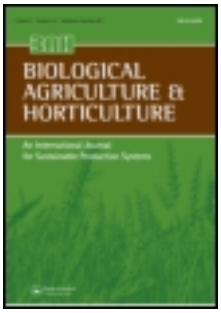

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Effect of UV protectants on the efficacy of *Pieris brassicae* granulovirus

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Fluorescent brighteners increase insect viral activity by providing protection against UV inactivation. The studies were conducted to evaluate the effect of some UV protectants on the efficacy of a localized potential *Pieris brassicae* granulosis virus (PbGV) isolate for its effective field usage. Laboratory studies determined the effect of UV protectants on median inactivation time of virus and inhibition of virus activity of the test isolate. UV protectants, robin blue, congo red, and tinopal were effective in minimizing the detrimental effect of UV radiations on PbGV in suspension. Promising combinations were evaluated under field conditions at two locations in India, Palampur (sub-tropical) and Sangla (temperate), on three cole crops (cabbage, cauliflower, and broccoli). Congo red followed by robin blue and tinopal was found to be the most promising candidate for enhancing the PbGV efficacy by protection from UV radiation.

Keywords: congo red; fluorescent brightener; *Pieris brassicae* granulosis virus; *Pieris brassicae*; robin blue; tinopal

Introduction

Baculoviruses have potential to be used as microbial control agents against insect pests owing to their high pathogenicity, host specificity, and environmental biosafety (Kondo & Maeda 1991; Rahman & Gopinathan 2003; Simon et al. 2004). They comprise a large family of insect-specific DNA viruses from two genera, nuclear polyhedrosis virus (NPV) and granulosis virus (GV) (Moscardi 1999; Blissard et al. 2000), based on occlusion body (OB) morphology. Baculoviruses have a long history as effective and environmentally benign insect control agents in field crops, vegetables, forests, and pastures (Moscardi 1999). However, baculoviruses used as biocontrol agents suffer from instability after exposure to solar radiation, especially due to quick inactivation of virus by natural sunlight, which hinders their satisfactory field utilization for pest management (Bullock 1967; Shapiro et al. 2009). UV-B (280–310 nm) in particular is considered responsible for the inactivation of insect pathogens in general and insect viruses in particular under natural conditions (Ignoffo et al. 1977; Asano 2005). The resulting loss of biological activity prolongs the rate at which insect pests are killed and, in many instances, the pathogens lose $>90\%$ of their original activity within days (David et al. 1968; Ignoffo et al. 1977; Shapiro et al. 2002). Sometimes, the efficacy of these microbials is greatly reduced within the first 24–48 h post-spraying (Bullock 1967; Young & Yearian 1986).

A *Pieris brassicae* granulosis virus (PbGV) was isolated and characterized from the temperate region (altitude of 2590 m amsl, 31°25′ N latitude, and 78°15′ E longitude) of

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Himachal Pradesh, India, and its pathogenicity against the pest has been proven (Sood 2004). The isolate has been tested against the pest alone and in combination with various botanical extracts under laboratory and field conditions (Bhandari et al. 2009; Sood et al. 2011) and found effective against the pest; however, the efficacy of the virus still requires further improvements for its successful field utilization owing to the above limitations.

Brighteners, white carbon, and antioxidants have been found to provide some level of UV protection in the case of insect viruses (Shapiro & Robertson 1990; Shapiro 1992). In view of this, studies were conducted to identify effective and economical materials as UV protectants for PbGV, so that the virus could be more efficiently used in the integrated pest management program of *P. brassicae*. The present studies were therefore conducted to evaluate the impact of known UV protectants in reducing the detrimental effect of UV radiations on PbGV efficacy under laboratory conditions against second instar larva of *P. brassicae* and under 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 diameter) over a UV-irradiated filter paper moistened with sterile distilled water (SDW) to prevent desiccation under laboratory conditions [temperature 25 ± 2°C; relative humidity (RH) 75–80%]. Newly hatched larvae were transferred to fresh cabbage leaves and surface sterilized with aqueous solution of sodium hypochlorite (0.05%) followed by three washings in SDW. The cabbage leaves were kept in ethanol-washed and UV-sterilized cages (15 × 15 × 15 cm). The first three larval instars were reared in the small cages (15 × 15 × 15 cm) while the later instars were reared in large 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 cages and were 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 flowering shoots of mustard as a pollen source. Potted cabbage plants were kept in each cage for egg laying whenever needed.

