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Evaluation of Resistance Status of *Cyamopsis tetragonoloba* and *Crotalaria juncea* Seedlings Raised under in vitro Conditions in Nutrient Medium Containing Phytoproteins from *Boerhaavia diffusa* or *Clerodendrum aculeatum* against Sunnhemp Rosette Virus (SRV) Infection

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ABSTRACT: Germination of seeds under in vitro conditions was appreciably better in medium containing phytoproteins. These plants under natural growth conditions exhibited better resistance to infection indicating that the beneficial effect was carried forward. Test hosts *Cyamopsis tetragonoloba* and *Crotalaria juncea* were raised in vitro, using a nutrient medium which contained the phytoproteins from *B. diffusa* and *C. aculeatum*. The in vitro raised plants were challenge inoculated with SRV and the degree of resistance induced was evaluated in terms of reduce lesion number in *C. tetragonoloba* and reduced symptom severity in *C. juncea*.

I. INTRODUCTION

The agarified nutrient media BM3 and BM4 were used for seed germination of *C. tetragonoloba* and *C. juncea* and comprised the control for this experiment. The phytoproteins from *B. diffusa* and *C. aculeatum* after filter sterilization were added to the autoclaved nutrient medium at concentrations of 4, 8, 12 and 16% (v/v). The surface sterilized seeds of *C. tetragonoloba* and *C. juncea* were used for inoculation. The aforesaid process was done under aseptic conditions. In vitro raised seedlings of *C. tetragonoloba* and *C. juncea* were also acclimatized in liquid nutrient medium BM5 alone as well as with different concentrations (4, 8, 12 and 16 % v/v) of phytoproteins from *B. diffusa* or *C. aculeatum*. After 20 days, seedlings of *C. tetragonoloba* and *C. juncea* were grown in potted soil under glasshouse conditions. Onto such plants, virus testing was done. The different dilutions (1:10, 1:20 and 1:50) of sunnhemp rosette virus (SRV) were inoculated on the leaves of in vitro-raised *C. tetragonoloba* seedlings. The lesions produced by SRV in *C. tetragonoloba* were counted on the third day after inoculation of virus. The SRV culture for the experiment was obtained by inoculating 1:2, 1:5 and 1:10 concentrations of SRV on *C. juncea* plants which produced severe mosaic symptoms after 10, 15 and 20 days of inoculation.

II. MATERIALS AND METHODS

In vitro stimulation of growth of plants and virus resistance using phytoproteins

Plant material The plants of *Cyamopsis tetragonoloba*, and *Crotalaria juncea* were raised from seeds germinated under tissue culture conditions. The excised shoots were subcultured under axenic culture conditions while seeds were surface sterilized prior to their culture.

The procedure for surface sterilization of seeds involved washing seeds under running water for 15 minutes and then treating them for 15 minutes in 5% Labolene (neutral liquid detergent, M/s Glaxo Smith Kline pharmaceuticals Ltd., Mumbai, India) containing Tween 20 (2 drops per 100ml), to remove dust adhering to the seeds. The seeds were again washed with water and then dipped for 10 seconds in rectified spirit. It was then washed with sterilized distilled water.

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These pre-treated seeds were further sterilised by immersing them in 0.1 % HgCl₂ solution for 10 minutes followed by rinsing in sterilized distilled water.

All the procedures concerned with culture work were conducted under aseptic conditions in laminar flow cabinet.

Culture media

Preparation of stock solutions

Analytical grade chemicals (BDH, Merck, Sigma, Qualigens) were used to prepare culture media. The stock solutions of macro and micro nutrients were prepared by dissolving the salts in double distilled water and then solutions were stored at 4°C. The stock solutions of growth hormones were made by dissolving it in respective solvent, i.e. alcohol or KOH, and stored at 0-2°C. The stock solutions were used within the period specified below:

Stock solution	Period in days
Inorganic salts	60
Trace elements	30
Amino acids and vitamins	10
Auxins, cytokinins and other growth substances	7

Na-Fe-EDTA solution was prepared by dissolving 557 mg FeSO₄.7H₂O in 100 ml warm solution containing 745 mg of Na₂EDTA in double distilled water followed by aeration. The stock solutions were checked before use and only clean and clear stock solutions were used.

Compositions of nutrient media

Compositions of nutrient basal media used are given in Table MM1.

