Pak. J. Biotechnol. Vol. 5 (1-2) 73-81 (2008) ISSN. 1812-1837

EFFECT OF NATURAL EXTRACTS OF SOME PLANTS ON THE ANTIVIRAL ACTIVITY OF TOMATO MOSAIC TOBAMOVIRUS

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ABSTRACT

The antagonistic activities of the extracts of black seed (*N. sativa*) and Neem leaves were determined against TMV using *N. glutinosa* and *D. metel* as local lesion hosts. The isolate of is study was first confirmed as it reacted with *Ch. quinoa* by appearing necrotic local lesion (NLL), forming amorphous inclusion bodies in the cytoplasm of TMV-infected tissue stained with 0.5% methyl green pyronin-Y and electron microscopy of partial purified particles. The molecular confirmation by RT-PCR confirmed the presence of coat protein gene of TMV. Data showed that the applied extracts were varied in their antiviral activities via the dilutions used. As interestingly, their crude extracts completely inhibited the inducing of NLL on the tested plants. By dilution of the extracts up to 1/2, 1/4, 1/8 and 1/16, and 1/32, the NLL appeared on the inoculated leaves. This could be due to the decreasing of the antiviral substance(s), which excreted out from the plant cells and released into the supernatants. The results paid an attention to the possibilities of extraction, purification and identification of such substances. Furthermore, this work also encourages the idea for the use of such active extracts as a bio-control agent for controlling plant pathogens, i.e, viruses. As a conclusion, the viral replication could be inhibited due to the inhibition of protein synthesis, inhibition of ribosomal frame shift, induction of viral nucleic acid hydrolysis and induction of apoptosis in the infected cells. These criteria are involved in the inhibition of the viral infection and decrease the viral propagation in plant.

Introduction

Several plants, such as *Pelargonium hortorum*, *Chenopodium album*, *C. amaranticolor*, *Capsicum frutescens* (Blaszczak *et al*., 1959), *Azadirachta indica* (Sangar and Dhingra, 1982), *Vitis vinifera* (Erkan and Yorganci, 1982), and *Rosa banktia* (Sangar *et al*., 1984), possess antiviral factors. Plant-derived antiviral compounds are active against plant, animal, and human viruses (Verma *et al*., 1995). According to Zipf (1995), plant antiviral compounds are grouped as furocoumarins, alkaloids, terpenoids, lignins, and specific proteins. The ribosome-inactivating proteins (RIPs) are widely distributed in higher plants, hold promise for agricultural and pharmaceutical applications (Mehta and Boston, 1998).

Virus disease can not be cured; therefore, control of plant viruses is very important to human beings. Antiphytovirals are substances exist that can affect the development of virus disease in plants and may be divided into two groups (antiviral substances and inducers of virus resistance) (Wood, 1982). The antiviral substances can originate from plants, microorganisms, and

insects or can be synthetic or semi-synthetic compounds. Antiviral may act directly through the host or they may affect the interaction between the virus and its host (Woods, 1982). Antiviral substances prevent the multiplication of viruses *in vivo*. Some chemicals have been reported to act as antiviral on virus replication in plants including several synthetic analogue of RNA and DNA bases or nucleotides and antibodies (Hirai, 1979).

Tobamoviruses are reported to be an economically important virus group infecting tomato (Van Regenmortel *et al.*, 2000). Brunt *et al.* (1996) reported that tobacco or tomato mosaic tobmovirus (TMV or ToMV) causes mosaic symptoms with financial loss ranged from 12 to 33 % of tomato yield. Singh *et al.* (1971) used insecticides, redicarb, carbofuran, diazinon, disulfoton, lindane phorate and alddicarp to prevent the spread of yellow mosaic virus via its vector *Bemisia tabaci*. Yoshizaki (1985) reported that three strains of TMV were inactivated by chlorine.

Based on the previous reports, this study aimed to pay an attention to focus on and rise up the effect of antiviral substances naturally occurred in plants (such as black seed and neem) and its role in the control of plant virus(es) (such as TMV).

Materials and methods

Virus Source: The TMV isolate and TMV-specific IgGs used in this study were provided by Prof. Mamdouh H. Abdel-Ghaffar, Laboratory of Virology, Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University.

