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ORIGINAL ARTICLE

Exploration of teratogenic and genotoxic effects of fruit ripening retardant Alar (Daminozide) on model organism *Drosophila melanogaster*

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ABSTRACT

Alar (Daminozide) is a plant growth regulator which is widely used as a fruit preservative for apple and mango to prevent pre-harvest fruit drop, promote color development and to delay excessive ripening. The aim of the present work was to demonstrate the effect of Alar on several life history traits, adult morphology, Hsp70 protein expression and *in vivo* DNA damage in the brain of the model organism *Drosophila melanogaster*. We assessed the life history and morphological traits including fecundity, developmental time, pupation height, egg-to-adult viability and mean wing length, body length, arista length and sternopleural bristle number of the emerging flies. The results showed a significant delay in the developmental milestones, increase in body length, wing length, arista length, a decrease in fecundity, pupal height and variation in sternopleural bristle number in the treated flies in comparison to the controls. Overexpression of Hsp70 protein suggests alar induced subcellular molecular stress and comet assay validates genotoxicity in the form of DNA damage in the treated larvae. Mutation screening experiment revealed induction of X lined lethal mutation.

KEY WORDS: Alar; drosophila melanogaster; life history traits; Hsp 70; comet assay; DNA damage

Introduction

Alar (Daminozide, Figure 1) is used as plant growth regulator and considered a "stop-drop" wonder chemical as its application prevents fruits from prematurely falling. Alar is manufactured by mixing succinic anhydride with 1,1-dimethylhydrazine (UDMH), a toxic component of rocket fuel. The chemical is widely used in processing major varieties of red apples (like Mclntosh, Cortand, Jonathan and Red Delicious) and mangos (like Langra and Dashehary, etc). Though it is not a pesticide, it earns popularity among the farmers as it provides economic benefits. Moreover, the Alar treated fruits appear lucrative to consumers owing to its cosmetically enhanced color and crunchiness. Alar was first used in the USA and was continued until 1989 when the US Environmental Protection Agency suggested a ban of the chemical owing to increasing reports on cancer incidence among consumers of Alar treated apples. Alar penetrates the apples' pulp and cannot be washed or peeled off. Thus the chemical enters the living system following its consumption with fruits. Moreover, upon heating for making apple sauce or sterilizing apple juice or following digestion, Alar is degraded into UDMH, which is 1000 times more carcinogenic than Alar itself (Gordon, 2011).

As far as published literature is concerned, studies on adversities of Alar in biological systems are tantalizingly lacking. The present work has been conducted to fill such a void with experimental outcome and consequent scientific understanding. As the mutagenicity of various compounds used in agriculture have been tested through assays performed on the model organism *Drosophila melanogaster*, we have taken this opportunity considering the ease of experimentation with this insect model and to cope with ethical issues. The use of *Drosophila* in research has been recommended by the European Centre for the Validation of Alternative Methods (Festing *et al.*, 1998). Our present work represents the very first attempt to prove that the fruit preservative Alar is not safe to consume and it inducts various teratogenic and genotoxic effects at molecular level in higher eukaryotes.

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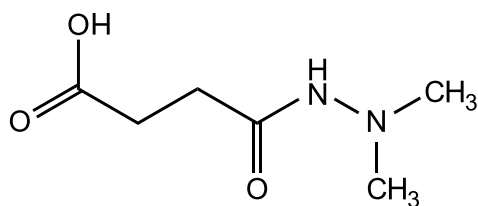
Figure 1. Chemical Structure of Alar [4-(2,2-dimethylhydrazinyl)-4-oxobutanoic acid].

