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Inhibitory Effects of *Acinetobacter* sp. KTB3 on Infection of *Tobacco mosaic virus* in Tobacco Plants

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During the screening of antiviral substances having inhibitory effects on *Tobacco mosaic virus* (TMV) infection on tobacco plants, we found a bacterial isolate KTB3, and identified it as *Acinetobacter* sp. which strongly inhibited the infection of TMV. When the culture filtrate from KTB3 was applied on the upper surface of the Xanthi-nc tobacco leaves at the same time, or 24 hours before TMV inoculation, almost complete inhibition was achieved. Likewise, 86% inhibition was achieved, when the culture filtrate was applied on the underside of the leaves. In field trials, transmission of TMV from diseased seedlings to healthy ones during transplanting work was reduced by 92%, when the culture filtrate was sprayed onto the tobacco seedlings, cv. NC82, 24 hours before transplanting. No toxic effect was observed on the tobacco plants. Antiviral substance from the culture filtrate was purified by ethanol precipitation, dialysis, DEAE-cellulose, and Sephadex G75 gel column chromatography. The partially purified active material which showed positive color reaction to sugar and protein inhibited TMV infection by 60% at 1 μg/ml.

Keywords **:** *Acinetobacter* sp., Inhibitory effects, TMV infection, Tobacco plants

Many plant diseases are caused by viruses, among them, *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), and *Potato virus Y* (PVY). These viruses damage the plants of commercial significance such as tobacco, tomato, sweet and bell pepper among others.

To control these viral diseases, researchers have investigated many inhibitors coming from various higher plants (Hudson, 1990; Stevens, 1992; Ito et al., 1992), microorganisms (Klement et al., 1966; Yeo et al., 1997), and some mushrooms (Aoki et al., 1993. They have also isolated and characterized a few of potent inhibitors.

However, effective materials are mostly proteins or polysaccharides, which have little potential to control viruses under field conditions, because their mass production and systemic translocation in plants are generally limited. To obtain a more potent inhibitor with systemic effect, we have tested various microorganisms, including fungi, actinomycetes, and bacteria. These tests led to the finding that culture filtrate of a bacterial isolate, KTB3, showed very high antiviral activity with systemic effects. This paper describes the inhibitory activity of culture filtrate from KTB3 on TMV, CMV, and PVY infection on tobacco plant.

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Materials and Methods

Taxonomy of the bacterial isolate. The bacterial isolate was identified according to Bergey's Manual of Systemic Bacteriology (Kreih and Halt, 1984). Physiological and biochemical studies were mainly performed with the bacterium grown in nutrient agar medium. The fatty acid composition of the cell wall was analyzed by using the Microbial Identification System (MIDI Inc. U.S.A) (Yang et al., 1993). The 16S rDNA secondary structures of isolate KTB3 were constructed with templates published in the Ribosomal Database Project (RDP) (Maidak et al., 1997) to aid in identifying homologous sequence positions. Multiple sequence alignments were performed manually in the CLUSTAL W (Yumoto et al., 1998).

Sample for assay. A loopful of KTB3 isolate, which was maintained on Mueller-Hinton-Agar (MH) slant, was transferred to 500 ml Erlenmeyer flask containing 200 ml of MH medium. This was cultured on a rotary shaker at 100 rpm for 4 days at 28°C. The culture was centrifuged at 10,000 rpm for 30 min. The supernatant was used for antiviral test.

Inhibitory activity of cultue filtrate. *Nicotiana tabacum* cv. Xanthi-nc was used for local lesion assay of TMV infection; while *N. tabacum* cv. NC82 and Burley 21 were used for systemic infection in the greenhouse and at the field.

For the virus inoculum, tobacco leaves (0.1 g) of NC82, systemically infected with TMV, were grounded in 20 ml of phosphate buffer (0.02 M, pH 7.3). The sap was filtered through two layers of cheesecloth. The extract was then centrifuged at 3000 rpm for 15 min to remove debris. The supernatant was used as TMV inoculum. The inocula of CMV and PVY were prepared

from *N. tabacum* cv. Burley 21 with similar method mentioned above.

Antiviral activity in local lesion host was tested by using the half-leaf method. The culture filtrate was applied with cotton swabs on the upper or underside surface of half leaf, while distilled water was applied on the remaining half as control. The virus was inoculated 24 hours after application of the culture filtrate by using ordinary Carborundum (600 mesh) methods.

For the antiviral activity in the systemic host, culture filtrate was sprayed onto the entire surface of the systemic hosts, tobacco cvs. NC82 and Burley21. Viruses were inoculated mechanically by hands on the underside leaf surface of each plant 24 hours after the spraying. The symptom on the inoculated plants was observed at 2 to 3 weeks after the inoculation, and the effects of the treatment were measured by comparing the number of diseased plants between the treated and untreated ones (control).

