Antiviral and Antiviroid Activity of MAP-Containing Extracts from *Mirabilis jalapa* Roots

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ABSTRACT

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Extracts of *Mirabilis jalapa* (Nyctaginaceae), containing a ribosome inactivating protein (RIP) called Mirabilis antiviral protein (MAP), were tested against infection by potato virus X, potato virus Y, potato leaf roll virus, and potato spindle tuber viroid. Root extracts of *M. jalapa* sprayed on test plants 24 h before virus or viroid inoculation inhibited infection by almost 100%, as corroborated by infectivity assays and the nucleic acid spot hybridization test. Antiviral activity of MAP extracts was observed against mechanically transmitted viruses but not against aphid-transmitted viruses. Purified MAP showed the same antiviral effect as the crude extracts.

Several plants, such as *Pelargonium hortorum*, *Chenopodium album*, *C. amaranticolor*, *Capsicum frutescens* (4), *Azadirachta indica* (20), *Vitis vinifera* (6), and *Rosa banktia* (21), possess antiviral factors (1). Plant-derived antiviral compounds are active against plant, animal, and human viruses (27,29). According to Zipf (30), plant antiviral compounds are grouped as furocoumarins, alkaloids, terpenoids, lignins, and specific proteins. Among plant-derived antiviral proteins, a group called ribosome-inactivating proteins (RIPs), which are widely distributed in higher plants, hold promise for agricultural and pharmaceutical applications (2,15). RIPs exist either as single-chain (type I) or double-chain (type II) proteins. Both type I and type II RIPs are basic proteins with isoelectric points above nine. Type II RIPs posses a biologically active polypeptide (A chain) and a B chain, which includes a galactose binding domain (15) that enables the RIP to bind to the cell wall galactose receptors and to internalize the A chain into the cell. Cell penetration of type I RIPs is enabled by wounds on plant cell walls caused by insects, mechanical contact among leaves, or previous viral infections (17). Recently, an acidic type III RIP, typified by maize b-32, has been identified (16). All RIPs inactivate ribosomes by modifying the 28S rRNA through its N-glycosidase activity, which is manifested by cleavage of the N-glycosidic bond at a specific adenine. The adenine at position 4324 of rat liver 28S rRNA and corresponding adenines on other eukaryotic or prokaryotic models are the target sites (24). Through this mechanism, the binding of elongation factor 2 is prevented, with the consequent arrest of protein synthesis.

Leaves and roots of the native Peruvian plant *Mirabilis jalapa* L. (14) were found to contain an antiviral protein which was active against the mechanical transmission of certain plant viruses (10,26). This protein, a type I RIP, was named Mirabilis antiviral protein (MAP; 8). MAP was purified to homogeneity and was revealed to be lysine rich and basic (pI 9.8), with a molecular weight close to 24.2 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 26). Purified MAP has been shown to inhibit the mechanical transmission of tomato mosaic virus (TMV) in tobacco, tomato, and pepper plants, and cucumber green mottle mosaic virus in cucumber plants (10). Moreover, MAP was also shown to inhibit protein synthesis in *Escherichia coli* as well as in eukaryotes (8) and to possess repellent properties against aphids and white flies (28). Kataoka et al. (9) showed that MAP was compartmentalized in *M. jalapa* vacuoles, sequestering its ribosome inactivating activity away from its own ribosomes.

Potential agricultural applications of RIPs include their use as plant antiviral and fungicidal agents (18,24). The genes coding for RIPs in some plants, such as *Phytolacca americana*, have been cloned and introduced into the potato and tobacco genome, allowing transgenic plants to show resistance against a broad spectrum of viruses and root-rot fungi (13,31). RIPs have also shown pharmaceutical potential as chimaeric toxins, which can be targeted to cancer cells (23,24). Moreover, it has been demonstrated that several RIPs inhibit the replication of human immunodeficiency virus (HIV), a finding that has stimulated clinical trials (12).

Here we report the development of an easily adaptable technology for controlling potato viruses by the pre-inoculation application of *M. jalapa* extracts.