Materials and Methods

Fly Strain

The wild type Oregon R strain and a transgenic strain Bg[9] of *Drosophila melanogaster* were used for this study. In the Bg[9] transgenic strain, the wild type hsp70 sequence is tagged with reporter gene construct through P-element induced insertional mutagenesis. Here the reporter gene is bacterial lacZ which expresses β-galactosidase along with induced expression of Hsp70 protein following exposure to stress. These stocks were maintained in drum vials measuring 30 mm in diameter and 105 mm in height and on standard yeast-agar-cornmeal culture medium at $24\pm1\,°C$ and 65–70% relative humidity (RH)in BOD incubator (Ashburner, 1989).

Chemicals

Analytical grade Alar or Daminozide, IUPAC Name 4-(2,2-dimethylhydrazinyl)-4-oxobutanoic acid (purity 99%) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were obtained from Sisco Research Laboratoty (Mumbai, India).Agarose, Low Melting Point Agarose, Ethidium Bromide and Collagenase were from Sigma Chemical Co. (St. Louis, MO USA). Phosphate Buffered Saline (PBS) (Ca^{2+}, Mg^{2+}free) was procured from Hi-Media Pvt. Ltd. (Mumbai, India). All other chemicals were of molecular biology reagent grade.

Preparation of Alar for treatment

Alar or 4-(2,2-dimethylhydrazinyl)-4-oxobutanoic acid was dissolved in distilled water to prepare a high concentration stock solution and mixed with standard *Drosophila* culture medium at different concentrations (200, 400, 10 000, 20 000 ppm) by making necessary dilutions from the stock solution.

Determination of acute LC_{50}

To investigate the acute toxicity of Alar in *Drosophila melanogaster* in terms of LC_{50}, twenty late 2nd instar larvae were introduced in each food vial containing 5 ml of standard *Drosophila* food mixed with different concentrations of Alar (2 000, 4 000, 8 000, 12 000 and 13 000 ppm). Similarly, control sets were set up with the food vials without Alar. Triplicate sets of each treated and

control group were maintained at 24°C and 65–70% RH till eclosion of the adult. Death of larvae and pupae were recorded by counting the pupae following final molt and by recording hatched imagoes, respectively.

Morphological and life history parameters

Flies were reared in culture medium containing Alar at a concentration of 400 ppm under standard laboratory conditions for successive five discrete generations (fresh vials for each generation) and freshly eclosed flies of generation 6 were then subjected to study. Simultaneously control sets were maintained in a culture medium prepared with distilled water instead of Alar solution. Uninterrupted rearing of the flies for five successive generations in Alar containing medium intuitively ensured homogeneous integration of Alar molecules in subcellular organization of treated flies. We collected 10 pairs of virgin males and females from the freshly eclosed 6th generation and transferred each pair to 10 respective vials containing Alar free medium. They were allowed to mate and oviposit for the next 24 hours. On the very next day the male fly from each vial was removed and the female fly was transferred to fresh vials every day (for 24 hours) for the next 20 days and the number of eggs laid daily by those 10 females was recorded for each day. We estimated total fecundity (in terms of number of eggs laid) and mean egg production by a female through the range of 20 days (Mukhopadhyay *et al.*, 2003)to take the accounts of respective life history traits for the entire period of reproductive age of the *Drosophila* females.

The pupal height (the level of puparium on the wall of glass vial) of each larva was measured as the distance from the surface of the food to the midpoint between the spiracles on the puparium (Sokolowski, 1985). Emergence of the adults from the puparium was counted to estimate egg-to-adult viability and sexing of adult was also done. All flies were observed carefully under stereo zoom microscope to find any morphological distortions such as changes in eye color, wing shape, body pigmentation, *etc.* and photographs were taken with Olympus stereo zoom microscope model SZ-11. A single wing was removed from each adult fly and the distance between the alula notch and the tip of the wing was measured using a dissecting microscope fitted with a graduated eyepiece. Besides, the mean wing length and body length of the emerging adults were measured following the method by Santos *et al.* (1994). The time needed to complete the pupation and imago emergence (the day of pupation and adult emergence) was recorded as parameter of developmental time. As the eggs are hatched at any time within 24 hours following oviposition, we synchronized our record on adult emergence time and day of pupation initiating the time count from the time of hatching of the first instar larvae. This experimental regimen was followed for all the replicates in treated and control categories.

