Toxicity of phosphine on the developmental stages of rust-red flour beetle, *Tribolium castaneum* Herbst over a range of concentrations and exposures

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Abstract The susceptibility of the developmental stages of rust-red flour beetle, *Tribolium castaneum* to a range of concentrations of phosphine over varying durations from 24 to 168 h was reconnoitered in the laboratory at 25 ± 2 °C. Responses of the life stages exposed to phosphine were compared with those of un-treated controls over 24, 48, 72, 96, 120, 144 and 168 h exposures and mortality was assessed after 14 days. Among the life stages tested, pupae were more tolerant to phosphine followed by the egg and the larval instars. At 24 h, the maximum LC_{50} value was observed in case of egg; 1.571 mgL^{-1} ; followed by the pupae, 6th instar, 4th instar and 2nd instar larvae with LC_{50} values of 1.184, 0.336, 0.212 and 0.081 mgL^{-1} respectively. However, continued exposure of the developmental stages to phosphine, recorded maximum LC values in the pupae followed by egg and the larval instars. A linear increase in the mortality response was witnessed in all the insect stages when the exposure periods were extended from 24 to 168 h with increasing concentrations of phosphine, conversely significant increase in mortality was greatly apparent during the initial treatment periods.

Keywords Phosphine · *Tribolium castaneum* · Developmental stages · Exposure period · Toxicity

Introduction

Stored food commodities are often prone to attack by a variety of insect pests resulting in qualitative and quantitative losses.

The damage to stored food products caused by insects accounts for about 5–10 % in the temperate regions of the world and 20–30 % in the tropical countries (Rajashekar et al. 2010; Nakakita 1998). Management of these stored product insect pests mainly involves the use of synthetic pesticides in the form of fumigants and as residual contact insecticides (Devi and Devi 2013; Jaya et al. 2012; Pandey et al. 2012). Fumigation is one such technique administered at the storage level to eradicate the pest population effectively (Homayouni et al. 2014); well known for its rapid toxicity and no/less residual effect on end use products. However, botanical formulations are well known for their insecticidal activity, repellence to pest, contact toxicity and fumigation effects (Kedia et al. 2013), their applicability to large scale fumigation is still uncertain. Phosphine is the major and widely used fumigant for the control of insect pests in various stored food commodities world over (Rajashekar et al. 2012) and has replaced many liquid fumigants, ethylene dichloride-carbon tetrachloride mixture and mixtures containing ethylene dibromide. In India, from the total production of 2500 MT of aluminium phosphide formulations, about 80 % is used for the protection of stored products; (Rajendran and Narasimhan 1994) indicating the dependency of stored product insect pest management on phosphine. Insect survival is an emerging problem particularly in large-scale phosphine fumigation of commodities attributed mainly by reduction in the treatment period and/or due to its poor toxicity on the tolerant egg and pupae of insect pests. The higher tolerance of both the eggs and pupae to phosphine at least partially has been attributed to the lower uptake of phosphine compared to larvae and adults. In general, a compromise in the exposure period is not entitled thereby increase in phosphine dose as the action on insects is known to be slow. In addition to this, it was reported that for phosphine toxicity, time period is considered imperative both as a dosage factor and as a response factor (Winks

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1986). A minimum of 5–7 days of exposure has been suggested for the effective PH_3 fumigation carried at 25 °C. For complete eradication of insects at lower temperatures, still longer exposures are required than the prescribed one (AFHB/ACIAR 1989). The evolution of phosphine resistance among the insect pest population asserts the need of increasing the exposure time further to control resistant populations. For instance it was 7 days (Taylor and Harris 1994; Bengston et al. 1997; Rajendran and Muralidharan 2001), later 8 days (Rajendran and Gunasekaran 2002; Collins et al. 2005), 6–9 days (Price and Mills 1988; Liang et al. 1999; Collins et al. 2002) and more than 7 days (Sayaboc et al. 1998) for phosphine-resistant *Rhyzopertha dominica*. The differences between the age-groups of the tolerant stages are extremely higher for phosphine fumigant (Howe 1973). The degree of tolerance to phosphine varies between the insect stages, especially known to alter during the developmental period of the pre-adult stages. In addition, it has been reported that the change in tolerance to phosphine in both the egg and pupal stage during their development is hasty and large (Lindgren and Vincent 1966; Nakakita and Winks 1981). Phosphine is more effective on the more susceptible stages of first instar larva and the adults, while high levels of kill can be expected in the tolerant stages, provided an adequate concentration is maintained for a period long enough for eggs to reach late egg stage or hatch, or for pupae to reach late pupal stage or moult to adults (Winks 1986).

