Effect of feeding stimulants on the insecticidal properties of **Pieris brassicae** *granulovirus (PbGV) against* **Pieris brassicae**

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Abstract Feeding stimulants (phagostimulants) increase the food uptake by the host insect larvae and thereby play a vital role in enhancing overall activity of insect viruses formulated as biopesticides. A local strain of granulovirus of *Pieris brassicae* (PbGV), a serious pest of cole crops worldwide, has shown potential as a biopesticide against this pest and with slight increase in its efficacy can be used successfully as a promising biocontrol agent in integrated pest management (IPM) programs of the pest. The addition of phagostimulants may enhance the efficacy but so far no specific phagostimulant for PbGV has been reported. In the present studies, some reported phagostimulants for insect viruses were tested for their impact on food utilization by host larvae and efficacy of the virus. The specified concentration of virus isolate was mixed with phagostimulants and laboratory studies were conducted to uncover their effect on consumption index (CI) and larval mortality. The studies revealed that phagostimulants, *viz*. boric acid and crude sugar, when mixed with PbGV, showed highest CI and larval mortality. Consumption index of 1.34, 1.49 and 1.43 mg/mg/day and corresponding larval mortality of 32.9%, 45.8% and 34.2% were recorded with the addition of 0.5% and 1% boric acid and 2% crude sugar, respectively, in PbGV. The leaf extracts of cabbage, cauliflower and broccoli at both the concentrations (1% and 2%) also enhanced the CI but were comparatively less effective than the boric acid and crude sugar. Field evaluation of PbGV alone and in combination with phagostimulants carried out at two geographically isolated locations, *viz*. Palampur (subtropical) and Sangla (temperate) on three cole crops (cabbage, cauliflower and broccoli) showed that boric acid was the most promising candidate for enhancing the PbGV efficacy under field conditions, followed by crude sugar.

Keywords Baculovirus · Boric acid · Cabbage butterfly · Crude sugar · Phagostimulants

P. Sood · A. Choudhary · C. S. Prabhakar · P. K. Mehta
Department of Entomology, CSK HP Agricultural University, Palampur 176 062 Himachal Pradesh, India

P. Sood (✉)
Crop Research Sub-Station, CSK HP Agricultural University, Sundernagar, Mandi 175 019 Himachal Pradesh, India
e-mail: pankajplp@rediffmail.com

A. Choudhary
Department of Entomology, Punjab Agricultural University, Ludhiana 141 004 Punjab, India

C. S. Prabhakar
Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085 Maharashtra, India

Introduction

Cole crops comprising cabbage, cauliflower and broccoli are the major vegetable crops grown in Himachal Pradesh, India. A number of insect pests attack these crops at different growth stages, causing severe damage.

Among the major pests cabbage butterfly, *Pieris brassicae* (Linnaeus) (Lepidoptera: Pieridae), is a destructive cosmopolitan pest (Bhandari *et al.* 2009; Feltwell 1978), whose young caterpillars feed gregariously on leaves – thus defoliating the plants (Ali & Rizvi 2007; Sachan & Gangwar 1980; Younas *et al.* 2004), making insecticidal application mandatory for their successful cultivation (Sood *et al.* 2011). Since the insecticides pose a number of undesirable side effects, such as resistance in the pest to insecticides, loss of natural enemies and beneficial fauna, and many environmental issues like pollution and residues in food, scientists around the globe have been working continuously to develop some alternatives to these synthetic pesticides. In the past years many biocides were developed like pesticides based on plant products, entomopathogens and new chemistries with novel modes of action. Of these, entomopathogens are the potential candidates and can be successfully included in the integrated pest management (IPM) programs to negate the ill effects of synthetic pesticides, especially in the case of lepidopteran insects.

Entomopathogenic viruses, especially nucleopolyhedroviruses (NPV) and granuloviruses (GV) belonging to the family Baculoviridae have an established history and are regarded as safe and effective bioinsecticides against a number of lepidopteran pests (Moscardi 1999). Baculoviruses are attractive biological insecticides due to their high virulence, host specificity, safety to the environment and nontarget organisms, and ease in application (Hunter-Fujita *et al.* 1998; Simon *et al*. 2004; Sood *et al.* 2011).