Virus strain

The PbGV used in the study was the local strain isolated and characterized from the dry temperate region of Himachal Pradesh, India, by Sood (2004). The virus was multiplied in the host larvae in the Postgraduate Laboratory of Department of Entomology, CSK HP Agricultural University, Palampur, India (32°6′ N latitude, 76°3′ E longitude, and 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 strength of 2×10^{12} OBs ml^{-1} (approximately) was prepared and stored in amber-colored bottles at 4°C. Serial dilutions (2×10^{3} to 2×10^{9} OBs ml^{-1}) were prepared from the quantified virus stock solutions in SDW. Second instar larvae were used in a leaf disk bioassay to examine the pathogenicity of PbGV. The lowest concentration recording about 50% mortality (2×10^{5} OBs ml^{-1}) was used for UV protection studies.

Dose mortality response of PbGV against P. brassicae

PbGV pathogenicity was determined using natural diet (cabbage leaves) following the procedure described by Sathiah et al. (2003). Leaf disks of diameter 7.5 cm were treated with 10 μl PbGV suspension of various concentrations using a Potter's tower. Both the surfaces were treated evenly with the viral suspension. Leaf disks were placed in Petri plates and one larva was released per Petri plate. Plates were maintained at 75% RH and 25 ± 2°C. After 24 h of feeding, the larvae were transferred to untreated cabbage leaves. Mortality was recorded at subsequent time intervals. Data were subjected to probit analysis to calculate median lethal concentration (LC_{50}) following the method of Finney (1971). The median lethal times (LT_{50}) were also calculated against the PbGV concentration giving 50% mortality.

Test material (UV protectants)

UV protectants, iron oxide (black), robin blue, tinopal, and congo red were evaluated at two test concentrations (1% and 2% w/v) with the viral suspension for bioassay studies.

UV Source and exposure conditions

A UV tube (15 W Philips G15T8) was used as the UV source. Cabbage leaf disks (7.5 cm diameter) were cut from 70% ethanol-washed fresh cabbage leaves collected from insecticide-free plots, dipped in the test solutions (virus suspension alone and/or with UV protectants), and exposed to UV light. The distance between the UV source and the foliage was adjusted to 50 cm. UV irradiation was performed on both sides of the foliage.

Evaluation of UV protectants against P. brassicae

Cabbage leaf disks (7.5 cm diameter) were dipped in the test solutions (virus suspension with UV protectants) for a few seconds, dried on a paper towel at room temperature, and exposed to UV light for 10 min. A treatment of PbGV without UV protectant was also exposed to UV light and maintained as control. Bioassays were then conducted against second instar larvae using the leaf. The disks were kept singly in the UV-irradiated Petri plates. Ten pre-starved larvae (24 h old) of second instar were released on treated cabbage leaf disks 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 leaves. Mortality counts were recorded 48 h after release and thereafter up to 5 days after treatment (DAT) and converted to percent mortality. 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.

A laboratory experiment was simultaneously conducted to ascertain the effect of UV protectants on the efficacy, inhibition of viral activity (IVA), and median inactivation time (IT_{50}) of PbGV on *P. brassicae* larvae when exposed to natural sunlight. A standard dose of PbGV (2×10^5 OBs ml^{-1}) alone and PbGV mixed with UV protectants (1% w/v) was each applied on parafilm (500 μl) separately and spread uniformly. The films were dried in the shade and exposed to sunlight for 3, 6, 12, 18, 24, and 30 h, considering 6-h daily active exposure starting from 10:00 to 16:00 h. After exposure, the virus deposit on the film was eluted with a known quantity of SDW and the suspension was stored in Eppendorf tubes at 4°C for further bioassay studies.