We observed distorted morphological features among some treated flies and subsequently evaluated whether those distorted features were hereditary by crossing two identical dysmorphic flies and by performing test cross

between F1 progeny and either of the parental flies. For all the experimental design the generation count was started following inception of the experiments.

Effects of temperature on adversity of Alar

We reared the 10 pairs of male and female flies in 200 ppm Alar-mixed culture medium at two different temperatures (23 °C and 28 °C) to test whether imperilments inducted by Alar get exacerbated in interaction with increasing temperatures. Other experimental conditions remained the same as mentioned in the life history and morphological study above.

In situ molecular stress assessment by Hsp70 expression level

As the Hsp 70, the molecular chaperon of eukaryotic system, exhibits overexpression under any kind of cellular stress, we intended to study the expression pattern of this protein under the exposure of Alar. Late 2nd instar larvae of Bg[9] stock of *Drosophila melanogaster* were reared in Alar-mixed culture medium (200, 400 and 10000 ppm) till their 3rd instar larval stage was reached. Wandering 3rd instar larvae feeding on different concentrations of Alar were collected and washed thoroughly in Poel's salt solution (PSS). These larvae were dissected, fixed in 2.5% glutaraldehyde followed by washing in 50 mM sodium phosphate buffer (pH 8.0), stained in X-gal staining solution (Sarkar *et al.*, 2015) in grooved slides, kept overnight at 37 °C in a humidified chamber and observed under stereo-zoom microscope for differential Hsp70 protein expression.

Genotoxicity and neurotoxicity assessment by alkaline comet assay

In vivo genotoxicity, DNA damage in particular and neurotoxicity of Alar in *Drosophila melanogaster* was assessed using single cell gel electrophoresis or comet assay following the method of Dhawan *et al.* (2009) with minor modifications. Comet slides were prepared by using 10 late 2nd instar larvae fed with sub-lethal (400 and 10000 ppm) and lethal (20000 ppm) concentration of Alar as well as control larvae for 60 hrs in culture vial at standard laboratory condition (24±1 °C temperature, 65–70% relative humidity). After 60 hrs, wandering 3rd instar larvae from both treated and control groups were removed from the culture medium and washed with Dulbecco's Phosphate Buffered Saline (PBS pH 7.4). Cerebral ganglia from 10 larvae each from control and treated groups were explanted in PSS (pH 6.8) and treated with 300 µl of collagenase (0.5 mg/ml in PBS, pH 7.4) for 15 min at 24±1 °C. The cells were then passed through nylon mesh (60 µm), washed with PBS with gentle shaking and finally suspended in 80 µl of PBS. For positive control, cells were exposed to 25 µM H_2O_2 for 5 min at this stage.

The cell suspension was then mixed with equal volume of 1.5% low melting point agarose (LMPA) and layered on top of the end-frosted slides that were precoated with 1% normal melting point agarose (NMA). The slides were cooled at 4 °C for 5 min. After lysis for 2 h at 4 °C in lysing solution (2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris, pH 10 and 1% Triton X-100, pH 10), the slides were transferred to a horizontal electrophoresis apparatus filled with freshly chilled electrophoresis buffer (1 mM of Na_2 EDTA and 300 mM of NaOH, pH>13) and left for 10 min to facilitate unwinding of DNA. Electrophoresis was performed for 15 min at 300 mA, 25 V at 4 °C. After electrophoresis, the slides were immediately neutralized with 0.4 M of Tris buffer (pH 7.5) and stained with ethidium bromide (20 µg/ml) for 10 min in dark. After washing in chilled distilled water the slides were examined under a fluorescent microscope (Leica, Germany) at 40X magnification and the images were analyzed using CASP version 1.2.3beta2 (Comet Assay Software Project, http://casplab. com/). Each experiment was performed in triplicate with 10 larvae and the slides were prepared in duplicate. Three different parameters were used as indicators of DNA damage – tail moment (TM; arbitrary units), tail DNA (%) and tail length (µm).

