



Research Article

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## A Possible Mechanism of Action for the Inhibition of Plant Viruses by an Antiviral Glycoprotein Isolated From *Boerhaavia diffusa* Roots

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### Abstract

Glycoprotein from *Boerhaavia diffusa* roots acts directly on virus (es), when it was mixed with virus inocula and incubated *in vitro*. The viruses were either inactivated by the aggregation of particles or by coating with glycoprotein and/or fracturing of virus particles. While *in vivo* glycoprotein prevents infection and multiplication of virus (es), by inducing systemic resistance in host plants, when applied 24 hrs prior to virus challenge. The virus infection in hypersensitive and systemic hosts was prevented/ arrested and a very few particles were seen in electron micrographs of samples taken from treated hosts and subsequently infected/ inoculated with different viruses. It is most probable that low molecular weight resistance inducing glycoprotein, from *B. diffusa* plants when applied to susceptible host plants, confers upon them the ability to fight viral attachment and inhibit or exclude the virus infection. *B. diffusa* glycoprotein stimulates host plants defense against virus infection. The induced resistance appears to be a form of expression of repressed pre-existing mechanism(s) which gets activated through this glycoprotein and prevents viral infection or virus spread.

### Keywords

Mechanism of action; Plant viruses; Glycoprotein; *Boerhaavia diffusa*

### Introduction

Many higher plants have the ability to resist pathogen attack including virus infection [1]. Some of the plants are known to contain endogenous proteins that act as antiviral agents [2]. There is no indication, however, that all the plants contain same type of inhibitor or that the antiviral mechanism is the same in all cases. Although attempts have been made to explain the mechanism of antiviral action of plant products on the infection of plant viruses [3]. However, no mechanism fully and satisfactorily explains the phenomenon. Obvious gaps exist about the mechanism of their action on viruses [4,5]. In most of the cases, the proposed mechanism for the antiviral action of the widely occurring proteinaceous inhibitors seems to be the result of the inhibitor ribosome-specific-N glycosides activity *in vitro* [6]. In the present communication efforts have been made to

explain the mode of action of an antiviral glycoprotein, isolated from the roots of *Boerhaavia diffusa* plants [7-10] which showed *in vivo* activity, where virus and inhibitor from plant do not come in physical contact.

*B. diffusa* is reported to possess many pharmacological, clinical, and antimicrobial properties, however, the potent antiviral efficacy of this plant was observed against phytopathogenic viruses. The antiviral agent isolated from this plant was found to be a glycoprotein with a molecular weight of 16–20 kDa [11-13]. Administered by foliar spraying in the field, this antiviral agent could protect some economically important crops against natural infection by plant viruses [14-16].

### Materials and Methods

#### Preparation of *B. diffusa* glycoprotein

The antiviral glycoprotein was isolated and purified from dried roots of *B. diffusa* plants, using latest biochemical tools for the identification of glycoprotein, following the method as described earlier [7,8,13]. The partially purified material was lyophilized and used for various experiments after diluting with the phosphate buffer [13,17,18].

**Source of inoculum:** Cultures of Barley stripe mosaic virus (BSMV), Barley yellow mosaic virus (BYMV), Brome mosaic virus (Br MV), Potato virus X (PVX), Red clover mottle virus (RCMV) and Tobacco mosaic virus (TMV) were maintained by regular passage in their respective systemic hosts.

**Preparation of virus inoculum:** Virus inocula were prepared by grinding 3-4 g of fresh leaves, showing severe disease symptoms, in a mortar with distilled water (1 g/ml) in each case separately. The pulp was squeezed through two folds of muslin cloth and the filtrate was centrifuged at 5,000 g for 15 minutes. The supernatant was diluted with distilled water to obtain 100-300 lesions per leaf after inoculation with the virus inoculum.

**Host plants:** Seeds of systemic and hypersensitive hosts of different viruses were sown in clay pots filled with sterilized soil. The seedlings were transplanted, in clay pots filled with sterilized compost, at four-leaf stage and placed in an insect-free greenhouse. For experimental work, plants showing hypersensitive reaction; *C. amaranticolor* Coste & Reyn, *G. globosa* L., *P. vulgaris* L., *N. tabacum* L. var. Samsun NN and *Z. mays* L. were used at 4 leaf stage while systemic host plants; tobacco (*N. tabacum* L. var. NP-31), potato (*S. tuberosum* L.), Barley (*H. vulgare* L.), clover (*T. incarnatum* L.), bromegrass (*B. inermis* L.) were used at 4-6 leaf stage. All experiments were performed with a minimum of three replications having 3 plants with minimum 4 inoculable leaves in each.