Hence there is a strict need for standardizing appropriate dosage with respect to the insect stage and the time factor for achieving the wholesome insecticidal potency of phosphine. In this context, the present study was undertaken to examine the effect of extended exposure periods on the mortality of the developmental stages of *T.castaneum*, a common stored product insect. Toxicity tests were conducted on eggs (0–1 day old), early 2nd instar (2–3 days old), mid 4th instar (8–9 days old) and late 6th instar (14–15 days old) larvae and pupae (1–3 days old).

Materials and methods

Insect culture

Culture of *Tribolium castaneum* (laboratory strain) was initiated from adults isolated from stock culture and reared on whole-wheat flour supplemented with 5 % powdered yeast (FAO 1975). The insect cultures were maintained in the laboratory at 30±2 °C and 70±5 % R.H. in a temperature humidity controlled chamber.

Collection of eggs

In order to obtain eggs of *T. castaneum*, 250 g of wheat flour sieved 5–6 times using 85-mesh standard sieves (180 µm pore

size) was taken in a separate glass bottle (500 ml) and about 1000 *T. castaneum* adults of mixed age and sex were released onto it. After 24 h, the adults were separated by sieving the wheat flour with a 25-mesh sieve (600 µm pore size). The eggs were then separated from the flour by sifting through 85-mesh sieve.

Collection of larvae

To obtain larval instars, after 3 days the adults were removed using 85 mesh size sieve, retaining the culture contents on to the same bottle and were incubated at 30±2 °C and 70±5 % R.H. held inside a temperature humidity controlled cabinet. On the 5th d, the culture contents were spread across Whatmanns filter paper circles of 15 cm diameter to collect 2nd instar larvae adhered to the filter circles. After collecting adequate number of 2nd instar larvae for the toxicity studies, the culture contents were held back in the same culture bottle and were incubated at 30±2 °C and 70±5 % RH. For the collection of 4th and 6th instar larvae, the culture contents were sifted using a 600 µm pore size sieve on 6th and 12th day respectively.

Collection of pupae

To obtain pupae for the toxicity tests, after 3 days the adults were removed using the 85 mesh size sieve, retaining the culture contents on to the same bottle and incubated at 30±2 °C and 70±5 % R.H. inside a temperature/humidity controlled cabinet. After 20 day, the contents were sifted using a 600 µm pore size sieve and pupae which remained on the surface of the sieve were collected.

Generation of phosphine gas

Phosphine gas was generated in a gas burette using commercial formulation of aluminium phosphide tablet suspended in 5 % sulphuric acid solution (FAO 1975). For dosing, the required volume of gas was drawn from the gas burette using a gas-tight syringe, and injected into desiccators through the self sealing septum in the lid of desiccators which were used as test chambers for exposing the life stages of insects.

Toxicity studies on eggs

Polystyrene microtiter plates of 12 cm×8 cm size were cut into two halves, so that each plate had 48 microwells in it. Eggs (1–2 days old), one each, were counted into individual micro-wells of the plates using a binocular microscope (Olympus SZ 61). The microtiter plates were then kept inside individual desiccators 2.8 L (test chambers). The eggs were treated to different concentrations of phosphine ranging from 0.25 mgL⁻¹ to 6.0 mgL⁻¹ for 4 different exposure periods viz.,

24, 48, 72 and 96 h with 4 replicates each along with equal number of controls. After the exposure period, the plate set up was held in a temperature/humidity controlled cabinet at 30 ± 2 °C and 70 ± 5 % R.H. Daily counts were made on the numbers hatched till there was no further hatching. Wherever, there was hatching, about 300 mg of whole wheat flour was added to the micro-well as food source for the emerging

larvae. Mortality rates on reaching 14 days (end point mortality) were determined.