Table MM1: Compositions of nutrient media used (mg l⁻¹)

Constituents	BM3	BM4	BM5
(NH ₄) ₂ SO ₄	—	—	—
NH ₄ NO ₃	—	500	500
KNO ₃	80	250	250
KCl	65	—	—
KH ₂ PO ₄	—	125	125
NaH ₂ PO ₄ .H ₂ O	16.5	—	—
CaCl ₂ .2H ₂ O	—	—	—
Ca(NO ₃) ₂ .7H ₂ O	200	500	500
MgSO ₄ .7H ₂ O	360	250	250
Na ₂ SO ₄	200	—	—
Na-Fe-EDTA (ml/l)	3	3	3
MnSO ₄ .4H ₂ O	4.5	22.30	22.30
ZnSO ₄ .4H ₂ O	1.5	8.60	8.60
H ₃ BO ₃	1.5	6.20	6.20
KI	0.75	0.83	0.83
Na ₂ MoO ₄ .2H ₂ O	—	0.25	0.25
CuSO ₄ .5H ₂ O	—	0.025	0.025
CoCl ₂ .6H ₂ O	—	0.025	0.025
Thiamine-HCl	0.1	—	—
Pyridoxine-HCl	0.1	—	—
Nicotinic acid	0.5	—	—
Ascorbic acid	—	—	—
m-Inositol	—	—	—
Glycine	3	—	—
Adenine sulphate	—	—	—

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Malt extract	—	—	—
Sugar (%)	2	—	—
pH	5.8	5.8	—
Agar (%)	0.75	0.75	—

* pH was adjusted with 0.1N HCl/KOH prior to autoclaving the nutrient media.

The media BM3; BM4 and BM5 are modifications of Murashige and Skoog; White and Knop's medium, respectively (Singh 2001). BM3 and BM4 were used for seed germination and growth of germinated plantlet. Plants grown in aseptic conditions were acclimatized and hardened in liquid medium BM5 (Chaturvedi and Mitra 1974). The two phytoproteins used in culture studies were incorporated into the culture media at concentrations 2, 4, 8, 12, 16 and 20% (v/v) by adding suitable aliquot of original stock preparation.

Sterilization

Nutrient media, cultures vessels (test tubes, conical flask), glasswares, like pipettes, glass rods, instruments (forceps, blade, scissors etc.) and distilled water were steam sterilized in an autoclave at 1.08kg/cm^2 for 15 minutes. Prior to use, instruments were cooled with sterilized single distilled water. The organic compounds like glucose, phytohormones and the phytoprotein of *B. diffusa* and *C. aculeatum* were filter sterilized using bacteria proof filters and added directly to the sterilized media.

Raising of Culture Plants

In-vitro cultured plantlets were kept at $27 \pm 1^\circ\text{C}$ with $70\% \pm 5\%$ relative humidity and given a photoperiod of 15h. The light intensity of 3000 lux ($25\mu\text{mol m}^{-2}\text{s}^{-1}$ quantum flux density) was obtained from fluorescent tubes supplemented with 40 W incandescent lamps in the culture room.

Hardening of plants Ascetically grown seedlings of *C. tetragonoloba* and *C. juncea* were washed under the filtered running tap water to remove the adhering nutrient agar. This was followed by rinsing plantlets with distilled water, and then placing them in BM5 medium. These, plants were kept for hardening under aforesaid culture room conditions except that humidity was kept relatively high (90%). This high humidity regime was maintained by covering the plantlets with plastic hoods and spraying water regularly. Humidity was gradually reduced to normal culture room conditions by gradual opening of the windows and finally removing the hood.

The roots of plantlets were washed thoroughly with single distilled water weekly. Small earthen pots containing a mixture of garden soil and farm yard manure in a ratio of 3:1 was used for initial growth of in vitro-raised hardened plants. The acclimatized plants were transferred to the soil mixture in pots and kept under identical incubation conditions as used for acclimatization. The potted plants of *C. tetragonoloba* and *C. juncea* were transferred to glasshouse. After 10 days of growth of these plants in glasshouse conditions, plants were challenged with preparation of virus (SRV) inoculums and assessed for resistance.

