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Research Article

Stability of *Bacillus thuringiensis* and NPV Microencapsulated Formulation under Sunlight

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ABSTRACT

Microencapsulation technology is used for the formulation of bio pesticides and is effective against the ultra-violet radiation of sunlight. The present research studied the stability of Bt and NPV formulations microencapsulated with gelatin and sodium alginate, individually or in combination. The formulations were evaluated in outdoor space and under sunlight on potted growing cabbage. The stability of each active ingredient tested in each formulation was studied at 0, 3, 7 and 10 days after spraying on cabbage infested with diamondback moth *Plutella xylostella* second instars larvae. Results showed that non-formulated and microencapsulated formulations not exposed to sunlight (time zero) had similar mortality. However, after being exposed to sunlight for three days, the non-formulated Bt and NPV resulted in a significantly lower mortality (less than 40%); compared with the microencapsulated bio pesticides (more than 70% mortality). Fifty percent (50%) mortality was reached in microencapsulated formulations after seven and ten days of exposure to sunlight, whereas there was no mortality in larvae exposed to unformulated treated plants after ten days. ANOVA analysis showed the highest larval mortality was achieved by the Bt+NPV gelatin microencapsulated formulation followed by gelatin coated Bt, sodium alginate coated NPV, sodium alginate coated Bt+NPV, gelatin coated NPV and sodium alginate coated Bt. The formulations showed no significant LT_{50} differences between microencapsulated versus unformulated Bt and NPV.

Keywords: Bt, microencapsulated formulation, NPV, Sunlight.

INTRODUCTION

One of the main challenges in developing crop biopesticides is to maintain and save the efficacy of the active ingredient after field application; especially after exposure to sunlight radiations (Bradford, 1976; Griego & Spence, 1978). In fact, UV radiation destroys Bt protein crystals which have pesticide features (Cohen *et al.*, 2001). There are various formulations of Bt available in

today's markets such as wettable powder, wettable granule and suspension concentrate, etc. However, the most suitable formulation is to coat the active portion of the biopesticides with a polymer in the microencapsulated form (Satinder *et al.*, 2006). In addition, the sunlight causes changes in the DNA of the virus and inactivates it; for this reason, many trials have been studied and searched to reduce the impacts of sunlight UV through the use of microcapsule techniques or adding UV protection materials and common formulation methods (Villamizar *et al.,* 2010). The microencapsulated formulation contains spherical granules in 10-12 micro dimensions and the microbial agent is placed into coating, usually made of natural polymers such as gelatin, starch, alginate, cellulose, acacia, hydroxypropyl methylcellulose phthalate in various concentrations; being nontoxic is the most important characteristic shared by all these coatings (Danielle *et al*., 2011).

Dankle and Shasha used starch to achieve more resistant formulations of Bt bacteria against UV radiation (1988). Coating Bt by increasing calcium alginate polymer can provide the necessary cover for Bt against UV (Murat, 1944). The water essence of green tea possesses UV absorption effects. Black tea and lignin were also tested in UV exposure test for 300 min; both showing good protection (Salamouny *et al*., 2009). After exposing Bt microencapsulated formulation with lignin, corn flour or both of them for 8 h in artificial sunlight and artificial rain (50 cm/50 min), the formulation with corn flour base on *Ostrinia nubilalis* yielded 75.3% mortality after artificial rain and 78.5% mortality after artificial sunlight, that was higher in efficiency than the lignin base or a combination of both (Tamez- Guera *et al*., 2000). Khorramvatan *et al*. defined the Bt microencapsulated formulation using sodium alginate, starch and gelatin in a two-phase emulsion method (2013). Gifani *et al* microencapsulated the NPV virus using the emulsion method and natural polymer (2015). To assess additives protecting *Plutella xylosetella* granulovirus (PLXyGV) against UV and increasing its efficiency, natural materials including molasses, lignin and powder milk in comparison with tinopal or virus were individually exposed to UV, for 30 and 60 min in lab conditions. The results showed that all additives were significantly effective in protecting the virus against UV (Dezianian *et al*., 2010*).* Combination of NPV or CPV with Bt on some pest insects such as diamondback moth, cotton bollworm has synergistic or incremental impacts (Magholli *et al.,* 2013; Marzban *et al.,* 2009). The objective of this research was to study the stability of Bt and NPV microencapsulated formulation individually or in combination both in outdoor and under sunlight**.**

