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Biosafety of Nanoemulsion of Hexanal to Honey Bees and Natural Enemies

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Abstract

One of the main drawbacks of mango is its post harvest losses. Hexanal, a volatile plant component inhibits Phospholipase D (PLD), the key enzyme involved in the initiation of plasma membrane deterioration to induce the ripening of fruits. Nanoemulsion of hexanal would be more effective than the conventional form of treatment owing to its smaller droplet size. Studies were conducted to develop nanoemulsion of hexanal and its biosafety to pollinators and natural enemies in mango ecosystem. Combination of hexanal, Tween 20, ethanol at 1:10:10, was found to have good emulsion. The average droplet size was 9.9 nm with the zeta potential of -20.0 mV. This combination was used for the biosafety studies on honey bees *Apis cerana indica* F., egg parasitoid *Trichogramma chilonis* Ishii and predator *Chrysoperla carnea* Stephens. Nanoemulsion of hexanal on honey bees and exposure of bees to hexanal treated mango varieties had no adverse effect on honey bees (0% mortality). Nanoemulsion at recommended field dose (0.04%) showed 96.53% parasitization and 96.61 per cent adult emergence of the egg parasitoid and recorded 85.05 per cent emergence in the predator. When the grubs were fed with hexanal sprayed *Corcyra* eggs as well as by direct spraying of hexanal nanoemulsion on the grubs, there was 100 per cent pupation and adult emergence.

Keywords: *Apis cerana indica* F., Biosafety, *Chrysoperla carnea* Stephens, Hexanal, Nanoemulsion, Phospholipase D (PLD), *Trichogramma chilonis* Ishii

1. Introduction

The 'King of Fruits', mango is most preferred by the world population. India ranks first in production of mango in the world[1]. One of the main drawbacks of mango is its post harvest losses. The ripening process of mango fruit involves a series of biochemical reactions, resulting in increased respiration and ethylene production, change in structural polysaccharides resulting in the softening of the skin, degradation of chlorophyll, developing pigments by carotenoids biosynthesis, change in carbohydrates or starch conversion into sugars, organic acids, lipids, phenolics and volatile compounds, thus leading to ripening of fruit with softening of texture to acceptable quality[2].

Many biologically active volatile compounds like hexanal is found to reduce the post harvest losses due to over ripening. Phospholipase D (PLD) is the key enzyme involved in the initiation of plasma membrane deterioration. In response to hormones and external stimulus, PLD binds to the plasma membrane, initiating cascade of metabolic reactions leading to the generation of several neutral lipids, resulting in destabilization of the membrane. Hexanal inhibits PLD in the fruit skin and delays the post-harvest deterioration. Hexanal is found to delay ripening, increase firmness in apple and enhanced shelf life in banana. In tomato shelf life is extended nearly for 2 months[3].

Nanoemulsion of hexanal would be more effective than the conventional form of treatment owing to uniform and extremely small droplet sizes, typically less than 100 nm range. In addition, high kinetic stability, low viscosity and optical transparency make them very attractive systems for product delivery[4]. Biosafety is another important aspect of nano product in order to prevent the adverse effect of non-target organisms and human beings.

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Mango is a cross-pollinated crop, adequate pollinators are needed for pollen transfer to increase fruit set. Honey bees play a major role in pollen transfer to increase fruit set. There are several natural enemies in mango ecosystem which helps in controlling red banded thrips, mango leafhoppers, aphids, mites, immature scales, mealy bugs and small caterpillars[5]. Therefore, the present research was undertaken to study the safety of nanoemulsion of hexanal.

2. Materials and Methods

Studies were conducted to prepare nanoemulsion of hexanal and to evaluate the toxicity of nanoemulsion of hexanal against honey bees, natural enemies of insect pests.

2.1 Preparation of Nanoemulsion of Hexanal

Nanoemulsion of hexanal was prepared with different combinations of hexanal, surfactants and co surfactants and the best combination was selected based on particle size and zeta potential which was used further for biosafety tests. Surfactants – Polysorbate 20 (Tween 20) co surfactant –ethanol[6] were used for the test combinations.

2.2 Combinations of Hexanal with Surfactants and Co-surfactants

Hexanal: Tween 20: Ethanol (1:10:10), (1:5:5), (1:4:4), (1:3:3).

