under a JEM 1010 electron microscope (JOEL, Japan) at 80 kV. Cultural and physiological characteristics of the bacterial isolate were examined by the usual methods for its classification (Schaad et al., 2001). Carbon source assimilation was examined using the Biolog GN test kit (Biolog Inc., Hayward, CA, USA) according to the manufacturer's specifications. Also to identify the bacterium at the gene level, polymerase chain reaction (PCR) was performed to amplify a part of the 16S rRNA gene of the bacterium using the 27mF and 1492mR universal primers (Brosius et al., 1978) and following the same projcedures described in the previous paper (Jeon et al., 2006). The nucleotide sequence of 16S rRNA gene of *P. putida* JB-1 was determined by an ABI Prism 3700 DNA Sequencer at National Instrumentation Center for Environmental Management (NICEM) in Seoul National University. It was compared with published 16S rRNA gene sequences by using a Blast Search at NCBI. The sequence of 16S rRNA gene was deposited at GenBank (Accession no. EF513733).

Preparation of antiviral materials from isolate JB-1. The culture supernatant of isolate JB-1 after grown in nutrient broth at 28°C for 4 days was added and mixed with the same volume of *n*-hexane to start dual solvent systems for separating antiviral materials (followed by chloroform-water of the prior water layer, ethyl acetate-water of the second water layer, and *n*-butanol-water of the third water layer). During the above separation of the materials, TMV-inhibitory activity was monitored by the half-leaf local lesion assay using dilutions of each layer extract equivalent to the original culture volume after solvent evaporation.

Characterization of antiviral compounds from isolate JB-1. To determine the thermal stability of antiviral compounds produced by JB-1, the water layer extracts equivalent to original concentration (1 ml in different tubes) were treated separately at 60°C for 15 min, 80°C for 15 min, kept in a boiling water bath (100°C) for 15 min, or autoclaved (at 15 psi, 121°C) for 20 min. The tubes were cooled and assayed for TMV-inhibitory activity as mentioned above. The water layer extract maintained at room temperature served as control.

To determine the stability of the antiviral materials from isolate JB-1 to pH, the water layer extracts of JB-1 were adjusted to different pH (from 4.0 to 10.0) with 1 N HCl or 1 N NaOH, followed by incubating at 30°C for one day, and the antiviral activity was assayed as described above.

Whether the antiviral materials produced by JB-1 are proteinaceous or not, inactivation test of the water-soluble compounds of the bacterial culture filtrate was conducted using a protein-degrading enzyme, proteinase K. Proteinase K (Type XIV, Sigma Chemical Co., MO, USA) was dissolved in 0.05 M Tris-HCl buffer, pH 7.3, with 0.1 M $CaCl_2$ at concentration of 5 mg/ml. The water layer extracts of JB-1 were incubated with proteinase K at final concentration of 500 μg/ml at 37°C for 60 min, and treated on tobacco leaves 1 hr before TMV inoculation. Distilled water and the water layer extract with no proteinase K treatment served as

negative and positive controls, respectively. The number of lesions formed on a treated leaf was measured and compared to that on control leaves at 3-4 days after inoculation as describe above. Each treatment was performed with three replications.

To test systemic or indirect inhibitory effect of the JB-1 culture filtrate in *N. tabacum* cv. Xanthi-nc, TMV was inoculated on adaxial half leaves opposite to the abaxial half leaves on which the bacterial culture supernatant was applied 1 hr before the TMV inoculation. Distilled water instead of the bacterial culture supernatant served as control. The number of lesions formed on a treated half-leaf was measured and compared to that on control half-leaf at 3-4 days after inoculation. Each treatment was performed with 3 replications.

Electron microscopy for examining interaction between antiviral materials of *P. putida* JB-1 culture filtrate and TMV. Interactions of TMV particles and antiviral materials of the bacterial culture filtrate were observed by electron microscopy using the purified TMV incubated for 1 hr with the bacterial culture supernatant. Formvar-coated grids were floated on drops of TMV solution or the mixture of the bacterial culture supernatant and TMV solution for 1 min, and the preparations were washed with distilled water and stained with 2% uranyl acetate for 1 min before the electron microscopic observation at 80 kV. Using the electron micrographs, widths of TMV particles were measured with an Axio Vision image analyzer (ver. 4.2, Carl Zeiss, Germany), replicating 19 times.