MATERIALS AND METHODS

Growing host plant

A thousand (1000) cabbage seeds, Takii variety were grown on plant trays containing Pit moss inside the greenhouse. The trays were covered by gunny cloths to save their moisture and ensure seeds are not washed out by watering. The plant trays were placed near heat source in the flower house. The trays were checked every day to ensure a certain level of moisture and observe seed budding. After producing 3 to 4 leaves, the cabbages were transported into pots of 20 cm diameters. To improve the quality of plants, the vermicompost of cow manure in soil (20/80) was used.

Diamondback Moth collection and growth

To proceed in this phase, the diamondback moth pupas were collected from Shahriar farms in Alborz Province and transported to the insectarium. The room was kept at 25±1°C, 65±1 relative humidity and 8:16 light/dark photoperiod. Two wooden cages of 100 x 100 cm were supplied with fence and the cabbage pots were placed inside them. The collected pupas were transported to those cages. After the emergence of adult insects, used fresh and leafy pots, water and 10% honey mix for feeding them was added. After

recording an increase in larva populations in the colony (after three generations), thin brushes were used to remove second instars larvae from the pots for bioassay.

Bt, NPV and Bt+NPV suspension

To prepare Bt suspension with similar concentration of microencapsulated formulation, 0.1 g of Bt powder was added to 300 ml Tween water 0.4%. To prepare NPV virus suspension with similar concentration of microencapsulated formulations; 0.03 g of HaNPV powder was weighed. The powder was supplied from Henan Jiyuan Baiyun Co. Ltd. a Chinese Company and was mixed in 300 ml water. In addition, Tween 80 was used, 0.4%. To prepare the Bt +NPV suspension, using a digital scale, 0.03 g of NPV powder and 0.1 g of Bt powder were measured and mixed in 600 ml water. The tween 0.04% was also used.

Preparation of Bt, NPV and Bt+NPV microencapsulated formulation

At the first phase, sodium alginate and gelatin microencapsulated containing Bt spore-crystal was carried out by the method of Khorramvatan *et al.* (2013). To prepare the water phase, 0.1 g of *Bacillus thuringiensis* spore was carefully weighted. Five percent sodium alginate or gelatin was then added to it and the mix was placed on a shaker for 24 h. To make the oleic phase, 63 ml of corn oil was poured into a 500 ml beaker and 200 micro liter span 80 was added to it. Before adding the water phase drops to the oleic phase, the stirrer was turned on inside the oleic phase for some minutes so the system would be fixed. The water phase drops were then added to the oleic phase in a homogenous form for a certain time (about half an hour).After adding the solvent to 37.5 ml ethanol, 37.5 ml calcium chloride (0.3 molar) and 1 ml acetic acid, the system needed one hour to allow the particles bind strongly. A stirrer with 2000 rpm was used. To prepare microencapsulated NPV, the above mentioned method was used with the only difference being that in the water phase preparation, 0.03 g virus powder was used; followed by the addition of 5% sodium alginate or gelatin and the mix was placed on a shaker for 24 h. In preparation of the water phase Bt+NPV microencapsulated, 0.1 g Bt powder and 0.03 g NPV virus powder were scaled carefully and then 5% sodium alginate or gelatin was added and placed on a shaker for 24 h.

Formulations stability in sunlight

At first, 30 pots were selected in three replicates and were wrapped with gunny cloth to prevent fast wilting of the cabbage shrubs. Those 30 pots were placed in a spot where they would be exposed to sunlight during the day. The tests were performed in September, a period when the daylight lasted for about 14 h. To save the plants from wilting, the pots were regularly watered. The pots were labeled and each sprayed with Bt, NPV and Bt+NPV microencapsulated with gelatin and sodium alginate formulations as well as with non-microencapsulated suspensions. A series of pots received no spraying and served as the control group. The above mentioned pots were placed under direct sunlight for 10 days after receiving spray. In the intervals of zero day (immediately after spraying), three, seven and ten days after spraying, one leaf was taken from each subject pot at random from the petiole and was cut into three equal pieces. Each piece was placed inside a paper cup and 10 s instars larva diamondback moth were transported inside the cup using a sterile brush. All cups were labeled by the specification of the formulations. The cups were placed in an insectarium in 25±1˚C, 65±1% humidity and 8:16 light conditions (light:dark). After 48 h, the lid of each cup was removed and fresh leaf without spray was put in access of the larva.

