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Antiphytoviral Activity of the *Plectranthus tenuiflorus* on Some Important Viruses

B.A. OTHMAN AND S.A. SHOMAN[1]†
Department of Microbiology, Faculty of Agriculture, Ain Shams University, Cairo–Egypt
†*Department of Microbiology, Faculty of Science, Ain Shams University, Cairo–Egypt*
[1]Corresponding author's e-mail: saharshoman@hotmail.com

ABSTRACT

Crude extract of *Plectranthus tenuiflorus* crude extract was tested as an antiphytoviral agent against different plant viruses like Tobacco Necrosis Virus (TNV), Tobacco Mosaic Virus (TMV), and Tomato Spotted Wilt Virus (TSWV). When it was applied onto *Phaseolus vulgaris*, *Datura stramonium*, and *Chenopodium amaranticolor* as pre-inoculation spray (*in vivo*), it reduced the infectivity of above viruses by 90.6, 85.8 and 77.7%, respectively. However, when the extract was mixed with the virus inoculum (*in vitro*), it inhibited the local lesion development by 100% after one hour of mixing for TNV, and three hours for both TMV and TSWV. Effect of *P. tenuiflorus* on the systemic TMV infection was also studied. It delayed the onset of the disease development from 4 to 5 days, although it had less apparent effect on the virus accumulation. Studying the effect of temperature and rate of dilution on the antiphytoviral activity revealed that the thermostable property of this extract as well as remaining of its antiphytoviral activity up to 10^{-2} dilution rate. In order to investigate the bioactive constituent of the *P. tenuiflorus*. Crude extract, its proteins, carbohydrates, and storage oil were separated and tested as an antiphytoviral agent. The results indicated that the antiviral activity was not mainly attributed to these constituents.

Key Words: Antiviral; *P. sp;* Plant virus

INTRODUCTION

The plant family Lamiaceae (mint family) represents a valuable pool of plant species, which contain biologically active molecules (Harley & Reynolds, 1992). The genus *P.* consists of about 350 species distributed from Africa through to Asia and Australia (Godd, 1985).

Many *P.* species are plants of economic and medicinal interest: *Plectranthus hereroensis* (Batista *et al*., 1995), *P. elegans* (Dellar *et al*., 1996), and *P. grandidentatus* (Teixeira *et al*., 1997). It has been reported that the medicinal value of these *P.* species attributed to the antibacterial, antifungal, and antiviral activities. These activities mainly depend on the phytochemical constituent characteristic of this genus (Abdel-Mogib *et al*., 2002).

Concerning the phytopathogenic viruses, various substances of natural and synthetic origins have been used for control of virus infection. However none of them possessed a satisfactory selective action, which could enables them to be used as a therapy of plant viral diseases (Allam *et al*., 1979; Verma & Baranwel, 1983; Barakat, 1988; Hansen**,** 1989; Takanami *et al*., 1990; Othman *et al*., 1991; Meyer *et al*., 1995; Yordanova *et al*., 1996; Eldougdoug, 1997; Shoman, 2002).

P. tenuiflorus is a small, downy, very leafy herb, with stems of about 60 cm long, found in Abha, Saudia Arabia (Collenette, 1985). Its medicinal value has been reported by Abulfatih (1987). This work was undertaken to study the effectiveness of *P. tenuiflorus* extract on different plant virus infections as an antiphytoviral agent. To our knowledge, this is the first study to address this topic in Egypt.

MATERIALS AND METHODS

Source of viruses. The viruses used in this study were Tobacco Necrosis Virus (TNV-D), Tobacco Mosaic Virus (TMV-8) and Tomato Spotted Wilt Virus (TSWV) isolates. The viruses were provided by Dept. of Microbiology, Faculty of Science, Ain Shams Univ. and Dept. of Agric. Microbiology, Faculty of Agriculture, Ain Shams Univ., Cairo, Egypt.