A granulosis virus of *Pieris brassicae* (PbGV) has been isolated and characterized from the temperate region (situated at an altitude of 2590 m amsl, 31°25′ N and 78°15′ E) of Himachal Pradesh, India (Sood 2004; Sood & Prabhakar 2012). Recently, studies were conducted for the successful use of this virus against *P. brassicae* larvae (Bhandari *et al.* 2009, 2010; Sood *et al.* 2010, 2011) but observations on the persistence and efficacy are still under way. There is scope to enhance the efficacy of a baculovirus by adding feeding stimulants which encourage phytophagous larvae to consume more foliage contaminated with viral occlusion bodies (OBs), thereby resulting in enhanced infection and improved pest control (Andrews *et al.* 1975; Ansari *et al.* 2012; Bell & Kanavel 1977; Shapiro *et al.* 2009). Castillejos *et al.* (2002) reported the enhanced efficacy of insect viruses under field conditions after addition of the phagostimulants. Therefore, in the present studies some known phagostimulants along with some host plants of *P. brassicae* (cabbage, cauliflower and broccoli) were screened for their possible effect as feeding stimulants. The impact on efficacy of PbGV against *P. brassicae* larvae was also studied under laboratory and field conditions.

Materials and methods

Rearing of experimental insect The initial culture of *P. brassicae* was started from field-collected eggs. The eggs were kept in sterilized petri plates (7.5 cm diam) over a UV irradiated filter paper moistened with sterile distilled water (SDW) to prevent desiccation under laboratory conditions (temp. 25 ± 2°C; r.h. 75–80%). Newly hatched larvae were transferred to fresh cabbage leaves, surface sterilized with aqueous solution of sodium hypochloride (0.05%) followed by three washings with SDW. The cabbage leaves were kept in an ethanol-washed and UV-sterilized rearing cage (15 × 15 × 15 cm). The first three larval instars were reared in the small rearing cages while the later instars were reared in large rearing cages (45 × 45 × 55 cm). Caterpillars in cages were provided with surface-sterilized fresh cabbage leaves daily. The full-grown caterpillars were transferred to the new cages for pupation. Two-day-old pupae were detached from the walls of the cage and kept in a batch of 20 pupae in each cage (60 × 60 × 70 cm) over a thick layer of UV-irradiated filter paper for adult emergence. The adults were held in cages (60 × 60 × 70 cm) provided with cotton swabs soaked in honey solution, SDW and some flowering shoots of mustard as pollen source. Potted cabbage plants were kept in each cage for egg laying whenever needed.

Virus strain The *Pieris brassicae* granulovirus (PbGV) used in the study was the local strain isolated and characterized from the dry temperate region of Himachal Pradesh by Sood (2004). The virus was multiplied in the host larvae in the Postgraduate Laboratory of the Department of Entomology, CSK HP Agricultural University, Palampur, India (1290 m amsl). The virus was purified and the concentration of OBs in stock suspensions was determined by direct count using a Helber bacteria hemocytometer (0.02 mm depth). A stock solution of PbGV with a strength of 2×10^{12} OBs ml^{-1} was prepared and stored in amber colored bottles at 4°C.

Desired concentrations were prepared from the quantified virus stock solution in SDW.