**Assay for virus inhibition:** Infectivity of viruses was assayed in their respective hypersensitive hosts viz. Barley stripe mosaic virus on *C. amaranticolor* Coste & Reyn, Barley yellow mosaic virus on *G. globosa* L., Brome mosaic virus on *P. vulgaris* L. and *Zea mays* L., Potato virus X on *G. globosa* L., Red clover mottle virus on *P. vulgaris* L. and Tobacco mosaic virus on *N. tabacum* L. var. Samsun NN.

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Antiviral activity of B. diffusa glycoprotein

**In vitro:** To detect the virus inhibitory activity, partially purified preparation of BD glycoprotein was dissolved (2 mg/ml) in phosphate buffer (pH7) and mixed with purified inocula of different viruses separately (1:1). The mixtures were incubated separately at room temperature for 0,15,30,60, & 120 minutes and then inoculated onto the two opposite leaves of hypersensitive host plants. Rest two opposite leaves of the same plant, in each case, were inoculated with virus inocula mixed with phosphate buffer instead of B.D. glycoprotein and served as control.

**In vivo:** The partially purified glycoprotein preparation, dissolved (2 mg/ 10 ml) in phosphate buffer (pH 7), was applied onto the two basal leaves of hypersensitive as well as systemic hosts. Virus(es) in each case were challenge inoculated 6, 12 and 24 hrs after B.D. glycoprotein application. An equal number of leaves on identical plants treated with phosphate buffer, instead of B.D. glycoprotein served as control. In hypersensitive hosts local lesions were counted on treated and control plants 4-6 days after virus inoculation. In case of systemic hosts disease symptoms were recorded after a week of virus inoculation till one month. The virus titre, in each case, was assayed on the hypersensitive hosts of the respective virus(es).

Electron microscopy

**Antiviral effect/virus inhibitory action in-vitro:** Electron microscopic studies were done by negative staining of a mixture (1:1) of B.D. glycoprotein and purified preparation of different viruses incubated separately for 15 minutes. Controls consisted of a mixture (1:1) of purified glycoprotein and buffer solution incubated for 15 minutes simultaneously.

Electronmicrographs of negatively stained preparation, of a mixture (1:1) of purified virus preparation and partially purified B.D. glycoprotein and a mixture of glycoprotein and buffer solution incubated for 15 minutes for each virus separately, were taken in a Hitachi H U 11/E electron microscope at the Institute fur Phytopathologie, AdL der DDR, 432, Ascherslaben, Germany. A droplet of the preparation was mixed on carbon coated copper grids with 2% uranyl Acetate (pH 4.5) and viewed at a magnification of X 64,500 at an accelerating voltage of 80 K.W.

**Antiviral effect /virus inhibitory action in-vivo:** The semi-purified B.D. glycoprotein (2 g/ 10 ml) was sprayed with the help of a glass atomizer onto the two basal leaves of respective systemic host plants after different intervals of glycoprotein treatment on all the inoculable leaves. An equal number of leaves of control plants (2 basal leaves) were sprayed with phosphate buffer served as control. After a month of virus(es) inoculation when disease symptoms appeared on control plants, leaves from each systemic host plants (treated and control) were taken separately and macerated with an equal amount (W/V) of phosphate buffer, pH 7. The sap was squeezed through two folds of muslin cloth and the clear solutions were taken. Virus(es) were purified following the standardized purification procedure for each (differential centrifugation) virus separately. Electronmicrographs of negatively stained preparation were taken in a Hitachi HU11/E electron microscope at the Institute fur Phytopathologie, AdL ds DDR.432, Ascherslaben, Germany. A droplet of the preparation was mixed on carbon coated grids with 2% uranyl acetate (pH 4.5) and viewed at a magnification of X 64,500 at an accelerating voltage 80KW.

Results and Discussion

The results presented in Table 1 show that the virus infectivity was not affected when the glycoprotein and viruses were mixed in vitro, and immediately inoculated, without incubating the mixture on susceptible hosts. However after 15 minutes of incubation in vitro all the six viruses under study were considerably inactivated and only a few local lesions appeared on the leaves of hypersensitive hosts (Figure 1). The percent decrease in virus infectivity was highly significant. If the incubation period was further increased (30, 60 and 120 minutes) the reduction in virus infectivity was 100% in case of each virus. The electron-micrographs of the virus(es), mixed separately with phosphate buffer instead of BD glycoprotein (controls), showed the presence of numerous particles (Figure 2 a,c,e and Figure 3 a,c,e ). However electron micrographs of the viruses

Table 1: Antiviral effect of B D glycoprotein when mixed with virus inocula and incubated for different time intervals before inoculation.