III. RESULTS

In vitro germination of seeds and growth of seedlings

Phytoproteins added in the medium increased germination of seeds of both the plant species (*C. tetragonoloba* and *C. juncea*). The increase was seen when 12% (v/v) phytoproteins were added. BM4 appeared to be better than BM3 (Plate 5.1 and Table 5.A.1). Viability of *C. tetragonoloba* seeds was more than of *C. juncea* seeds, when the phytoproteins were present either from BD or CA. Phytoprotein from *B. diffusa* improved seed germination up to 92% in *C. tetragonoloba* and 88% in *C. juncea*. Similarly, *C. tetragonoloba* responded better to phytoproteins from *C. aculeatum* showing seed germination up to 96%, while it was 92% in case of *C. juncea*. With the increase in concentration of phytoproteins, percentage of germination as well as condition of seedlings progressively improved. The seedlings had perfect healthy stem with green leaves at the optimum concentration of 12% (v/v) of phytoproteins from BD or CA. Although seed germination as well as growth of seedlings declined at 16% (v/v) concentration of phytoproteins, it was better than the control.

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Acclimatization and growth of seedlings

20-day-old seedling were acclimatized ex vitro in BM5 inorganic salt solution added with 4-16% (v/v) of phytoproteins of either from *B. diffusa* or *C. aculeatum* for 20 days. Better seedlings of *C. tetragonoloba* and *C. juncea* were developed in all the concentrations of phytoproteins from BD or CA than the control (Plate 5.2). Seedlings progressively increased in length and green leaves developed with the increasing concentration of the phytoprotein up to 12% (v/v), but during nurturing of plants, growth rate declined when concentration of phytoproteins increased up to 16% (v/v).

After 20 days of nurturing, the plantlets were transferred to potted soil for 10 days under Culture Room conditions, where they picked up growth and were then transferred to glasshouse for 5 days before the virus was inoculated.

Challenge treatment

SRV at varying amount was used to challenge the in vitro-raised and hardened plants of *C. tetragonoloba* and *C. juncea*. Results are presented in Plate 5.3; Table 5.A.2. Phytoproteins from BD and CA were found to decrease the number of lesions in the local lesion host *C. tetragonoloba* and disease incidence in the systemic host, *C. juncea* (Plate 5.4; Table 5.A.3). In vitro-raised plants from increased level of phytoproteins in the nutrient medium exhibited maximum reduction in lesion number (80% reduction) at 16% concentration of BD phytoproteins and 69% reduction at 16% of CA phytoproteins when SRV was given at a dilution of 1:50 (Fig 5.1). *C. juncea* showed the least disease incidence at 16% (v/v) concentration of phytoproteins from BD or CA, where SRV was given at a dilution 1:10. Increase in the concentration of phytoproteins from 4 to 16%, increased the antiviral activity of both phytoproteins, but BD phytoprotein showed better results in comparison to CA phytoprotein against SRV at different dilutions, i.e., 1:10, 1:20 and 1:50 on in vitro-raised plants of *C. tetragonoloba* under glass house conditions.

The SRV produced severe symptoms on in vitro-raised plants of *C. juncea* usually after 10 days of inoculation at the dilutions of 1:2, 1:5, and 1:10 under glass house conditions. Concentrations of phytoproteins at 4-16% (v/v) showed mild, feebly perceptible or no symptoms on *C. juncea*, and delayed time of symptom appearance when treated with 12% and 16% (v/v) concentrations of phytoproteins from BD or CA. Phytoprotein-treated *C. juncea* showed no severe mosaic symptoms in any of the plants.

IV. DISCUSSION

So far, phytoproteins extracted from *Boerhaavia diffusa* and *Clerodendrum aculeatum* have been studied for their virus inhibiting effects in the virology laboratory of Lucknow University, Lucknow and elsewhere. Phytoproteins from other plant species like, *Mirabilis jalapa* (Verma and Kumar 1980); *Cuscuta reflexa* (Awasthi 1981); *Bougainvillea spectabilis* (Verma and Dwivedi 1984), *Pseuderanthemum atropurpureum* (Verma et al. 1985) and *Clerodendrum inerme* (Prasad et al. 1995) have also been reported to inhibit viruses. However, their growth promoting effects on plants have not been studied in details earlier. Effect of phytoproteins from *B. diffusa* and *C. aculeatum* on virus resistance capability and vegetative growth of cultured tissue was also seen by Verma et al. (1998), Gupta (1999), Srivastava (1999). Effect of some chemical and biological constituents on production of some secondary metabolites, which include phenols imparting to disease resistance, have been studied on some plants like, *Phytolacca americana* (Misawa et al. 1975), *Agrostemma githago* (Takayama et al. 1987) and *Mirabilis jalapa* (Ikeda et al. 1987, 1988). When added to the culture nutrient medium, effect of *B. diffusa* and *C. aculeatum* phytoproteins on vegetative growth as well as on systemic resistance inducing activity against viruses has already been reported under field and green house conditions. It has been shown that these two phytoproteins are highly potent in enhancing the vegetative growth of crops like tomato, okra, brinjal, chilli, mungbean, bottle gourd and ginger, etc. (Verma et al. 1979, 1980, 1982, 1984; Awasthi et al. 1984; Verma et al. 1985, 1995a,b, 1999). Also, the phytoproteins have been demonstrated to enhance the systemic resistance of otherwise susceptible plants, like, tobacco, guar, Bottle gourd and sunnhemp against a number of plant viruses when sprayed on test plants (Verma et al. 1982, 1983b, 1984; Verma and Prasad 1983, 1995, 1998). At a present stage of investigation of phytoproteins from *B. diffusa*, *C. aculeatum* and many other plant spp. listed above do have useful role to play in enhancing growth as well as virus resistance in certain plants. In this way it may be possible to create virus resistant plant without resorting to preying somatic hybridization or transgenesis. In any case, such