Test material Laboratory reared second instar *P. brassicae* larvae showing no symptoms of virus infection were used in leaf disc bioassay. Aqueous extracts of cabbage, cauliflower and broccoli were prepared by using powder of shade-dried leaves of the plants of these crops. Powder of a plant was first sieved through a muslin cloth and stock solution of 50% strength (w/v) was prepared by soaking 50 g of dried powder in 100 ml of SDW. After 24 h of soaking, the solution was filtered through muslin cloth and the final volume was made up to 100 ml. Working solutions (A1 and A2 with strengths of 2% and 4%, respectively) were prepared from this stock solution. Similarly to the stock solution of PbGV, working solution B with a strength of 3.6×10^4 OBs ml^{-1} was prepared. Both the working solutions A1 and A2 were diluted with equal volumes of B to achieve final concentrations of 1% and 2% of phagostimulants and a virus concentration of 1.8×10^4 OBs ml^{-1} (v/v). Boric acid and crude sugar were mixed (w/v) as per desired concentrations directly in PbGV solution of 1.8×10^4 OBs ml^{-1}. These combinations were evaluated initially under laboratory conditions for their utility in enhancing the efficacy of virus strain against *P. brassicae* larvae. The best combinations were further evaluated for the field efficacy at two geographically isolated locations in India.

Bioassays Leaf disc bioassays as described by Ballard *et al.* (2000) were carried out with second instar *P. bassicae* larvae offered with various treatments applied on cabbage leaf discs. A leaf disc of 4.5 cm diam was dipped in PbGV + phagostimulants solutions for 10 s, dried on a paper towel at room temperature and used for bioassays. A treatment of PbGV without phagostimulants was used as positive control, and cabbage leaf discs treated with SDW alone was the negative control, kept during all sets of experiments. The discs were kept singly in the UV-irradiated petri plates. Ten pre-starved second instar larvae (24 h) were released on treated cabbage leaf discs for each treatment separately. Each treatment was replicated five times. After 24 h of feeding, the larvae were transferred to 70% ethanol-washed untreated cabbage leaf discs. Mortality counts were recorded at 48 h after release and thereafter to work out the percent larval mortality in each treatment up to 7 days of treatment. Observations on PbGV infection were recorded by identifying the peculiar viral symptoms (virosis). In case of uncertainty, larvae were prodded with a needle in order to provoke the characteristic disintegration of the larval tissue.

Consumption index (CI) In the same experiment observations on weight of cabbage leaves consumed and larval weight were also recorded. The cabbage leaf discs were weighed on an electronic balance (Denver APEX series APX-200) before and 24 h after treatment. The leaf discs were replaced daily and observations on the amount of food consumed during every 24 h and weight of larvae were taken daily until 120 h of treatment. The data taken were used to calculate CI of *P. brassicae* larvae on a fresh (wet) weight basis following Waldbauer (1968) and Jalali *et al.* (1988). Correction for water evaporation was obtained by holding ten cabbage leaf discs under identical conditions.

$$\text{Consumption Index(CI)} = \frac{F}{T \times A}$$

where

F fresh weight of food eaten
T duration of feeding period (days)
A mean fresh weight of larvae during feeding

Field studies The best treatments in laboratory experimentation were again evaluated under field conditions at two geographically isolated locations in India, *viz*. the experimental farm of the Department of Entomology, HP Agricultural University, Palampur (subtropical, situated at an altitude of 1290 m amsl, 32°6′ N and 76°32′ E) and the Mountain Agricultural Research and Extension Centre, Sangla (temperate, situated at an altitude of 2590 m amsl, 31°25′ N and 78°15′ E). The crops (cabbage, cauliflower and broccoli) were raised using the standard package of practices except for the insect pest control applications. The experiment was conducted in a randomized block design with three replications. After the initiation of *P. brassicae* activity the crops were sprayed two times at a 14-day interval with PbGV 500 LE (larval equivalent) per hectare and in combinations with earlier tested best phagostimulants (boric acid, crude sugar and cauliflower leaf extract). A positive control using a recommended insecticide (cypermethrin 0.01%) and a negative check (SDW) were also maintained. The experimental set up at Sangla was similar to that at Palampur with the exception that only one spray of the treatments was made after the appearance of pest at Sangla. The observations were recorded on pretreatment and post-treatment larval count (at 7, 14 and 22 days at Palampur and 7 and 14 days at Sangla) to work out the percent reduction in larval population. Percent reduction in larval population was calculated by the formula of Fleming & Retnakaran (1985).

