

Effect of Phytoproteins with bio-enhancers on agents Causing Natural Infection and Reducing Yield of Some Vegetable/Pulse by using *Boerhaavia diffusa* and *Clerodendrum aculeatum*

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Abstract: The degree of enhancement among different bio-enhancers was variable. Mixing of milk protein gave best response among all the bio-enhancers. The viral inhibitory potential of phytoprotein can be enhanced to a limited extent only – increasing strength of bio-enhancer beyond 2.0% did not increase the viral inhibitory potential any further. The effectiveness of bio-enhancer appears to be influenced by the host genotype as observed in case of chilli and mungbean, where addition of papain to the phytoproteins gave better response than milk protein, which otherwise gave better response in many plants viz. brinjal, cowpea/lobia.

I. INTRODUCTION

The viral diseases are of immense importance as they cause extensive crop damage and severe losses in terms of yield and quality of produce. Hence, their management has been of great interest to the researchers in the field of plant virology. Further, because of the peculiar nature and characteristics of plant viruses in the host system, any therapeutic method to control them has not been found very successful. Where resistant varieties are available, long term and effective protection has been achieved, but for a number of crops no resistant variety is available. However, it has been observed that preventive measures, if adopted, can be of great help in controlling/avoiding the viral diseases. Several reports are available on the effectiveness of a biotic and biotic agents as elicitors of resistance in susceptible plant hosts. These agents appear to act by sensitizing the plant for activation of resistance mechanism. Natural plant substances have also been shown to protect plants against infections from viruses and also to prevent the establishment of viruses in the host (Verma *et al.* 1982, 1985). Such plant substances are capable of inducing resistance in non-treated leaves of the plant and have been called as systemic resistance inducers (SRIs) (Verma *et al.* 1985, 1986, 1992, 1995; Gupta *et al.* 2004; Srivastava *et al.* 2004). The induction of resistance is expressed as reduction in lesion number in hosts reacting hypersensitivity to virus infection. Though induced resistant state of plant lasts for a short duration (depends on plant species) it can be prolonged either through the use of concentrated SRIs or addition of modifiers or through repeated treatments at weekly intervals (Verma *et al.* 1993; Verma and Baranwal 1999).

Brinjal [*Solanum melongena*, family: Solanaceae] a vegetable crop, is severely infected by egg plant mottle crinkle virus and brinjal mild mosaic virus due to which yield and fruit quality are drastically reduced. Chilli [*Capsicum annum*, family: Solanaceae] is used as vegetable, condiment and spice and is susceptible to chilli veinal mottle virus and chilli mosaic disease. Cowpea/Lobia [*Vigna sinensis*, family: Leguminosae] is an important pulse and vegetable crop. The crop is severely infected by cowpea mosaic virus, cowpea stunt virus, yellow vein mosaic virus etc.

Mungbean [*Vigna radiata*, family: Leguminosae] is a very important pulse yielding crop, and is highly susceptible to mung bean yellow mosaic virus (Varma *et al.* 1992; Verma *et al.* 1993; Biswas *et al.* 2005).

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II. MATERIALS AND METHODS

The experiments were performed in field conditions. The vegetable/pulse yielding crop was sown in separate beds arranged in randomized block design. Upon germination, the seedlings, depending upon plant species and bed size were thinned to approximately 25-100 plants/bed. Border weeds along the side of the bed were routinely removed to facilitate the free flow of virus vectors. Two bio-enhancers viz., milk protein and papain were added separately to each of the phytoproteins at a strength of 2%. Observations on disease incidence with respect to plant height, flowering and fruiting were made weekly. Fruit length, number of fruits/plant, number of root nodules/plant (in case of legumes) and total crop yield was recorded when the crop was mature. These attributes taken together were treated as indices of disease incidence. Extraction of proteins from roots of *B. diffusa* and leaves of *C. aculeatum* was carried out, essentially according to Verma *et al.* (1980, 1984) with desired modifications. In brief, the roots of *B. diffusa* were washed with distilled water, cut into small pieces, dried under natural sunlight and ground to fine powder in a grinder. The root powder was then mixed with 0.1 M phosphate buffer saline (PBS, pH 7.2) containing 0.1% 2-mercaptoethanol in the ratio of 1:5 and left overnight at 4°C. It was then filtered through two folds of muslin cloth and filtrate so obtained was centrifuged at 8000 rpm for 15 minutes to obtain supernatant 60% (w/v) ammonium sulphate was added to the supernatant with continuous stirring and left overnight at 4°C. The mixture was centrifuged at 5000 rpm for 15 minutes and supernatant was discarded. The precipitate was retained and suspended in small amount of buffer [20g fresh weight/ml of 0.1M PBS (pH 7.2)] and then dialyzed to obtain total protein fraction. The dialyzed protein fraction was either diluted as per requirement or was concentrated through freeze-drying. Lyophilized protein was stored at -20°C. Essentially a similar protocol was adopted for obtaining a protein rich fraction from the leaves of *C. aculeatum*.