Virus	Host (Hypersensitive)	Incubation period (Minutes)/ Percent reduction in virus activity				
		0	15	30	60	120
Barely stripe mosaic virus (BSMV) (Anisometric)	Chenopodium amaranticolor Coste & Reyn	19 <sup>b</sup>	84 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>
Barely yellow mosaic virus (BYMV) (Anisometric)	Gomphrena globosa L.	26 <sup>b</sup>	98 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>
Brome mosaic virus (BrMV) (Isometric)	1-Phaseolus vulgaris L.	24 <sup>b</sup>	98 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>
	2-Zea mays L. Var.golden gaint sugar	12 <sup>a</sup>	98 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>
Potato Virus X (PYX) (Anisometric)	Gompherena globosa L.	23 <sup>a</sup>	98 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>
Red clover mottle virus (RCMV) ( Isometric)	Phaseolus vulgaris L.	-16 <sup>a</sup>	98 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>
Tobacco Mosaic Virus (TMV) (Anisometric)	N. tabacum L. Samsun NN	27 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>

Note: Differences due to treatment with B.D. glycoprotein are significant (a) at 1% level; and (b) at 5% level.

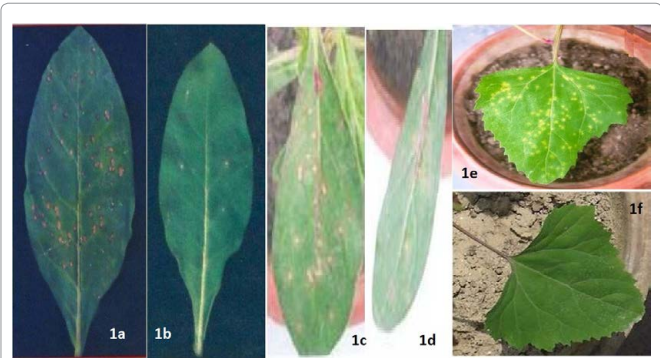
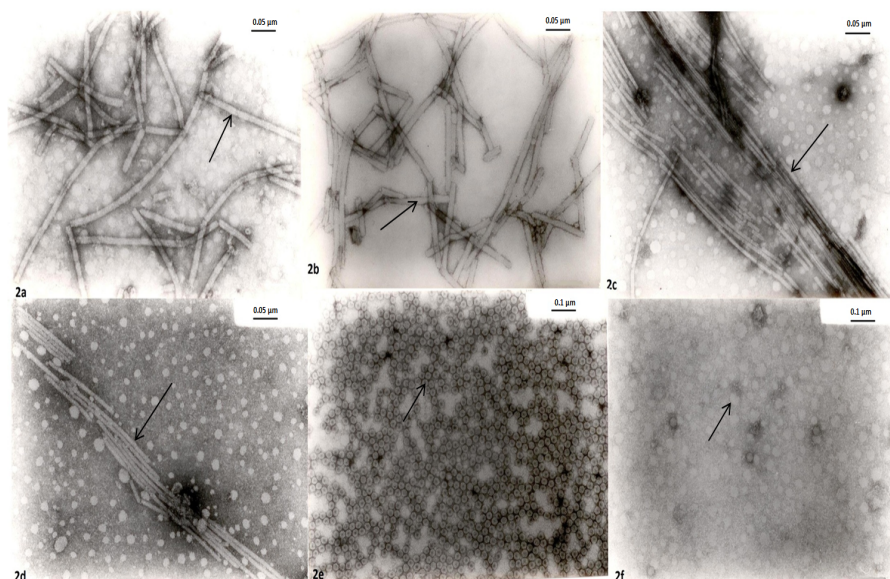
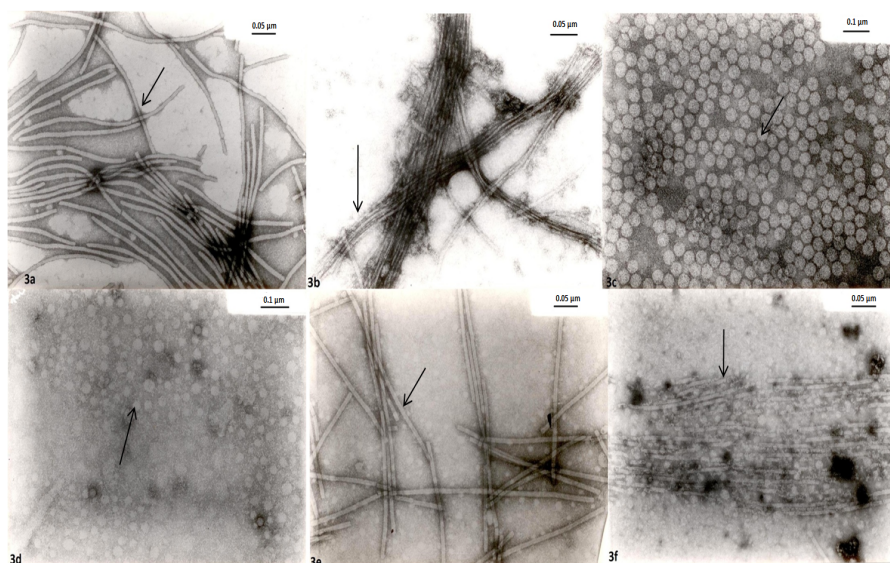


Figure 1: Antiviral effects of B.D. glycoprotein when mixed in vitro with virus and inoculated after incubation on hypersensitive hosts: 1a- TMV/ Nicotiana tabacum samsun NN, control and 1b- incubated with B.D. glycoprotein; 1c- PVX/ Gomphrena globosa, control and 1d- incubated with B.D. glycoprotein and 1e- BSMV/Chenopodium amraticolor, control and 1f- incubated with B.D. glycoprotein.