Statistical analysis The percent GV infection was calculated based on the number of larvae infected out of the total number of larvae observed (infected, healthy and others). The data obtained were then subjected to arcsine transformation and analyzed statistically by using the technique of analysis of variance for completely randomized design as described by Gomez & Gomez (1984). The field data, however, were analyzed using randomized block design. The treatment means were compared at 5% level of significance by the least significance difference test ($LSD_{0.05}$) described by Gomez & Gomez (1984).

Results

Laboratory studies: Effect of phagostimulants on PbGV efficacy The data (Table 1) revealed that the highest consumption index (CI) of 1.49 mg/mg/day, leading to the highest *P. brassicae* larval mortality (45.8%), was observed when boric acid was mixed with PbGV @ 1.0% w/v. Boric acid at lower concentration (0.5% w/v) recorded a CI value of 1.34 mg/mg/day with corresponding mortality of 32.9% and was at a par with the former treatment. Crude sugar (2% w/v) added to PbGV recorded a CI value of 1.43 mg/mg/day with larval mortality of 34.2%. The leaf extract of cauliflower (2.0% v/v) was effective in enhancing the food intake by *P. brassicae* larvae as evident by a CI value of 1.37 mg/mg/day and corresponding larval mortality of 32.7%. All the above mentioned combinations were, however, statistically at a par with each other and superior to PbGV alone and negative control (cabbage leaf discs with SDW). Plant extracts of the two other crops (cabbage and broccoli) at both the concentrations tested, 2.0% and 1.0% v/v also showed some additive effect in enhancing food intake and CI over control, but the differences were statistically non-significant. Boric acid was the most effective and statistically superior to PbGV alone and negative control with respect to CI at both the tested concentrations (0.5% and 1.0% w/v), while crude sugar and cauliflower leaf extracts were significantly superior to PbGV alone and negative control at only the higher concentration of 2.0% w/v.

Field studies At Palampur, promising treatment combinations on the basis of laboratory studies were evaluated for their effect on PbGV efficacy under field conditions against *P. brassicae* on three crops, *viz*. cabbage, cauliflower and broccoli. It was observed that the trends of efficacy were similar in all the three crops (Fig. 1). Data revealed that the PbGV combinations with boric acid and crude sugar evaluated in the study increased the efficacy of PbGV against the pest on cabbage, cauliflower and broccoli, as evident from the enhanced reduction in pest population over control to that of PbGV alone. The trends

Table 1 Effect of phagostimulants on the efficacy of *Pieris brassicae* granulovirus against *P. brassicae* larvae

Treatment	Concentration	CI[x] (mg/mg/day)	Larval mortality (%)
PbGV alone [1.8×10^4 OBs ml^{-1}]		1.07	19.5
PbGV + Cabbage leaf extract[z]	1%	1.21	23.2
	2%	1.29	29.9[b]
PbGV + Cauliflower leaf extract[z]	1%	1.32	25.1[b]
	2%	1.37[a]	32.7[b]
PbGV + Broccoli leaf extract[z]	1%	1.18	22.6
	2%	1.23	27.5[b]
PbGV + Boric acid[y]	0.5%	1.34[a]	32.9[b]
	1%	1.49[a]	45.8[b]
PbGV + Crude sugar[y]	1%	1.29	26.3[b]
	2%	1.43[a]	34.2[b]
Sterile distilled water (SDW) (Negative control)	-	1.12	7.9
$LSD_{0.05}$	-	0.26	3.9

[a,b] Within columns, values differ significantly from control (PbGV alone)