MATERIALS AND METHODS

Plant material and protein separation. *M. jalapa* plants were collected at the botanical garden of the Universidad Nacional Agraria La-Molina, Lima, Peru. A modified protein extraction protocol was used (22). Briefly, root extracts were prepared by homogenizing 0.1 g of lyophilized tissue in 2.5 ml of ice-cold extraction buffer (25 mM $NaPO_4$, pH 7.0, with 250 mM NaCl, 10 mM EDTA, 10 mM thiourea, 5 mM dithiothreitol [DTT], 1 mM phenyl methyl sulfonyl fluoride, and 1.5% polyvinylpolypyrolidone). The recovered supernatant was filtered through glass microfiber filters and subsequently precipitated in 20% ammonium sulfate. After centrifugation, samples were desalted on an Economac 10DG column (Promega, Madison, WI), then buffer exchanged on 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; pH 8.0). HS/M POROS cation-exchange columns (Perspective Biosystems, CA) in conjunction with a Waters 600E high-performance liquid chromatography (HPLC) system equipped with a U6K injector and a photodiode array (Waters, MA), were used for protein separation. Equilibration, loading, and washing were carried out in 25 mM HEPES (pH 8.0), and elution was accomplished using a linear salt gradient (0 to 300 mM NaCl) in 10 column volumes. The flow rate was 5 ml/min throughout the procedure.

Protein purity was assessed by SDS-PAGE, which was performed on 13.5% acrylamide discontinuous gels according to Laemmli (11) using a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Richmond, CA) following the manufacturer's instructions.

Viruses and viroid. Potato virus X strain cp (PVXcp) was maintained in *Nicotiana glutinosa* plants. The necrotic and common strains of potato virus Y (PVY^N and PVY^O) were maintained in *N. occidentalis* plants. Systemically infected

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Table 1. Antiviral effect of crude extracts from *Mirabilis jalapa* on the infectivity of potato virus X in *Gomphrena globosa* plants

Source of extract	Dilution	No. of local lesions[a]	Inhibition (%)
Root	1:50	74.2 ± 5.1	25
	1:5	15.9 ± 1.4	84
Leaf	1:50	57.1 ± 1.2	42
	1:5	1.3 ± 0.1	99
Control	Phosphate buffer	98.2 ± 5.2	…
Root	1:50	71.0 ± 3.6	20
	1:5	0.5 ± 0.01	99
Leaf	1:50	101.7 ± 6.8	–15
	1:5	1.1 ± 0.08	99
Control	Sterile water	88.6 ± 5.7	…
Root	1:50	69.2 ± 4.2	9
	1:5	2.5 ± 0.1	97
Leaf	1:50	71.1 ± 3.7	7
	1:5	2.3 ± 0.1	97
Control	Non-sterile water	76.2 ± 5.9	…

[a] Local lesions were counted 14 days after virus inoculation. The number of local lesions corresponds to the average of 15 plants used per treatment. The percentage of inhibition was calculated comparing the number of local lesions of the treatment with the control. The experiment was repeated three times. Phosphate buffer, sterile water, and non-sterile water were rubbed as controls on the leaves 24 h prior to virus inoculation.

leaves were used as sources of inoculum in all experiments. Potato spindle tuber viroid (PSTVd) inoculum consisted of infected *Lycopersicum esculentum* cv. Rutgers leaves. Potato leafroll virus (PLRV) was maintained in infected potato plants, which were used as the inoculum source.

Nucleic acid spot hybridization. The nucleic acid spot hybridization (NASH) test was conducted according to Salazar and Querci (19) using a Riboprobe system-SP6 kit (Promega Corp., Madison WI). Treated and control leaves were pressed onto nitrocellulose membrane using uniform manual pressure for 15 to 30 sec. The membranes were then hybridized with specific viral probes. Specific radioactive (P^{32}) probes were utilized for PVX (pX61), PVY (pGEM4), PLRV (pSP65), and PSTVd (pSP65-B2) (19).