Biological confirmation of the viral isolate under investigation: Virus inoculation was prepared by triturating infected leaf tissues of tobacco plant in a mortar and pestle using 0.01 M Tris buffer pH 7.2. The sap was filtered through two layers of cheesecloth and used for mechanical inoculation of differential hosts. Mechanical inoculation was carried out according to Allam *et al.* (2000). After 30 minutes from inoculation, plants were sprayed with water and kept under greenhouse conditions ((30°C±2). The inoculated plants were examined daily for visual symptoms up to 21 days.

Symptoms and diagnostic hosts: Different diagnostic hosts were mechanically inoculated with TMV-infectious sap are: *Chenopodium amaranticolor*, *Ch. quinoa*, *Chenopodiaceae***),** *Datura metel, D. stramonium, N. glutinosa, N. rustica* and *N. tabacum* cvs. Samsun, White Burley (*Solanaceae*). The internal symptoms, i.e., the presence of amorphous inclusions were detected as mentioned by Allam *et al*. (2000).

Morphology of virus particles: The virus was partially purified from TMV-infected tobacco leaves according to Glasa *et al.* (2000). Virus preparations were tested by negatively staining according to Griffin (1990) by electron microscopy.

ELISA confirmation: The indirect ELISA was carried out according to Koening (1981) to confirm the viral isolate of this study. The plates were read at 405 nm using ELISA reader (Bio-Tec Instruments) each half an hour till 2 hours, the average reading were calculated.

Chemical confirmation using RT-PCR: For RT-PCR of the *cp* gene of TMV one primer pair **F** and **R** was used for flanking a PCR fragment of a size of **bp**. These primers were kindly obtained from AGERI, ARC, Giza, Egypt.

RNA extraction and purification: RNA was extracted from 50-100 mg of the leaf samples according to the procedure described in Tripure Isolation Reagent Manual (Roche Diagnostics Coporation, IN, USA), all the equipment used during

the isolation procedure were made RNase-free.

RT-PCR reaction: In this experiment, protocol of the AccessQuick™ RT-PCR System was successfully used for RT-PCR isolation of *cp* gene using the extracted RNA, the *Tfl* DNA polymerase, the upstream and downstream primers, *AMV* reverse transcriptase and nuclease-free water. The reaction was conducted in a volume of 50 µl contains: nuclease free water (18µl), *AMV*/*Tfl* 5X reaction buffer (10µl), dNTP mix (10mM each dNTP) (1µl), TMV-F primer (50pmol) (1µl), TMV-R primer (50pmol) (1µl), 25 mM $MgSO_4$ (1µl), *AMV* reverse transcriptase (5U/µl) (2µl), *Tfl* DNA polymerase (5U/ µl) (1µl), RNA sample (15µl).

The mix was subjected to one cycle at 48°C for 1 hr; one cycle at 95°C for 2 min and 30 cycles, each consists of 95, 50 and 72°C for 45 sec, 45 sec and 1 min, respectively. The final cycle was extended for 10 min. The PCR product was analyzed by agarose gel electrophoresis of 1.2% in TAE buffer stained with ethidium bromide and the product was then visualized by transillumination as recommended by Sambrook *et al.* (1989).

Preparation of sap extracts from some natural plants: In this experiment, 15 grams of the black seeds (*Nigella sativa*) and 30 grams of Neem leaves were separately ground into a fine powder by liquid nitrogen in a mortar and pestle. Five duplicate dilutions (1/2, 1/4, 1/8, 1/16 and 1/32) from each powder was prepared by adding three grams from each powder to six ml of 0.002M $NaPO_4$, pH, 7.2 to create ½ dilution, and then, three ml from ½ dilution were added to fresh three ml buffer to create ¼ dilution, and so on.

Determination of antiviral-inhibitory activities of the prepared saps: The antiviral activities of each dilution were determined by mixing equal volumes from the dilution and virus-infectious sap and then the reaction of TMV isolate treated with the different dilutions were tested by mechanically inoculation of one ml of the mixture on five leaves of *Nicotiana glutinosa* or *Datura metel* as will be mentioned in the results part. The +ve Control was average number of local lesions produced when the leaf inoculated with TMV-crude infectious sap, while, -ve Control was average number of local lesions produced when the leaf inoculated with extraction buffer.

All treated and untreated plants were kept at a controlled greenhouse conditions at AGERI, ARC, Giza, Egypt for developing of the necrotic local lesions (NLL) for 15 days. The average number of NLL was calculated from the numbers of NLL produced on the five leaves for each treatment. The level of resistance was suggested as follows: 100-(Average number of L.L for each dilution/50) X (%).