The viral inocula were prepared from infected frozen leaves of *Phaseolus vulgaris*, *Nicotiana tabacum,* var white barley and *Chenopodium amaranticolor* previously infected with TNV, TMV and TSWV, respectively.

Five grams of leaf material was ground with 50 mL of distilled water in a mortar. The extract was strained and the filtrate made up to 200 mL with distilled water. This dilution of sap extract was found to give a suitable number of discrete local lesions on test plants.

Source of *P. tenuiflorus.* Part of the stem of plant (about 15 cm) was obtained from Saudia Arabia and planted in 10 cm pots, kept in the glass green house at 25±5°C. Parts of the new branches were taken and subcultured to obtain a large number of the plant material.

Preparation of the antiphytoviral. To obtain the crude extract, 10 g of the small, downy, herbaceous leaves of *P.*

tenuiflorus were ground completely in porcelain mortar with 20 mL of distilled water then clarified

Plant materials. Seeds of *Datura stramonium*, *Chenopodium amaranticolor* and *Nicotiana tabacum*, var white barley were sown in trays, maintained in a glass house at 25±5°C and watered as required. When the seedlings were large enough, they were planted individually into 10-cm plastic pots. They were selected for testing when they had 4-6 true leaves. Seeds of *Phaseolus vulgaris* were potted directly in the plastic pots, plants were selected when they had the full expanded primary (cotyledonary) leaves. Care was taken to ensure that the experimental plants were as uniform in size as possible.

Assay of the antiphytoviral activity

In vitro. To investigate the effect of the antiphytoviral agent on the viruses, 1.0 mL of each virus suspension was mixed with 1.0 mL of the *P. tenuiflorus.* crude extract. The mixture was left for 0, 1, 2, and 3 h at room temperature. The samples (mixtures of both viruses & antiphytoviral agent) were inoculated mechanically by dipping the forefinger in inoculum and rubbing once over the upper surface of the local lesion host leaves (*Phaseolus vulgaris* for TNV, *Datura stramonium* for TMV, & *Chenopdium amaranticolor* for TSWV), using carborandum as abrasive material. The number of local lesions on the inoculated leaves was counted 5-6 days after inoculation. The antiphytoviral inhibitory effect on the infectivity of viruses was assayed using the following equation: % of inhibition=number of local lesions on control-number of local lesions on treated / number of local lesions on control X 100. For control, distilled water was used instead of the antiphytoviral agent. Four to six plants of each host were used for each treatment.

In vivo. This experiment was carried out by applying different dilutions (10^{-1}, 10^{-2}, & 10^{-3}) of antiphytoviral extract to the local lesion hosts (*Phaseolus vulgaris, Datura stramonium,* and *Chenopdium amaranticolor)* for 2 successive days prior to TNV, TMV, and TSWV inoculation respectively. *Nicotiana tabacum,* var white barley was used also as a systemic host for assay of the antiphytoviral activity of *P. tenuiflorus.* With systemic infection.

ELISA. TMV was serologically detected in its systemic hosts using ELISA. The tests were done as described by Clark and Adams (1977). After two weeks of TMV inoculation the sap of the upper leaves of both *P. tenuiflorus* extract-treated and untreated tobacco plants were prepared by homogenizing leaf tissues in 0.02 M phosphate buffer saline containing 0.05% tween 20 and 2% polyvinylpyrrolidine.

TMV antiserum was obtained by the repeated injections of 50 µg of purified virus preparations (provided from Dept. of Agric. Microbiology, Faculty of Agriculture, Ain Shams University) into Newzland healthy rabbits. The purified preparation of virus was measured with spectrophotometer (230-320 nm) as a quick and convenient method confirm on the purity of the virus (Donbrow, 1967). The doses were applied at weakly intervals. The TMV suspension was emulsified with incomplete then complete adjuvants, finally antiserum was collected and partially purified by using saturated ammonium sulphate solution.