Protection of crops from viral diseases by inducing immunity in plants

Various plants, like vegetable yielding, pulse yielding (legumes), cash crops and fruit yielding plants, were selected for conducting different experiments and induction of resistance was evaluated on hosts both reacting hypersensitivity (local lesion) or systematically to viruses.

Treatment of plants with phytoproteins

The dialyzed total protein fraction was suitably diluted with distilled water (1:5, *B. diffusa*; 1:2, *C. aculeatum*) and this fraction was rubbed on the upper surface of the lower (site) leaves of the test plants. The upper (remote site) leaves were left untreated. In field trials, aqueous extract of phytoproteins was sprayed on test plants with the help of a pneumatic hand sprayer.

Disease incidence and disease severity was assessed respectively with respect to number of diseased plants and symptom severity in systemic host. The active virus titer in the infected plant was measured by infectivity assay on local lesion hosts. The induced resistance was expressed as reduction in lesion number in host reacting hypersensitivity to virus infection.

III. RESULTS

The vegetable/pulse yielding crops are affected by viral diseases that cause significant loss in yield. Studies were further extended to plants like, brinjal, chilli, cowpea/lobia and mungbean category of pulses which are commonly infected by egg plant mottle crinkle virus and brinjal mild mosaic virus, chilli veinal mottle virus and chilli mosaic disease, cowpea yellow vein mosaic virus and mungben yellow vein mosaic virus. Milk protein and papain, were used as bio-enhancers.

Various treatments were found to suppress disease incidence. The plants treated with phytoproteins mixed with milk protein or papain exhibited minimum damage. The beneficial effects were variable in different treatments.

BD mixed with milk protein/papain:

Addition of milk protein/papain to phytoprotein solution was found to increase the potential of BD phytoprotein mediated induction of resistance in plants of *Solanum melongena*, *Capsicum annum*, *Vigna sinensis* and *Vigna radiata* against viral infections. The height of treated plants was increased. In most of the plants increase was about 120% of control plants. Height of plants treated with modified phytoprotein (bio-enhancer + phytoprotein) was more than plants treated with BD phytoprotein alone. The phytoprotein from BD mixed together with milk protein was more efficacious in protecting crops than BD alone. Enhanced flowering, fruiting, nodule production and crop yield was recorded for

plants treated with BD phytoprotein only. With the use of modified BD phytoprotein a further increase in the above parameters was recorded. The addition of milk protein/papain decreased disease severity and also the number of diseased plants (Plate 2.4, 2.5, 2.6 and 2.7; Table 2.C.1, 2.D.1, 2.E.1, 2.F.1; Fig. 2.1). Addition of modifier to BD phytoprotein induced better resistance as compared to BD alone. However, BD phytoprotein in combination with milk protein was more effective than either BD alone or BD with papain.