These combinations were sonicated at 20 kHz in the sonicater (Sonics) for 15 min for a good emulsion from aggregation[7] and their size and stability was studied using particle size and zeta potential respectively in particle size analyzer (Horiba Scientific SZ - 100).

2.2.1 Treatments Tested for Biosafety

T_1 - Nanoemulsion of hexanal @ 0.02%.
T_2 - Nanoemulsion of hexanal @ 0.04%.
T_3 - Nanoemulsion of hexanal @ 0.06%.
T_4 - Pure Hexanal @ 0.02%.
T_5 - Pure Hexanal @ 0.04%
T_6 - Pure Hexanal @ 0.06%.
T_7 - Ethanol @ 0.2%.
T_8 - Tween 20 @ 0.2%.
T_9 – Control.

2.3 Studies on the Safety of Nanoemulsion of Hexanal to Honeybees

2.3.1 Toxicity of Nanoemulsion of Hexanal to Honeybees Dry Film Toxicity

A laboratory experiment was conducted to assess the toxicity of nanoemulsion of hexanal with mentioned treatments, replicated three times against honey bees as per the guidelines given by Environmental Protection Agency (EPA)[8]. Honey bees, Apis cerana indica F., were obtained from Apiary, Tamil Nadu Agricultural University (TNAU), Coimbatore. Healthy bees were collected from the same hive. Immediately after collection, bees were anesthetized by keeping in refrigerator (10°C) for no longer than 3 min. Experiment was conducted with 1 to 7 days old worker bees and bees used in the test were assigned randomly to treatments and controls. Whatman 40 filter paper was impregnated in the respective concentration of hexanal as referred in 2.2.1 and placed in the plastic container. To this plastic container 20 honey bees were released and closed tightly and kept undisturbed for one hour. After contact period of one hour the honey bees were transferred to fresh container and observation was taken at 1, 3, 6, 12, 24 and 48 hours after treatment and per cent mortality was recorded. A 50 per cent sugar/water solution was provided ad libitum throughout the holding and test periods. Experiment was conducted in control lighting and other environmental variables. Temperature was maintained at 25 ± 2°C, with relative humidity between 70 and 80 per cent. Test bees were maintained in the dark except during dosing and observations.

2.3.2 Effect of Hexanal Treated Mango Fruit Varieties against Honey Bees

The contact effect of hexanal through the hexanal treated mango fruit varieties on honey bees were assessed using the fruits treated with hexanal at different intervals i.e., sprayed 30 days before harvest, 15 days before harvest, 30 and 15 days before harvest.

The freshly harvested mango fruits of varieties *viz*., Neelum, Bangalora and Banganapalli were collected from Cumbum and stored at 15°C. Two fruits from each treatment were taken in the polythene cover and 10 honey bees were released into the polythene covers and tightly

closed and kept undisturbed and sufficient pin holes were made for aeration. Observation was taken at 1, 3, 6, 12, 24 and 48 after treatment hours and mortality (%) was recorded.

2.4 Toxicity of Nanoemulsion of Hexanal to Parasitoid *Trichogramma chilonis* Ishii

2.4.1 Adult Emergence

Trichogramma egg cards were impaired with different treatments as referred in 2.2.1 and were cut into bits of 1 x 1 cm^2. Treated egg cards were dried and put into separate test tubes (18 x 150 mm) and closed tightly with cotton. The number of wasps that emerged from each treatment was recorded after 48 hrs and the emergence (%) was worked out.

The mortality data was categorized as follows[9]:

Mortality (%)	Category
<50	Harmless
50-79	Slightly harmful
80-89	Moderately harmful
>90	Harmful

2.4.2 Parasitisation Study

Corcyra egg cards were sprayed with hexanal solution at different treatments as referred. The treated egg cards were cut into bits of 2 x 1 cm^2 and the Trichogramma parasitized egg cards into 1 x 1 cm^2. One bit of treated egg card and one bit of parasitized egg card were put into a test tube (18 x 150 mm) and the number of parasitized eggs (eggs appearing black and plumpy) was recorded after 48 h using optical microscope.

2.5 Study of Toxicity of Hexanal against Predator *Chrysoperla carnea* Stephens

2.5.1 Grub Emergence

Chrysoperla carnea Stephens's egg cards were sprayed with different concentration of hexanal as indicated earlier and were cut into bits of 1 x 1 cm^2. Treated egg cards were dried under shade and put into separate test tubes (18 x 150 mm) and the number of adults that emerged from each treatment was recorded after 48 hrs using optical microscope.