Effects of JB-1 culture filtrate on TMV infection in a susceptible host. To check the possibility of JB-1 culture filtrate for use in controlling TMV of commercial tobacco plants, most of which are infected systemically with mosaic symptoms, the bacterial culture supernatant was tested using a systemic symptom host plant, *N. tabacum* cv. NC82 at 6-8 true leaf stages by applying the culture supernatant 1 hr before TMV (infected plant sap) inoculation or by inoculating the mixtures of the culture supernatant and infected plant sap on the lower leaves of the tobacco plant. TMV inoculation alone served as control. The inoculated plants were placed at 25-27°C in the greenhouse, and systemic mosaic symptom development on leaves was examined from 7 days up to 21 days after inoculation.

At ten days after inoculation, two leaf disks were cut with a cork borer (diameter 6 mm) each from inoculated leaf to upper leaf of three replicate pots, and were used for testing by a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). DAS-ELISA testing was carried out using the PathoScreen kit for *Tobacco mosaic virus*-c as described by manufacturer's specifications (Agdia, USA). The binding of alkaline phosphatase (AP)-labeled antibodies were revealed by the use of 1 mg/ml p-nitrophenyl phosphate substrate with an incubation period of 1 hr at room temperature. The enzyme-substrate reactions were measured at A_{405nm} using a Multiscan ELISA reader (Tecan/Spectra, USA). In all ELISA formats, plates were washed three times between all incubation steps by filling the well with washing buffer for 3 min.

Results

Screening of antiviral bacteria. Out of 50 bacterial isolates screened for inhibition of TMV infection, five showed relatively high efficacies in inhibiting the local lesion formation on *N. tabacum* cv. Xanthi-nc leaves, among which JB-1 had the strongest TMV-inhibitory effect (Fig. 1A). In JB-1, the number of local lesions was reduced by 93.3% compared to the control, indicating 93.3% control value, showing numerous lesions formed on untreated control half leaves but almost no lesions on the treated half leaves (Fig. 1B).

Identification of Isolate JB-1. The bacterial isolate JB-1 was rod-shaped with lophotrichous flagella (Fig. 2). It was Gram-negative, aerobic, and produced fluorescent pigment on King's B medium, but formed no aerial mycelium and levan, showing morphological and cultural characters similar to *Pseudomonas* sp. (Table 1). Assimilation tests using the Biolog system identified the isolate JB-1 as *Pseudomonas putida* with similarity index of 0.66 (data not shown). Also, the nucleotide sequences of 16S rRNA gene of the isolate JB-1 (GenBank accession no. EF513773) matched well with those of another isolate (GenBank accession no. AF094740) with similarity of 99%, confirming the bacterial

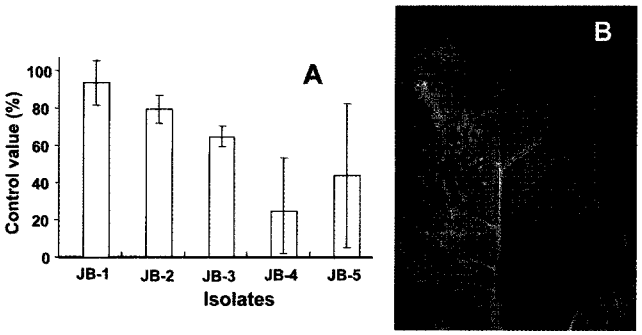

Fig. 1. Inhibitory effect of culture filtrates of bacterial isolates against TMV infection on a local lesion host, *N. tabacum* cv. Xanthi-nc (A), showing numerous local lesions on the left control half leaf but almost no lesions on the right JB-1-treated half leaf (B). Control values are the percentages of the number of lesions formed on the treated half leaves relative to that of the untreated control half leaves. Vertical bars and lines are averages and standard deviations of three replications.