CA mixed with milk protein/papain

Addition of milk protein/papain to phytoprotein solution increased the potential of CA phytoprotein in inducing resistance in *Solanum melongena*, *Capsicum annum*, *Vigna sinensis* and *Vigna radiata* against common viral infections. The height of treated plants increased by nearly 30% as compared to controls. Increase in height of plants treated with modified phytoprotein was more than plants treated with CA phytoprotein alone. The differences were statistically insignificant. As in the previous experiment, in this case too, the treated plants showed increase in flowering, fruiting, root nodule formation and crop yield as compared to the controls. Here too the addition of bio-enhancer to the CA phytoprotein, further improved overall growth of the plants as compared to crops sprayed with CA phytoprotein only. The addition of milk protein/papain decreased disease severity and also the number of diseased plants (Plate 2.4, 2.5, 2.6 and 2.7; Table 2.C.2, 2.D.2, 2.E.2, 2.F.2; Fig. 2.1).

Addition of modifier induced better resistance as compared to CA alone. However, CA phytoprotein in combination with milk protein or papain were statistically insignificant.

IV. DISCUSSION

The antiviral potential of root extract of *Boerhaavia diffusa* and leaf extract of *Clerodendrum aculeatum* has been established through the extensive work conducted by various workers (Verma *et al.* 1973, 1974, 1975, 1978, 1979, 1980, 1982, 1983, 1984; Awasthi *et al.* 1984, 1985; Verma *et al.* 1985, 1986; Awasthi *et al.* 1989; Khan and Verma 1990; Verma *et al.* 1992; Baranwal 1992; Verma *et al.* 1995a,b; Prasad *et al.* 1995; Srivastava 1995; Verma *et al.* 1996, 1998, 1998a, 1999; Gupta *et al.* 2004; Srivastava *et al.* 2004; Singh *et al.* 2004; Awasthi *et al.* 2006). Among these, Prof Verma's work deserves special credit for recognizing antiviral potential and popularizing it among the virologists of various institutes. The virus inhibitory potential of phytoprotein was demonstrated by the ability of phytoprotein to prevent the formation of necrotic lesions in local lesion hosts and in delaying the development of disease specific symptom in systemic hosts when the treated host plants were challenged with virus. This type of induction of resistance in plants has been referred to as induced systemic resistance (ISR). Many agents such as natural plant products, bacteria like *Pseudomonas* spp. etc., and chemicals like benzothiadiazole, are known to elicit this resistance phenomenon.

PLATE 2.4

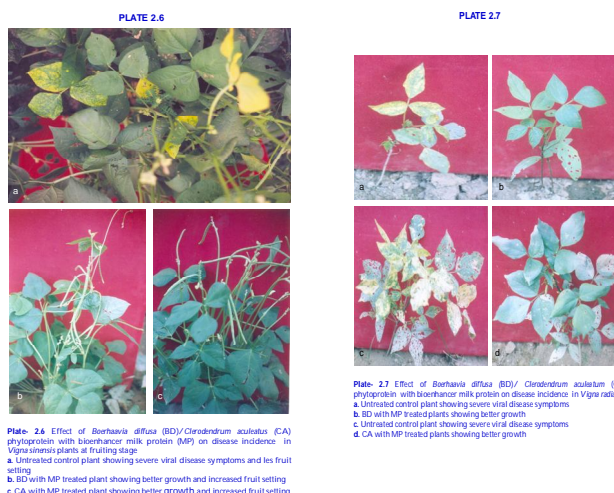


Plate 2.4 Effect of *Boerhaavia diffusa* (BD)/*Clerodendrum aculeatum* (CA) phytoprotein with bioenhancer milk protein (MP)/papain (P) on yield and disease incidence in *Solanum melongena*
a. BD with MP treated plant showing better growth and flowering (left) and untreated control plant showing poor growth and feebly perceptible viral infection (right)
b. CA with P treated plant showing better growth and fruiting (in micro plot)
c. Untreated control plant (for CA) showing viral infection (in micro plot)
d. CA with MP treated plant showing high fruit setting (in macro plot)
e. Untreated infected plant showing less fruit setting (in macro plot)

PLATE 2.5



Plate 2.5 Effect of *Boerhaavia diffusa* (BD)/*Clerodendrum aculeatum* (CA) phytoprotein with bioenhancer milk protein (MP)/papain (P) on disease incidence in *Capsicum annum*
a. Untreated control plant showing severe viral disease symptoms (left) and Treated plants (BD+MP) showing better growth and no disease symptoms (right)
b. Untreated control plant showing severe viral disease symptoms (left) and Treated plants (CA + P) showing better growth and flowering no disease symptoms (right)