Results and Discussion

Ibrahim *et al*. (2005) reported that ribosome-inactivating proteins (RIPs) are homogenous family of plant proteins. It is of special interest due to its unique activity. All RIPs possess a highly specific rRNA N-glycosidase activity and capable of catalytically inactivating ribosome and so inhibit the protein biosynthesis. The ricin, a RIP, was extracted and purified from castor bean (*Ricinus communis*) seeds and its effect on tobacco mosaic virus (TMV) infectivity was studied.

In the present study, the antagonism activities of the extracts of black seed (*N. sativa*) and Neem leaves were determined against TMV using *N. glutinosa* and *D. metel* as local lesion hosts. Data in Figures 1, 2, 3 and 4 showed that the biological confirmation of TMV isolate are under investigation, as it reacted with *Ch. quinoa*

by appearing necrotic local lesion (NLL) (Figure-1), forming amorphous inclusion bodies in the cytoplasm of TMV-infected tissue stained with 0.5% methyl green pyronin-Y and stained with red color while, the nucleus stained with blue color (Figure-2). This results in full agreement with that found by Allam *et al*. (2000).

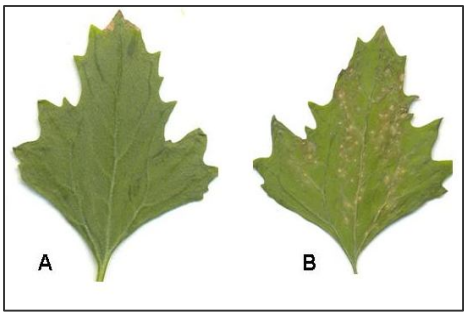

Figure-1: Reaction of TMV isolate of this study on *Chenopodium quinoa* plant leaf (B). A, negative control (Leaf inoculated with buffer). Note: appearance of local

Figure-2: Amorphous inclusion (AI) induced by the TMV isolate under investigation in leaf cell of *Nicotiana tabacum*, 15 days post virus inoculation. Stripe was stained with methyl green pyronin-Y stain (1%) for 15 min. AI appeared with red color while, nucleus stained with blue color.

Results in Figure-3 showed that the presence of rod shaped virus particles in electron micrograph of the virus preparation negatively stained with 2% uranyl acetate. This result in harmony with that found by several investigators studied on TMV strains.

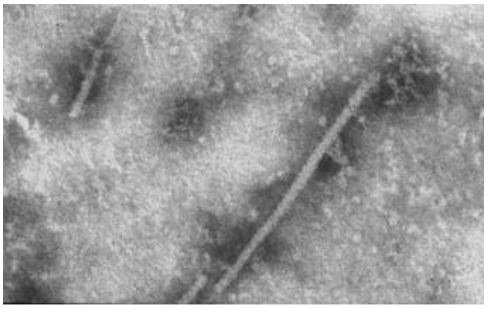

Figure-3: Electron micrograph shows negative staining of clarified virus preparation. Rod shaped particle appeared.

At the molecular level, RT-PCR results in Figure-4 confirmed the presence of coat protein gene of TMV as a fragment with a size of about bp was amplified.

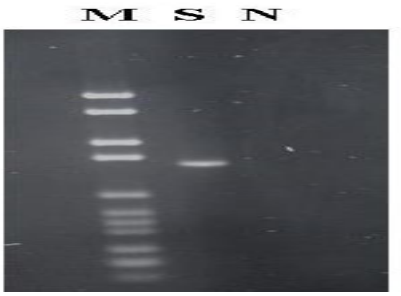

Figure-4: Confirmation of TMV isolate under investigation using reverse transcription-polymerase chain reaction (RT-PCR). A PCR fragment was amplified from the *cp* gene of TMV genome using two specific primers. M: DNA marker, S: sample (TMV-RNA), and N: PCR with no RNA template as a negative control.

The natural roles of RIPs have been proposed in an attempt to understand the function of RIPs in nature. First, defensive roles against plant viruses by arresting protein synthesis, cell death and prevention of viral replication and spread (Barieri *et al.*, 1992). Also, RIPs found to inhibit the frame shifting of the ribosomes and so can inhibit the virus proteins expression (Tumer *et al.*, 1998). According to the unique property of the RIPs, they have different application in both agricultural and medical fields. The applications in agriculture include the screening for their actions on viral infection (Chen *et al.*, 1991), insect attack and transformation of plant with the RIPs for viral resistance (Lodge *et al.*, 1993).