Preventive treatment with *M. jalapa* extracts. *Gomphrena globosa* plants were used as local lesion hosts to monitor the

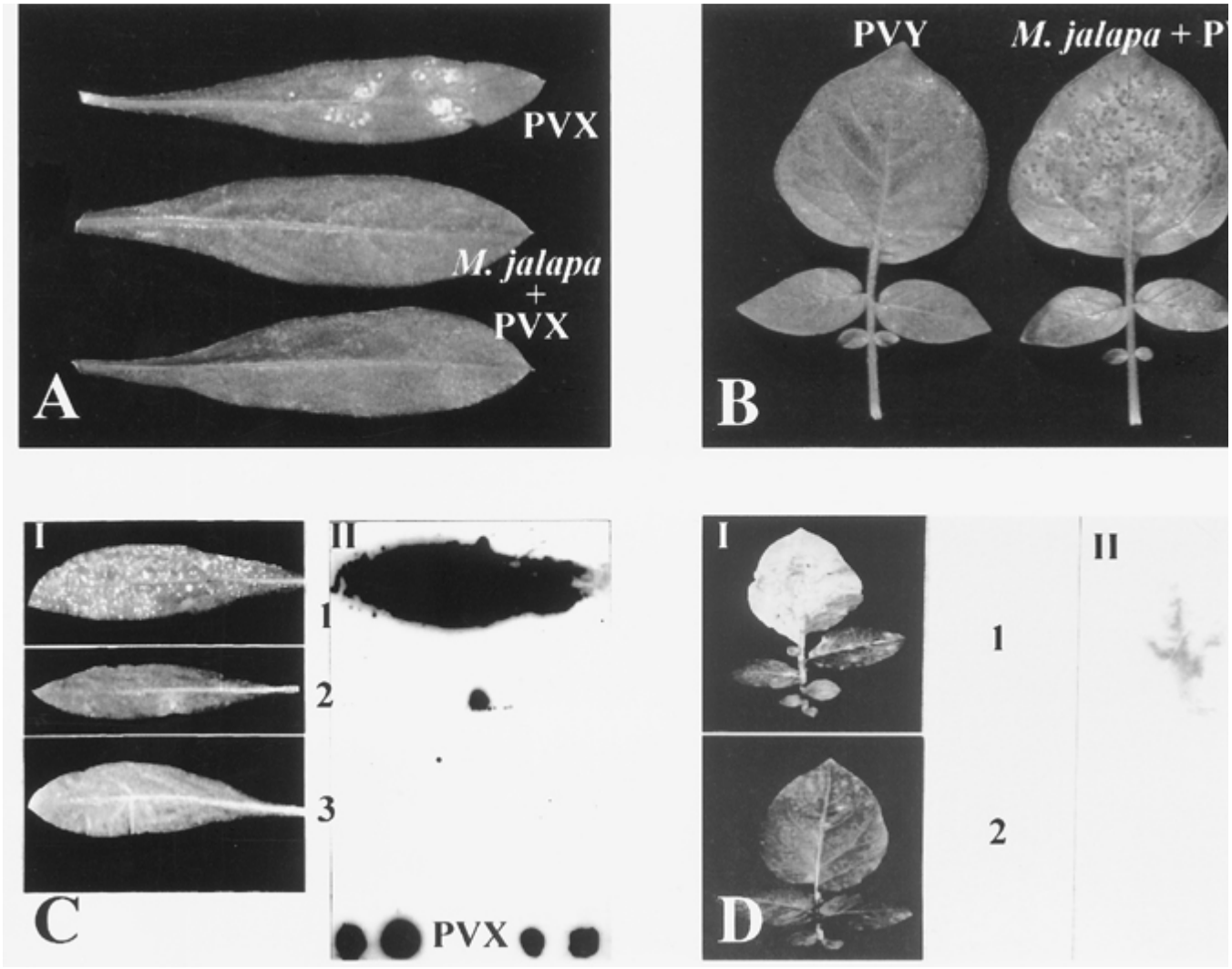

Fig. 1. Effect of *Mirabilis jalapa* extracts against **(A)** potato virus X strain cp (PVXcp) on *Gomphrena globosa* leaves and **(B)** potato virus Y, common strain (PVY^O) on *Solanum tuberosum* var. Aquila clone A-6 leaves. **(CI)** Reaction of *G. globosa* leaves to PVX; 1 = leaf inoculated with PVX (control); 2 and 3 = leaves inoculated with PVX 24 h after the spray with *M. jalapa* extracts. **(CII)** Hybridization test to detect PVX. **(DI)** Reaction of *S. tuberosum* var. Aquila clone A-6 leaves; 1 = leaf inoculated with PVY^O (control); 2 = leaf inoculated with PVY^O 24 h after the spray with *M. jalapa* extracts. **(DII)** Hybridization test performed on the same leaves against PVY.

effect of the treatments on PVX. *Chenopodium amaranticolor* and *Solanum tuberosum* var. Aquila clone A-6 plants were used as hypersensitive hosts for PVY. In each treatment, four leaves per plant were inoculated. The effect of MAP on PSTVd replication was monitored using *Lycopersicum esculentum* var. Rutgers as the susceptible host. The *S. tuberosum* clone DTO-33, susceptible to all viruses tested, was also included in some experiments.

M. jalapa root and leaves were blended and diluted (1:5, wt/vol) in tap water, sterile water, or 25 mM phosphate buffer (pH 7.2). Total protein concentration in the diluted extracts was 100 µg/ml. The procedure used in these experiments consisted of pretreatment of the host leaves with *M. jalapa* extracts (100 µg/ml) either by hand rubbing or spraying, followed by viral inoculation 24 h later. Viruses and the viroid were mechanically inoculated to plants dusted with Carborundum, 600-mesh, as an abrasive. The inoculum consisted of virus or viroid in leaf sap