The leaf disk bioassays were performed against second instar larvae using the virus eluted from the parafilm as described earlier. Control treatments using SDW and PbGV without exposure were also maintained simultaneously. Mortality data were recorded after 36 h (near to LT_{50} calculated in earlier experiments). The mortality data were converted to percent mortality based on the number of larvae infected to the total number of larvae observed. The percent mortality data were then analyzed statistically using analysis of variance for completely randomized design as described by Gomez and Gomez (1984). The treatment means were compared at 5% level of significance by the least significance difference (LSD) test described by Gomez and Gomez (1984).

IT_{50} was also calculated using probit analysis (Finney 1971), while the IVA was calculated using the following formula:

$$\text{IVA}\,(\%) = \frac{\text{percent larval mortality before exposure} - \text{percent larval mortality after exposure}}{\text{percent larval mortality before exposure}} \times 100.$$

Field studies

A field experiment was conducted for two seasons in the experimental farm of the Department of Entomology, CSK HP Agricultural University, Palampur, India, situated at an altitude of 1280 m amsl, 32°6′ N latitude, and 76°3′ E longitude. The crops, cabbage, cauliflower, and broccoli, were raised using the standard package of practices except insecticide application. The experiment was conducted in a randomized block design with three replications. After the initiation of *P. brassicae* activity, the crops were sprayed twice at 14-day intervals with PbGV alone 500 larval equivalents (LEs) and its combinations with the four UV protectants, congo red, robin blue, tinopal, and iron oxide at 1% (w/v). A positive control using recommended insecticide (cypermethrin 0.001%) and negative check (SDW) were also maintained.

The same set of experiments was also replicated in the experimental farm of Mountain Agricultural Research and Extension Centre, Sangla (CSK HP Agricultural University), India, situated at an altitude of 2590 m amsl, 31°25′ N latitude, and 78°15′ E longitude. However, at Sangla, only one spray of the treatments was made after the appearance of the pest. The observations were recorded on pretreatment and posttreatment larval count after 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 following Fleming and Retnakaran (1985) as:

$$\text{Reduction in population}\,(\%) = \left[\left(1 - \frac{\text{posttreatment population in treatement}}{\text{pretreatment population in treatement}} \times \frac{\text{pretreatment population in check}}{\text{posttreatment population in check}}\right) \times 100\right].$$

Results

Dose mortality response of PbGV against P. brassicae

On the basis of LT_{50} values (20.5 h), it was observed that the first instar larvae were highly susceptible to PbGV, as all the larvae died within a day (Table 1). The LT_{50} values increased with the age of the larvae. A similar trend was observed with respect to LC_{50} values (Table 2).

Table 1. LT_{50} of PbGV against *P. brassicae* larvae.

P. brassicae larval instars	LT_{50} (h)	Fiducial limit (h)	χ^2	Slope
First	20.5	17.08–23.75	1.14	3.63 ± 0.34
Second	32.0	28.91–34.64	0.25	6.39 ± 0.99
Third	63.1	60.54–65.33	0.51	15.41 ± 2.04
Fourth	71.6	67.45–74.72	0.22	9.95 ± 1.50

Table 2. LC_{50} of PbGV against *P. brassicae* larvae.