The mortalities were registered. In this arrangement, the treatment tests (Total 10 treatments with control) in three replications and four time intervals (0, 3, 7 and 10 days after spraying) were performed in the template of a split plot plan in time.

DATA ANALYSIS

The mortalities percent were modified using the Abbott method (Abbott, 1925) and then were analyzed using the mstat-c statistical software. The means were compared by Duncan tests in 95% certainty coefficient. The LT50 was calculated for each one of the formulations using the SAS statistical software.

RESULTS AND DISCUSSION

The data variance analysis showed significant differences between the formulations which were The microencapsulated formulations showed considerable mortality in the third day as well. In the pots treated with microencapsulated formulations, after 7 days to 10 days of exposure to sunlight, more than 50% mortality was still observed in the larvae while the non-microencapsulated formulations had lost all their effects after 10 days (Table 1).

Based on the results of Table 2, comparing the lethal time 50% of formulations (LT_{50}) showed there are significant differences between the time for 50% killing of the 9 formulations subject of test and they are in two groups. Microencapsulated Bt+NPV formulation with gelatin coat had LT_{50} in 2.60 days and was placed in Group A; followed by Bt formulations with gelatin coat, non-microencapsulated Bt, non-microencapsulated Bt+NPV, Bt with sodium alginate coat and gelatin; in addition, non-microencapsulated NPV had the highest LT50 and were placed in Group B.

In Bt+NPV with gelatin coat, and Bt+NPV with sodium alginate coat in day zero and day three from spraying, less than 10% of larva developed into pupa. Moreover, the insects that evolved from those pupas after one week were all deformed, could not fly and died fast. In the 7^{th} day and 10^{th} day post-spraying test, approximately half of the insects that emerged from pupas were defected and were not able to fly. In few cases, the pupas turned black after 10 days. These deformed full insects that emerged from pupas were observed in lower percent in the microencapsulated Bt formulations alone, and NPV alone too.

used in terms of their mortality F= (8,18)=134.4014; P < 00001. In addition, different times of the tests showed significant differences F= (3, 8)=314.899; P < 00001.The results showed that all formulations subjected to the test had considerable mortality immediately after spraying and no significant difference was found between them. After three days exposure to sunlight, the mortality of the non-microencapsulated formulations reduced considerably, in comparison with the microencapsulated formulations and reached less than 40%. Therefore, it could be concluded that Bt and NPV formulations to some extent are able to disrupt the insect's life cycle and reduce the population of pests in addition to their direct effects in destroying larva.Using Bt-based pesticides in farms has some restrictions, as its stability reduces significantly when exposed to UV. In fact, sunlight UV destroys the Bt protein crystals that have toxic characteristics (Cohen *et al.*,2001). Microencapsulation of Bt by calcium alginate polymer was compared to free spores.

Results showed that the spores in the microencapsulated particles have higher resistance against UV (Murat, 1944). Although NPV has some restrictions outside the host's body, by accumulation of effective particles of the virus inside a specific nucleocapsid protein in the microencapsulated form could become stable and show effectiveness in reducing pest population (Moscardi, 1999; Bonsal, 2004; Farrar *et al.*, 2007). A bioassay of Bt microencapsulated formulations by Sodium alginate (5% w/w) showed the highest viabilities of 86 and 90% after long term exposure to Ultra Violet (UVB 385 nm) and Ultra Violet in the short term (UVC 254 nm) radiation, respectively, while viabilities of non-microencapsulated spores under these conditions were 40 and 50%, respectively. The crystal activities (mortality) of irradiated and non irradiated none encapsulated formulations on second-instars larvae of *Ephestia kuehniella* were 15 and 93%, respectively (Khorramvatan *et al.*, 213).