2.5.2 Larval Feeding Method

Eggs of *C. cephalonica* were exposed to UV radiation of 15 W for 15 min to kill the embryo. The UV killed *Corcyra* eggs were taken and sprayed with different treatments as mentioned above. The treated eggs were shade dried for 15 min and then transferred to plastic tray (plates) with 24 wells @ 1 cc per well. Second instar *C. carnea* Stephens grubs were transferred to these wells @ 1 grub per well. After the grubs completely fed the hexanal treated eggs, the grubs were provided with untreated *Corcyra* eggs till pupation. Observations were made on the grub mortality (12, 24 and 48 h after treatment), pupation and adult emergence.

2.5.3 Direct Spray Method

Another study was conducted by spraying hexanal directly on the grubs using an atomizer. Eggs of *C. cephalonica* were exposed to UV radiation of 15 W for 15 min to kill the embryo. Second instar *C. carnea* grubs were taken and sprayed with concentration of hexanal as indicated. Hexanal treated grubs were left in the plastic tray (plates) containing 24 wells @ 1 grub per well and provided with 1cc of UV treated *Corcyra* eggs per well.

Observations were made on the grub mortality (12, 24 and 48 h after treatment), pupation and adult emergence.

3. Results and Discussion

Studies were conducted to develop a nanoemulsion of hexanal using various combinations of surfactants and co-surfactant and its effect on biosafety against natural enemies, honey bees. The results of the research are discussed in this chapter. An effort has been made to interpret the research findings with suitable reasoning.

3.1 Nanoemulsion of Hexanal

Nanoemulsion was prepared using different combinations of hexanal, surfactant and co-surfactant. The size of the nanoemulsion was measured using dynamic light scattering of particle size analyzer (Horiba Scientific SZ-100). The combination of hexanal, surfactant (Tween 20), co-surfactant (ethanol) (1:10:10) ratio showed good emulsion and the average droplet size of 9.9 nm (Figure 1) with maximum stability of -20.0 mV (Figure 2) measured by zeta potential, among all the combinations (Table 1). As hexanal: Tween 20:ethanol (1:10:10) combination

has smallest droplet size and high zeta potential, it was selected for the biosafety studies. Studies show that, the larger droplet size of neem nanoemulsion shifted to smaller with the increase in concentration of Tween 20. The present investigation also indicated that reducing the concentration of surfactant resulted in bigger droplets[10]. Tween 20 was found to be the best surfactant for β-carotene nanoemulsion resulting in smallest droplet size[11]. Since, there was stable nanoemulsion with good stability and smaller droplet size (9.9 nm), it would result in a better delivery as well as converge of hexanal on the mango fruits and ultimately could help in effective absorption inside the fruits to interfere with biochemical process of ripening.

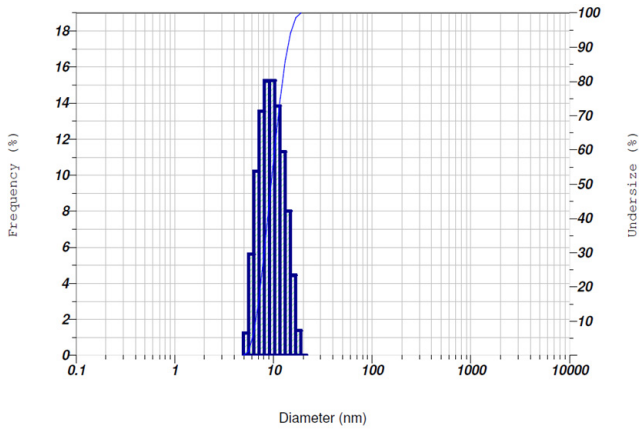

Figure 1. Particle size analysis for hexanal nanoemulsion (1:10:10).