In all tests microplate ELISA plates were used with coating TMV-antiserum at 10 µg/mL and antibody-enzyme conjugate at 1:1000. The enzyme substrate paranitrophenyl phosphate at concentration 0.6 mg/mL was allowed to act for 30 min at room temperature. The reaction was then stopped by adding 50 µL of 3N NaOH and the absorbance was measured at 405 nm with a spectrophotometer.

Effect of dilution and heating on the antiphytoviral agent. The crude extracts of *P. tenuiflorus.* was serially diluted with distilled water to 10^{-1}, 10^{-2} and 10^{-3}. For heat treatment, test tubes each containing 2 mL of the crude extract were heated in water bath for 10 min at 40, 50, 60, 70, 80, 90 and 100°C and cooled immediately with tap water. Each treatment of dilutions and heating was assayed for the antiphytoviral activity.

Extraction of *P. tenuiflorus* Proteins. The total soluble proteins of *P. tenuiflorus* leaves were extracted as described by Parent and Asselin (1984). Protein concentration was estimated by using Sigma diagnostic kit and bovine serum albumin (BSA) as standard. To investigate the possible role of the *P. tenuiflorus* proteins as antiphytoviral, soluble solutions of different protein concentrations were prepared and applied to the host plant for two successive days before TNV inoculation.

Extraction of *P. tenuiflorus* Carbohydrates. The total water-soluble carbohydrates of *P. tenuiflorus* were extracted according to the method of Cerning and Guilbot (1973). The Quantitative determination of the total soluble carbohydrates was carried out by using Anthron reagent. The developing colour was measured in a colourimeter at 620 nm (Umbreit *et al*., 1969). Different concentrations of these extractable carbohydrates were prepared and tested as antiphytoviral agents as previously described.

Extraction of *P. tenuiflorus* Oil. Extraction of storage oil and determination of its concentration in the dry leaf tissues of *P. tenuiflorus* was carried out according to AOAC (1990) using petrolium ether as a solvent (40-60 BP) for 16 h by Soxhelt apparatus.

Soluble solution of oil in distilled water was prepared for testing the antiphytoviral effect. To overcome the insolubility of the oil in water, appropriate amount of oil was mixed with 0.1 mL of tween 80 containing 0.05 mL of ethanol and subsequently made up to 100 mL with distilled water. Different concentrations of the oil were prepared. The control one was water/ ethanol/tween 80.

Statistical analysis. The significance of difference between mean value for treatment and control was estimated statistically using one tailed student T-test.

RESULTS

Effect of *P. tenuiflorus* crude extract on the TNV, TMV, and TSWV infectivity

***In vitro* assay.** Three RNA viruses (TNV, TMV, and TSWV) were selected from different virus groups. *In vitro* tests showed that the infectivity of each virus was affected by treating with crude extract of *P. tenuiflorus*. When the extract and the virus were mixed and inoculated directly (at zero time) into their local lesion hosts, the percentage of viral inhibition was 54.5% for TNV infection, while with TSWV it was 2.5% (Table I). Treatment of TMV revealed that there was no inhibition detected at this time (Table I). Once the virus particles were incubated for longer time with the *P. tenuiflorus* extract, the inhibitory effect was rapidly increased. The reduction of local lesions reached to 100% after one hour of mixing with TNV and three hours for both TMV and TSWV (Table I).

In vivo assay. Results with TNV showed that the *P. tenuiflorus* extract (antiphytoviral) induced resistance against infection by this virus, where the mean number of local lesions reduced by 90.6% (Table II). In both TMV and TSWV infections, the resistance also developed in their treated hosts. The degree of this induced resistance was varied according to the plant-virus system used (85.8% for TMV and 77.7% for TSWV infections). The positive correlation was statistically significant between the *P. tenuiflorus* extract and the induced resistance when the different dilutions of the antiphytoviral were applied (10^{-1}, 10^{-2} & 10^{-3}) and the percentage of inhibition declined with these serial dilutions (Table II).