**Figure 2:** Electron micrographs of virus particles- Barley stripe mosaic virus mixed with phosphate buffer alone (a) and BD glycoprotein (b), Barley yellow mosaic virus mixed with phosphate buffer alone (c) and BD glycoprotein (d) and Brome mosaic virus mixed with phosphate buffer alone (e) and BD glycoprotein (f) *in vitro*.



**Figure 3:** Electron micrographs of virus particles- Potato virus X mixed with phosphate buffer alone (a) and BD glycoprotein (b), Red clover mottle virus mixed with phosphate buffer alone (c) and BD glycoprotein (d) and Tobacco mosaic virus mixed with phosphate buffer alone (e) and BD glycoprotein (f) *in vitro*.

mixed separately with glycoprotein and incubated for 15 minutes, showed particles of abnormal shape and size. In case of viruses with anisometric particles (Figure 2b,d,b and Figure 3f), there were a few shorter particles, which may have been produced by fracturing of normal particles due to BD glycoprotein. These particles were dull in appearance as compared to sharp and very clear particles in controls. In addition there was strong binding of viruses with BD glycoprotein as the particles seem to have some coating like appearance on them. Beside, aggregation of particles many broken particles were also seen (Figure 3f). In case of isometric particles (Figure 2f and Figure 3d), the inhibitory action of BD glycoprotein was very much pronounced. The electron micrographs of incubated samples revealed only a few

intact particles while majority of particles were seen as only protein coat shells without nucleic acid (Figure 2f and Figure 3d).

Results on *in-vivo* action of BD glycoprotein have clearly indicated that it had no inhibitory effect on virus (es) infection when applied up to 6 hrs prior to virus challenge. However, some protection was observed when it was applied 12 hrs prior to virus challenge, on to the basal leaves of hypersensitive as well as systemic hosts. Maximum protection against viruses infection was observed when BD glycoprotein was applied 24 hrs prior to virus challenge on to the two basal leaves of test hosts. Since the protection was observed in upper non-treated leaves of test hosts it is believed that the protection was systemic (Table 2). In case of systemic hosts, the appearance

**Table 2:** Antiviral effect of B.D. glycoprotein when applied at different time intervals before virus challenge on to the two basal leaves of test hosts.

Virus	Host (Hypersensitive/ Systemic)	Percent reduction in virus activity/ time interval before virus challenge		
		6	12	24
Barely stripe mosaic virus (BSMV) (Anisometric)	1- <i>Chenopodium amraticolor</i> Coste & Reyn	26 <sup>b</sup>	48 <sup>b</sup>	96 <sup>a</sup>
	2- <i>Hordeum vulgare</i> L.	S.S. (-6)	V.M.S. (86 <sup>a</sup> )	N.S. (98 <sup>a</sup> )
Barely yellow mosaic virus (BYMV) (Anisometric)	1- <i>Gomphrena globosa</i> L.	14	38 <sup>a</sup>	84 <sup>a</sup>
	2- <i>Hordeum vulgare</i> L.	S.S. (-18)	V.M.S. (42 <sup>a</sup> )	V.M.S. (82 <sup>a</sup> )
Brome mosaic virus (BrMV) (Isometric)	1- <i>Phaseolus vulgaris</i> L.	28 <sup>a</sup>	39 <sup>a</sup>	100 <sup>a</sup>
	2- <i>Bromus inermis</i> Layss	S.S. (5)	V.M.S. (37 <sup>a</sup> )	V.M.S. (79 <sup>a</sup> )
Potato Virus X (PYX) (Anisometric)	1- <i>Gomphrena globosa</i> L.	16	38 <sup>a</sup>	88 <sup>a</sup>
	2- <i>Solanum tuberosum</i> L.	S.S. (-20)	S.S. (-14)	V.M.S. (76 <sup>a</sup> )
Red clover mottle virus (RCMV) ( Isometric)	1- <i>Phaseolus vulgaris</i> L.	6	28 <sup>a</sup>	98 <sup>a</sup>
	2- <i>Trifolium incarnatum</i> L.	S.S. (-29 <sup>b</sup> )	M.S. (12)	N.S. (88 <sup>a</sup> )
Tobacco Mosaic Virus (TMV) (Anisometric)	1- <i>N. tabacum</i> L. Samsun NN	20 <sup>a</sup>	51 <sup>a</sup>	100 <sup>a</sup>
	2- <i>N. tabacum</i> L. var. N.P. 31	S.S. (-43 <sup>a</sup> )	M.S. (-16)	V.M.S. (78 <sup>a</sup> )