diluted 1:20 (wt/vol) in 10 mM phosphate buffer (pH 7.2). Local lesions were counted 14 days after virus inoculation. Control treatments consisted of plants pretreated with water or phosphate buffer and subsequently inoculated with the purified virus or sap from virus-infected plants without any pretreatment.

The percentage of viral inhibition in the treatments was analyzed utilizing a random distribution model, using each plant as an experimental unit. Percentage of viral inhibition was employed to analyze the data.

Persistence of antiviral effect. The residual inhibitory effect of the *M. jalapa* extracts was tested against PVX infection on a group of *G. globosa* plants that were sprayed with the extracts of *M. jalapa* (pretreatment). From this group, three plants per day were then inoculated with PVXcp, starting 24 h after pretreatment and continuing on a daily basis for a month.

Effect of MAP on aphid transmission. Virus-free, apterous, *Myzus persicae* aphids maintained in *Brassica pekinensis* were placed for 24 h on two sets of potato plants infected with PVY and PLRV, respectively. Meanwhile, a group of susceptible potato plants was sprayed with *Mirabilis jalapa* extracts. After 24 h, the PVY and PLRV infectious aphids were transferred to the leaves of potato plants (10 per virus) and then killed with a contact insecticide 24 h later. Infection was assessed by the NASH test.

Table 2. Inhibitory effect of the *Mirabilis jalapa* extracts against potato virus X (PVX) and potato virus Y (PVY) infection on susceptible *Solanum tuberosum* clone DTO-33 plants

	PVX[a]		PVY[a]	
Days after inoculation	**Control**	***M. jalapa***	**Control**	***M. jalapa***
15	3/5	0/5	3/5	0/5
30	3/5	0/5	5/5	0/5
45	3/5	0/5	5/5	0/5
60	4/5	0/5	5/5	0/5

[a] Number of plants infected/number of plants inoculated. Plants were tested by nucleic acid spot hybridization.