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studies provide ample scope for further investigations which can be utilized for improvement of plant spp. to increase the useful products from them, be their fruit yield or active principles.

PLATE- 5.1

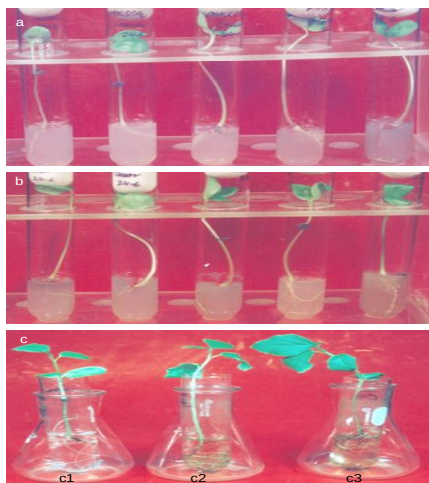


Plate- 5.1 Effect of addition of phytoprotein (control, 4%, 8%, 12% and 16% v/v) obtained from *Boerhaavia diffusa* (BD)/*Clerodendrum aculeatum* (CA) on *in vitro* germination of seeds of *Cyamopsis tetragonoloba*
a. Phytoprotein from BD (left to right; control, 4%, 8%, 12% and 16%)
b. Phytoprotein from CA (left to right; control, 4%, 8%, 12% and 16%)
c. Acclimatization in liquid nutrient medium
c1. Control **c2.** Treated BD (12%) twice **c3.** Treated CA (12%) twice

PLATE- 5.2

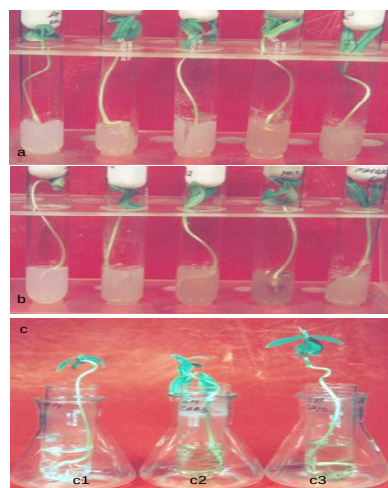


Plate- 5.2 Effect of addition of phytoprotein (control, 4%, 8%, 12% and 16% v/v) obtained from *Boerhaavia diffusa* (BD)/*Clerodendrum aculeatum* (CA) on *in vitro* germination of seeds of *Crotalaria juncea*
a. Phytoprotein from BD (left to right; control, 4%, 8%, 12% and 16%)
b. Phytoprotein from CA (left to right; control, 4%, 8%, 12% and 16%)
c. Acclimatization in liquid nutrient medium
c1. Control **c2.** Treated BD (12%) twice **c3.** Treated CA (12%) thrice

PLATE- 5.3

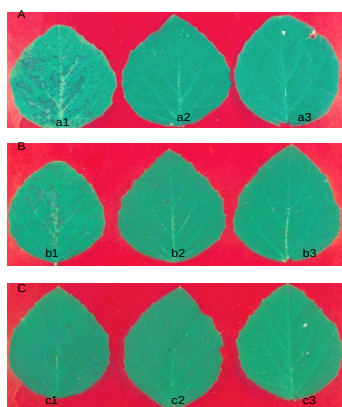


Plate- 5.3 Resistance inducing activity of optimum concentration of phytoprotein (16% v/v) of *Boerhaavia diffusa* (BD)/*Clerodendrum aculeatum* (CA) on leaves of *in vitro* raised plantlets of *Cyamopsis tetragonoloba* against localised infection of SRV
a1,b1,c1. Control
a2,b2,c2. BD treated
a3,b3,c3. CA treated