Toxicity studies on larval instars and pupae

Early 2nd instar (0–1 day old), mid 4th instar (8–9 days old) and late 6th instar (14–15 days old) and pupae (2–3 days old)

Table 1 Toxicity of phosphine against immature stages of *T. castaneum* over different exposure periods at 25 ± 2 °C and 70 ± 5 % R.H

Insect Stage	LC ₅₀ (mg L ⁻¹)	Fiducial limits	LC ₉₉ (mg L ⁻¹)	Fiducial limits	Slope (b)±SE	χ ² (d.f)
24 h						
Egg	1.571	1.255; 1.865	33.366	21.563; 63.968	1.753±0.177	82.15 (36)
2 nd instar larvae	0.081	0.076; 0.086	0.263	0.226; 0.322	4.556±0.324	17.93 (23)
4 th instar larvae	0.212	0.181; 0.243	2.025	1.575; 2.797	2.372±0.169	23.49 (18)
6 th instar larvae	0.336	0.296; 0.380	6.799	5.063; 9.732	1.781±0.096	39.25 (38)
Pupae	1.184	0.948; 1.497	66.963	35.576; 159.256	1.323±0.111	27.51 (19)
48 h						
Egg	0.306	0.267; 0.344	2.140	1.757; 2.757	2.755±0.197	9.63 (11)
2 nd instar larvae	0.064	0.054; 0.073	0.238	0.190; 0.332	4.090±0.441	31.37 (15)
4 th instar larvae	0.124	0.111; 0.137	0.880	0.716; 1.131	2.730±0.156	21.75 (20)
6 th instar larvae	0.118	0.097; 0.140	2.332	1.555; 4.015	1.793±0.138	57.23 (34)
Pupae	0.509	0.239; 0.794	6.866	3.933; 20.023	2.059±0.337	28.22 (9)
72 h						
Egg	0.235	0.209; 0.258	0.763	0.644; 0.983	4.554±0.496	10.80 (6)
2 nd instar larvae	0.031	0.023; 0.037	0.092	0.064; 0.239	4.859±1.078	64.09 (12)
4 th instar larvae	0.073	0.066; 0.081	0.361	0.293; 0.473	3.371±0.253	15.96 (14)
6 th instar larvae	0.074	0.061; 0.086	0.693	0.498; 1.099	2.388±0.209	39.54 (22)
Pupae	0.203	0.166; 0.242	2.824	2.010; 4.457	2.033±0.168	9.59 (13)
96 h						
Egg	0.217	0.123; 0.281	1.051	0.706; 3.016	3.395±0.695	27.01 (7)
2 nd instar larvae	0.021	0.016; 0.026	0.151	0.095; 0.367	2.740±0.428	35.15 (16)
4 th instar larvae	0.051	0.043; 0.059	0.237	0.177; 0.380	3.506±0.422	26.77 (15)
6 th instar larvae	0.069	0.060; 0.078	0.612	0.459; 0.906	2.449±0.207	26.81 (20)
Pupae	0.108	0.089; 0.126	0.914	0.677; 1.402	2.507±0.242	19.28 (12)
120 h						
2 nd instar larvae	0.021	0.012; 0.029	0.174	0.091; 1.08	2.517±0.600	119.46 (20)
4 th instar larvae	0.037	0.263; 0.044	0.092	0.070; 0.198	5.848±1.327	39.26 (9)
6 th instar larvae	0.044	0.031; 0.056	0.425	0.263; 0.985	2.350±0.336	66.78 (20)
Pupae	0.070	0.047; 0.094	0.909	0.525; 2.476	2.094±0.311	46.05 (15)
144 h						
2 nd instar larvae	0.018	0.007; 0.024	0.063	0.041; 0.403	4.226±1.252	56.50 (9)
4 th instar larvae	0.034	0.027; 0.041	0.160	0.109; 0.336	3.446±0.529	21.98 (10)
6 th instar larvae	0.030	0.021; 0.038	0.219	0.147; 0.437	2.677±0.366	20.67 (11)
Pupae	0.049	0.027; 0.076	0.577	0.304; 2.100	2.189±0.378	46.04 (10)
168 h						
2 nd instar larvae	0.015	0.009; 0.019	0.064	0.043; 0.169	3.599±0.741	58.51 (12)
4 th instar larvae	0.024	0.021; 0.027	0.138	0.110; 0.190	3.062±0.278	15.34 (17)
6 th instar larvae	0.028	0.018; 0.042	0.991	0.415; 5.012	1.504±0.230	63.62 (19)
Pupae	0.040	0.032; 0.048	0.549	0.386; 0.885	2.043±0.177	18.30 (11)