Control efficacy of culture filtrate in field was examined with NC 82 tobacco plants. The tobacco seedlings, cultivated in the greenhouse at 4-6 leaf stage, were sprayed with 4 day-old culture filtrate 24 hours before transplanting. Virus inoculation was induced from TMV-infected tobacco seedlings during transplanting work. Two and three weeks after inoculation, systemic mosaic symptom appearances were examined and compared with the control with no treatment of the culture filtrate. The 90 plants were tested for each treatment with 3 replications.

Results and Discussion

Taxonomy of the bacterial isolate. Strain KTB3 was an aerobic Gram-negative, non-sporulating rod (0.8-1.0 × 1.0-1.2 μm) with rounded ends. It showed weak motility and exhibited good growth at 27-37°C, and colonies on nutrient agar medium were moderate in growth, circular, convex, smooth, and slightly opaque with entire margins. Diffusible figments were not observed. The major fatty acids of strain KTB3 are $C_{18:1}$ (27.78%), summed feature 3 (27.24%), $C_{16:0}$ (25.95%), and ubiquinone Q-9 was analyzed as isoprenoid quinone.

Based on the above characteristics and partial 16S rDNA sequence analysis (data was not shown), the strain KTB3 showed similar properties of the strains that are registered species of the genus *Acinetobacter,* and was designated as *Acinetobacter* sp. KTB3.

Productivity of antiviral material. A time course for the production of antiviral material is shown in Table 1. *Acinetobacter* sp. KTB3 was cultured in 500 ml Erlenmeyer flasks containing 200 ml of Mueller Hinton medium (30°C/100 rpm). As seen, the maximum cell growth and antiviral activity of the culture filtrate were observed at 72 hours of culture.

Persistence of antiviral activity of *Acinetobacter* sp. KTB3. To examine the persistence, the upper surfaces of half-leaves of Xanthi-nc tobacco plants were treated with the culture filtrate, viable cells, and dead cell suspensions prepared from 3-day-old culture broth of KTB3, and TMV was inoculated from 1 to 9 days after treatment of those materials mentioned above.

Table 1. Time course of antiviral activity of *Acinetobacter* sp. KTB3 culture

Cultivation time (hours)[a]	Viable cell counts (ml^{-1})[b]	Antiviral activity (%)[c]
0	3.80×10^6	0
3	2.15×10^7	0
6	5.75×10^7	50.0
12	6.35×10^9	62.6
24	1.07×10^{12}	83.7
48	2.00×10^{12}	91.7
72	4.52×10^{12}	92.3
96	2.26×10^{12}	89.0
120	1.46×10^{12}	96.8
144	1.20×10^{12}	94.8
168	5.27×10^{11}	94.4

[a] Mueller-Hinton broth, 100 rpm, 28°C.
[b] 1 ml of culture broth was serially diluted and smeared on Mueller-Hinton agar plate.
[c] Biological assay on Xanthi-nc tobacco leaves with half-leaf method.

When the culture filtrate was applied before TMV inoculation, 100% inhibitory efficacy was achieved and persisted up to 5 days. This was lowered to 46% at 7 days after treatment (Table 2). Viable cells also showed similar antiviral effects with culture filtrate and its antiviral activity persisted 90% up to 7 days. Antiviral efficacy of dead cells treatment was 89% up to 1 day after treatment, but its persistence sharply decreased 2 days after treatment.

Inhibitory activity of culture filtrate. As shown in Table 2, the culture filtrate completely inhibited the TMV local lesion formation by application onto the upper surface of tobacco leaf 24 hours before the virus inoculation. It also inhibited TMV infection by 100% when applied as mixture

Table 2. Persistence of antiviral activity of strain KTB3 against TMV infection in tobacco plant

Time of TMV inoculation after treatment (day)[a]	Inhibitory activity (%)[b]		
	Culture filtrate	Viable cells[c]	Dead cells[d]
1	100	100	89
3	100	100	43
5	100	98	0
7	46	90	0
9	0	75	0

[a] TMV was inoculated at 1-9 days after treatment of sample solutions.
[b] Half-leaf methods using Xanthi-nc tobacco plants.
[c] 4 day-old culture was centrifuged and cell cake was suspended with distilled water.
[d] Heat-treated viable cells (100°C/15 min).

Table 3. Effects of the time of treatment on inhibitory activity

Time of treatment (hours)[a]	No. Local lesions/Half-leaves[b]		Inhibition (%)
	Treated	Untreated	
-168	74	289	46
-120	0	302	100
-72	0	269	100
-24	0	233	100
0	0	376	100
+1	266	446	40
+3	218	426	48
+6	296	332	10
+12	356	357	0

[a] The culture filtrate was applied to the upper surface of half-leaves of Xanthi-nc tobacco before (–) or after (+) TMV inoculation.
[b] Mean number of local lesions on 3 half-leaves.

the underside of the half-leaf of tobacco, virus infection onto the upper surface was also inhibited by 86% (data not shown). This result indicated that the inhibitory effects of culture filtrate were induced not only by barrier effects, but also by some other unclear antiviral mechanism. The systemic movement or PR gene induction of antiviral materials may be considered as mode of action.