Effect of *P. tenuiflorus* crude extract on the TMV infectivity in its systemic host. Since the previous observation indicated that *P.* crude extract had a major inhibitory effect on the hypersensitive viral infection, one might expect that it would have similar effect on the systemic disease symptoms. Surprisingly, this proved not to be the case. Tobacco plants sprayed with crude extract of *P. tenuiflorus* for 2 days prior to TMV inoculation consistently showed a delay in the onset of systemic symptoms compared with untreated plants. Faint necrotic spots were developed on TMV-inoculated leaves in 3-4 days for treated or untreated plants. The typical mosaic symptoms were first evident on the new foliage of untreated plants 8 to 10 days. However, the average time of appearance systemic symptoms was delayed by 4 to 5 days, especially for plants treated with high concentration of *P. tenuiflorus* extract.

Detection of TMV in systemic hosts by ELISA. The specificity of TMV-antiserum was preliminary confirmed with two-fold serial dilutions of purified viral samples using ELISA. The results revealed the fidelity of TMV-antisera used when the average absorption at A_{405} nm decreased with increase in viral dilutions (Fig. 1).

Detection of TMV particles in the treated tissues of tobacco plants was examined using these specific-tested antisera. It was showed that TMV particles accumulated in the leaves of both *P. tenuiflorus* extract –treated and untreated plants where the average of absorbance at 405 nm was slightly changed from 0.285 to 0.308 OD, respectively. However, there was a delay in the appearance of systemic symptoms TMV accumulation weakly was affected as compared with control even with using serial dilutions of *P. tenuiflorus* extract (Table III).

Effect of dilutions and heat treatments on the inhibitory activity of the *P. tenuiflorus* crude extract. The dilution rate of the *P. tenuiflorus* crude extract (antiphytoviral) exhibited important role in the viral inhibition. The percentage of inhibition was 96.2, 82.4 and 24.1% when the

Table I. Effect of *P. tenuiflorus* crude extract on TNV, TMV, and TSWV infectivity (*in vitro* assay)

Hours post mixing with Plectranthus extract	TNV		TMV		TSWV	
	Mean of L. L±SEM.	% of inhibition	Mean of L. L±SEM	% of inhibition	Mean of L. L±SEM	% of inhibition
Control	33.0±6.8		40.2±4.0		11.8±2.4	
Zero time	14.6±2.2**	54.5	45.5±4.9	-13.1	11.5±2.1	2.5
One hour	_	100	20.5±5.1**	49.0	10.3±1.7	12.7
Two hours	_	100	1.6±0.4 **	96.0	2.4±0.9**	79.6
Three hours	_	100	_	100	_	100

Mean of L.L= the mean number of local lesions; SEM=Standard Error Mean; **=High Significance reduction in disease compared with control

Table II. Effect of *P. tenuiflorus* crude extract on the induced resistance of the host plant against TNV, TMV, and TSWV (*in vivo* assay)

Dilution of applying Plectranthus extract	TNV		TMV		TSWV	
	Mean of L. L±SEM	% of inhibition	Mean of L. L±SEM	% of inhibition	Mean of L. L±SEM	% of inhibition
Control	21.5±5.0		40.9±6.6		12.6±3.0	
10^{-1}	1.6±0.4**	90.6	5.8±1.1	85.8	2.8±0.3**	77.7
10^{-2}	5.6±0.9**	71.7	15.5±2.0**	62.1	7.6±1.6**	39.6
10^{-3}	12.2±2.0**	42.8	26.8±4.9 *	34.4	9.2±1.9*	26.9

**, *=High Significance, Significance reduction in disease compared with control

Table III. Analysis of TMV using ELISA in both treated tobacco with serial dilutions of *P. ten.* crude extract and untreated (control) plants. The numbers are the average A_{405}nm of 4 replicates

Treatment	Average absorbance at 405 nm±SEM
Control	$0.308\pm3.3\times10^{-2}$
Plectranthus extract at 10^{-1}	$0.285\pm2.2\times10^{-2}$ NS
Plectranthus extract at 10^{-2}	$0.311\pm8.8\times10^{-3}$ NS
Plectranthus extract at 10^{-3}	$0.297\pm2.4\times10^{-2}$ NS