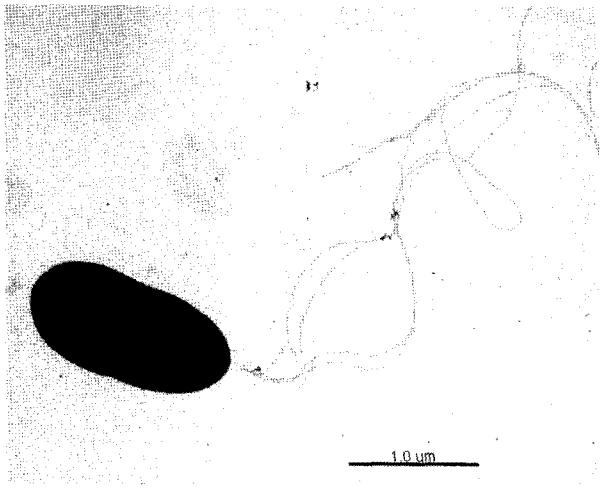

Fig. 2. Electron microscopy of isolate JB-1 negatively stained with 2% uranyl acetate, showing a rod-shaped bacterium having thread-like lophotrichous flagella (typical morphological characters of plant bacteria including *Pseudomonas*).

Table 1. Identification of JB-1 based on cultural and physiological characters

Characteristics	Present isolate JB-1	*P. putida*[a]
Gram stain	–[b]	–
Yellow or orange colonies in NGA, YDC or NBY media	–	–
Fluorescent pigment on KB	+	+
Grows anaerobically/aerobically	–/+	–/+
More than four peritrichous flagella	–	–
Growth on D-1 M agar	–	–
Spore formed	–	–
Aerial mycelium	–	–
Leven formation	–	–
Gelatin liquefaction	–	–
Growth at 4°C	–	V
Growth at 41°C	–	–

[a]Data from the Laboratory Guide for Identification of Plant Pathogenic Bacteria by Schaad et al. (2001).
[b]+, positive; –, negative; V, variable

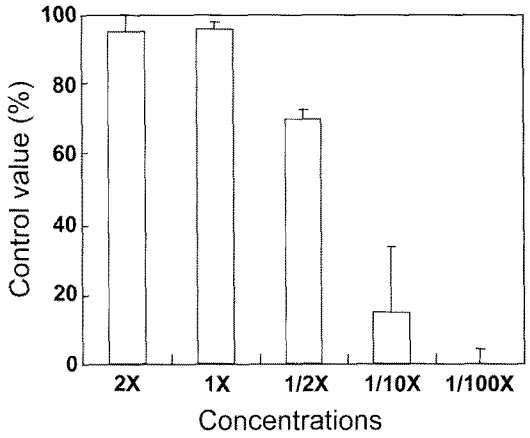

Fig. 3. Inhibitory effect of *P. putida* JB-1 culture filtrate at different concentrations against TMV infection on a local lesion host, *N. tabacum* cv. Xanthi-nc. Control values are the percentages of the number of lesions formed on the treated half leaf relative to that of the untreated control half leaf. Vertical bars and lines are averages and standard deviations of three replications.

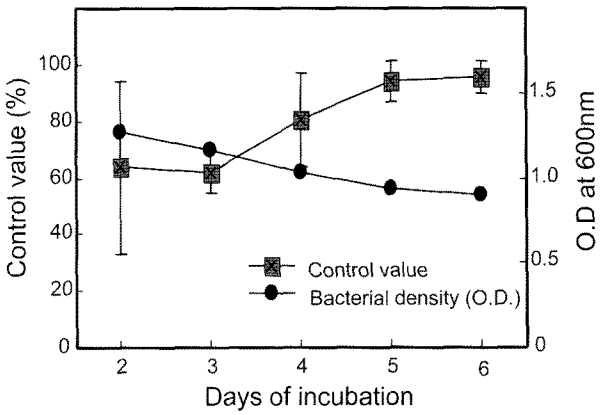

Fig. 4. Inhibitory effect of *P. putida* JB-1 culture filtrate with different time periods of incubation against TMV infection on a local lesion host, *N. tabacum* cv. Xanthi-nc and the bacterial densities shown by spectrometry. Control values are the percentages of the number of lesions formed on the treated half leaf relative to that of the untreated control half leaf. Markers and vertical lines are averages and standard deviations of three replications.

isolate as *P. putida*.