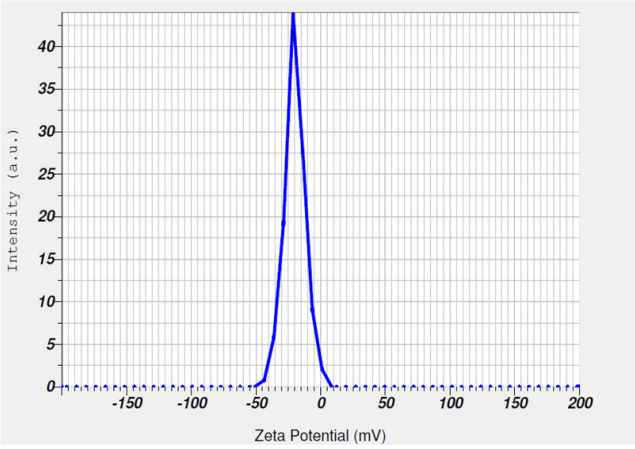

Figure 2. Zeta potential value for hexanal nanoemulsion (1:10:10).

3.2 Evaluation of Toxicity of Nanoemulsion of Hexanal to Honey bees - *Apis cerana indica* F

3.2.1 Dry Film Toxicity

The toxicity of hexanal treatments to the honey bee workers was studied in the laboratory and the results are presented in Table 2. Observation was taken at of 1, 3, 6, 12, 24 and 48 Hours After Treatment (HAT). All the treatments were found to be non toxic to honey bee worker population. There was no mortality even after 48 HAT.

Table 1. Characteristics of nanoemulsion prepared using different combinations of hexanal, surfactant and co-surfactant

Hexanal	:	Surfactant (Tween 20)	:	Co-surfactant (Ethanol)	Emulsion droplet size (nm)	Zeta potential (mV)
1	:	10	:	10	9.9	-20.0
1	:	5	:	5	8.3	-9.9
1	:	4	:	4	8.5	-10.1
1	:	3	:	3	22.1	-6.6

Table 2. Toxicity of nanoemulsion of hexanal to the honey bees *Apis cerana indica* F. – Dry film toxicity

Treatment	Mortality (%)*					
	1 HAT	3 HAT	6 HAT	12 HAT	24 HAT	48 HAT
T_1	0	0	0	0	0	0
T_2	0	0	0	0	0	0
T_3	0	0	0	0	0	0
T_4	0	0	0	0	0	0
T_5	0	0	0	0	0	0
T_6	0	0	0	0	0	0
T_7	0	0	0	0	0	0
T_8	0	0	0	0	0	0
T_9	0	0	0	0	0	0

* Mean of three replications; HAT – Hours after treatment.

3.2.2 Toxicity of Hexanal Treated Mangoes on Honeybees

The toxicity of hexanal treated mangoes to honey bee workers was studied and the results are presented in Table 3. Observations were made at period of 1, 3, 6, 12, 24 and

Table 3. Toxicity of hexanal treated mangoes varieties to the honey bees *Apis cerana indica* F

Treatment	Mortality (%)*																	
	Neelum						Bangalora						Banganapalli					
	(HAT)																	
	1	3	6	12	24	48	1	3	6	12	24	48	1	3	6	12	24	48
Hexanal spray 30 days before harvest	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hexanal spray 30 days and 15 days before harvest	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hexanal spray 15 days before harvest	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control (Unsprayed)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Mean of three replications; HAT – Hours after treatment.

48 HAT. There was no mortality of honey bees was not found in any of the treatments even after 48 HAT. The results of present study are in comparable with Flesar J.[12] who reported that acute oral toxicity of the most active natural products was determined on adult honey bees, showing them as non-toxic at concentrations as high as 100 µg/bee. Studies were made to compare direct and residual, contact and oral toxicities to honey bees of sweet corn insecticides and of Bt-sweet corn. Bt-sweet corn tassels had no impact on honey bee mortality. Pollen collected from insecticide field treated corn and fed to honey bees had no impact on mortality. As worker bees are encountering several volatiles during foraging including hexanal at lower concentrations from various plant species[13]. Hence it could be inferred that hexanal had not shown any adverse effect on honey bee workers.

3.3 Toxicity of Nanoemulsion of Hexanal to *Trichogramma chilonis* Ishii - Adult Emergence and Parasitization

The effect of hexanal treatments on the adult emergence and parasitization of *T. chilonis* are summarized in the Table 4. The results had indicated that all the treatments exerted lesser impact on the emergence of adults. The mean adult emergence of two experiments ranged from 97.15 to 93.05 per cent in different treatments. The hexanal treatments had little impact on the parasitization of *T. chilonis*. The mean parasitization of two experiments ranged from 96.94 to 92.85 %, in different treatments.