PLATE- 5.4

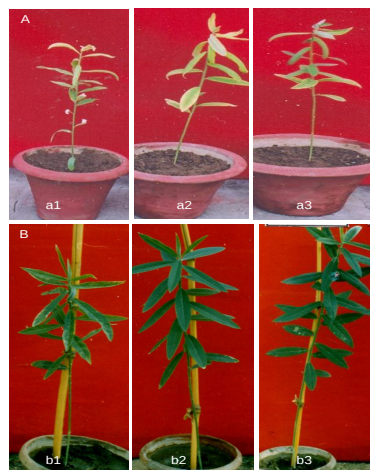


Plate- 5.4 Resistance inducing activity of optimum concentration of phytoprotein (16% v/v) of *Boerhaavia diffusa* (BD)/*Clerodendrum aculeatum* (CA) on leaves of *in vitro* raised plantlets of *Crotalaria juncea* against systemic infection of SRV
a1,b1. Control (sever symptoms)
a2,b2. BD treated (feebly perceptible symptoms)
a3,b3. CA treated (mild symptoms)

V. CONCLUSION

It was concluded from the above experiment that 12% to 16% (v/v) phytoproteins from BD or CA not only enhanced the seed germination of *C. tetragonoloba* and *C. juncea*, but also improved further growth of these seedlings when added to the nurturing media. Under the influence of phytoproteins at optimum concentrations lesser number of local lesions on *in vitro*-raised plantlets of *C. tetragonoloba* and delayed appearance of symptom on *in vitro*-raised plantlets of *C. juncea* were seen.

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Table 5.A.1. Effect of different concentrations of phytoproteins obtained from roots of *Boerhaavia diffusa* and leaves of *Clerodendrum aculeatum* on in vitro germination of seeds of *Cyamopsis tetragonoloba* and *Crotalaria juncea* in agarified nutrient medium BM3 and BM4

* Data based on average of 25 replicate seedling; where number of '+' signs denotes intensity of response

Treatment			Response*			
			Incubation: 20 days			
Nutrient medium	Phytoproteins obtained from plant species	Conc. of phytoprotein (v/v)	Cyamopsis tetragonoloba		Crotalaria juncea	
			Germination (%)	General conditions of seedlings	Germination (%)	General conditions of seedlings
BM3	B. diffusa	0%	12	+	8	+
		4%	16	+	12	+
		8%	16	++	20	++
		12%	20	+++	20	+++
		16%	12	+	16	+
	C. aculeatum	0%	12	+	8	+
		4%	16	++	12	++
		8%	20	++	20	++
		12%	24	+++	20	+++
		16%	16	++	12	++
BM4	B. diffusa	0%	76	+	72	+
		4%	80	++	76	+

Table 5.A.2: Resistance inducing activity of phytoproteins of *Boerhaavia diffusa* and *Clerodendrum aculeatum* on in vitro-raised seedlings of *Cyamopsis tetragonoloba* under glass house conditions.

*Data represent average number of local lesions formed on 12 leaves from 6 replicate plants $\pm$ SEM.

†1.0 g of SRV infected leaf tissue in 10/20/50 ml distilled water [virus-SRV (1:10, 1:20 and 1:50)]

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Table 5.A.3: Resistance inducing activity of phytoproteins of *Boerhaavia diffusa* and *Clerodendrum aculeatum* on in vitro raised seedlings of *Crotalaria juncea* under glass house conditions

†Dilution of virus (SRV)	Time interval in days after virus inoculation	Response* Disease severity									
		Concentration of phytoproteins (v/v)									
		Control	B. diffusa					C. aculeatum			
		0%	4%	8%	12%	16%	4%	8%	12%	16%	
(1:2)	10	+++	+++	++	++	+	+++	++	++	++	
	15	++++	+++	++	++	++	+++	++	++	++	
	20	+++++	+++++	+++	++	++	+++++	+++	+++	++	
(1:5)	10	++	++	+	+	+	++	++	+	+	
	15	+++	++	++	+	+	++	++	+	+	
	20	++++	+++	++	+	+	+++	++	++	+	
(1:10)	10	+	+	—	—	—	+	+	—	—	
	15	++	+	+	—	—	++	+	+	—	
	20	++	+	+	—	—	++	++	+	—	