Antiviral effect of various culture concentrations on TMV infection. Antiviral effect of *P. putida* JB-1 culture filtrate was higher at the higher concentrations than at the lower concentrations, showing over 70% TMV inhibition at two times (1/2X) diluted concentration (Fig. 3). No control efficacy was detected at a hundred times (1/100X) diluted concentration of the bacterial culture filtrate.

Effects of incubation periods of JB-1 on the production of antiviral materials. The antiviral activity of JB-1 culture filtrate increased significantly from 4 days, and was strongest at 5 and 6 days after culturing (Fig. 4). However, the bacterial population density was slightly decreased gradually from 2 days after culturing, indicating cell degradation. This indicates the antiviral materials were produced at the later stage of the bacterial growth or accumulating in the culture media during the bacterial culturing.

Preparation of antiviral materials inhibitory to TMV infection. In the preparation procedures of the antiviral

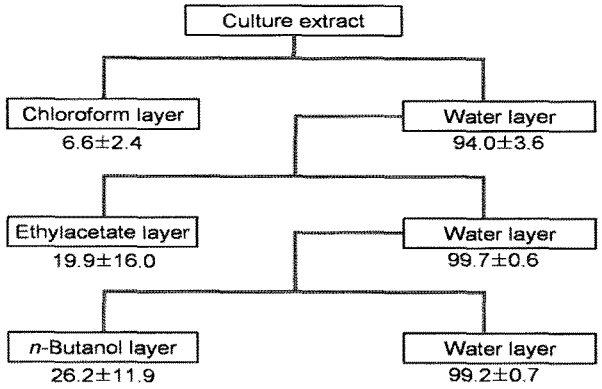

Fig. 5. Procedures and separating antiviral materials using equal volumes of two solvents in a separation funnel and control values of separation layers against TMV infection on a local lesion host, *N. tabacum* cv. Xanthi-nc. Figures are averages and standard deviations of 3 replications.

materials from the JB-1 culture filtrate, the TMV-inhibitory effect was observed only in the water layer extracts, while their relevant chloroform, ethyl acetate and *n*-butanol layer ones had no evident antiviral effects (Fig. 5), indicating that the antiviral materials may be water-soluble. Antiviral activities maintained very high in all water layer extracts like the original bacterial culture filtrate during the separation procedures, almost completely blocking the TMV infection.

Stability of the antiviral compounds produced by JB-1 to heat and pH treatment. The water layer extract of *P. putida* JB-1 culture filtrate subjected to high temperature treatments and even boiling (100°C) showed the control efficacy of about 100% as untreated control (Fig. 6A). Although the antiviral activity was somewhat decreased by autoclaving (at 15 psi, 121°C), it was still high enough to reduce TMV infection more than 70%. Also, the antiviral materials appeared to be very stable in a wide range of pH,

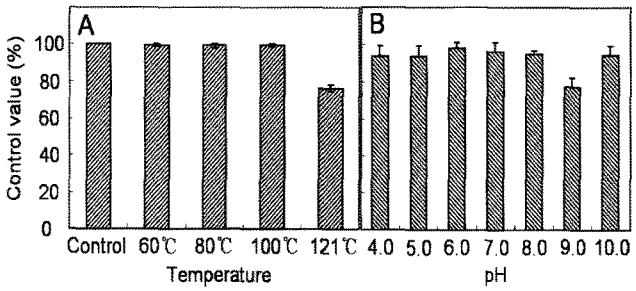

Fig. 6. Effect of temperature (A) and pH (B) on the antiviral materials of *Pseudomonas putida* JB-1 culture filtrate for controlling TMV infection on a local lesion host, *Nicotiana tabacum* cv. Xanthi-nc. Vertical bars and lines are averages and standard deviations of 3 replications.

