

# Research Studies on Phytoremediation of Natural Infection of *Carica papaya* by Regular Spray of Phytoproteins Obtained from *Boerhaavia diffusa* and *Clerodendrum aculeatum* and Bio-Enhancer Protein

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**ABSTRACT:** The protection studies were extended to plants grown in the open field in order to evaluate the efficacy of phyto-protein mediated protection under field conditions. The fruit yielding crop was sown in separate beds that were arranged in randomized block design. Upon germination the seedlings were thinned to 50 plants/bed. Border weeds along the side of the bed were routinely removed to facilitate the free flow of virus vectors. The bio-enhancer milk protein at a strength of 2.0% was added to each of the phyto-protein i.e. *B. diffusa* and *C. aculeatum*. The crop plants were sprayed with (BD/CA) phytoproteins or BD/CA phytoproteins with MP. A total of 8 sprays were administered at weekly intervals. Alternate treatments with either BD/CA alone or BD/CA in combination with MP were administered. The plants were sprayed for a duration of 2 months, especially when the plants were in the susceptible phase. Addition of milk protein to the phytoproteins induced a greater degree of resistance than when either of the phytoproteins was used alone. The treated plants developed mild symptoms and showed marked decrease in disease incidence. The number of plants showing systemic infection was also reduced. The treated plants were taller, with better flowering and fruiting and the crop yield was also increased upon addition of milk protein to phytoproteins.

## I. INTRODUCTION

Phytoremediation, the use of plants and their associated microbes for environmental cleanup, has gained acceptance in the past 10 years as a cost-effective, noninvasive alternative or complementary technology for engineering-based remediation methods. Plants can be used for pollutant stabilization, extraction, degradation, or volatilization. Phytoremediation technologies are reviewed here, including their applicability for various organic and inorganic pollutants, and most suitable plant species. To further enhance the efficiency of phytoremediation, there is a need for better knowledge of the processes that affect pollutant availability, rhizosphere processes, pollutant uptake, translocation, chelation, degradation, and volatilization. The remaining gaps in our knowledge, and the practical implications for designing phytoremediation strategies. Transgenic approaches to enhance these processes are also reviewed and discussed. Papaya [*Carica papaya*, family: Caricaceae] is an economically important fruit crop. Papaya fruits are highly valued for their edibility and proteolytic enzymes-papain and chymopapain (Medora *et al.* 1979). Limitations encountered in papaya cultivation is its susceptibility to a number of viral diseases. Papaya plants are commonly infected by papaya ring spot virus and papaya leaf curl virus. The commercial cultivation of papaya has become unprofitable owing to the severity of the viral disease (Prasad and Sarkar 1989; Kudada and Prasad 2000, 2002; Prasad and Kudada 2005). India's share in the world market is very meager, i.e., only 7.25 % (Sathiyamoorthy *et al.* 1995).

The viral diseases are of immense importance as they cause extensive papaya damage and severe losses in terms of yield and quality of produce. 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).

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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 hyper sensitively 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).

The roots of *B. diffusa* and leaves of *C. aculeatum* have been shown to contain potent endogenous virus inhibitory proteins called as BD-SRIP and CA-SRIP, respectively (Verma and Baranwal 1999; Verma *et al.* 1996). These phytoproteins confer strong systemic resistance in several plants against a number of plant viruses (Verma *et al.* 1984, 1996, 1999; Gupta *et al.* 2004; Srivastava *et al.* 2004). Phytoproteins isolated from *B. diffusa* and *C. aculeatum* have molecular masses of 30 and 34 kDa, respectively. The phytoproteins are highly stable and could be purified (Verma *et al.* 1996). The effect of antiviral resistance inducing activity of isolated phytoproteins and enhancement in their activities by various bioenhancers like milk protein, black pepper powder, khand, turmeric powder, ginger powder, papain and jaggery (Singh, 2006).

## II. MATERIALS AND METHODS

The roots of *B. diffusa* were collected from fully mature plants growing in the district Kushinagar, Uttar Pradesh, India, whereas, the leaves of *C. aculeatum* were collected from plants growing as hedge in the University campus of Lucknow. These plant parts were used to obtain phytoproteins (Plate MM1). 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 total protein fraction was suitably diluted with distilled water (1:5, *B. diffusa*; 1:2, *C. aculeatum*). 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. To evaluate the efficacy of phyto-protein mediated resistance under field conditions, regular sprays with these phytoproteins were administered till the plants were in the susceptible phase. Observations on disease incidence were made weekly with respect to plant height, flowering and fruiting. Finally, when number of fruits per plant and other yield attributes were also included in assessing efficacy of phyto-protein.