RESULTS

Inhibitory activity of *M. jalapa* extracts against PVX and PVY. *M. jalapa* root extracts were applied to the leaves of *G. globosa*, an indicator plant which reacts hypersensitively to PVXcp infection. Results show that the root and leaf extracts diluted 1:5 (vol/vol) in sterile water were strongly inhibitory to PVX infection, because almost 100% inhibition was observed (Table 1). The inhibitory activity of MAP was not affected by the three diluents; even extracts diluted with tap water gave an inhibitory effect. Similar effects were found by using leaf or root tissues. Purified MAP also showed antiviral inhi-

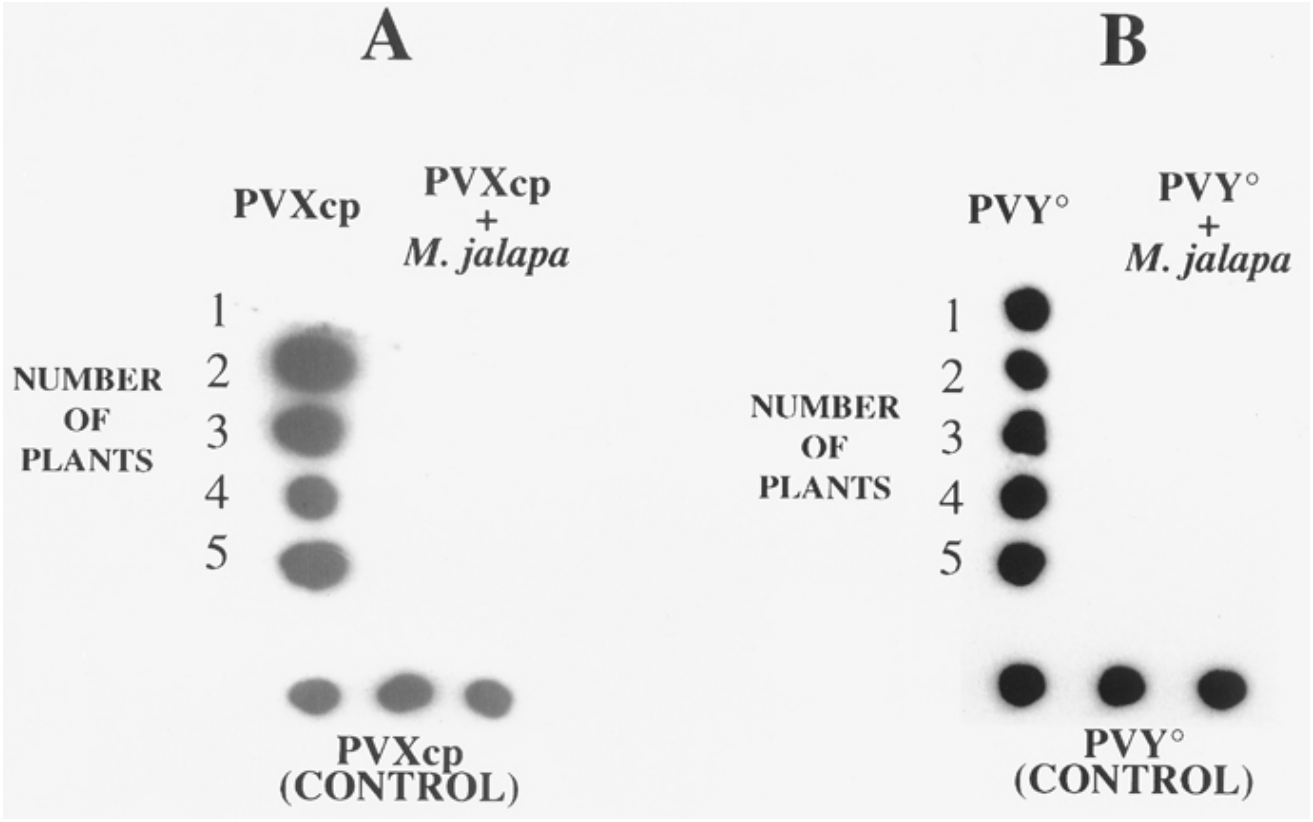

Fig. 2. Inhibitory effect of the preventive spray of *Mirabilis jalapa* root extracts against **(A)** potato virus X (PVX) and **(B)** potato virus Y (PVY) infection on the susceptible *Solanum tuberosum* clone DTO33. The nucleic acid spot hybridization test was performed on the leaves of five *S. tuberosum* plants 45 days after viral inoculation.

bition (Fig. 1A). Similar levels of inhibition were observed against PVY on *C. amaranticolor* and on *S. tuberosum* var. Aquila clone A-6 (Fig. 1B).