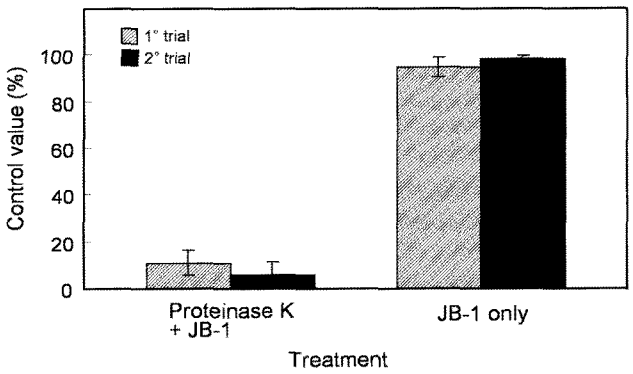

Fig. 7. Effect of proteinase K on the antiviral materials of *P. putida* JB-1 for controlling TMV infection on a local lesion host, *N. tabacum* cv. Xanthi-nc. Vertical bars and lines are averages and standard deviations of 3 replications.

showing almost complete inhibition of TMV infection in all acidic and basic conditions tested except pH 9.0 that was somewhat decreased but still high enough to be similar to other pH in the TMV-inhibitory activity (Fig. 6B).

Effects of proteinase K on the antiviral activity of water-soluble materials produced by *P. putida* JB-1. When the water layer extracts of the bacterial culture filtrate were treated with proteinase K, they lost the antiviral activity almost completely, showing almost no protection of the plant from TMV infection (Fig. 7). This indicates that proteinase K may have inactivated the antiviral materials in the bacterial culture filtrate.

Systemic or indirect inhibitory effects of JB-1 against TMV infection. Several local lesions were formed on the adaxial half leaves of *N. tabacum* cv. Xanthi-nc of which the opposite abaxial half leaf surfaces were treated with *P. putida* JB-1 culture filtrate, showing its systemic or indirect inhibitory effect more than half of the direct inhibitory effect for which the complete inhibition of TMV infection was achieved in this experiment (Table 2). There was no

Table 2. Comparison of direct and systemic or indirect antiviral activity of *P. putida* JB-1 culture filtrate against TMV infection on a local lesion host, *N. tabacum* cv. Xanthi-nc

Treatment[a]	No. of lesions/3.0 cm²		Control value (%)
	Treated half-leaf	Control half-leaf	
Direct effect	0.0±0.0	29.0±14.7	100.0±0.0
Indirect effect	9.7±7.6	27.3±24.0	63.0±14.6
Distilled water	32.7±11.7	35.0±7.7	8.8±7.7

[a]Direct effect: treated on inoculated half-leaf surface (adaxial), Indirect effect: treated on the opposite (abaxial) surface of the inoculated half-leaf.