[z] Concentrations made as v/v

[y] Concentrations made as w/v

[x] Consumption index

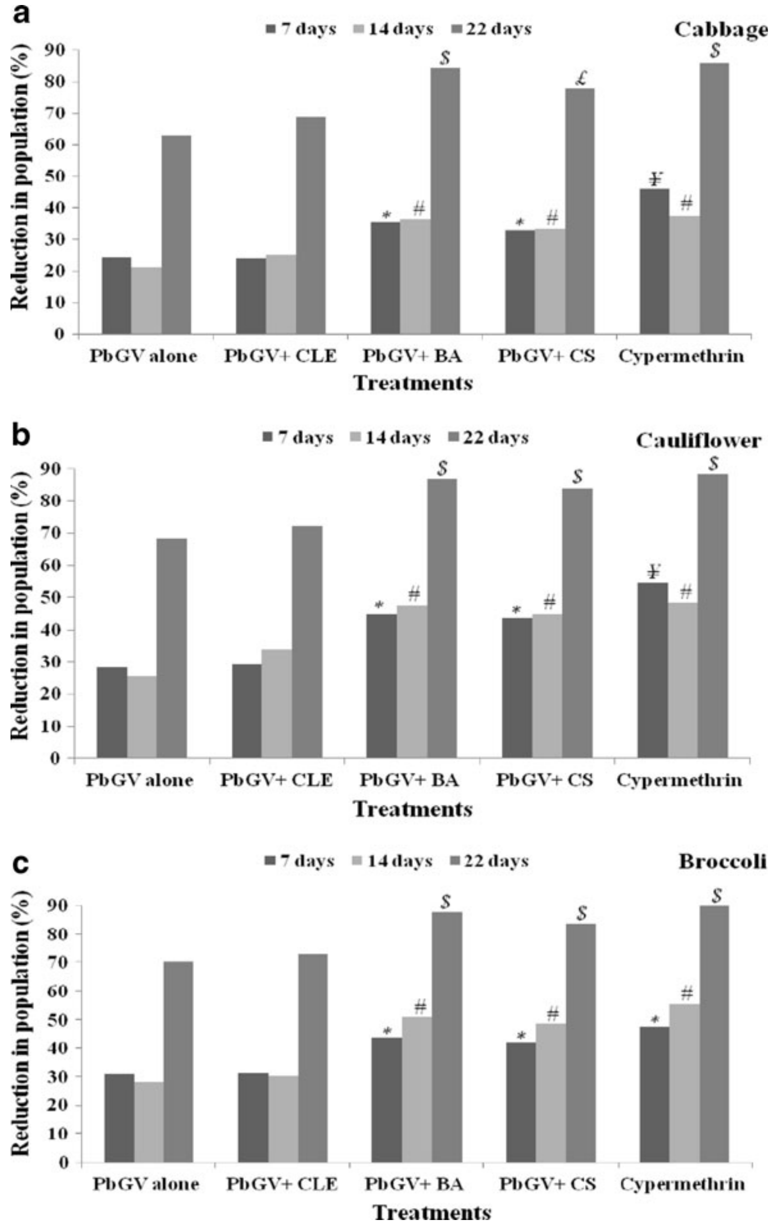

Fig. 1 Effect of phagostimulants on PbGV efficacy against *Pieris brassicae* on (A) cabbage, (B) cauliflower, and (C) broccoli under field conditions at Palampur. PbGV applied @ 500 LE [larval equivalent] ha^{-1}; cauliflower leaf extract (CLE) @ 2% v/v; crude sugar (CS) @ 2% w/v; boric acid (BA) @ 1% w/v; cypermethrin applied @ 0.01%. Bar with */#/$/£/¥ indicates significant difference from PbGV alone at 5% level of significance

in efficacy of phagostimulants at 14 DAT also behaved almost similarly to that of 7 DAT as evident from increased reduction in the pest population except for cypermethrin (in case of cabbage and cauliflower) where percent reduction in pest population at 14 DAT decreased compared to 7 DAT. There was, however, a significant increase in percent reduction in pest population at 22 DAT compared with 7 and 14 DAT in all the treatments. Although the evaluated phagostimulants were inferior to cypermethrin initially at 7 and 14 DAT, however, at 22 DAT it was boric acid (1% w/v) in combination with PbGV which was equally effective to the positive check (cypermethrin) and significantly superior to PbGV alone and other treatments. It was followed by crude sugar (2% w/v) in terms of reduction in pest population. In the case of cauliflower and broccoli also, the trends of efficacy were almost the same as that of cabbage.