## III. RESULTS

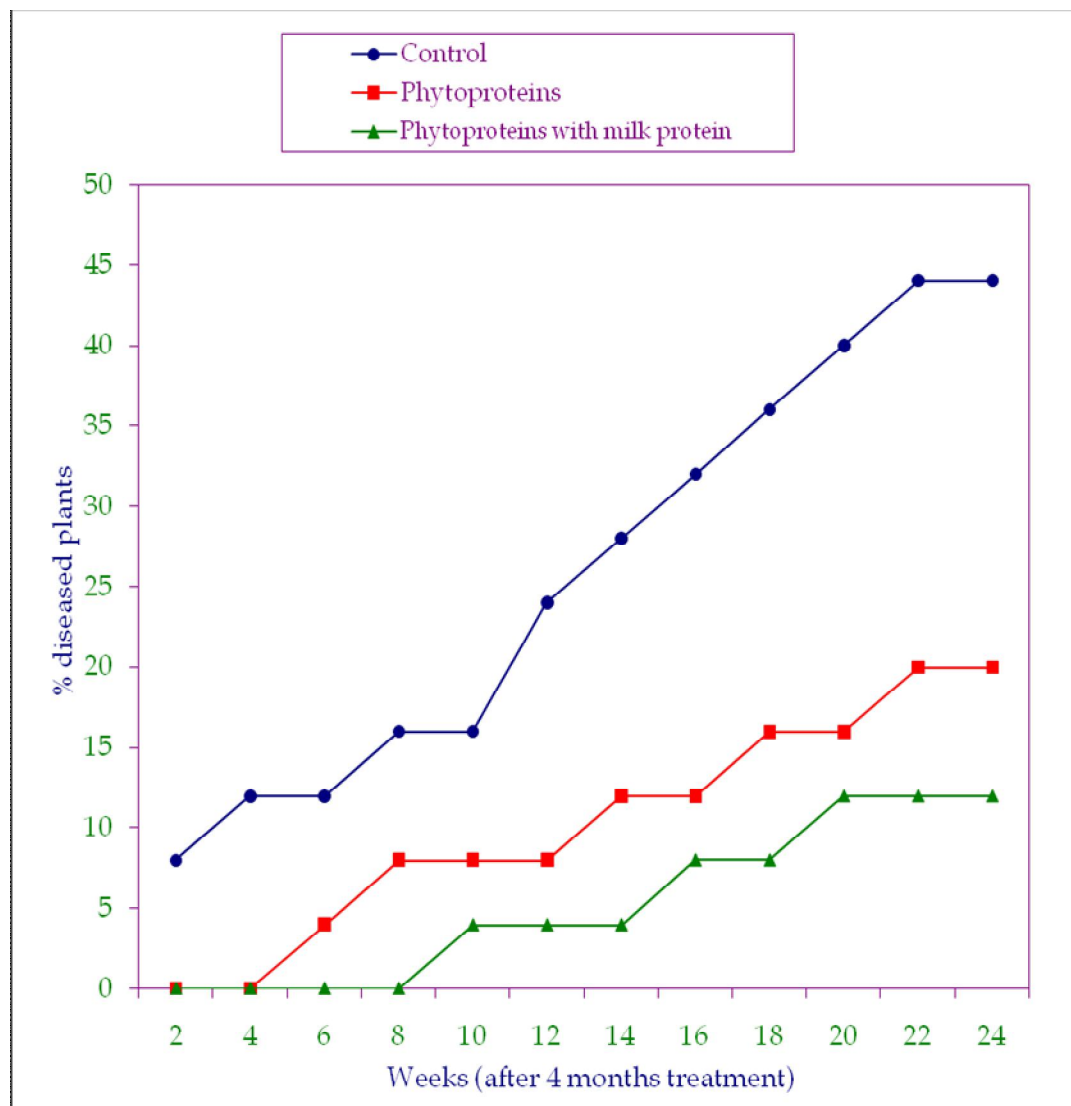
The treatments decreased disease incidence. The plants of *Carica papaya*, treated alternately with BD and CA phyto-protein mixed with milk protein, showed improved growth (Plate 3.1 Table 3.A.1). Treated plants did not exhibit any visual disease symptoms. They exhibited an increase in height (144% of control) and number of leaves (129% of control). 16% disease incidence was observed in papaya plates belonging to control distilled water treated set, due to infection by two commonly prevalent virus diseases, papaya leaf curl and papaya ring spot. Thus, the treated plants showed resistance towards virus diseases and improved plant growth. The symptoms observed due to virus infection in control plants of papaya were of leaf curling and cupping of leaves, followed by vein clearing and thickening. Enations developed in the form of frills on green veins. The affected leaves became leathery and brittle and the petioles became twisted in a zig - zag manner. Fruits on diseased plants were few and small. These were distorted in shape and fell prematurely. A delay in symptom appearance was noted in plants treated with phytoproteins +milk protein. Though some plants showed symptoms at later stages, the disease severity was mild as compared to control. Reduced disease severity was observed in treated plants. The treated plants were able to reach reproductive stage and showed flowering. Phyto-proteins amended with milk protein gave better protection than when either of the phyto-proteins was