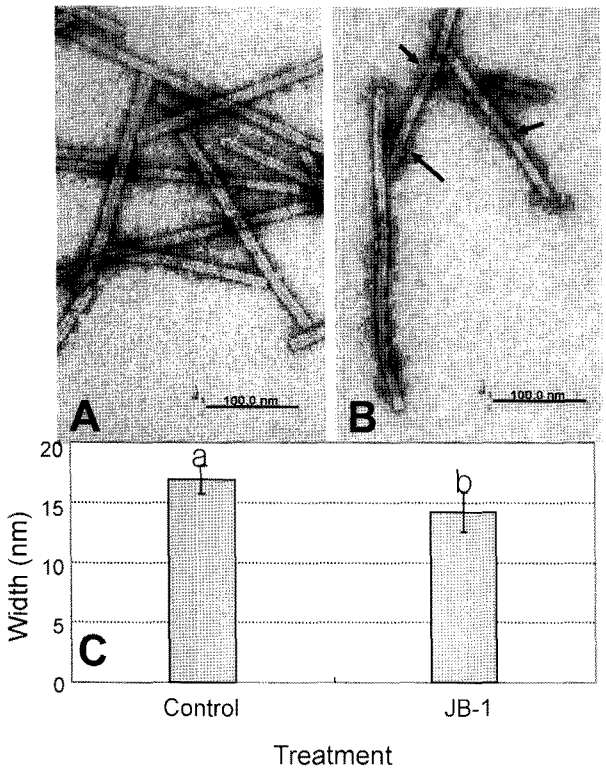

Fig. 8. Electron microscopy of purified TMV with no treatment (A) and treated with *P. putida* JB-1 culture filtrate (B). Arrows: granule-like materials attached to TMV particles. (C): Widths of TMV particles with no treatment (Control) and treated with *P. putida* JB-1 culture filtrate (JB-1). Bars and vertical lines are averages and standard deviations of 19 replications. Different letters above the bars denote a significant difference at $P=0.01$ by least significant difference test.

significant difference in local lesion numbers on the half leaves among the controls for respective treatments and the untreated control check.

Electron microscopy for examining interaction between antiviral materials of *P. putida* JB-1 culture filtrate and TMV. Typical tobamovirus type rod-shaped virus particles, straight and looking intact with a width of about 16-18 nm, were viewed by the transmission electron microscopy of the untreated purified TMV, while the virus particles incubated with *P. putida* JB-1 culture filtrate for 1 hr appeared 13-16 nm in width to be somewhat significantly contracted approximately one eighth to maximum one fourth (Fig. 8). In the treated TMV, granule-like materials were attached on terminal and lateral regions of the virus particles.

Effects of JB-1 culture filtrate on TMV infection in a systemic symptom host. Apparent mosaic symptoms appeared on the systemic symptom host plants, *N. tabacum*

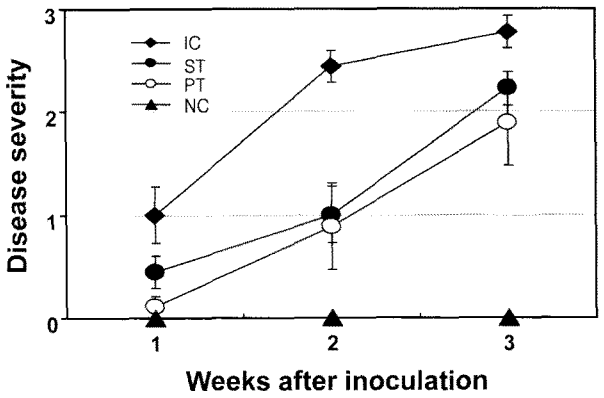

Fig. 9. Control effect of *P. putida* JB-1 culture filtrate against TMV infection on a susceptible host, *N. tabacum* cv. NC 82. Markers and vertical lines are averages and standard deviations of 3 replications. IC: inoculated control, ST: simultaneous treatment of *P. putida* JB-1 culture filtrate on inoculated leaves, PT: pretreatment of *P. putida* JB-1 culture filtrate on inoculated leaves, NC; negative control (uninoculated healthy plants). Disease severity: 0; no disease, 1; 10-20%, 2; 30-60%, 3; >70% diseased leaves (modified from Park et al. (1994)).

cv. NC 82, from one week after inoculation. At this time after inoculation, about 20% of leaves in a plant (upper leaves) showed mosaic symptoms in the untreated control, while only about 5% and almost no symptoms appeared on the plants treated with the bacterial culture filtrate simultaneously at and 1 hr before TMV inoculation, respectively (Fig. 9). Mosaic symptoms expanded and appeared on almost all inoculated plants with disease severity of over 65% in no treatment from 2 weeks after inoculation, while in the treated plants appeared less than 20% and around 50% at 2 and 3 weeks after inoculation, respectively. Symptom development was somewhat more retarded by the pretreatment of the bacterial culture filtrate than by the simultaneous treatment.

TMV concentrations in inoculated leaves and their first, second and third upper leaves were examined by DAS-ELISA (Fig. 10). In the inoculated leaves, TMV concentration was not significantly different between TMV inoculation alone and the treatments at 10 days after inoculation; however, the viral concentration decreased proportionally in order from upper to lower leaves in the plants treated with the bacterial culture filtrate, especially by its pretreatment. Consequently, in the first upper leaves (the lowest leaves above the inoculated leaves), no virus was detected in the pretreatment of the bacterial culture filtrate, showing the optical density values below negative control (healthy plant sap) by DAS-ELISA (Fig. 10).