Effects of the time of treatment. The culture filtrate was applied on the upper surface of leaves of Xanthi-nc tobacco before and after TMV inoculation to determine the effects of the time of treatment. As shown in Table 3, no appreciable inhibition was found when the treatment was done 12 hours after inoculation, although complete protection was observed when the treatment was done 120 hours before inoculation.

Systemic effects in the local lesion host. As mentioned above, application of the culture filtrate on the underside of the leaf potently inhibited TMV infection on the upper surface. So we did further experiments about systemic effects of culture filtrate from strain KTB3 in the Xanthi-nc tobacco plants. The results are shown in Table 4. TMV

Table 4. Systemic inhibitory effects of the culture filtrate on TMV infection

Inoculation time after treatment (hours)	Inhibitory activity (%)[a]			
	Treated[b] lower leaves	Untreated upper leaves		
		1st	2nd	3rd
0	99	23	2	4
24	100	56	13	3
48	100	45	8	-11

[a] Half-leaf method using Xanthi-nc tobacco plants.
[b] Culture filtrate was applied to only lower 3 leaves and then, TMV was inoculated to the treated, and untreated upper 3 leaves, respectively.

Table 5. Inhibitory activity of culture filtrate of strain KTB3 in virus-systemic host combinations

Virus-Tobacco plants	No. of infected plants[a]		Inhibition (%)
	Treated	Control	
TMV-NC82	3	20	85
CMV-NC82	4	17	76
CMV-Burley 21	7	17	59
PVY-NC82	8	20	60
PVY-Burley 21	10	18	44

[a] The symptoms on 20 plants were observed and infected plants were counted 2 weeks after virus inoculation.

infection on the 1st untreated upper leaf which was located just above the treated leaf, was significantly reduced up to 56%. But no significant effect was observed on the 2nd and 3rd untreated upper leaves.

Antiviral effects in the systemic host. Antiviral effects of culture filtrate in systemic hosts are summarized in Table 5. When the culture filtrate was sprayed 24 hours before virus inoculation, TMV, CMV, PVY infections through the underside leaves of their systemic host were remarkably reduced in greenhouse condition. This result showed that the inhibitory activity of culture filtrate was also systemic in tobacco plants.

Control of TMV in field. With treatment of the culture filtrate, TMV incidence was remarkably decreased, with 92.2% (Table 6) control efficacy at 2 weeks after inoculation. The control efficacy persisted above 91% up to 3 weeks after treatment.

Antiviral material from culture filtrate of KTB3. Antiviral material from culture filtrate was isolated and partially purified by ethanol precipitation, dialysis, Sephadex G75 column chromatography. The purified antiviral material was white powder, soluble in water only, and showed positive color reaction to sugar and protein. Table 7 shows the antiviral activity of partially purified material. Detailed studies on physicochemical properties and structural elucidations of antiviral material from strain KTB3 are currently being studied.

It has been known that higher plants and fungi widely contain antiviral substances, and some of these substances

Table 6. Control efficacy of culture filtrates of KTB3 against TMV infection in field

Treatment[a]	No. of infected plants (control efficacy, %)	
	2 weeks	3 weeks
Culture filtrate	18(93)	24(91)
Control	267(0)	270(0)

[a] Culture filtrate of 4 day-old was sprayed on the seedlings of NC82 cultivars 24 hours before TMV inoculation.

Table 7. Inhibitory effects of antiviral material from KTB3

Antiviral material (ppm)[a]	Local lesions/Half-leaves[b]		Inhibitory effects (%)
	Treated	Control	
500	0	214	100
100	0	252	100
10	16	404	96
1	136	332	60

[a] Antiviral material dissolved in distilled water and diluted serially.
[b] Mean number of lesions on 3 half-leaves of Xanthi-nc tobacco.

were isolated and characterized. But the antiviral substances from bacteria were rarely reported and inhibitory activity in the field has not been reported yet. The present study definitely shows that the *Acinetobacter* sp. strain KTB3 contains a potent inhibitor of TMV infection on tobacco plants. The culture filtrate of this strain showed 93.2% inhibitory activity when it was sprayed on tobacco plant 24 hours before virus inoculation in field.

The field control effect of the culture filtrate from KTB3 may be related to physical stability and systemic effect of the responsible material. Although the culture filtrate showed weak systemic effects in the local lesion host under the greenhouse conditions, the activity in systemic host at the field was noteworthy. The antiviral materials showed systemic effect both in local lesion and in the systemic host that was partially purified from culture filtrate. This material showed positive color reaction to sugar and protein, and are heat stable at 121°C for 15 min.

From these physicochemical properties with the analysis of ^{1}H-NMR spectrum data (data not shown), we tentatively conclude that the antiviral materials are glycoproteins. The mode of action, structural elucidation of antiviral principles from culture filtrate of strain KTB3 will be the subject of future reports.

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