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used alone. While a decrease was observed in number of diseased plants (1/3 of control) (Fig. 3.1), increases were observed for plant height (135% of control), number of leaves (130% of control), number of flowers (139% of control), fruiting/plant (150% of control), number of fruits (170% of control) and total fruit yield (about 2 times of control).



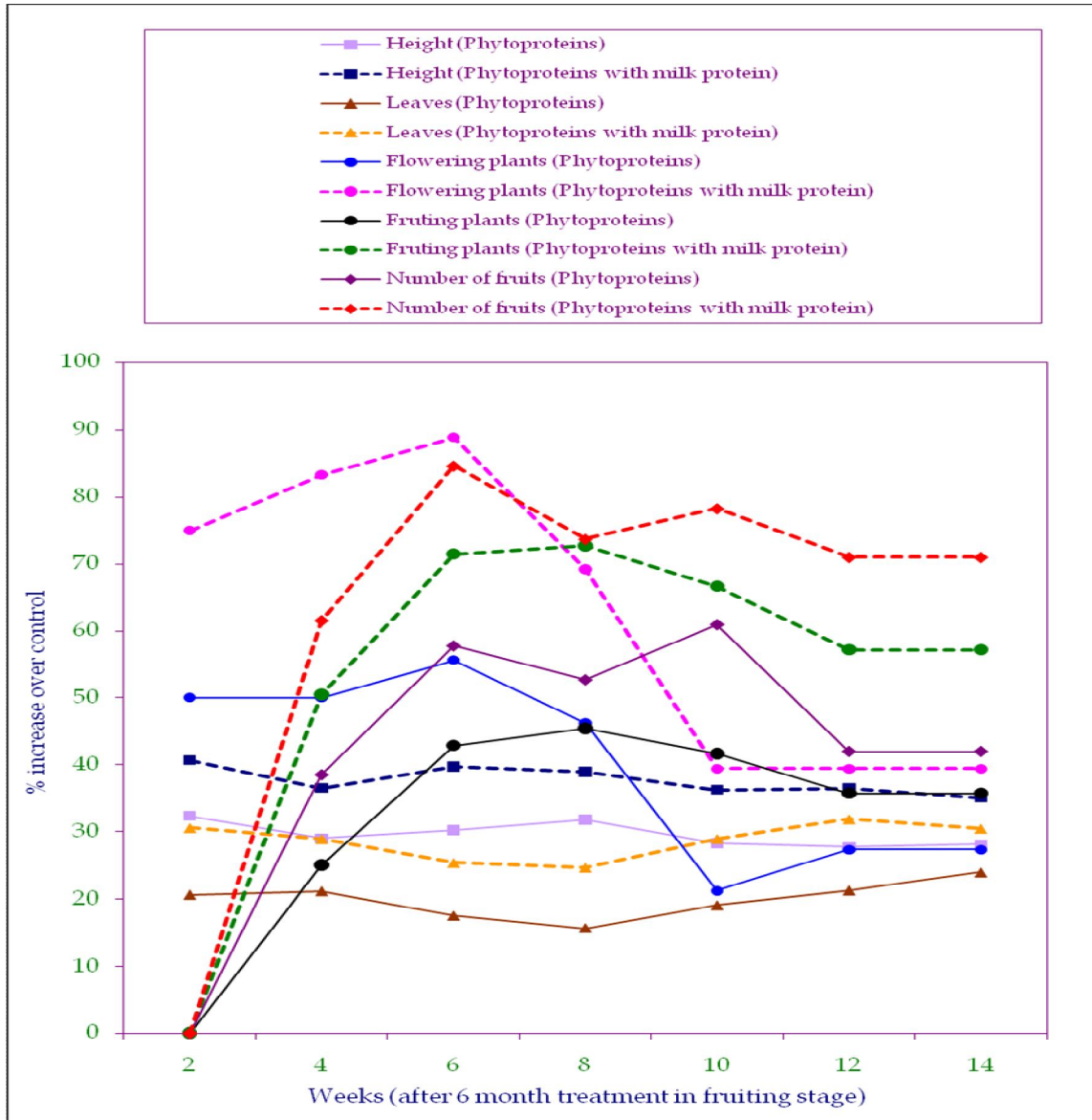
**Fig 3.1** Effect of addition of milk protein to the phytoproteins from *Boerhaavia diffusa* and *Clerodendrum aculeatum* on protection of *Carica papaya* against viral infection