The protection is shown to be induced systemically in various plants viz., tobacco, sun hemp, bottle gourd, chilli and moonbeam etc. The antiviral potential of extracts of these plants has been proven against many viruses like TMV (Verma *et al.* 1973, 1975, 1979, 1980, 1983, 1984, 1985), CMV (Verma and Kumar 1979, 1980), CGMMV (Verma and Kumar 1979, 1980; Verma and Dwivedi 1983; Verma and Khan 1984; Verma *et al.* 1984), GMV (Verma and Mukerji 1975; Verma and Awasthi 1979; Verma *et al.* 1979), SRV (Verma and Mukerji 1975; Verma and Awasthi 1979; Verma *et al.* 1979; Verma and Khan 1984; Verma *et al.* 1984; Verma and Khan 1985) TRSV (Verma and Awasthi 1979; Verma *et al.* 1979), Tm YMV (Verma and Kumar 1979; 1980; Verma and Dwivedi 1983; Verma *et al.* 1984), PLRV (Verma and Kumar 1979, 1980) and others.

The antiviral properties of the plant extracts have been shown due to certain novel proteins present in the extracts. These proteins can be applied in the form of aqueous extracts of the plant part as purified proteins, the latter being more effective. The antiviral potential has been shown to exist in extracts of plants like, *Euphorbia hirta* (Verma *et al.* 1979), *Mirabilis jalapa* (Verma and Kumar 1980); *Cuscuta reflexa* (Awasthi 1981); *Clerodendrum aculeatum* (Verma *et al.* 1984), *Bougainvillea spectabilis* (Verma and Dwivedi 1984), *Pseuderanthemum atropurpureum* (Verma *et al.* 1985), *Boerhaavia diffusa* (Verma and Baranwal 1988), *Celosia cristata* (Baranwal and Verma 1992) and *Clerodendrum inerme* (Prasad *et al.* 1995).

Though precise mechanism of protection offered by these phytoproteins has not been worked out but it has been shown that application of phytoproteins, induces formation of some virus inhibiting agent (VIA) in the treated plants. The VIA formation is sensitive to Actinomycin D, implying that VIA is a protein (Verma and Awasthi 1980; Verma and Dwivedi 1984; Prasad *et al.* 1995; Srivastava 1995).

The gene for the antiviral resistance inducing phytoprotein from *C. aculeatum* has been isolated and cloned to study various aspects of its functioning as antiviral agent or inducer of VIA (Kumar *et al.* 1997). It has also been shown that antiviral potential of these phytoproteins can be further enhanced by mixing certain biological compounds (papain or trypsin) to these phytoproteins (Varsha and Verma 1995). Such antiviral potential enhancing compounds (adjuvants) have been referred to as modifier/bio-enhancer. However, this aspect has not been extensively studied.

A systematic approach in this regard will help the farmers to avoid viral diseases in their crops, as no other effective control measure is available against viral diseases. The development of disease resistant variety itself can provide the best option but breeding for disease resistance varieties is a tedious job and also time consuming. Moreover, to develop disease resistance varieties for every crop is practically difficult (Grumet 1995; Lecoq 1998; Fraser 1990).