Five antimoulting compounds when exposed to growth regulators at 4 days after parasitisation of *Trichogramma chilonis,* had drastically affected the development of the immature stages and the effects were more pronounced by triflumuron and HOE 607. Exposure at 7th day after parasitisation had very little effect on the emergence of adults but decreased the fecundity of emerged adults[14]. Nasreen[9] studied the toxicity of seven insecticides and classified the products (used at their recommended rates) as harmless (<50% mortality), slightly harmful (50-79 % mortality), moderately harmful (80-89 % mortality), harmful (>90%). The present study on the safety of hexanal nanoemulsion clearly revealed that the hexanal being a plant component was not toxic to the parasitoid *T. chilois* even at 0.06 per cent which is evidenced by higher parasitisation and adult emergance.

3.4 Toxicity of Nanoemulsion of Hexanal to Predatory Green Lacewing - *Chrysoperla carnea* Stephens

The mean egg hatchability observed in untreated check was 95.30% (Table 4). Among the treatments, nanoemulsion @ 0.02 per cent treated cards showed higher adult emergence of 91.97% followed by recommended dose (0.04%) of nanoemulsion of hexanal (85.05%). Pure hexanal at 0.02% showed 84.87% emergence and nanoemulsion of hexanal at 0.06% showed 82.80% emergence. Then was 82.21% emergence in Tween 20 treated cards, followed by pure hexanal 0.04% and 0.06% treated cards recording an adult emergence of 81.45 and 80.05 % adult emergence respectively. The least egg hatchability was recorded in ethanol (0.2%) treated cards, 79.55%.

3.4.1 Toxicity of Nanoemulsion of Hexanal to the Grubs of Chrysoperla carnea Stephens

3.4.1.1 Larval Feeding Method

The toxicity of hexanal to *C. carnea* Stephen was determined by diet contamination or larval feeding bioassay method revealed that hexanal treatments were not toxic to the predator. There was no grub mortality in

Table 4. Effect of nanoemulsion of hexanal to *Trichogramma chilonis* Ishii, *Chrysoperla carnea* Stephens

Treatment	*Trichogramma chilonis* Ishii.		*Chrysoperla carnea* Stephens		
	Adult emergence	Parasitization	Grub emergence	Larval feeding Method	Direct spray Method
	Mean (%)*		Adult Emergence (%)*		
T_1	97.15	96.94	91.97	100	100
T_2	96.61	96.53	85.05	100	100
T_3	95.78	95.59	82.80	100	100
T_4	95.56	95.15	84.87	100	100
T_5	94.51	94.24	81.45	100	100
T_6	94.31	93.68	80.07	100	100
T_7	93.05	92.85	79.55	100	100
T_8	94.04	95.86	82.21	100	100
T_9	99.13	99.03	95.30	100	100

* Mean of three replications

any of the treatments *viz* 0.02 – 0.06 per cent and showed 100 per cent pupation and adult emergence (Table 4).

3.4.1.2 Direct Spray Method

The safety of to hexanal treatments to the *Chrysoperla* grubs was studied using direct spray method. The result revealed that hexanal treatments were not toxic to the predator. There was no grub mortality in any of the treatments and showed 100 per cent pupation and adult emergence (Table 4).

Rezaei[15] studied the effects of imidacloprid, propargite and pymetrozine on the common green lacewing, *C. carnea*. Imidacloprid had no significant effect on fecundity, but propargite and pymetrozin caused significant reductions ($p < 0.05$). Imidacloprid was found to be harmless (E = 27.44%); propargite (E = 49.78%) and pymetrozine (E = 66.9%) were slightly harmful. Commercial formulation of azadirachtin (Align) on *C. carnea* adults to determine its effects on reproduction. It proved to be harmless to newly emerged adults. Tests showed that males were not involved in the reduction of oviposition[16].

Larval feeding studies carried out by Krishnamoorthy[17] reported that the newly hatched larvae of *C. carnea* were more susceptible to organophosphates, carbamates and pyrethroids when compared to the egg stage. Unlike the chemical insecticides, hexanal a naturally occurring alkyl aldehyde would not have affected the natural enemy owing to their co-evolution along with plant species. Hence adverse effects were not recorded.

4. Conclusion

Biosafety studies of hexanal carried out on honey bees, natural enemies revealed that hexanal being a naturally occurring plant component is non toxic. Hence, it can be inferred that spraying of hexanal nano emulsion at 400 ppm can be safer.

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