Table IV. Effect of dilutions on the viral inhibitory activity of *P. tenuiflorus* crude extract

Dilutions	Mean no. of L.L ±SEM	% of inhibition
Control	41.6±4.8	
10^{-1}	1.6±0.2**	96.2
10^{-2}	7.3±1.6**	82.4
10^{-3}	31.6±6.7*	24.1

Table V. Effect of temperature on the viral inhibitory activity of *P. tenuiflorus* crude extract

Temperature	Mean no. of L.L. ±SEM	% of inhibition
Control	48.6±7.4	
Room temp.	6.9±0.9**	85.8
40C°	7.8±1.7**	83.9
60C°	10.1±3.4**	79.2
80C°	14.0±2.2**	71.1
100C°	16.8±4.1**	65.4

**=High Significance reduction in disease compared with control

Table VI. Effect of *P. tenuiflorus* phytochemical constituents as antiphytoviral against TNV

Phytochemical	Concentration /mg	Mean of L.L ±SEM	% of inhibition
Proteins	0	51.6±7.1	
	0.1	69.6±9.9	-34.8
	0.01	56.6±6.3	-9.6
	0.001	79.3±5.0	-47.8
Carbohydrates	0	40.8±4.5	
	5.0	34.8±6.1	14.7
	0.5	19.2±4.4**	52.9
	0.05	20.4±4.9**	50.0
Oil	0	49.6±5.6	
	18.0	24.0±4.5**	51.6
	1.8	32.0±6.6*	35.4
	0.18	30.8±6.8*	37.9

**, *=High Significance, Significance reduction in disease compared with control; NS= non significant

virus treated with the serial dilutions of the antiphytoviral 10^{-1}, 10^{-2}, and 10^{-3}, respectively (Table IV).

The effect of heat on antiphytoviral activity of the extract of *P. tenuiflorus* was examined at different degrees of temperature. The results in table (V) show that the inhibitory antiphytoviral activity of *P. tenuiflorus* was slightly affected by heating. The extract boiled for 10 minutes at 100°C still reduced the number of local lesions by 65.4% whereas the percentage of inhibition was 85.8, 83.9, 79.2, and 71.1 at room temperature, 40, 60, and 80°C, respectively.

Effect of certain *P. tenuiflorus* phytochemical constituents on viral infectivity

Effect of *P. tenuiflorus* proteins. It was found that each 100 g of *P. tenuiflorus* leaf tissues contained 0.93 mg of soluble proteins. Various concentrations (0.1, 0.01 and 0.001 mg) of these proteins were prepared and for their inhibitory effect against TNV. The results in Table (VI) indicated that these proteins had no inhibitory effect on TNV infectivity, rather it caused enhancement in its infectivity that was independent on the protein concentrations.

Effect of *P. tenuiflorus* carbohydrates. About 1.5 g of carbohydrates was obtained form 100 g of *P. tenuiflorus* leaf tissues. The infectivity of TNV for producing local lesions on the host plant was slightly decreased by 14.7% at 5.0 g of carbohydrates. Surprisingly, this low inhibitory effect was moderately increased to 50% with decrease in carbohydrates concentration at 0.05 g (Table VI).

Effect of *P. tenuiflorus* oils. Presence of high oil content in *P. tenuiflorus.* (9.08 g/100 g tissues) encouraged us for testing its inhibitory effect as antiphytoviral agent. The results in Table VI showed that there are significant difference between the number of lesions on control and oil–treated plants, 51.6% inhibition relative to control.. Moreover, there was a trend towards more lesions as the oil concentration decreased (Table VI).