P. brassicae larval instars	LC_{50} (OBs ml^{-1})	Fiducial limit	χ^2	Slope
Second	7.82×10^4	$(4.63–12.74) \times 10^4$	1.85	0.93 ± 0.13
Third	0.95×10^7	$(0.35–2.53) \times 10^7$	2.34	0.88 ± 0.09
Fourth	1.65×10^8	$(1.18–2.27) \times 10^8$	2.58	0.89 ± 0.07

Effect of UV protectants on PbGV efficacy

All the UV protectants, when evaluated under laboratory conditions along with PbGV at two concentrations each against second instar larvae of *P. brassicae*, resulted in enhanced efficacy of the virus isolate compared with that of PbGV alone. At 1%, congo red gave greater larval mortality than iron oxide but did not differ significantly from tinopal or robin blue. At 2%, there was no significant difference in larval mortality between the four UV protectants (Table 3).

Evaluating UV protectants at different concentration along with PbGV under laboratory conditions by exposing them to sunlight for different times and testing their bioefficacy against second instar larvae (Table 4) revealed that with the increase in exposure time, the viral activity decreased due to inactivation caused by UV in sunlight. PbGV bioefficacy against *P. brassicae* second instar larvae decreased from an initial 98.5% to 14.7% after 30-h exposure to natural sunlight. In the case of PbGV mixed with UV protectants, however, the bioefficacy did not decrease to the same extent. The corresponding larval mortality after 30 h of exposure in case of congo red, robin blue, tinopal, and iron oxide was 18.5%, 18.4%, 17.3%, and 16.9%, respectively. Congo red gave the greatest protection to PbGV as the larval mortality was significantly higher than with the other UV protectants and PbGV alone after different exposure times. Data presented in Table 5 also revealed that PbGV mixed with UV protectants were more effective against *P. brassicae* than PbGV alone as UV protectants mixed with PbGV gave IVA of 81.3–82.2% compared with 85.1% with PbGV alone after 30-h exposure. The IT_{50} also showed the UV protection efficacy of congo red, robin blue, tinopal, and iron oxide when mixed with PbGV. The IT_{50} recorded with protectants was 12.5–14.8 h compared with 10.0 h with PbGV alone (Table 6).

Field studies

At Palampur, when UV protectants were evaluated for their effect on PbGV efficacy under field conditions against *P. brassicae* on cabbage, cauliflower, and broccoli, a similar trend in efficacy was observed in all the three crops (Figure 1). Data revealed that congo red, tinopal, and robin blue significantly increased the efficacy of PbGV against the pest as evident from the enhanced reduction in pest population compared with that of PbGV alone

Table 3. Effect of UV protectants on PbGV efficacy against second instar *P. brassicae* larvae after UV irradiation treatment.

Treatments[a]	UV protectant concentration (% w/v)	*P. brassicae* larval mortality (%)
PbGV alone		25.2
PbGV + iron oxide	1	43.4
	2	58.2
PbGV + robin blue	1	49.6
	2	64.0
PbGV + tinopal	1	50.6
	2	60.0
PbGV + congo red	1	52.8
	2	64.2
$LSD_{0.05}$[b]		6.49

[a] PbGV applied at 2.0×10^5 OBs ml^{-1}.
[b] $LSD_{0.05}$ = LSDs between treatments at 5% level of significance.

at 7, 14, and 22 DAT in cabbage and cauliflower, whereas congo red and robin blue, but not tinopal, had a significant effect in broccoli. Cypermethrin (positive control) was statistically superior to all UV protectants used in this study along with PbGV alone at 7 and 22 DAT in cabbage, cauliflower, and broccoli.

At Sangla, as only one spray of the treatments was made, the data were taken only up to 14 DAT. Figure 2 shows that in cabbage and cauliflower, congo red, robin blue, and tinopal were significantly superior to PbGV alone in terms of reduction in the percent pest population at 7 and 14 DAT. Only congo red and robin blue in combination with PbGV were found significantly superior over PbGV alone at 7 and 14 DAT in broccoli. Cypermethrin (positive control) was statistically superior to all UV protectants used in this study along with PbGV alone at 7 DAT in cabbage and cauliflower; however, cypermethrin efficacy was drastically reduced and statistically inferior to all the UV protectants used in combination with PbGV in cabbage and cauliflower at 14 DAT.