Better plant height, number of leaves, flowering and consequent fruiting was observed in all treated sets of beds (Fig. 3.2). Thus, result show increased fruit yield and better fruit quality (Plate 3.2; Table 3.A.2) in papaya sprayed with BD/CA phytoproteins mixed with milk protein.

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**Fig. 3.2** Effect of addition of milk protein to the phytoproteins from *Boerhaavia diffusa* and *Clerodendrum aculeatum* on various vegetative and reproductive parameters of *Carica papaya*

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**Plate- 3.1** Effect of alternate sprays of *Boerhaavia diffusa/Clerodendrum aculeatum* phytoprotein with bioenhancer milk protein on disease incidence in *Carica papaya*  
**a.** Untreated control plant showing severe viral disease symptoms at early stages  
**b.** Treated plant showing better growth and no viral disease symptoms  
**c.** Severe viral disease symptoms in untreated plant at reproductive stage  
**d.** Treated plant remained healthy at reproductive stage

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**Plate- 3.2** Effect of alternate sprays of *Boerhaavia diffusa*/*Clerodendrum aculeatum* phytoprotein with bioenhancer milk protein on disease incidence in *Carica papaya* after 10 months of last spray

a. Untreated control plant showing severe viral disease symptoms and less fruit setting

b. Treated plant showing better growth and increased fruit setting

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**Table 3.A.1.** Effect of addition of milk protein to phytoproteins obtained from *B. diffusa* and *C. aculeatum* on the disease development and growth of papaya (*Carica papaya*) crop

Response*												
Duration	Diseased plant (%)			Disease severity			Plant height (cm)			Number of leaves/plant		
Weeks	Control	Phytoproteins	Phytoproteins + Milk protein	Control	Phytoproteins	Phytoproteins + Milk protein	Control	Phytoproteins	Phytoproteins + Milk protein	Control	Phytoproteins	Phytoproteins + Milk protein
2	—	—	—	—	—	—	30±4.32	35±3.52	37±3.49	5.6±0.32	6.1±0.36	6.3±0.34
4	4.0	—	—	+	—	—	34±4.12	41±3.92	44±6.53	6.0±0.48	6.6±0.42	6.8±0.42
6	4.0	—	—	+	—	—	39±4.32	47±5.16	51±5.43	6.6±0.64	7.3±0.46	7.7±0.36
8	8.0	—	—	+	—	—	45±5.17	55±6.18	59±7.13	7.1±0.53	8.2±0.89	8.6±0.94
10	8.0	—	—	+	—	—	52±4.12	68±6.36	73±6.24	7.6±0.52	8.7±0.86	9.2±0.56
12	8.0	—	—	+	—	—	59±4.34	75±6.89	81±6.24	8.1±0.58	9.4±0.92	9.8±0.62
14	12.0	—	—	++	—	—	66±4.92	85±7.42	92±7.41	8.9±0.46	10.6±0.02	11.2±0.76
16	12.0	4.0	—	++	+	—	74±5.02	98±8.72	104±9.56	9.8±0.63	11.8±1.21	12.7±1.24
18	16.0	8.0	—	++	+	—	81±6.56	108±10.46	115±12.62	11.0±0.98	13.5±1.32	14.6±1.67
20	20.0	8.0	4.0	+++	+	+	88±6.96	116±11.21	123±11.32	12.1±1.06	15.4±1.26	16.5±1.52