To confirm that lack of symptoms corresponded to blockage of virus replication in the treated leaves, the NASH test was used. No virus was detected in asymptomatic areas, confirming the inhibitory effect of the extracts (PVX and PVY, Fig. 1C and D, respectively).

To determine the effect of *M. jalapa* root extracts against PVXcp and PVY^{O} in a systemic-infected host, the susceptible potato clone DTO-33 was used. Subsequently, the plants were screened for virus presence by NASH, using probes specific for PVXcp and PVY. The screening was performed every 12 to 15 days for almost 2 months. All plants pretreated with *M. jalapa* root extracts were not infected by PVXcp and PVY, respectively (Table 2 and Fig. 2). Sprouts from tubers obtained from DTO-33 plants infected with PVXcp were homogenized and the sap inoculated onto *G. globosa* plants (*data not shown*). This back-test confirmed the total absence of PVX in the tested plants. Local lesions were produced on *G. globosa* leaves inoculated with "control" tuber sprouts but not on the leaves inoculated with tuber sprouts from plants previously sprayed with *M. jalapa* extracts. The inoculated leaves were also analyzed against PVX and PVY by NASH, with the same negative results. The experiments were repeated twice and similar results were obtained.

Inhibitory activity of *M. jalapa* extracts against PSTVd. The effect of *M. jalapa* root extracts was also tested against PSTVd in the susceptible host *L. esculentum* cv. Rutgers. Symptoms typical for PSTVd infection developed 15 days after inoculation in control plants pretreated with water. No symptoms were observed in the five plants pretreated with *M. jalapa* extracts (Fig. 3A). The results were confirmed when the experiment was repeated on 10 more plants; PSTVd infected none of the *L. esculentum* cv. Rutgers plants when treated with *M. jalapa*. All 15 plants pretreated with *M. jalapa* 24 h prior to mechanical inoculation with PSTVd were negative by NASH, indicating that no PSTVd replication occurred. The above experiments were also confirmed on the susceptible potato clone DT0-33 (Fig. 3B).

Effect of *M. jalapa* extracts against aphid-mediated viral infection of PLRV and PVY. The effect of *M. jalapa* extracts was tested against aphid-mediated transmission of PLRV and PVY on two sets of 10 susceptible potato clone DTO-33 plants, using the aphid *Myzus persicae* as vector. In this case, no inhibitory effect was observed in the plants previously treated with *Mirabilis jalapa* extracts (*data not shown*). Control and treatment plants showed similar levels of viral infection when tested by NASH.

Persistence of antiviral effect. The persistence of the antiviral effect (residual effect) was tested during a time course experiment, in which *G. globosa* plants sprayed with the extract were inoculated with PVXcp at different intervals after the treatment. Approximately 90 and 50% inhibition remained in plants inoculated 15 or 30 days, respectively, after pretreatment with the *M. jalapa* extracts (Table 3).

DISCUSSION

M. jalapa is a member of the Nyctaginaceae family, which includes *Bouganvillea spectabilis* and *Boerhaavia diffusa*, both of which have antiviral properties (10). Grasso et al. (7) pointed out the order Chenopodiales and the sub-class Centrospermae as a source of antiviral agents.