Note:  
 (A) Differences due to treatment with B.D. glycoprotein are significant (a) at 1% level; and (b) at 5% level.  
 (B) S.S. = Severe symptoms; M.S. = Mild symptoms; V.M.S. = Very mild symptoms;  
 N.S. = No symptoms  
 Data in ( ) = Virus titre was assayed on the hypersensitive hosts of respective viruses.

of disease symptoms was delayed and symptoms produced, if any, were very mild. Active virus titre assayed on hypersensitive hosts of respective viruses 25 days after inoculation, revealed significant decrease in virus titre, in plants treated 24 hrs earlier with BD glycoprotein. In plants treated 12 hrs earlier with glycoprotein; symptoms were mild as compared to control and no marked effect was observed in plants treated 6 hours earlier with glycoprotein. The disease symptoms in these plants were as severe as in control and the virus titre in such plants was also very high (Table 2).

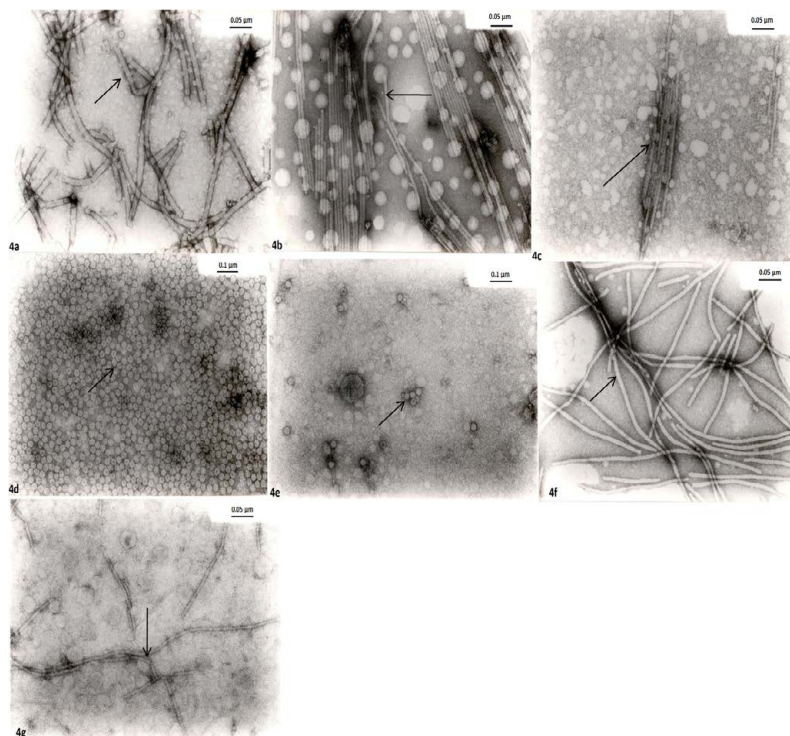
Electron micrographs of different viruses, from non-treated (control) and treated host (challenged with BD glycoprotein) at different hours before virus inoculation, showed that the preparations from control systemic hosts revealed numerous virus particles (Figure 4a, b, d, f and Figure 5a, c) whereas, from those pre-treated with BD glycoprotein 6 hours or 12 hours before virus challenge, comparatively less number of particles were observed. However, electron micrographs of viruses, isolated from systemic host plants, treated 24 hours earlier with glycoprotein revealed that in case of Barley stripe mosaic virus, no particles could be seen. The active virus titre in its systemic host (*H. vulgare*) was also very low (Table 2). Electron micrographs of Barley yellow mosaic virus, Potato virus X and tobacco mosaic virus (anisometric) revealed only a few particles. The active virus titre in case of all the viruses was greatly reduced (Table 2 and Figure 4c,g and Figure 5d). The effect of glycoprotein was more pronounced with isometric viruses (Brome mosaic and Red clover mottle viruses). Electron micrographs of these viruses revealed a very low virus titre as only a few particles were seen. Active virus titre assayed was also very low (Figure 4e and Figure 5b). Brome mosaic and Red clover mottle viruses when inoculated on hypersensitive or systemic host plants, pretreated 24 hours earlier with glycoprotein did not produce local lesions or systemic disease symptoms.