Discussion

TMV is exceptionally infectious and stable (Kim et al.,

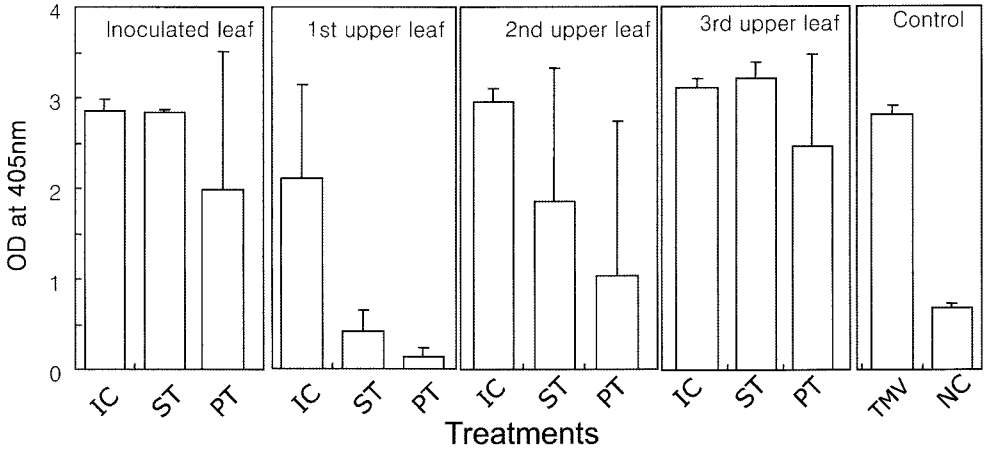

Fig. 10. TMV concentrations in the leaves of a susceptible host, *N. tabacum* cv. NC 82, examined by DAS-ELISA at 10 days after inoculation. Vertical bars and lines are averages and standard deviations of 3 replications. IC; inoculated control, ST; simultaneous treatment of *P. putida* JB-1 culture filtrate on inoculated leaves, PT; pretreatment of *P. putida* JB-1 culture filtrate on inoculated leaves, TMV; purified TMV, NC; negative control (healthy plant sap).

1996) so that its practical control can be achieved most of all by removal of the inoculum source. Spraying the plants with or dipping them in milk provides some control of TMV by inhibiting its infection (Agrios, 2005). However, milk may have little systemic activity for the inhibition of the viral multiplication or movement in plants. In this sense, the culture filtrate of *P. putida* JB-1 may be as potentially applicable as or over milk for the control of TMV in field conditions as it has strong direct inhibition capability of TMV infection like milk and efficient systemic or indirection antiviral activity in addition. Also, the antiviral materials of *P. putida* JB-1 may be very stable to harsh weather conditions so that their antiviral activity should be durable in fields because little activity reduction occurred by the heat treatments (even boiling) and in a wide range of pH, and by exposure to organic solvents during the separation procedures.

The culture filtrate of *P. putida* JB-1 definitely inhibited TMV infection and symptom development in the susceptible tobacco cultivar showing systemic mosaic symptoms to TMV infection, although it could not prevent its infection completely probably because of high TMV's transmissibility (Lucas, 1975). TMV fully multiplied and translocated into whole plants in 10 days after inoculation in the susceptible tobacco plants because a high TMV concentration was detected by DAS-ELISA in the first upper leaf above the inoculated leaf, the last virus translocation and multiplication place (Agrios, 2005). On the other hand, TMV was not detected in the first upper leaf above the inoculated leaf especially by pretreatment, suggesting that the virus transmission should have been retarded significantly. In field conditions, a small amount of decrease in TMV inoculum (infectivity) may result in a significant decrease in disease development as shown in the control of TMV in tobacco stumps by urea application in which the reduction of infectivity from 8.5% to 6.9% (infectivity reduction rate of 18.8%) reduces disease incidences in fields from 44.1% to 19.9% (disease incidence reduction of 54.9%) (Park et al., 1994). In this sense, the bacterial culture may be developed as a good potential biocontrol agent against TMV infection in field conditions. The more control effect was noted in the pretreatment of the bacterial culture filtrate than in the simultaneous treatment probably because the systemic antiviral activity of the antiviral materials was added more to the direct antiviral activity in the pretreatment than in the simultaneous treatment. Therefore, spraying the susceptible tobacco plants with or dipping them in the bacterial culture filtrate before cultural practices such as transplanting and soiling may provide a good control of TMV.