The effect of MAP against virus infection suggests a mechanism by which MAP

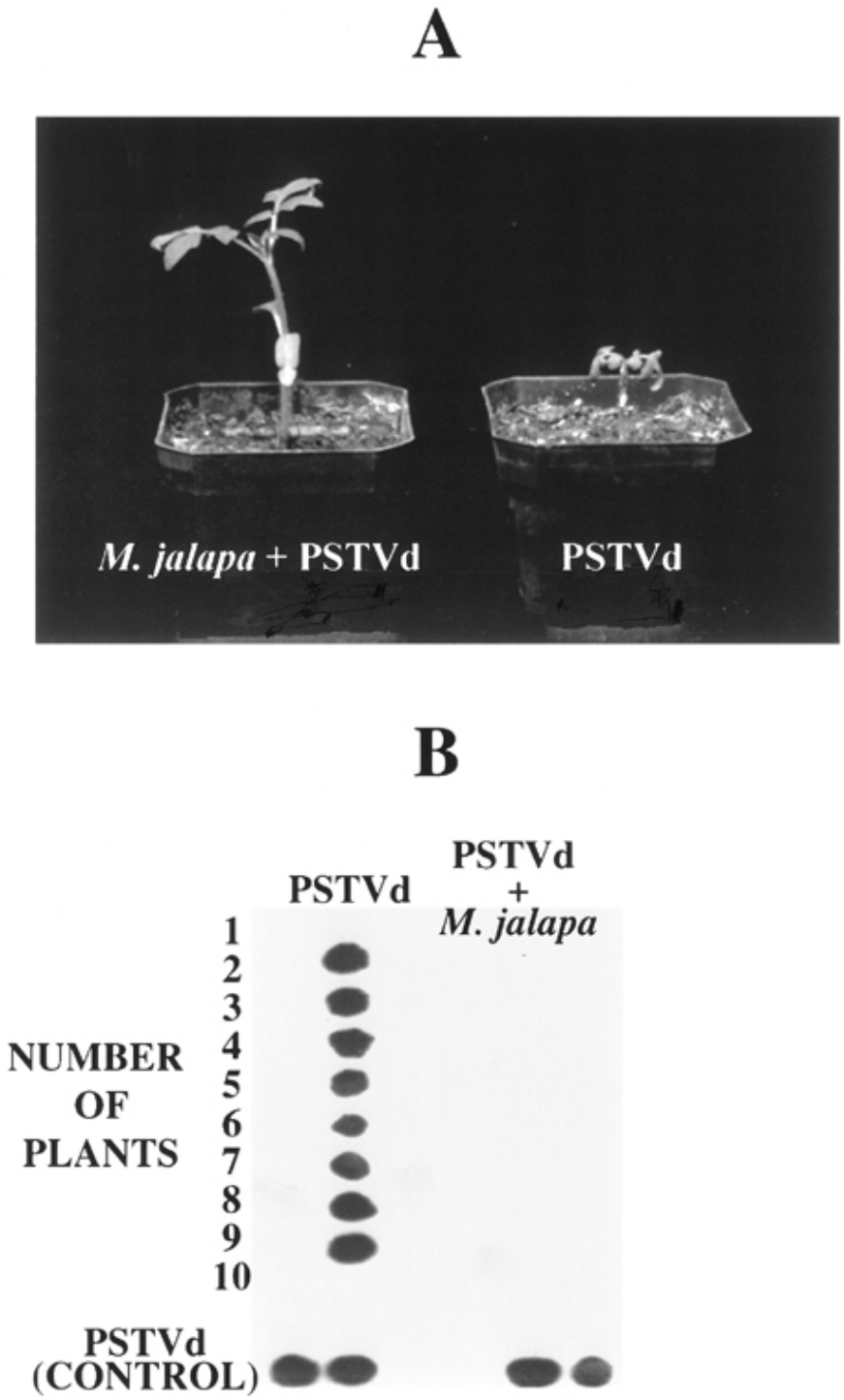

Fig. 3. Inhibitory effect of *Mirabilis jalapa* extracts against potato spindle tuber viroid (PSTVd) infection on **(A)** the susceptible *Lycopersicum esculentum* plant (left) shown 10 days post inoculation and **(B)** *Solanum tuberosum* clone DTO33, as shown by the nucleic acid spot hybridization test performed on leaves of 10 *S. tuberosum* plants 45 days after viroid inoculation.

penetrates the upper layers of the epidermis and situates itself in the intercellular spaces. When the plants are wounded during virus infection, MAP is able to penetrate epidermal and other leaf cells, where it deglycosilates the 28S rRNA. This prevents viral replication at an early stage by deactivating the cell protein synthesis machinery. This hypothesis accounts for the lack of inhibition against viruses, such as PLRV, which are transmitted by aphids in a persistent manner. Apparently, MAP translocation was not fast enough to reach the phloem cells, where PLRV replicates.