*Data based on average of 6 replicate plants; where number of '+' signs denotes intensity of disease severity, '—' no symptoms (healthy plants)

† 1.0 g of SRV infected leaf tissue in 2/5/10 ml distilled water [virus–SRV (1:2, 1:5 and 1:10)]

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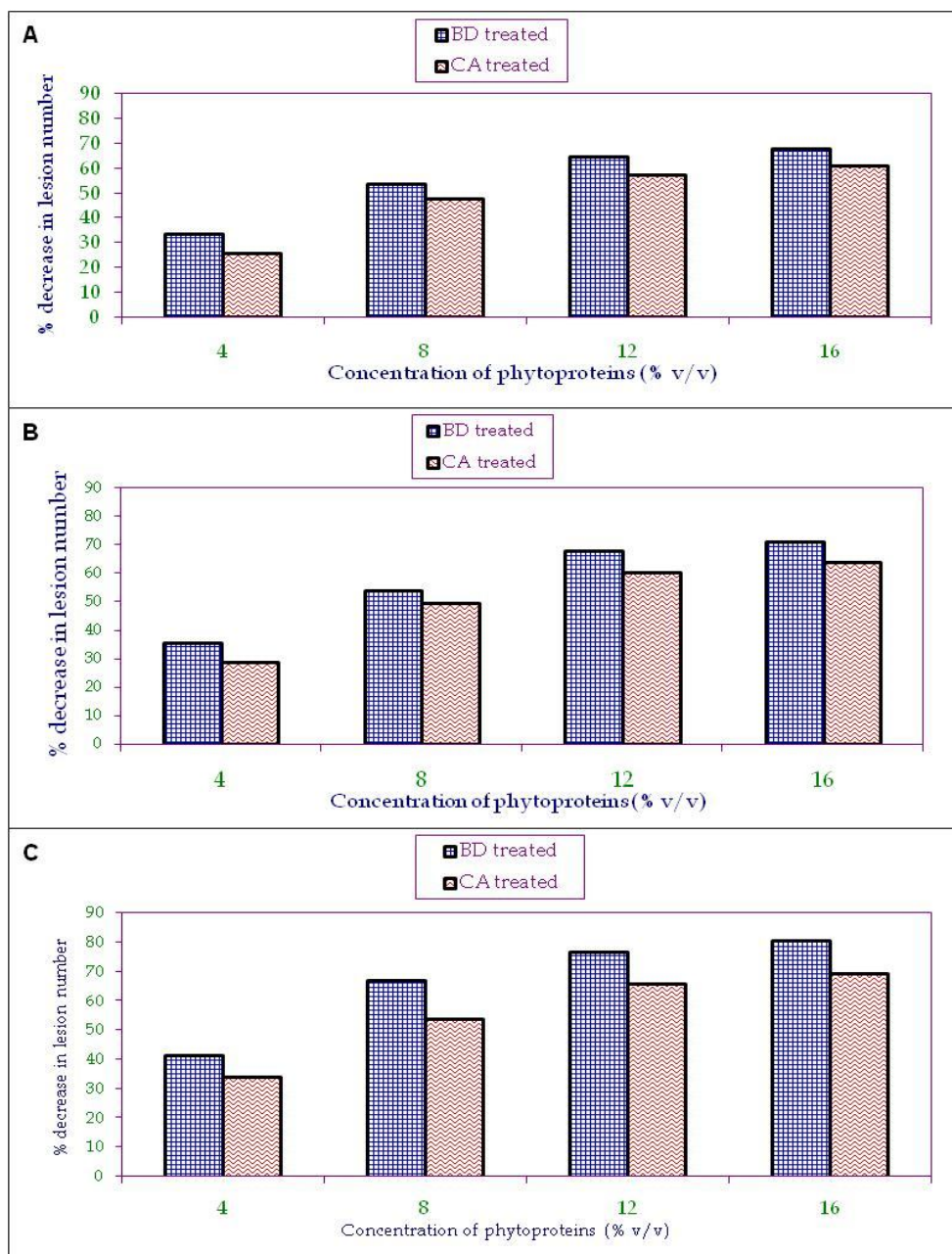


Fig. 5.1 Effects of virus challenge on *Cymopsis tetragonoloba* plants raised under *in vitro* conditions with different concentrations of phytoproteins of *Boerhaavia diffusa* (BD)/*Clerodendrum aculeatum* (CA) added to the culture medium.

A. SRV used at concentration of 1:10

B. SRV used at concentration of 1:20

C. SRV used at concentration of 1:50

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