Discussion

The susceptibility of *P. brassicae* larvae decreased with the age of the larvae as both the concentration and time required for killing 50% of the population increased with age in this study. This may be due to the less developed defense system of insects at their young stage than at the older stage and greater permeability of the midgut peritrophic membrane, which allows the viral particles to enter and infect the susceptible cells causing virosis in the insect. These results are in line with the findings of Matthews et al. (2002), Grzywacz et al. (2008), and Bhandari et al. (2009) in *Lacanobia oleracea*, *Spodoptera exempta*, and *P. brassicae*, respectively, who have also reported that early instar larvae were more susceptible than the late instar larvae to GVs, i.e., susceptibility is inversely proportional to the age of the insect.

Optical (fluorescent) brighteners, initially described by Paine et al. (1937) and later by Eggert and Wendt (1939), are a group of substituted stilbenes that are routinely utilized in many industrial processes for lightening or brightening paints, fibers, and coatings. Numerous adjunct uses have been described for these compounds including fluorochromes for micro-organisms (Darken 1961; Dougherty et al. 1996). All the fluorescent brighteners used in this study enhance viral activity and larval mortality almost 400-fold compared

Table 4. Effect of UV protectants in reducing the effect of natural sunlight on PbGV efficacy.

Treatments[a]	*P. brassicae* larval mortality after different hours of exposure (%)						
	0	3	6	12	18	24	30
PbGV alone	98.5 (9.97)	80.0 (8.99)	67.7 (8.28)	50.7 (7.18)	34.1 (5.92)	24.8 (5.07)	14.7 (3.95)
PbGV + tinopal	97.5 (9.92)	82.7 (9.14)	75.5 (8.74)	65.9 (8.17)	40.4 (6.43)	33.1 (5.83)	17.3 (4.28)
PbGV + congo red	99.0 (10.01)	83.4 (9.18)	76.9 (8.82)	67.5 (8.27)	45.7 (6.83)	38.3 (6.27)	18.5 (4.41)
PbGV + iron oxide	98.3 (9.95)	81.6 (9.16)	71.1 (8.68)	60.7 (7.93)	39.4 (6.39)	32.7 (5.74)	16.9 (4.16)
PbGV + robin blue	98.2 (9.96)	82.7 (9.14)	73.4 (8.62)	68.9 (8.36)	43.2 (6.64)	34.8 (5.98)	18.4 (4.36)
$LSD_{0.05}$[b]	NS	0.11	0.10	0.05	0.07	0.22	0.04

Note: Values in parentheses are arcsine transformed.
[a] PbGV applied at 2.0×10^5 OBs ml^{-1}; UV protectants applied at 1% w/v.
[b] $LSD_{0.05}$ = LSDs between treatments at 5% level of significance.

Table 5. Effect of UV protectants on IVA of PbGV.

Treatments[a]	IVA after different hours of exposure (%)					
	3	6	12	18	24	30
PbGV alone	18.8	31.3	48.6	65.3	74.8	85.1
PbGV + tinopal	15.3	22.6	32. 5	58.5	66.1	82.2
PbGV + congo red	15.8	22.4	31.9	53.8	61.3	81.4
PbGV + iron oxide	17.0	27.7	38.2	59.9	66.8	82.8
PbGV + robin blue	15.9	25.3	29.8	56.0	64.6	81.3
$LSD_{0.05}$[b]	NS	2.16	3.58	3.71	4.07	1.97

[a] PbGV applied at 2.0×10^5 OBs ml^{-1}; UV protectants applied at 1% w/v.
[b] $LSD_{0.05}$ = LSDs between treatments at 5% level of significance.

Table 6. Effect of UV protectants on IT_{50} of PbGV.