Another explanation could be that MAP situates itself on the surface of the leaf, remaining stable due to its basic nature (2). When viral inoculation is performed, the virus penetrates the cell along with MAP. Once inside the cell, MAP and the virus compete for the active sites on the ribosomes. MAP depurinates the 28S rRNA and thus inactivates the protein chemistry of the cell. If this is the case, our results suggest that MAP reaches the active site of the ribosomes first, preventing viral infection at an early stage, probably before viral de-encapsidation. Chen et al. (5), while performing inhibitory experiments against TMV with pokeweed antiviral protein (PAP), a type I RIP from *Phytolacca americana*, reported that the TMV coat protein release involves the action of the functional host ribosomes. Once the virus has been uncoated and translation of some viral RNA has taken place, the infection process may not be sensitive to PAP (5). This is consistent with the effectiveness of the preventive treatment we found using *M. jalapa* extracts against potato viruses X, Y, and PSTVd viroid.

Based on our results, we hypothesize that prevention of PVX and PVY infection is due to the inhibition of viral protein and enzyme synthesis. It has been reported that some RIPs, such as Saporin-L1, a RIP from *Saponaria officinalis*, release many adenine residues not only from rRNA but from other tested RNAs, from poly(A), and from herring sperm DNA (25). Similarly, MAP may have a direct nuclease activity against PSTVd. Furthermore, the ability of RIPs to inhibit viral replication without damaging host cell protein chemistry has been observed (15,25).

The MAP could behave as a signal molecule that turns on a cascade response, activating a series of defense mechanisms before viral infection takes place (31). The availability of plant model systems, such as *Arabidopsis* spp., will help to elucidate this hypothesis. Experiments are currently being conducted to clarify this possible RIP-related activity.

MAP is a good candidate for transformation of potato varieties and has already been established with other RIPs in tobacco (13). With adequate promoters, the MAP gene could be expressed in the vacuoles or extracellular spaces of the potato plant; thus, a broad spectrum virus-resistant plant could be produced. Alternatively, MAP antiviral activity could be used in simple crop-protection methods in low-input agricultural systems, such as the spraying of root extracts on leaves of various crops to prevent or control viral infection. To our knowledge, this is the first time that antiviroid activity has been attributed to RIP-containing extracts.

Table 3. Effect of *Mirabilis jalapa* extracts on infection of *Gomphrena globosa* plants mechanically inoculated with potato virus X

	No. of local lesions/plant[a]		
Day[b]	Treatment	Control	Inhibition (%)
1	0.5	35.5 ± 1.2	98.6
2	0.5	31.0 ± 0.6	98.4
3	2.0	73.0 ± 1.9	97.0
4	5.5 ± 0.2	78.5 ± 4.1	93.0
5	8.0 ± 0.6	103.5 ± 7.2	92.3
6	20.0 ± 1.2	117.5 ± 6.1	83.0
7	29.5 ± 2.5	121.5 ± 4.2	76.0
8	9.5 ± 0.7	121.0 ± 3.4	92.1
9	8.0 ± 0.6	120.0 ± 3.8	93.4
10	20.0 ± 1.1	120.0 ± 5.3	83.4
12	16.0 ± 0.9	125.0 ± 7.4	87.2
14	70.0 ± 3.1	170.0 ± 8.9	58.8
15	13.0 ± 0.9	140.0 ± 5.8	90.7
16	59.0 ± 3.1	140.0 ± 6.4	57.8
18	53.0 ± 3.8	120.0 ± 4.1	53.8
21	32.0 ± 2.7	120.0 ± 3.2	73.3
23	67.0 ± 4.5	150.0 ± 5.1	55.3
24	80.0 ± 5.6	150.0 ± 6.3	46.6
28	62.0 ± 4.1	150.0 ± 4.1	58.67

[a] Lesions were counted 14 days after each virus inoculation. The experiment was repeated three times.

[b] Plants were inoculated 1 to 28 days after treatment with the extract.

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