Most of the initial work on virus inhibitors from plant was carried out by incubating the plant extract with virus and evaluating the inhibitory activity by half leaf method [2,19-21]. However, no

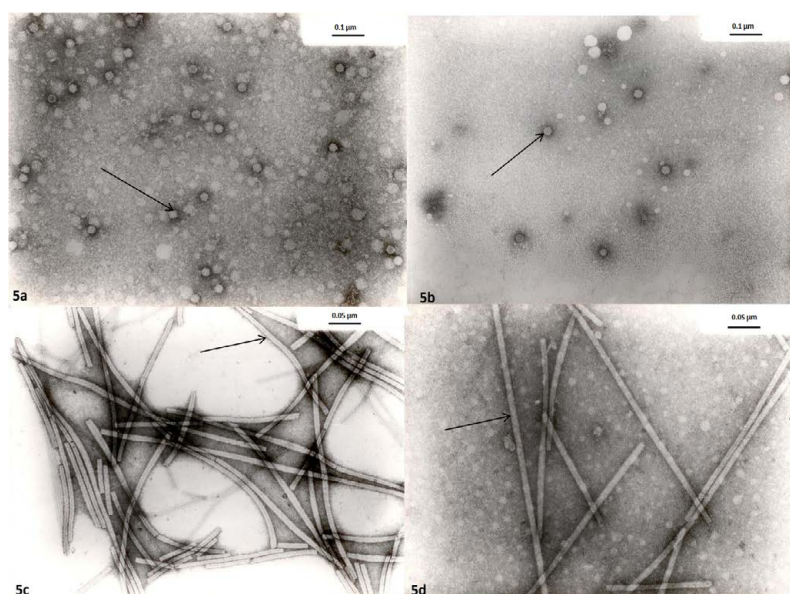
conclusive evidence was presented on the exact mode of action of virus inhibitors. Bawden [22] suggested that most inhibitors of plant origin inhibited virus infection and virus inhibiting process, several theories were put forth. There seems to be three possible mechanisms 1) by a direct effect on virus inactivating or denaturing the virus or making loose complex with virus particles, 2) by acting on the first stage of virus infection process or 3) by affecting the susceptibility of the host by altering the host cell metabolism- by forming virus inhibitory substances, which inactivate the viruses.

In the first process, the host is not involved in the suppression of virus disease symptoms but the effect of inhibitor is directly on the virus particles, which are necessary for their infectivity [23,24]. Aggregation of viruses by extracts of *Cucumis sativus*, *Pelargonium hortorum* and *Prunus persica* [25-27] and the coating of tobacco mosaic virus by polysaccharide from *Physarum polycephalum* and *Abutilon striatum* has been shown under electron microscope [28,29]. These studies have indicated that some inhibitors of plant origin act on the surface of virus particles. It has been observed in present study also that *B. diffusa* glycoprotein acts directly on particles of Barley stripe mosaic virus, Barley yellow mosaic virus, Potato virus X and Tobacco mosaic virus and thus the aggregation of virus particles as well as glycoprotein coating on virus particles and fracturing of rods were seen in electron micrographs. In case of viruses with isometric particles viz. Brome mosaic virus and Red clover mottle virus, heavy coating of glycoprotein was observed and only a few normal particles could be seen. In case of all the six viruses studied, inactivation of viruses was caused directly by *B. diffusa* glycoprotein. Virus(es) titre or the infectivity of inhibitor and virus (es) complex, incubated for 15 minutes or more, indicated that virus with isometric as well as anisometric particles were inactivated *in-vitro* and their infectivity was lost upto 100%, as only a few local lesions were produced on the leaves of hypersensitive hosts of different viruses (Table 1). Several naturally occurring compounds like tannins, phenolics and saponins have also been reported to interact with viruses forming loose





**Figure 4:** Electron micrographs of virus particles- Barley stripe mosaic virus mixed with phosphate buffer alone (a), Barley yellow mosaic virus mixed with phosphate buffer alone (b) and BD glycoprotein (c), Brome mosaic virus mixed with phosphate buffer alone (d) and BD glycoprotein (e) and Potato virus X mixed with phosphate buffer alone (f) and BD glycoprotein (g) *in vivo*.



**Figure 5:** Electron micrographs of virus particles- Red clover mottle virus mixed with phosphate buffer alone (a) and BD glycoprotein (b) and Tobacco mosaic virus mixed with phosphate buffer alone (c) and BD glycoprotein (d) *in vivo*.

combination with viral RNA, causing aggregation of virus particles or denaturing the nucleocapsid [25,30].

Virus inhibitors of second category suppress virus disease symptoms by interfering with the infection process without altering or irreversibly changing the virion or host susceptibility [30]. Such

substances interfere with either of two phases which constitute infection *viz.*, the establishment, or multiplication phase, inhibitors acting on the establishment phase possibly block the receptor surface [30-33]. Owens (1973) proposed a possible mechanism for inhibition of plant viruses by a polypeptide from *Phytolacca americana* when

mixed with the virus *in vitro* and advocated that *in vivo* inhibition of virus replication resulted from the interference with protein synthesis on host cell ribosome's. Several forms of pokeweed antiviral proteins viz., PAP, PAPII, and PAP-S, dianthins, the crude extract from *Bryonia dioica* seeds, and ribosome inhibiting proteins from *Phytolacca decandra*, *Spinacea oleracea*, *Chenopodium amaranticolor* and *Dianthus barbatus* have been reported to be inhibitors of protein synthesis in *in-vitro* translation system, thus indirectly preventing virus replication [34-38].