In a previous work, the leaves of *Nictoniana tabacum* cv. Xanthi were rubbed with a mixture of the virus supernatant and the PAP (RIP from *Phytolacca americana*) in an equal volume (Chen *et al.*, 1991). The infection with TMV was retained if the protein separated from the virus supernatant. After different times course, they determined the numbers of local lesions that indicate the degree of infection (Chen *et al.*, 1991). Their results indicated that the antiviral activity of PAP depends on the concentration of PAP and not on virus. They also showed that the RIP in general could be effectively used as antiviral agent because the activity of RIP does not depend on the type of the virus.

In this study, the data in Tables 1, 2 and 3 and illustrated by Figures 5, 6 and 7 showed that the applied extracts were varied in their antiviral activities via the dilutions used. As interestingly, their crude extracts completely inhibited the inducing of NLL on the tested plants. By dilution of the extracts up to 1/2, 1/4, 1/8 and 1/16, and 1/32, the NLL appeared on the inoculated leaves. This could be due to the decreasing of the antiviral substance(s), which excreted out from the plant cells and released into the supernatants. The results paid an attention to the possibilities of extraction, purification and identification of such substances. Furthermore, this work also encourages the idea for the use of such active extracts as a bio-control agent for controlling such plant pathogens, i.e, viruses.

Table-1: Reaction of TMV isolate treated with different dilutions (1/2, 1/4, 1/8 and 1/16, D, C, B and A, respectively) of sap extracted from black seeds (*Nigella sativa*) on *Nicotiana glutinosa*. Control: untreated leaf.

Dilutions	Replicates	No. of Local lesions	Suggested rate of resistance (%)
1/2	R1	2	96
	R2	2	
	R3	1	
	R4	2	
	R5	3	
	Average	2	
1/4	R1	14	72
	R2	15	
	R3	16	
	R4	12	
	R5	13	
	Average	14	
1/8	R1	17	62
	R2	19	
	R3	19	
	R4	20	
	R5	20	
	Average	19	
1/16	R1	32	40
	R2	30	
	R3	28	
	R4	31	
	R5	29	
	Average	30	
+ve Control	50		00
-ve Control	00		100

+ve Control: Average number of local lesions produced when the leaf inoculated with TMV-crude infectious sap.
-ve Control: Average number of local lesions produced when the leaf inoculated with extraction buffer.
Level of resistance: 100-(Average number of L.L for each dilution/50) X (%).

Table-2: Reaction of TMV isolate treated with different dilutions (1/2, 1/4, 1/8, 1/16 and 1/32, E, D, C, B and A, respectively) of sap extracted from black seeds (*Nigella sativa*) on *Datura metel.*

Dilutions	Replicates	No. of Local lesions	Suggested rate of resistance (%)
1/2	R1	2	98.3
	R2	2	
	R3	0	
	R4	1	
	R5	5	
	Average	2	
1/4	R1	16	88.3
	R2	12	
	R3	15	
	R4	13	
	R5	14	
	Average	14	
1/8	R1	28	79.8
	R2	23	
	R3	22	
	R4	31	
	R5	21	
	Average	25	
1/16	R1	41	66.6
	R2	40	
	R3	42	
	R4	40	
	R5	37	
	Average	40	
+ve Control	120		00
-ve Control	000		100

+ve & -ve Controls: Refer to previous Table.

Level of resistance: 100-(Average number of L.L for each dilution/120) X (%).

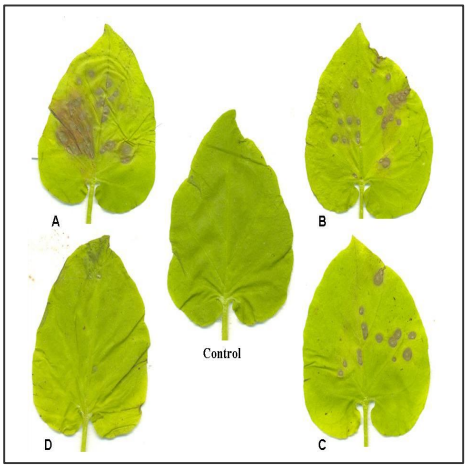

Figure-5: Reaction of TMV isolate treated with different dilutions (1/2, 1/4, 1/8 and 1/16, D, C, B and A, respectively) of sap extracted from black seeds (*Nigella sativa*) on *Nicotiana glutinosa.* Control: untreated leaf.