Assessment of the potential of Alar for inducing X-linked lethal mutation

We employed attached X method (Figure 9) to screen any X-lined lethal mutation induced by Alar exposure. Freshly eclosed 50 male flies of attached X stock (BDSC stock no. 43329) were isolated. Males were kept in separate vial having culture media mixed with Alar of concentration 1 000 ppm for 48 hours. Individual males were then crossed with two virgin females of attached X stock and reared at 24±1 °C and 65–70% RH for 48 hours and then the flies were removed from the vials. The vials were kept till the emergence of F1 flies. The F1 flies were examined thoroughly for the presence of males. We scored lethality following the deviation of female:male from 1:1 value.

Statistical analysis

Probit analysis was performed for the determination of LC_{50} of Alar in late 2nd instar larvae of *Drosophila melanogaster*. The treated and control sets were compared using 2×2 ANOVA for the morphological parameters having sex specific difference. We performed 'two-way ANOVA' to find if there was an interaction between culture temperature and Alar exposure on the morphological and life history trait of *Drosophila melanogaster*. One-way ANOVA was performed for life history parameters, where sex is not a significant variable. We recorded mean and median fecundity and compared the values for treated and control sets using t-test. Daywise fecundity is also recorded and compared using one-way ANOVA. Variations in the number of sternopleural bristles on both left and right body sides of control and treated flies (males and females separately) were compared and analyzed by t-test. For testing male lethality we compared the female:male ratio value by Chi square method.

The values of three selected parameters for DNA damage (Tail Length, % Tail DNA and Tail Moment) through comet assay were compared using Student's t-test. Statistical analyses were performed in SPSS (for Probit analysis), VassarStats (for ANOVA) and GraphPad (for t-test) statistical software.

Results

Determination of acute LC$_{50}$

The Probit parametric estimates are presented in Table 1. Figure 2 displays the Probit line graph of acute toxicity of Alar in *Drosophila melanogaster* larvae and pupae. The estimated mortality for control set was nearly zero. The calculated lower and upper limits for the LC$_{50}$ values were 6705.600 and 45181.211 ppm, respectively for
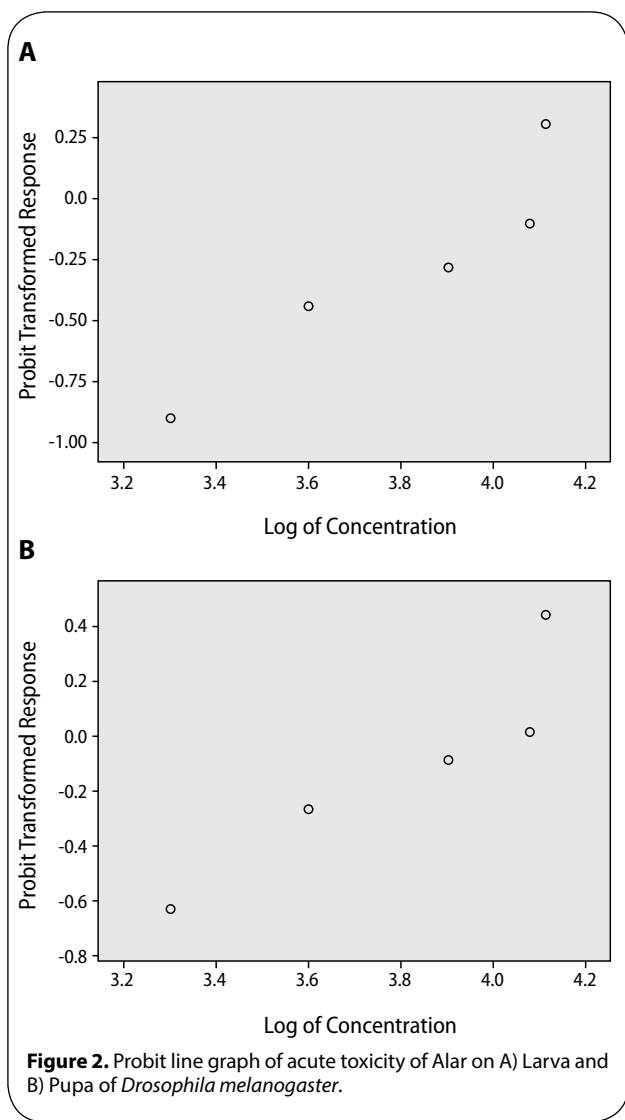
larvae and 4280.964 and 27921.125 ppm, respectively for pupae at 95% confidence interval. This suggests that *Drosophilia melanogaster* showed 50% pupal death at the lower concentration (8011.601 ppm) of Alar treatment in comparison to larvae, which exhibited 50% death at higher concentration (10842.477 ppm). There is a positive relation between toxicity and dose of Alar (Figure 2).