\*Data based on average of 25 replicate plants ± SEM; experiment was repeated thrice; after about 2 months of growth Treatment involved 1 spray per week of BD followed by 1 spray per week of CA in succession.

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**Table 3.A.2.** Effect of addition of milk protein to phytoproteins obtained from *B. diffusa* and *C. aculeatum* on the disease development, reproductive parts and yield of papaya (*Carica papaya*) crop

Duration Weeks	Diseased plant (%)			Disease severity			Plant height (cm)			Number of leaves/plant		
	Control	Phytoproteins	Phytoproteins + Milk protein	Control	Phytoproteins	Phytoproteins + Milk protein	Control	Phytoproteins	Phytoproteins + Milk protein	Control	Phytoproteins	Phytoproteins + Milk protein
2	24.0	8.0	4.0	+++	++	+	96±8.76	127±11.26	135±12.65	13.1±1.14	15.8±1.25	17.1±1.95
4	28.0	12.0	4.0	+++	++	+	107±10.29	138±14.02	146±13.37	14.2±1.32	17.2±1.89	18.3±2.16
6	32.0	12.0	8.0	+++	++	+	116±11.26	151±14.29	160±14.31	15.4±1.62	18.1±1.46	19.3±2.32
8	36.0	16.0	8.0	++++	++	+	126±11.92	166±12.90	175±13.29	16.6±2.11	19.2±1.78	20.6±3.22
10	40.0	16.0	12.0	++++	++	+	138±13.32	177±13.20	188±15.37	17.3±1.94	20.6±2.01	22.3±2.86
12	44.0	20.0	12.0	++++	+++	++	148±13.84	189±14.94	202±14.65	17.9±2.41	21.7±2.12	23.6±3.64
14	44.0	20.0	12.0	++++	+++	++	157±13.16	201±16.12	212±15.12	18.4±2.24	22.8±2.32	24.0±3.45

\*Data based on average of 25 replicate plants ± SEM; experiment was repeated thrice; after about 8 months of growth Treatment involved 1 spray per week of BD followed by 1 spray per week of CA in succession.

**Contd.**



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**Table 3.A.2 Contd.**

Duration Weeks	Flowering plants (%)			Fruiting plants (%)			Number of fruits/plant			Total crop yield (kg)		
	Control	Phytoproteins + Milk	Phytoproteins + Milk protein	Control	Phytoproteins	Phytoproteins + Milk protein	Control	Phytoproteins	Phytoproteins + Milk protein	Control	Phytoproteins	Phytoproteins + Milk protein
15	16.0	24.0	28.0	—	—	—	—	—	—	—	—	—
30	24.0	36.0	44.0	16.0	20.0	24.0	1.3±.24	1.8±0.29	2.1±0.24	—	—	—
45	36.0	56.0	68.0	28.0	40.0	48.0	2.6±0.21	4.1±0.28	4.8±0.76	—	—	—
60	52.0	76.0	88.0	44.0	64.0	76.0	3.8±0.41	5.8±0.45	6.6±0.89	—	—	—
75	66.0	80.0	92.0	48.0	68.0	80.0	4.6±0.64	7.4±0.63	8.2±1.02	—	—	—
90	66.00	84.0	92.0	56.0	76.0	88.0	6.2±0.56	8.8±0.91	10.6±1.16	—	—	—
105	66.00	84.0	92.0	56.0	76.0	88.0	6.2±0.56	8.8±0.96	10.6±1.16	93.0	146.0	178.0

\*Data based on average of 25 replicate plants ± SEM; experiment was repeated thrice; after about 8 months of growth Treatment involved 1 spray per week of BD followed by 1 spray per week of CA in succession.

### IV. DISCUSSION

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 phyto-protein 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.

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