In the present research, the mortality of Diamondback moth larvae showed 37 and 53% decrease; respectively, after 10 days exposure to sunlight when microencapsulated Bt formulation with gelatin and sodium alginate was used. On the other hand, in the non-encapsulated formulation, the mortalities showed 83% reduction in sunlight exposure.

The combination of Bt and NPV in the microencapsulated formulation as well as in the non-microencapsulated formulation created higher mortality than each one of them individually. Gelatin microencapsulated formulation had better protection against sunlight than sodium alginate. In Iran, due to lack of cloudy sky, sunlight UV reaches the earth significantly during September and October- that is, cabbage growth months and in spite of this, the microencapsulated formulations which were used proved to be of good efficiency in this condition.

One must note that in Iran, the highest sunshine radiation on earth occurs during June and July and the efficiency of these formulations might change.

In the meantime, the tests were carried out in urban conditions that might differ with farming conditions in height and climate which is effective in sunlight UV absorption. Using microencapsulated Bt and NPV formulations, particularly in a combination in farms could have positive economic and environmental effects in the integrated pest management of Diamondback moth.

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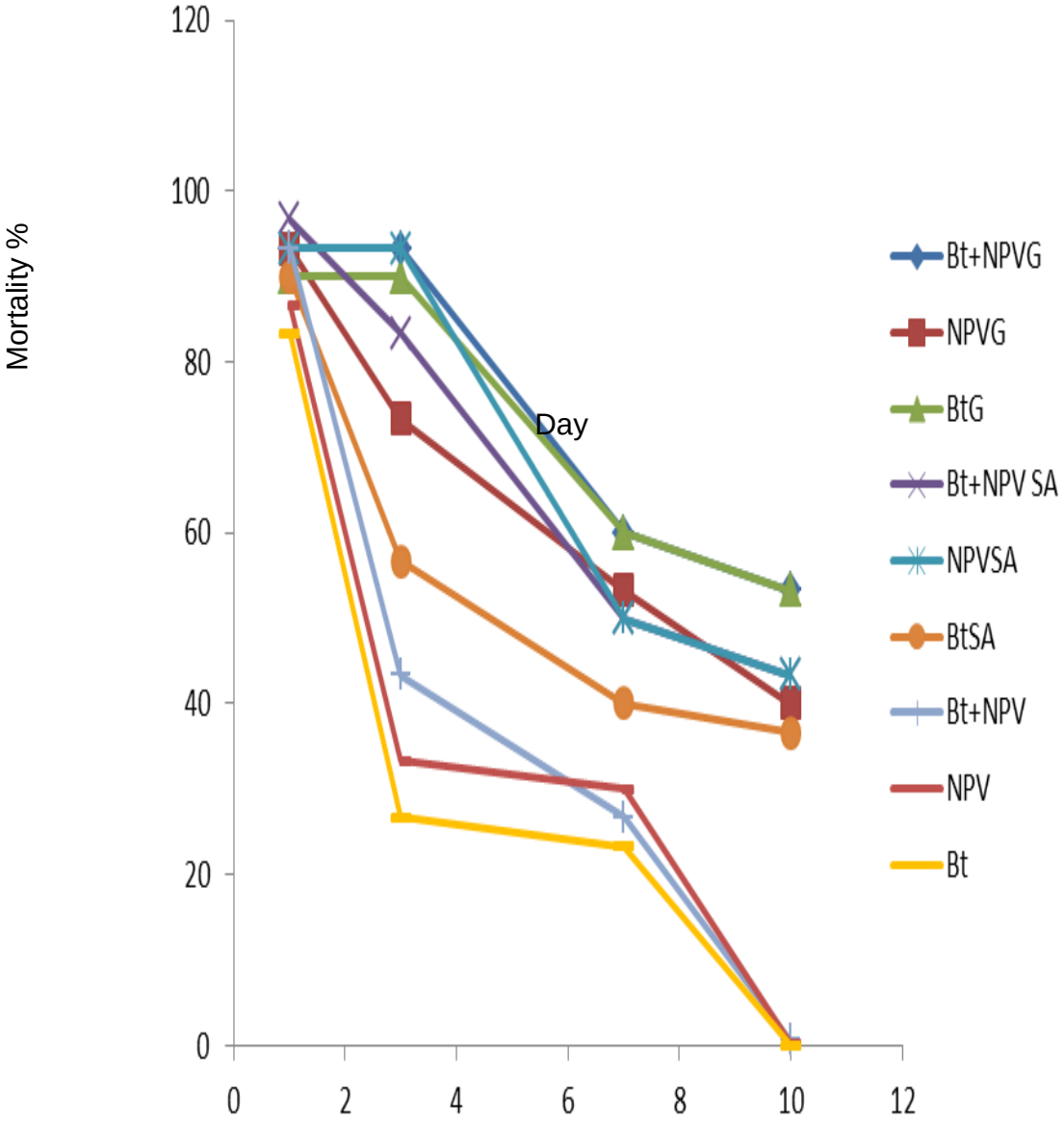