Morphological and life history parameters

Summary of the results is presented in Table 2. Alar treated flies exhibit a significant delay in pupation time and adult emergence time (*p*=0.003 and <0.0001 respectively in One-way ANOVA) in all experimental conditions than do the controls. We scored pupation time for control group as 4.9±0.2 days in contrast to treated group which attained pupal phase at 6.0±0.2 days reared in 400 ppm Alar containing medium. Similarly, the treated group needed 9.9±0.3 days to emerge as imago in contrast to 8.93±0.2 days for the control. We observed a significant reduction in pupation height in Alar treated flies (0.4±0.2 cm.) in contrast to the control (4.0±0.04 cm) in 400 ppm Alar medium. Similar results were obtained in other experimental conditions too (Table 2). We recorded a significant reduction (*p*=0.0009 in One-way ANOVA) in mean daily egg production in Alar treated groups with a span of over 20 days (Tables–3; Figure 3). Egg to adult survival rate was also decreased significantly in treated groups when compared to control (Tables 2–3; Figure 3).
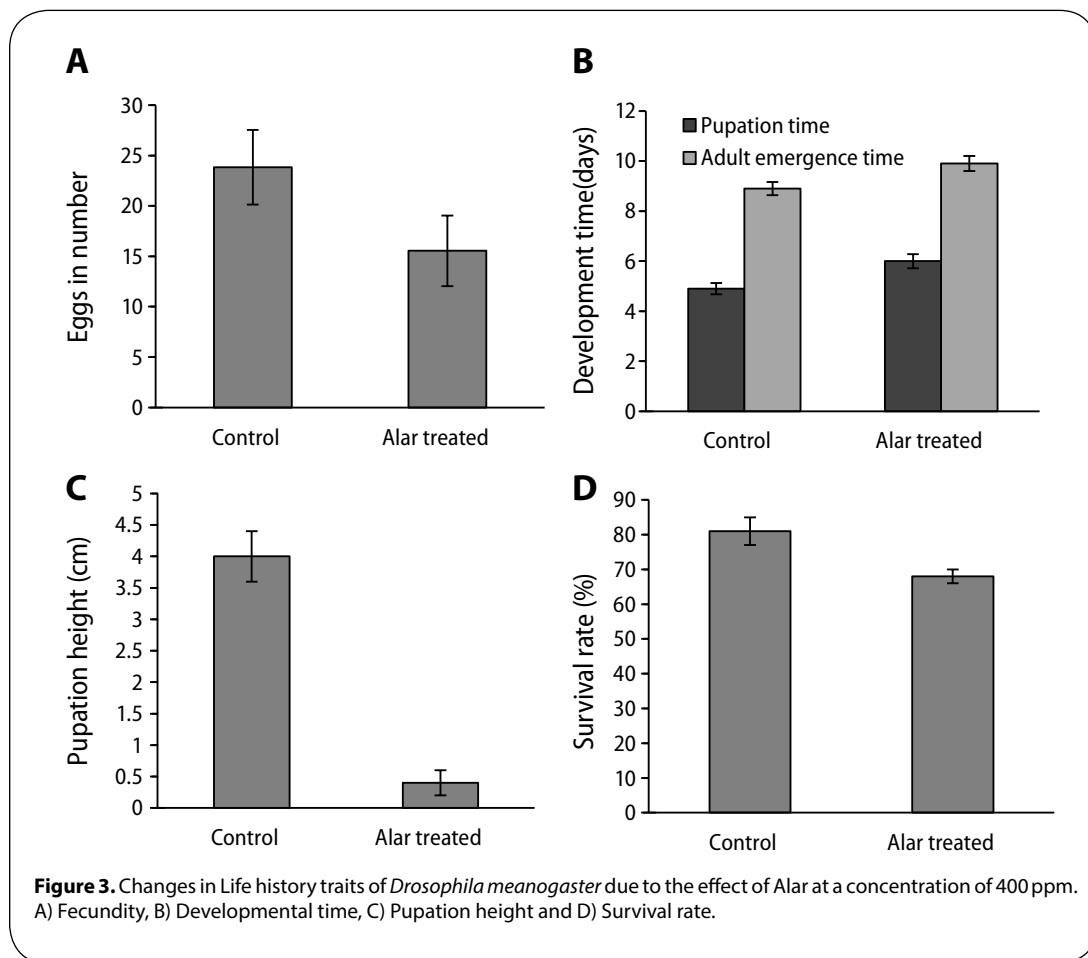
We assessed various morphometric traits including wing length, body length and arista length in sex specific manner. We scored female arista length for control as 274±0.5 μm and for treated 294±2.3 μm. For male sex, the estimates are 251±1.6 μm for control in contrast to 260±0.8 μm in treated reared in 400 ppm Alar containing medium. Similarly, body and wing also exhibited increase in length (Tables 2 and 4).