were taken in 7×12 cm size glass tubes separately. There were 30 insects in a tube (replicate). In the tubes containing larvae and pupae about 2 g of culture medium was added to avoid starvation. The open-end of the tubes were covered with pieces of muslin cloth held by rubber rings. The tubes containing the insects were placed individually in desiccators (test chamber) of 2.8 L capacity for PH_3 treatment. Four replicates were maintained for each life stage for each exposure period (total 7 exposure periods) with equal number of untreated controls. Each life stage was exposed to phosphine for 24, 48, 72, 96, 120, 144 and 168 h respectively. At the end of the exposure period, the life stages were removed from the test chambers and were transferred to tubes containing 5 g of culture medium and incubated at $30\pm2^\circ\text{C}$ and $70\pm5\%$ R.H. Mortality assessments were carried out for larval stages after 14 days of recovery period. The pupae were under regular observation, till adults started emerging. Adult emergence was then recorded on alternate days till no more adults emerged.

Statistical analysis

The corrected mortality data for the life stages were subjected to probit analysis (Polo Plus, Ver. 2.0 LeOra Software 2002) to determine LC_{50} and LC_{99} values together with fiducial limits and Chi-square tests were performed.

Results and discussion

The susceptibility of eggs, the larval instars and pupae increased with the length of exposure to phosphine as evident from the LC_{50} and LC_{99} values given in Table 1. The LC_{50} and LC_{99} values derived from the mortality assessment data after 14 days of recovery period showed that the extended exposures led to a linear decrease in the (LC_{50} and LC_{99}) concentrations obtained irrespective of the developmental stages. However, the difference in the LC_{50} and LC_{99} concentrations obtained after the treatment period of 144–168 h did not show any remarkable change. The data also revealed that the effect of phosphine on the immature stages vary in their response to stepwise increase in concentration as well as exposure periods. At 24 h exposure, the maximum LC_{50} value was observed in case of egg; 1.571; followed by the pupae, 6th instar, 4th instar and 2nd instar larvae with LC_{50} values of 1.184, 0.336, 0.212 and 0.081 mgL^{-1} respectively. However, at 48, 72 and other subsequent treatment periods (96, 120, 144 and 168 h), the pupae proved to be tolerant among the developmental stages recording higher LC values. When the exposure periods were lengthened, considerable decrease in the LC_{50} and LC_{99} values were noticed in the dormant stages, the egg and the pupae. The possible reason for extending the exposure time is that the phosphine tolerant immature stages,

Fig. 1 Dose mortality response of eggs (1–2 days old) exposed to phosphine over different exposure periods

the egg and pupa will develop to susceptible stages, i.e., larva and adult respectively during the course of fumigation and succumb (Winks and Ryan 1990). On the other hand, the susceptible larval instars showed a steady decrease in the LC_{50} and LC_{99} values. Similarly, it was reported that the LC_{50} values obtained after 24 h exposure to phosphine to be as 0.120 mgL^{-1} for the eggs, 0.003, 0.039, 0.090, 0.008 and 0.006 mgL^{-1} for 3, 4, 12, 19 and 25 days old larvae and 0.018 and 0.008 mg/L for the pupae and adults of *T. castaneum* respectively (Muthu 1974).