At Sangla, the data were recorded only up to 14 DAT as only one spray of the treatments was done. The data depicted in Figure 2 again revealed that all the treatments increased the efficacy of PbGV against the pest, as evident from the enhanced reduction in pest population

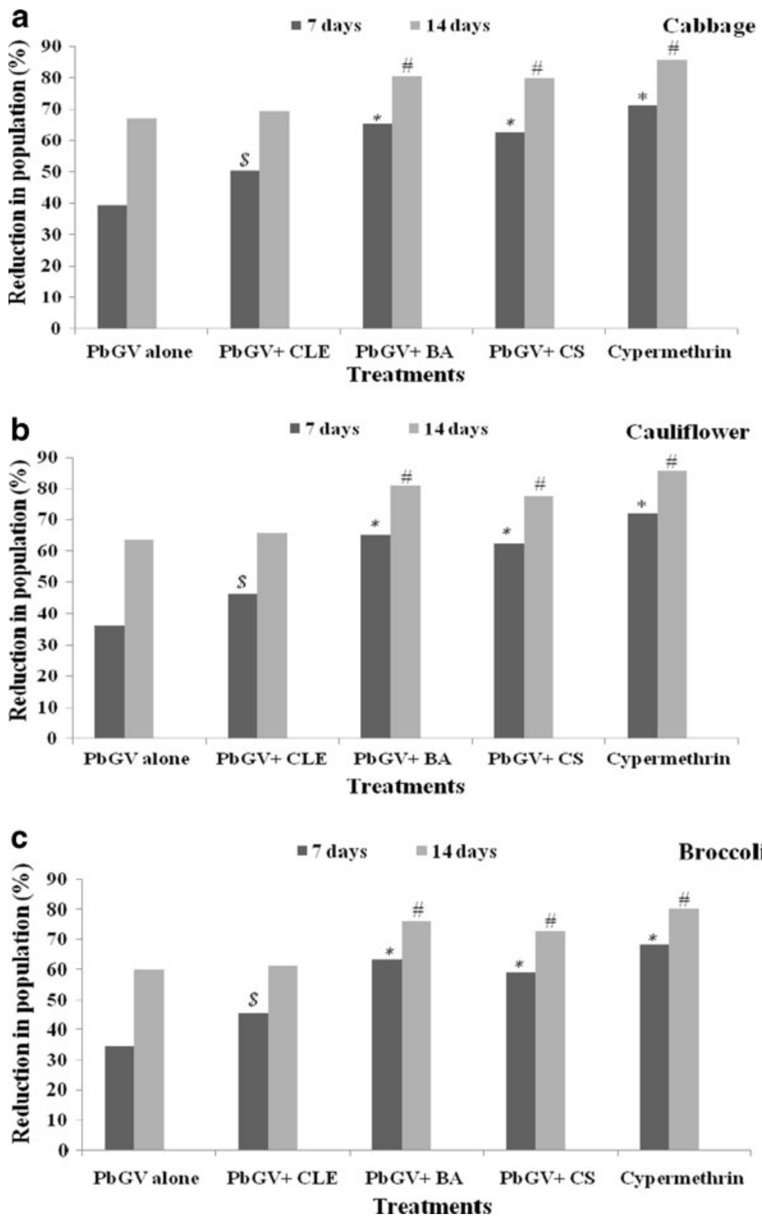

Fig. 2 Effect of phagostimulants on PbGV efficacy against *Pieris brassicae* on (A) cabbage, (B) cauliflower, and (C) broccoli under field conditions at Sangla. PbGV applied @ 500 LE [larval equivalent] ha^{-1}; cauliflower leaf extract (CLE) @ 2% v/v; crude sugar (CS) @ 2% w/v; boric acid (BA) @ 1% w/v; cypermethrin applied @ 0.01%. Bar with */#/$/£/¥ indicates significant difference from PbGV alone at 5% level of significance

over control to that of PbGV alone. At 7 and 14 DAT, it was boric acid (1% w/v) and crude sugar (2% w/v) which were equally effective to the positive check (cypermethrin) and significantly superior to PbGV alone. In case of cauliflower and broccoli also, the trends of efficacy were almost similar to that of cabbage.