Fig 1- Stability of Bt and NPV microencapsulated formulations in sunlight exposure (per number of days)

Table 1- Mortality of *P. xylostella second* instars larvae in various formulations

Treatment (formulation)	Days expose to sun	Mean mortality (±SE)	Group
Bt+NPVSA	0	96/67 ± 3/34	A
NPV+Bt G	0	93/33 ± 3/34	AB
Bt+NP	0	93/33 ± 3/34	AB
Bt+NPVG	3	93/33 ± 6/67	AB
NPVG	0	93/33 ± 6/67	AB
NPVSA	0	93/33 ± 3/34	AB

NPVSA	3	93/33 ± 3/34	AB
BtG	3	90/00 ± 0/01	AB
BtSA	0	90/00 ± 5/78	AB
BtG	0	90/00 ± 5/78	AB
NPV	0	86/67 ± 3/34	AB
Bt	0	83/33 ± 3/34	AB
Bt+NPVSA	3	83/33 ± 3/34	AB
NPVG	3	73/33 ± 3/34	BC
Bt+NPVG	7	60/00 ± 5/78	CD
BtG	7	60/00 ± 5/7	CD
BtSA	3	56/66 ± 3/34	CDE
NPVG	7	53/33 ± 3/34	DEF
Bt+NPVG	10	53/33 ± 3/34	DEF
BtG	10	53/33 ± 3/34	DEF
NPVSA	7	50/00 ± 5/78	DEFG
Bt+NPVSA	7	50/00 ± 0/01	DEFG
Bt+NPV	3	43/33 ± 3/34	DEFGH
Bt+NPVSA	10	43/33 ± 3/34	DEFGH
NPVSA	10	43/33 ± 3/34	DEFGH
NPVG	10	40/00 ± 0/01	EFGHI
BtSA	7	40/00 ± 5/78	EFGHI
BtSA	10	36/67 ± 3/34	FGHI
NPV	3	3/33 ± 3/343	GHI
NPV	7	30/00 ± 0/01	HI
Bt	3	26/67 ± 3/34	HI
Bt+NPV	7	26/67 ± 3/34	HI
Bt	7	23/33 ± 0/34	I
Bt+NPV	10	0/67 ± 3/34	J
NPV	10	0/33 ± 3/34	J
Bt	10	0/00 ± 0/00	J

Table 2- LT50 values for formulations on *P. xylostella* second instar larvae

Treatment	Df	LT_{50}	95% limit Lower - Uper	Slope	Intercept	X^2	P
Bt+NPVG	7	2/60	2/35 – 2/82	9/67	-4/07	5/14	0/64
BtG	7	2/89	2/60 – 3/20	7/12	-3/28	8/68	0/28
Bt	10	2/99	2/54 – 3/41	4/31	-2/05	4/02	0/95
Bt+NPV	10	3/03	2/73 – 3/32	6/90	-3/33	7/86	0/64
BtSA	7	3/06	2/82 -3/28	12/24	-5/96	4/36	0/74
Bt+NPVSA	10	3/07	2/81 – 3/31	9/10	-4/44	7/89	0/64
NPVSA	7	3/07	2/84 – 3/27	13/78	-6/72	1/78	0/97
NPV	10	3/23	2/91 – 3/53	6/97	-3/54	10/27	0/42
NPVG	10	3/27	3/01 – 3/51	10/17	-5/23	13/42	0/20