Treatments[a]	IT_{50} (h)	Slope	χ^2	Fiducial limits (h)
PbGV alone	10.0	1.84 ± 0.17	3.98	8.62–11.43
PbGV + tinopal	13.4	1.85 ± 0.17	3.42	9.10–20.23
PbGV + congo red	14.8	1.74 ± 0.17	3.66	9.80–24.60
PbGV + iron oxide	12.5	1.92 ± 0.17	3.67	9.18–18.97
PbGV + robin blue	13.9	1.75 ± 0.17	3.93	8.43–22.88

[a] PbGV applied at 2.0×10^5 OBs ml^{-1}; UV protectants applied at 1% w/v.

with the control (PbGV alone) under laboratory conditions. However, the trend of UV protectant after 30 h of sunlight exposure for larval mortality showed that congo red was more effective in enhancing the efficacy of PbGV followed by robin blue, tinopal, and iron oxide, though all were superior to PbGV alone. The increase in virulence of PbGV due to addition of fluorescent brighteners might also be due to a peculiar mode of action as several fluorescent brighteners such as tinopal and robin blue are known to interfere with cellulose and chitin fibrillogenesis (Monobrullah 2003). The peritrophic membrane is composed of chitin microfibrils, which presumably protect midgut cells from abrasion by food particles and serve as a barrier for the invasion of micro-organisms, including insect viruses (Inglis et al. 1995; Monobrullah 2003). Therefore, fluorescent brighteners used in this study probably disintegrated the larval peritrophic membrane and permitted a large number of virions to pass into the ectoperitrophic space and ultimately invade susceptible cells and replicate, thus accounting for the high mortality in the treatment where fluorescent brighteners were used. Many materials have been tested as UV protectants for insect pathogens and have shown success under laboratory and the field conditions. These materials include UV absorbers (Ignoffo & Batzer 1971; Felton 1999; Cohen et al. 2001) and UV reflectants (Foster 2000; Deleo 2005). Optical (fluorescent) brighteners such as tinopol, robin blue, and blankophor were reported to be effective UV protectants in many lepidopteron insect viruses including *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV) (Shapiro 1992; Shapiro & Robertson 1992; Argauer & Shapiro 1997), *Anticarsia gemmatalis* (Hübner) multiple nucleopolyhedrovirus (AgMNPV) (Morales et al. 2000), and *Spodoptera litura* (Fabricius) nucleopolyhedrovirus (SlNPV) (Okuno et al. 2003). Whereas iron oxide and congo red were reported as UV protectants by Jiang et al. (2007) and Inglis et al. (1995), respectively.