The dose mortality response of eggs to phosphine over different exposure periods is given in Fig. 1. Significant increase in mortality of eggs was observed during initial treatment period of 24–72 h, the effectiveness increasing with doses that cause higher mortality. However, inhibition in egg hatch was observed in the egg which survived phosphine treatment at 24–72 h. At a treatment period of 96 h, 100 % mortality of eggs was observed at 2.5 mgL^{-1} . Exposure of 1, 2 and 3 days old eggs of *T. castaneum* over 24 h, inhibited hatching at 58 and 115 ppm concentrations (Rajendran and Muthu 1991). To support this view, it has been reported that the susceptibility of insects to fumigants is known to vary considerably during their growth and to differences in the

Fig. 2 Dose mortality response of 2nd instar larvae (2–3 days old) exposed to phosphine over different exposure periods

Fig. 3 Dose mortality response of 4th instar larvae (8–9 days old) exposed to phosphine over different exposure periods

metabolic process of different stages (Bell and Glanville 1973).

The significant increase in larval mortality, irrespective of the larval instars was apparent during the initial treatment period of 24–120 h, the extent of increase varying with dose and the exposure period as shown in Figs. 2, 3 and 4. Among the larval instars, the 2nd instar larvae (2–3 days old) proved susceptible to phosphine, which recorded 0.187 mgL⁻¹ as the LC₉₉ value at 24 h exposure, which dropped down to 0.042 mgL⁻¹ when the exposure period was further extended to 168 h. In the case of mid larvae (8–9 days old), the obtained LC₉₉ value was about 1.046 mgL⁻¹ over 24 h exposure. Continued exposure of mid-larvae to 168 h brought down the LC₉₉ value to 0.083 mgL⁻¹.

The dose mortality response of pupae over exposure to phosphine for different exposure periods is depicted in Fig. 5. The LC₉₉ values for the pupae showed a gradual decrease in their levels from 20.33 mgL⁻¹ at 24 h, to 0.28 mgL⁻¹ at 144 h exposure. However, no significant difference in the LC₉₉ values was observed between 144 and 168 h exposure to phosphine. It was also observed that the treatment of pupae to phosphine led to the development of deformed adults which could survive only a few days. The pupae of *T. castaneum* and *T. confusum* were tolerant than larvae or adults (Bang and Telford 1966). The effect of phosphine is more in long than in shorter periods, where the

Fig. 4 Dose mortality response of 6th instar larvae (14–15 days old) exposed to phosphine over different exposure periods

Fig. 5 Dose mortality response of pupae (2–3 days old) exposed to phosphine over different exposure periods

additional time in exposure will improve the chance that the tolerant stage will reach a susceptible stage and succumb while being fumigated (Bell 1979). In support to the view of the present study, it has been suggested that the exposure time with respect to phosphine fumigant is vital than its dosage in many cases (Annis and Banks 1993).

Conclusion

To summarize, it is imperative that insects surviving repeated ineffective exposure, may give rise to resistant populations. While, the survival of phosphine tolerant immature stages may ascertain their ability to gain higher resistance compared to the susceptible stages surviving the treatments. Hence, increase in toxic effect of phosphine on *T. castaneum* may be achieved over increasing exposure periods irrespective of the insect stage. Though, considering larvae as the susceptible stage, wide variation in mortality response was observed among the larval instars. The results also suggest that, considerable difference in the mortality may be achieved during 24–120 h of initial treatment periods, especially in the phosphine susceptible, larvae and adults. Further, the extension of exposure period up to 144–168 h would increase the probability of phosphine tolerant egg and pupae to exterminate during their course of development. The implication of the above investigations indicate the need to extend the length of exposure period sufficiently in fumigations involving phosphine to preclude the chances of survival of immature stages especially the tolerant egg and the pupae during field applications.

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