Discussion

In laboratory bioassays, boric acid, crude sugar and cauliflower leaf extracts were identified as potential feeding stimulants for *P. brassicae* larvae. Earlier boric acid had been reported as a feeding stimulant and also used for potentiating the activity of many entomopathogens, *viz. Bacillus thuringiensis* and several baculoviruses (Cisneros *et al.* 2002; Morris *et al.* 1995; Shapiro & Bell 1982). Crude sugar as a phagostimulant significantly enhanced the larval mortality of *P. brassicae* as it is an important gustatory stimulant in many lepidopteran species (Bell & Kanavel 1978); however, its efficacy has been observed to vary with the concentration used (Ballard *et al.* 2000).

With respect to CI, boric acid and crude sugar were the most effective treatments. The CI was maximum in case of boric acid (0.5% & 1% w/v), followed by crude sugar (2% w/v), which implies that addition of boric acid and crude sugar in the PbGV resulted in enhanced uptake of virus – which consequently resulted in high CI value and larval mortality. In case of cabbage, cauliflower and broccoli leaf extracts, the CI recorded was higher than that of PbGV alone and negative control but the differences were statistically non-significant except for that of cauliflower extract @ 2% v/v. It was, however, observed that a slight (though nonsignificant) increase in CI value resulted in a significant increase in *P. brassicae* larval mortality. The addition or combination of phagostimulants with PbGV resulted in enhanced efficacy of the virus even at the half dose of PbGV applied. It is a well established fact that the efficacy of entomopathogens which act by ingestion can be improved by the use of formulations that include feeding stimulants. The phagostimulants increase the intake of the pathogen and ultimately enhance the pathogen efficacy and improve pest control (Bell & Kanavel 1977; Farrar & Ridgway 1994). The usefulness of host plants as phagostimulants, and increased efficacy of entomopathogens has been demonstrated by many workers (Ansari *et al.* 2012; Lasa *et al.* 2009; Morales-Ramos *et al.* 1998; Shapiro *et al.* 2009).

Under field conditions, boric acid and crude sugar were highly effective among feeding stimulants and significantly superior to PbGV alone in all the crops under study at both the locations (Sangla and Palampur). However, the time period for the mortality of pest larvae was less with cypermethrin than that of PbGV alone and PbGV with phagostimulants. The efficacy of PbGV alone and PbGV with phagostimulants increased with the passage of time after spray, which may be due to the differential mode of action of materials used in the study. Cypermethrin is a chemical insecticide with acute action and hence killed the larvae in a short period of time, whereas PbGV alone and in combination with phagostimulants had chronic action in which efficacy increased with the passage of time after application. Despite the fact that the potentiating effect of boric acid on baculoviruses has been recognized for several decades (Yadava 1970), there have been very few field studies to evaluate virus with boric acid formulations. Bujjur *et al.* (1991) reported a fourfold improvement in control of *Helicoverpa armigera* (Hübner) on sunflower treated with *H. armigera* nucleopolyhedrovirus (HaNPV) + boric acid (0.5%), while Chundurwar *et al.* (1990) reported improved control of *H. armigera* on chickpea treated with HaNPV + boric acid (0.5%). In maize, improved control of *Spodoptera frugiperda* (Smith) with *S. frugiperda* nucleopolyhedrovirus (SfNPV) + boric acid has been reported by Cisneros *et al.* (2002). Overall it can be concluded that the phagostimulants, *viz*. boric acid, crude sugar and cauliflower leaf extracts, could be successfully used to enhance the PbGV efficacy even at a low concentration of the virus under field conditions. This not only will help in the development of early, as well as durable pest management practices for the effective management of the pest, but will also minimize the insecticidal impact on the environment. The present findings open up new issues for research and experimentation on compatibility of these phagostimulants with entomopathogens so that a viable formulation can be developed with enhanced efficacy.

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