Plant virus inhibitors of third category have shown to exhibit their antiviral effect even when they do not come in physical contact with virus. They exert their inhibitory effect at the site of application or at a remote site and can be grouped as substances inducing localized or systemic induced resistance. Induced resistance by such inhibitors is dependent on formation of new antiviral substances sensitive to actinomycin D. Verma and Awasthi [7] also reported that the action of *B. diffusa* glycoprotein was sensitive to actinomycin D. Actinomycin D could reverse the resistance induction, when applied along with glycoprotein but not when applied 12-24 hours, after treatment with glycoprotein. They were able to isolate a highly potent antiviral protein from such resistant leaves [9].

Virus inhibitors of plant origin preventing infection of virus in the untreated parts of test plants were first observed by Mc keen [39]. He found inhibition of cucumber mosaic virus infection in the untreated opposite primary leaf of cowpea plants whose other primary leaf was treated with an extract of Pepper (*Capsicum frutescens*). No tests were done on upper leaves. He speculated that the inhibitor had translocated from one primary leaf to other leaves. However, no conclusive evidence was given of the transportability of virus inhibitor.

Verma and Awasthi [7] were able to demonstrate a strong and highly potent resistance inducing agent from roots of *B. diffusa*, the agent induced resistance in several host-virus combinations. The induced resistance was sensitive to actinomycin D treatment, indicating thereby an induced synthesis of translocable virus inhibitory substances in treated host plants. The antiviral agent from *B. diffusa* roots was purified and characterized and was found to be a glycoprotein [7,12,13,17,18], which could induce high degree of resistance as compared to crude root extract.

Singh [40] and Singh and Awasthi [41] reported that the aqueous root extract of *B. diffusa* effectively reduced mung bean yellow mosaic and bean common mosaic virus disease in mungbean and urd bean alongwith increased grain yield in field conditions. Later, Awasthi and Kumar [42,43], Kumar and Awasthi [44,45] found that weekly sprays significantly prevented infection, multiplication and spread of Cucumber mosaic virus, Bottle gourd mosaic virus, Cucumber green mottle mosaic virus and Pumpkin mosaic virus in cucurbitaceous crops. Kumar and Awasthi [46] reported that infection and spread of cucumber mosaic disease was also prevented. Significant management of viral diseases of legumes and tomato was studied by seed treatment followed by foliar sprays with *Boerhaavia diffusa* root extract [15,16,47]. Attempts have been made to explain the induced resistance mechanism involved in cross protection [48] and the resistance induced by chemicals and micro organism [49,50]. However, only little is known about the mechanism of induced resistance by substances of plant origin.

According to Mc Keen [39] the activity of virus inhibitors having the ability to act at a distance (remote site) might be because they

are conjugate proteins whose reactive components are selectively absorbed at the leaf and cell surfaces and subsequently move to block virus establishment and multiplication. Alternatively the protein itself, at the epidermal surface, may produce a chain reaction, which alters cell metabolism so that protein synthesis is diverted from virus formation. However, the induction of systemic resistance was sensitive to Actinomycin -D and was correlated with the production of new substances [51]. Attempts to understand the mechanisms involved in induced resistance have focused on the production of new proteins in uninfected resistant parts. Loebenstein and Ross [52] were able to isolate a protein with antiviral capacity from non-infected leaves of TMV-infected *Datura stramonium* plants. However antiviral factor (AVF) from TMV infected *N. glutinosa* reported by Sela [53] did not alter virus infectivity per-se. Induction of systemic resistance by higher plants was demonstrated for the first time by Verma and Mukerjee [54], Verma [55] and Verma [56]. Verma and Awasthi [7] were able to demonstrate a strong and highly potent antiviral agent from roots of the *B. diffusa* plants inducing systemic resistance in several hosts – virus combinations.