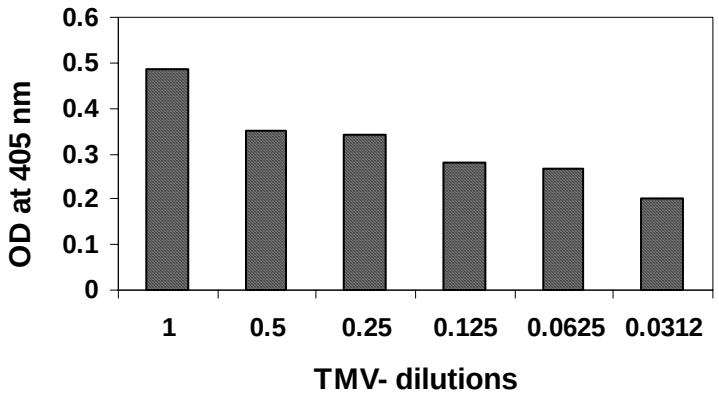
DISCUSSION

The use of natural resources from plant species in the treatment of plant viral diseases is an extensively explored area. *P. tenuiflorus* was proven to have a powerful inhibitory microbial activity against several bacterial and fungal pathogens (Dellar *et al*., 1996) and viruses affecting vertebrates (Batista *et al*., 1995). *P. tenuiflorus* also showed to possess inhibitory effects against phytopathogenic viruses. The previous reports support our observation that *P. tenuiflorus* effectively inhibited infection by a wide range of viruses (TNV, TMV, and TSWV) from groups with different genome components and replication strategies.

The mechanisms of *P. tenuiflorus* antiviral activity could be attributed to either inactivation of viral particles or inhibition of virus through inducing resistance response in the host (Zinnen & Fulton, 1986; Yalpani *et al*., 1993). In the present investigation, *P. tenuiflorus* as an antiphytoviral showed both mechanisms through *in vitro* and *in vivo* assays toward the three tested viruses. The possibility of other mechanism for the antiviral action enable it to be more potent inhibitory agent may be there.

Our results also indicated that the antiphytoviral activity in case of the local infection was higher than in systemic infection. This may be attributed to several possibilities; the virus replication might faster than the inducing resistance in treated plants (Fraser, 1979), and the antiphytoviral might have an effect on the virus movement

Fig. 1 Histogram representing the serological relationship between the serial dilutions of purified TMV and OD read by ELISA reader corresponding to the homologous TMV and TMV–antibody complex that result in a color change



more than virus multiplication thereby delaying the onset of systemic symptoms without significant effect on the virus accumulation. Similar assumption has been reached by Naylor *et al*. (1998).

The antiphytoviral of *P. tenuiflorus* extract was found to be thermostable and was active up to dilution of 1:100. A possible explanation for these unique properties lies in its active component which is a stable complex in the *P. tenuiflorus* extract. It might also be the occurrence of this substance in the crude extract which exhibits the antiviral activity and their concentration decreased with increase of dilution rate.

Several reports indicated that proteins may have antiviral agents (Smookler, 1971; Irvin *et al*., 1980; Mousa, 1986; Schonfelder *et al*., 1992, Verma *et al*., 1996; Shoman *et al*., 2002). The results of this study showed the weak antiviral activity of *P. tenuiflorus* proteins and the lower content of it in the *P. tenuiflorus* tissues. This suggests the involvement of other phytochemical constituents of *P. tenuiflorus* in this viral inhibitory activity. These might be the diterpenoids, essential oils or phenolic compounds (Abdel Mogib *et al*., 2002) which is consistent with the role of diterpens of different *Plectranthus* sp. as antimicrobial agents (Teixeira *et al*., 1997). This is supported by the fact that different extracts of many plants were tested for antiviral activity and it was explained due to their content of diterpenoids and essential oils (Remero *et al*., 1989; Bishop, 1995).

In conclusion, the study provided preliminary information that the application of the *P. tenuiflorus* extract (antiphytoviral) was a potential candidate for the protection of plants in the field or greenhouses. In particular, it is characterized by the broad spectrum effectiveness, stability, and comparative safety for the environment.

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