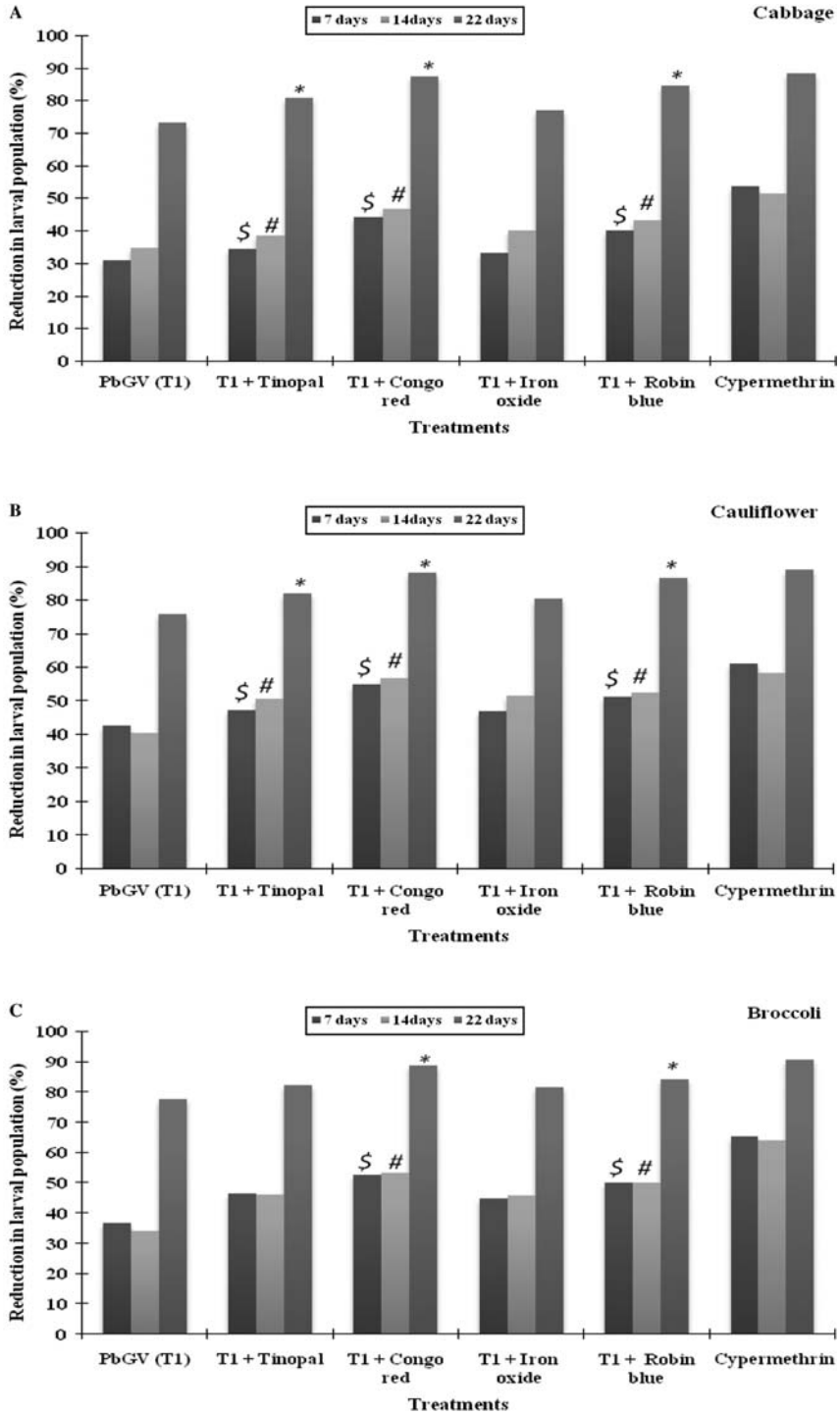

Figure 1. Effect of UV protectant on PbGV efficacy against *P. brassicae* on (A) cabbage, (B) cauliflower, and (C) broccoli under field conditions at Palampur. PbGV applied at 500 LEs ha^{-1}; UV protectants applied at 1% w/v; cypermethrin applied at 0.001%. Bar with ∗, #, and $ indicates significant difference from PbGV alone at 5% level of significance.