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Using a range of bio-enhancers gave some promising results. These bio-enhancers were found to increase the antiviral potential of extract or phytoprotein and also to increase the plant growth. The enhanced growth was reflected by substantial increase in height, leaf area, fresh shoot weight, fresh root weight, increased number of flowers and better resistance towards the development of systemic disease symptoms of viral infection. The results are in accordance with Verma and Varsha (1995). These workers reported similar results with *C. aculeatum* phytoprotein mixed with modifiers/enhancer (trypsin or papain). When used alone, these bio-enhancers were not effective at lower concentrations/dilutions. The best results were obtained when milk protein or papain was used as bio-enhancer. It was found that a certain threshold concentration (2.0%) of phytoprotein and bio-enhancer was required. Further increase in concentration of bio-enhancer did not increase the antiviral potential of phytoprotein appreciably. This further indicates that resistance in plants was due to some indigenous factors that responded positively to these inducers. The variation in the extent of protection of various plants against viruses also indicates influence of plant genotype on the degree of ISR. Amongst the two, the phytoprotein from *B. diffusa* roots was found to be more promising in combating viral infection. The same trend appeared when different bio-enhancers were added to them. However, when the two phytoproteins were used alternately, the degree of resistance induced in the plants was better than when they were used alone. This probably indicates that different components of resistance response are stimulated by the two phytoproteins and when used alternately gave better results due to coordination among the resistance mechanism. These experiments have provided two approaches to manage the viral diseases to a greater extent: one is to add bio-enhancer to the phytoprotein solution and the second is to use the bio-enhancer added phytoprotein alternately. This priming with resistance inducers can be done during *in vitro* culture of plants (Conrath *et al.* 2001; Oostendorp *et al.* 2001).

V. CONCLUSION

Addition of milk protein or papain to the phytoproteins induced a greater degree of resistance than when either of the phytoproteins was used alone. Phytoprotein from BD in combination with milk protein was most effective in inducing antiviral resistance. The treated plants developed mild symptoms with a significant decrease in disease severity in these plants. The number of plants showing systemic infection was also reduced. The treated plants were taller, had better flowering and fruiting and the crop yield was also increased. Addition of milk protein to phytoproteins gave better response than papain, however in some crops (tomato, chilli, and mungbean) addition of papain to phytoproteins was more effective.

Table 2.C.1. Effect of added bio-enhancers on virus resistance inducing activity of phytoprotein obtained from roots of *Boerhaavia diffusa* on protection of brinjal (*Solanum melongena*) crop

S. No.	Parameters	Response*			
		Control	Treated		
		DW	BD	BD+MP	BD+P
†1	Diseased plants (%)	27.5	12.5	5	7.5
2	Disease severity	+++	++	+	+
3	Flowering plants (%)	92.5	95.0	97.5	97.5
4	Fruiting plants (%)	85	92.0	97.5	95
5	Number of Fruits/Plant	4.6±0.5	6.4±0.6	7.1±0.6	6.9±0.7
6	Plant height (cm)	75±6.2	90±8.1	93±7.6	92±9.1
7	Total crop yield (kg)	17.4	26.9	32.8	31.6

*Data based on average of 40 replicate plants ± SEM; experiment was repeated thrice. Where number of '+' signs denotes intensity of disease severity; † after 60 days of growth

Table 2.C.2. Effect of added bio-enhancers on virus resistance inducing activity of phytoprotein obtained from leaves of *Clerodendrum aculeatum* on protection of brinjal (*Solanum melongena*) crop

S. No.	Parameters	Response*			
		Control	Treated		
		DW	CA	CA+MP	CA+P
†1	Diseased plants (%)	30.0	15.0	10.0	10.0
2	Disease severity	++++	++	+	+
3	Flowering plants (%)	90	95.0	95.0	97.5

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4	Fruiting plants (%)	87.5	92.5	95.0	95.5
5	Number of Fruits/Plant	5.1±0.62	7.2±0.6	7.8±0.7	7.5±0.6
6	Plant height (cm)	76±5.2	95±7.4	98±8.2	93±9.1
7	Total crop yield (kg)	18.2	30.8	33.8	32.5

*Data based on average of 40 replicate plants ± SEM.; experiment was repeated thrice. Where number of '+' signs denotes intensity of disease severity; † after 60 days of growth

Table 2.D.1. Effect of added bio-enhancers on virus resistance inducing activity of phytoprotein obtained from roots of *Boerhaavia diffusa* on protection of chilli (*Capsicum annuum*) crop