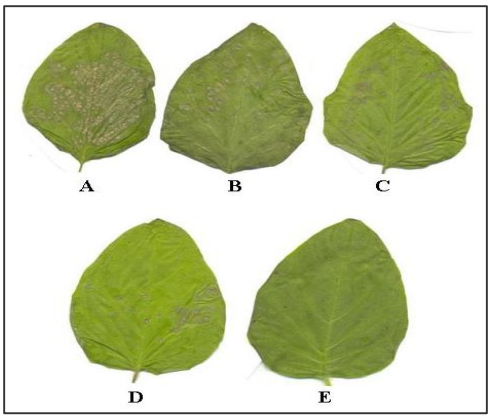

Figure- 6: Reaction of TMV isolate treated with different dilutions (1/2, 1/4, 1/8, 1/16 and 1/32, E, D, C, B and A, respectively) of sap extracted from black seeds (*Nigella sativa*) on *Datura metel.*

Table-3: Reaction of TMV isolate treated with different dilutions (1/2, 1/4, 1/8, 1/16, 1/32 and 1/64, F, E, D, C, B and A, respectively) of sap extracted from Neem plant on *D. metel*.

Dilutions	Replicates	No. of Local lesions	Suggested rate of resistance
1/2	R1	0	99.3
	R2	2	
	R3	1	
	R4	0	
	R5	2	
	Average	1	
1/4	R1	12	89.3
	R2	18	
	R3	16	
	R4	13	
	R5	16	
	Average	15	
1/8	R1	24	82.1
	R2	25	
	R3	21	
	R4	27	
	R5	28	
	Average	25	
1/16	R1	40	71.4
	R2	38	
	R3	37	
	R4	42	
	R5	43	
	Average	40	
1/32	R1	120	14.3
	R2	114	
	R3	122	
	R4	118	
	R5	126	
	Average	120	
+ve Control	140		00
-ve Control	00		100

+ve & -ve Controls: Refer to previous Table. Level of resistance**:** 100-(Average number of L.L for each dilution/140) X (%).

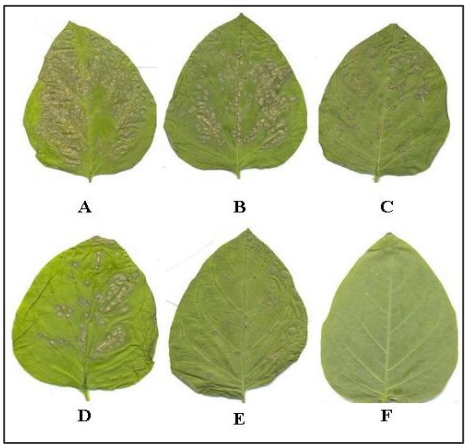

Figure-7: Reaction of TMV isolate treated with different dilutions (1/2, 1/4, 1/8, 1/16, 1/32 and 1/64, F, E, D, C, B and A, respectively) of sap extracted from Neem plant on *D. metel*.

As a conclusion, the viral replication could be inhibited due to the inhibition of protein synthesis, inhibition of ribosomal frame shift, induction of viral nucleic acid hydrolysis and induction of apoptosis in the infected cells. These criteria are involved in the inhibition of the viral infection and decrease the viral propagation in plant. Furthermore, according to the results, as a plant protecting protein against viral infection, more attention must be taken due to the high toxicity of the substances as a plant protecting protein against viral infected.

Acknowledgement: A lot of thanks are due to Prof. Dr. Mamdouh Hussein Abdel-Ghaffar, Prof. of Agric. Virology, Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University and Mr. Attia O. Attia, AGERI, ARC for their sincere help to accomplish this study.

REFERENCES

Allam, E.K. and Sohair I. El-Afifi Comparative study on methods of immunizing rabbits with free antigen of tobacco mosaic virus and potato virus X. Egyptian J. of Microbiol. 8:135-148. (1973).

Barieri, L., Ferreras J.M., Barraco A., Ricci P. and Stripe F. Some ribosome-inactivating proteins depurinate ribosomal RNA at multiple sites. Biochem. J. 286: 1- 4. (1992).