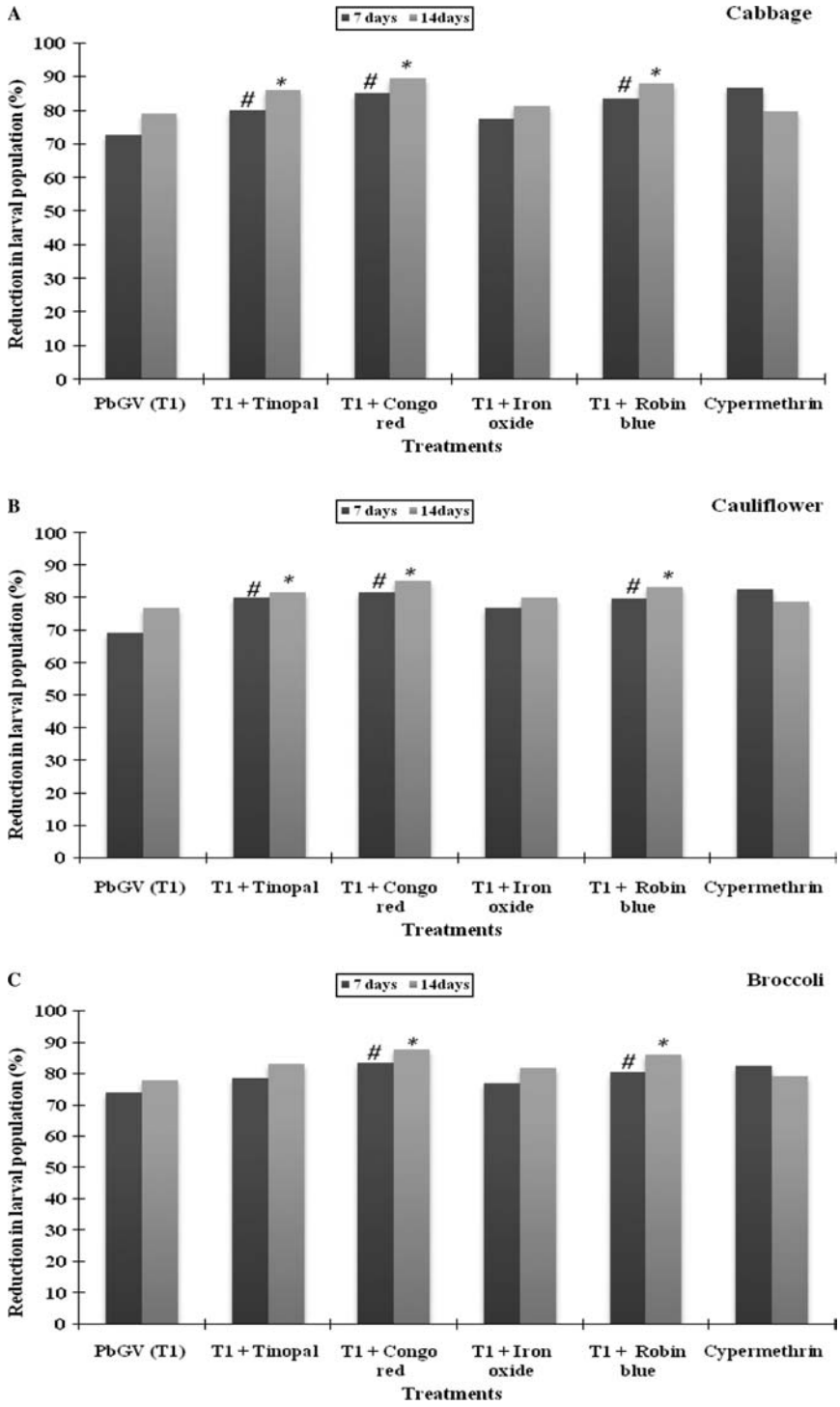

Figure 2. Effect of UV protectant on PbGV efficacy against *P. brassicae* on (A) cabbage, (B) cauliflower, and (C) broccoli under field conditions at Sangla. PbGV applied at 500 LEs ha^{-1}; UV protectants applied at 1% w/v; cypermethrin applied at 0.001%. Bar with ∗, #, and $ indicates significant difference from PbGV alone at 5% level of significance.

Under field conditions, congo red and robin blue were highly effective among UV protectants 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 with PbGV alone and PbGV with UV protectants. The trend of efficacy of PbGV alone and with UV protectants increased with the time after spray. However, the reverse was observed in the plot where cypermethrin was sprayed. This may be due to the different mode of action of the materials used in the study. Cypermethrin is a chemical insecticide with acute action and hence killed the larvae in short period of time, while PbGV alone and in combination with UV protectants had chronic action with increasing efficacy with passage of time after application. These findings open new researchable issues and experiments on compatibility of chemical insecticides, and entomopathogenic viruses should be carried out for the development of early, as well as durable, pest management practices for the effective management of the pest with low insecticide impact on the environment. Overall, it can be concluded that all of these UV protectants congo red, robin blue, tinopal, and iron oxide could be successfully used for the protection of PbGV against UV irradiation under field conditions for better management of the pest.

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