S. No.	Parameters	Response*			
		Control	Treated		
		DW	BD	BD+MP	BD+P
†1	Diseased plants (%)	36.0	20.0	12.0	16.0
2	Disease severity	+++	++	+	+
3	Flowering plants (%)	84.0	92.0	96.0	96.0
4	Fruiting plants (%)	80.0	88.0	92.0	88.0
5	Number of Fruits/Plant	14.2±2.35	23.3±2.62	28.3±2.7	25.2±3.16
6	Plant height (cm)	35±4.2	42±6.1	45±5.6	44±5.1
7	Total crop yield (kg)	2.8	3.6	4.4	4.1

*Data based on average of 25 replicate plants ± SEM.; experiment was repeated thrice. Where number of '+' signs denotes intensity of disease severity; † after 50 days of growth

Table 2.D.2. Effect of added bio-enhancers on virus resistance inducing activity of phytoprotein obtained from leaves of *Clerodendrum aculeatum* on protection of chilli (*Capsicum annuum*) crop

S. No.	Parameters	Response*			
		Control	Treated		
		DW	CA	CA+MP	CA+P
†1	Diseased plants (%)	32.0	24.0	16.0	12.0
2	Disease severity	+++	++	+	+
3	Flowering plants (%)	88.0	92.0	96.0	96.0
4	Fruiting plants (%)	84.0	88.0	92.0	96.0
5	Number of Fruits/Plant	15.2±2.65	25.3±2.74	32.3±2.50	27.2±3.37
6	Plant height (cm)	36±4.0	44±7.12	46±5.68	48±6.12
7	Total crop yield (kg)	2.7	3.9	4.2	4.6

*Data based on average of 25 replicate plants ± SEM; experiment was repeated thrice. Where number of '+' signs denotes intensity of disease severity; † after 50 days of growth

Table 2.E.1. Effect of added bio-enhancers on virus resistance inducing activity of phytoprotein obtained from roots of *Boerhaavia diffusa* on protection of cowpea/lobia (*Vigna sinensis*) crop

S. No.	Parameters	Response*			
		Control	Treated		
		DW	BD	BD+MP	BD+P
†1	Diseased plants (%)	32.0	16.0	8.0	8.0
2	Disease severity	++++	++	+	+
3	Flowering plants (%)	92.0	96.0	96.0	100.0
4	Fruiting plants (%)	88.0	92.0	96.0	96.0
5	Number of roots nodules/plant	32.6±3.24	47.5±4.62	54.2±4.32	49.8±5.42
6	Plant height (cm)	106±9.58	122±13.36	125±15.63	128±14.46
7	Total crop yield (kg)	3.2	4.7	5.2	4.8

*Data based on average of 25 replicates ± SEM; experiment was repeated thrice. Where number of '+' signs denotes intensity of disease severity; † after 55 days of growth

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Table 2.E.2. Effect of added bio-enhancers on virus resistance inducing activity of phytoprotein obtained from leaves of *Clerodendrum aculeatum* on protection of cowpea/lobia (*Vigna sinensis*) crop

S. No.	Parameters	Response*			
		Control	Treated		
		DW	CA	CA+MP	CA+P
†1	Diseased plants (%)	32.0	24.0	12.0	16.0
2	Disease severity	++++	++	+	+
3	Flowering plants (%)	88.0	92.0	96.0	96.0
4	Fruiting plants (%)	84.0	92.0	96.0	92.0
5	Number of roots nodules/plant	33.5±3.56	48.6±4.90	57.2±4.64	52.8±6.38
6	Plant height (cm)	105±9.06	126±14.46	132±14.83	130±15.52
7	Total crop yield (kg)	3.2	5.1	5.9	5.5

*Data based on average of 25 replicate plants ± SEM; experiment was repeated thrice.

Where number of '+' signs denotes intensity of disease severity; † after 55 days of growth

Table 2.F.1. Effect of added bio-enhancers on virus resistance inducing activity of phytoprotein obtained from roots of *Boerhaavia diffusa* on protection of mung (*Vigna radiata*) crop

S. No.	Parameters	Response*			
		Control	Treated		
		DW	BD	BD+MP	BD+P
†1	Diseased plants (%)	44.0	20.0	12.0	16.0
2	Disease severity	++++	++	+	+
3	Flowering plants (%)	80.0	88.0	92.0	96.0
4	Fruiting plants (%)	72.0	80.0	88.0	88.0
5	Number of roots nodules/plant	16.4±1.84	25.3±2.56	28.4±3.46	26.9±3.14
6	Plant height (cm)	25.2±3.46	32.4±3.42	34.6±4.21	35.7±3.89
7	Total crop yield (kg)	1.8	2.7	3.4	3.1

*Data based on average of 25 replicate plants ± SEM; experiment was repeated thrice.