Blaszczak, W., Ross, A., and Larson, R. H. The inhibitory activity of plant juices on the infectivity of potato Virus X. Phytopathology 49:784-791. (1959)

Brunt, A.A.; Crabtree K.; Dallwitz M.J.; Gibbs A.J. and Watson L. Virus of Plant (Description and lists from the VIDE Database), pp. 1033-1251. CAB International Press, Walling- ford , UK. (1996).

Chen, Z.C., White R.F., Antoniw J.F. and Lin Q. Effect of pokeweed antiviral protein (PAP) on the infection of plant viruses. Plant Path. 40: 612-620. (1991).

Erkan, S., and Yorganci, U. Investigations on the inhibition of Potato Virus X (PVX) infectivity by some plant extracts. J. Turk. Phytopathol. 11:61-75. (1982).

Hirai, T. Action of antiviral agents, In: Plant Dis. An Advanced Treatise, ed. By J.G. Horsball and E.B. Cowling. Vol. 1, Academic press, 285-306. (1979).

Ibrahim, N.E.; Elneairy, M.; Salama, M.; Abo El-Saad, M. and Abdou, S. Effect of Ribosome-Inactivating protein from castor bean on tobacco mosaic tobamovirus. Pakistan Journal of Biotechnology 2(1-2): 52-57. (2005).

Koenig, R.C. Indirect ELISA methods for broad specifi- city detection of plant viruses. J. of Gen. Virol. 55:53-62. (1981).

Lodge, J.K., Kaniewski W.K. and Tumer N.E. Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. Proc. Natl Acad Sci. USA, 90: 7089-93 (1993).

Mehta, A. D., and Boston, R S. Ribosome-Inactivating proteins. Pages 145-152 in: A Look Beyond Transcription: Mechanisms Determining mRNA Stability and Translation in Plants. J. Bailey-Serres and D. R. Gallie, eds. American Society of Plant Physiology, Rockville, MD. (1998).

Sambrook, J.; Fritsch, E.F. and Maniatis, T. Gel electophoresis of DNA. In: Molecular Cloning:A Laboratory Mannual,Part 6, pp. 1-15, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. (1989).

Sangar, R. B. S., Dhingra, M. K., Kumar, S., and Parihar, M. P. Inhibitor of Potato Virus X by *Rosa banktia* leaf sap. J. Indian Potato Assoc. 2:134-135. (1984).

Sangar, R.B.S., and Dhingra, M.K. Potato Virus inhibitor from Neem leaf extract. J. Indian Potato Assoc. 9:143-149. (1982).

Singh, H.; Sandhu G.S. and Mavi G.S. Control of yellow mosaic virus in soybean. Glycine max L. Merrill by the use of granular insecticides. Indain J. Entomol. 33(3): 272-278. (1971).

Tumer, N.E., Parikh B.A., Li P. and Dinman J. The pokeweed antiviral protein specifically inhibits *Ty1*-directed+1 ribosomal frameshifting and retrotransposition in Sacchro-myces cerevisiae. J. Virol. 72: 1036-42 (1998).

Van Regenmortel, M.H.V.; Fauquet C.M.; Bishop D.H.L.; Carstens E.B.; Estes M.K.; Lemon S.M.; Maniloff J.; Mayo

M.A.; McGeoch D.J.; Pringle C.R. and Wickner R.B. Virus Taxonomy: The Classification and Nomenclature of Viruses, pp.151-155. The 7th Rep. of the Inter. Comm. on Taxonomy of Viruses. Academic Press, San Diego. (2000).

Verma, H. N., and Kymar, V. Prevention of potato plants from viruses and insect vectors. J. Indian Potato Assoc. 6:157-161.Verma, H. M., Varsha, and Baranwal, V. K. 1995. Endogenous virus inhibitors from plants, their physical and biological properties. Pages 1-22 in: Antiviral Proteins in Higher Plants. M. Chessin, D. Deborde, and A. Zipf, eds. CRC Press, Boca Raton, FL. (1979).

Wood, R.R.S. Active Defense Mechanisms in Plants. Plenum Press, New York. pp. 275-297. (1982).

Yoshizaki, T. Inactivation of 3 strains of TMV by chlorine. J. Hokk. Univ. Educ., 35:87-96. (1985).

Zipf, A. Mechanisms of antiviral protein activity in higher plants. Pages 133-146 in: Antiviral Proteins in Higher Plants. CRC Press, Boca Raton, FL. (1995).