The active components of *P. putida* JB-1 culture filtrate responsible for inhibiting TMV infection appear to be water-soluble proteinaceous materials as revealed by TMV infection assays in this study. As infectivity comes from the nucleic acid (RNA) of the viral composition, the loss of infectivity may be either related to the damages of the viral RNA or the damages of the viral coat protein, inhibiting its uncoating to free the RNA, or derived from both damages. In electron microscopy of the TMV particles treated with the bacterial culture filtrate, the shrinkage of the virus particles with attachment of granule-like materials was viewed, suggesting that the viral coat protein might have been disrupted structurally and/or in its assembly. This would cause the uncoating of the protein coat hindered to have the viral RNA freed, reducing the viral infectivity. Also any RNA-binding or inactivating materials from the

bacterial culture filtrate or other plant sources might readily gain access to the nucleic acid covered with the collapsed protein coat.

Proteinase K is an endolytic protease discovered in extracts of the fungus *Tritirachium album* that cleaves peptide bonds (Ebeling et al., 1974). The fact that the antiviral materials from *P. putida* JB-1 culture filtrate were inactivated by the proteinase indicates that they may be any of simple and complex proteinaceous molecules covalently linked by peptide bonds such as proteins, glycoproteins, peptides, and even peptaibols. RNA-binding proteins from *Saccharomyces cerevisiae* proteins inhibit RNA virus infection (Zhu et al., 2007). Analogs of melittin correlated with sequence similarities of TMV coat protein induce conformational changes resulting on interaction of the peptides and RNA, which leads to inhibiting the virus infection (Marcos et al., 1995). Ribonucleases (RNases), which have function of determining vector specificity and host resistance to beetle transmission of plant viruses (Gergerich and Scott, 1988; Gergerich et al., 1986, 1991), may be involved in the inhibition of the viral infection by directly inactivating the TMV RNA. Glycoproteins are also a potential candidate as a partially purified glycoprotein material from the bacterium *Acinetobacter* sp. inhibited TMV infection (Kim et al., 2004). However, the antiviral materials from *P. putida* JB-1 may hardly be such simple or complex proteins of which the molecules are too large and complicatedly structured to be systemic, even a short distance, into plants in a short period of time. Therefore, the *P. putida* JB-1-producing antiviral materials are likely to be small molecular peptides or peptaibols.

Peptides and peptaibols with antimicrobial actions including antiviral activity have been identified from various sources (Grigoriev et al., 1995; Kim et al., 2002; Lehrer et al., 1993; Matsuzaki et al., 1995; Yun et al., 2000). The main mechanism of peptide or peptaibol materials for antimicrobial activity is to form pores through membranes (Gessmann et al., 1999; Hara and Yamakwwa, 1996; Menestrina et al., 1986; Nagaoka et al., 1996; Skehan et al., 1990); however, this mechanism can hardly applicable to the viruses composed of coat protein and rhibonucleic acid but not of phospholipids as in cell membrane. Kim et al. (2000) reported the direct inhibition of TMV infection by the peptaibols derived from *Apiocrea* sp. 14T, but their systemic effect was not indicated. Also these peptaibols are little soluble in water like many other peptaibols. Therefore, the antiviral materials produced by *P. putida* JB-1 are most likely to be peptides; however, more studies using instrumental analyses are required for the exact identification of the antiviral materials of *P. putida* JB-1 culture filtrate.

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