Where number of '+' signs denotes intensity of disease severity; † after 40 days of growth

Table 2.F.2. Effect of added bio-enhancers on virus resistance inducing activity of phytoprotein obtained from leaves of *Clerodendrum aculeatum* on protection of mung (*Vigna radiata*) crop

S. No.	Parameters	Response*			
		Control	Treated		
		DW	CA	CA+MP	CA+P
†1	Diseased plants (%)	40.0	28.0	20.0	16.0
2	Disease severity	++++	++	+	+
3	Flowering plants (%)	84.0	88.0	92.0	96.0
4	Fruiting plants (%)	80.0	84.0	88.0	92.0
5	Number of roots nodules/plant	15.8±1.64	27.4±2.86	30.5±3.29	28.2±3.67
6	Plant height (cm)	26.0±4.06	34.9±4.36	37.5±5.25	38.8±5.35
7	Total crop yield (kg)	1.9	2.9	3.5	3.8

*Data based on average of 25 replicate plants ± SEM; experiment was repeated thrice.

Where number of '+' signs denotes intensity of disease severity; † after 40 days of growth

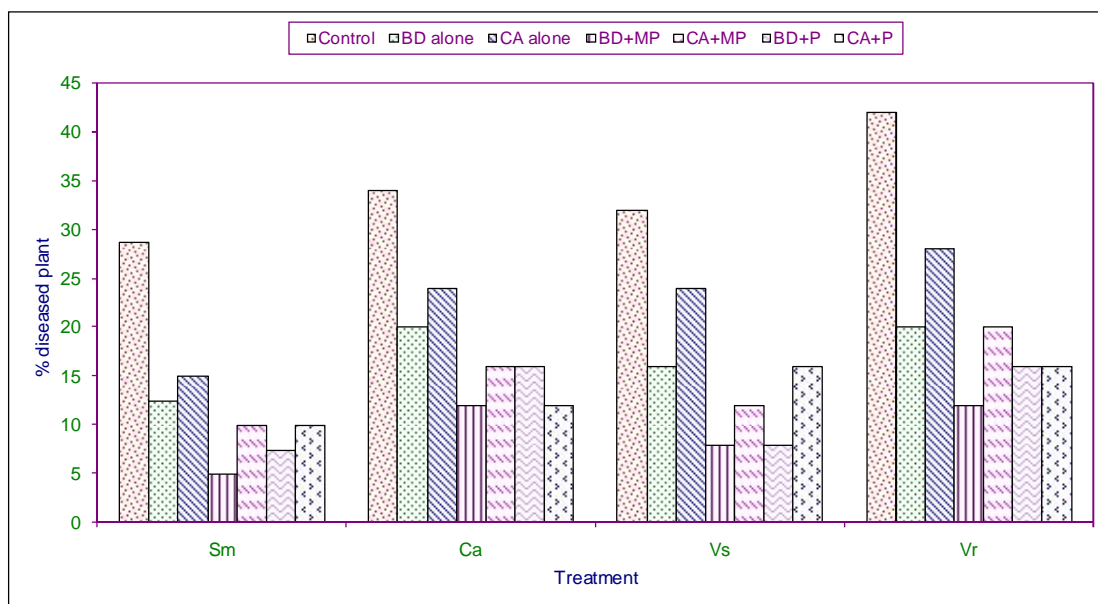


Fig. 2.1. Effect of various treatment of *Boerhaavia diffusa* (BD)/*Clerodendrum aculeatum*(CA) with milk protein (MP)/papain (P) on protection of susceptible different crops against viral infection

Sm: *Solanum melongena*

Ca: *Capsicum annum*

Vs: *Vigna sinensis*

Vr: *Vigna radita*

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