The protective effect was due to formation and translocation of some diffusible low molecular weight "Protective substances" in the host plants treated with *B. diffusa* glycoprotein. The protective substance triggered by the treatment with *B. diffusa* has been demonstrated to be low molecular weight heat labile proteins. Since these proteins inactivated the virus *in-vitro*, it has been called as virus inhibitory agent (VIA). VIA production is not host specific. They are synthesized both in the hosts reacting hypersensitively and systemically to different viruses. Their synthesis commences a couple of hours after treatment. After 2-3 days there was a steady decline, in the synthesis and no VIA was detectable. On the other hand, if the concentration of glycoprotein was increased, the decline was prolonged [9]. Verma and Dwivedi [57] and Khan and Verma [58] could further demonstrate the presence of induced virus inhibitory agent (VIA) in host plants treated with leaf extract of *Bougainvillea glabra* and *Pseuderan themum* biocolor. Ostermann [59] observed that a very short period was required to induce antiviral state in hosts by carnation leaf extract. They believed that the translocated information signal induced by the extract altered the membrane systems likely to be associated with virus receptor structures. They also thought that the transmitted signal induced biochemical events leading to an active cellular defense reaction. Studies have shown that antiviral proteins from *Phytolacca americana* (PAP) were weakly absorbed to the cell wall of *Nicotiana tabacum Xanthi* [60] and no absorption of PAP to protoplasts was observed. Root extract of *Boerhaavia diffusa* induced strong systemic resistance in susceptible host plant. In the study, we found that the BD-SRIP induced resistance against TMV infection [61].

Electron micrographs taken from the systemic test hosts inoculated with different viruses, which have been treated with *B. diffusa* glycoprotein prior to challenge inoculation by virus(es) revealed that the concentration of BSMV, BYMV, PVX, RCMV and TMV was decreased in plants pretreated with *B. diffusa* glycoprotein 6-12 hours before. No or only a few virus particles in each case could be seen in test hosts treated 24 hours prior to challenge inoculation by virus(es), as compared to non-treated control test hosts, where many virus particles were seen (Figure 4a-g and Figure 5a-d).

Ready [62] and Frotschl [63] also found by electron microscopy that the antiviral proteins of *Phytolacca americana*, *Dianthus barbatus*, *Spinacea oleracea* and *Chenopodium amaranticolor* bound with in the

cell wall matrix of the receptive plant tissues, heavily sequestered in case of pokeweed. Any breakage in the cell, as required for plant virus infection, could allow entrance of the protein into the cytoplasm.

It appears that *B. diffusa* acts on viruses directly (*in vitro*) by fracturing of virus particles, aggregation of virus particles and coating of virus particles with *B. diffusa* glycoprotein as evidenced by electron micrographs and also indirectly (*in vivo*) through hosts by inducing the formation of some antiviral substances thereby the process of virus replication is altered or inhibited, the production of antiviral substances is time dependent and requires at least 12 hours for its production in sufficient amount. *B. diffusa* glycoprotein when applied 24 hours before virus challenge on to the leaves of host plants, prevented infection of all the six viruses under study. It is presumed that *B. diffusa* glycoprotein interferes with establishment and multiplication phase of these viruses and thereby preventing the infection and multiplication, as evidenced by the number of local lesions produced on the leaves of hypersensitive hosts and disease symptoms on systemic host, which have been pretreated with *B. diffusa* glycoprotein (Table 2).

The systemic resistance inducing proteins (SRIPs) were extremely thermo-stable and retained biological activity upon incubation with pronase, trypsin and pepsin. The SRIPs were non-phytotoxic, promoted plant growth, improved crop yield and quality and showed broad spectrum protection against viruses [16]. The work has demonstrated for the first time that the inducible plant defense system against viruses can be switched on, after treatment with certain highly specific basic phytoproteins and has opened a new field of 'Plant Immunology'. This more recently developed novel strategy of immunizing plants using the phytoproteins, shows great promise as it is versatile, non specific and entirely risk free. A meaningful virus disease control, through host plant resistance, using non pathogen product has been achieved. The SRIPs indirectly prevent virus infection and perhaps are active in signaling the activation of defense mechanisms in susceptible hosts. Upon treatment these systemic resistance inducing proteins (SRIPs) provoke the plant to produce a new defensive protein in the treated plants, which presumably is the actual virus-inhibitory agent (VIA). These SRIPs, like interferon, are the only natural substances with the proven ability to inhibit *in vivo* virus infection and replication and will be very useful for immunization of susceptible plants against commonly occurring viruses. This strategy of defense in plants, in outcome, but not mechanism is comparable to the inducible defense system in animals. Although specific immunoglobulins have not been found in plants, but many new defensive proteins are formed in plants following treatment with a large number of different agents. These defensive proteins effective against cellular pathogens such as fungi and bacteria are more common and induced in greater quantities and number, whereas, induced protein(s) effective against viruses are formed in smaller quantities and hence their detection and purification was difficult. Cellular pathogens during attachment have the ability to elicit defense responses in plants, whereas, viruses since are directly delivered into the cell, the attachment process is by passed and hence they are not able to elicit strong defense response to produce detectable amounts of defensive proteins. These phytoproteins, which have some chemical and structural similarity to viruses, when applied externally in proper concentrations, have the capacity to elicit defense response by producing more quantity of defensive proteins effective against viruses. Plant virus interactions are mechanistically distinctive from other biotic agents and thus, the basis of resistance is likely to differ as well.

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