Beside morphometric variations between control and treated groups, we recorded various distortions and teratogenic effects on wing structure (Figure 4). These traits were found to be non hereditary in nature upon crossing between flies of similar trait up to F2. Abnormality in the formation of pupa was also found in F1 generation (Figure 5). Variation in the number and arrangement of sternopleural bristles (both unilateral and bilateral asymmetry) was found to be significant (*p*<0.0001 in t-test) between control and Alar treated F1 flies.

Figure 2. Probit line graph of acute toxicity of Alar on A) Larva and B) Pupa of *Drosophila melanogaster*.

Table 1. Parameter estimates for the Probit analysis.

PROBIT[a]	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
For Larval LC$_{50}$	Concentration	1.203	0.139	8.627	0.000	0.930	1.476
	Intercept	−4.854	0.536	−9.062	0.000	−5.390	−4.318
For Pupal LC$_{50}$	Concentration	1.043	0.134	7.773	0.000	.780	1.306
	Intercept	−4.070	0.513	−7.933	0.000	−4.583	−3.557

[a] PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10 000 logarithm)

Figure 3. Changes in Life history traits of *Drosophila meanogaster* due to the effect of Alar at a concentration of 400 ppm. A) Fecundity, B) Developmental time, C) Pupation height and D) Survival rate.

Table 2. Effect of Alar on morphological and life history traits of *Drosophila melanogaster* at different culture temperatures.

Morphological traits	Sex	Control at 23 °C	Treated at 23 °C 400 ppm	Treated at 23 °C 200 ppm	Control at 28 °C	Treated at 28 °C 200 ppm
Body length (mm)	Female	2.51±0.02	2.58±0.07	2.58±0.07	2.46±0.05	2.53±0.09
	Male	2.17±0.03	2.2±0.14	2.21±0.12	2.06±0.06	2.10±0.15
Wing length (mm)	Female	2.4±0.004	2.44±0.01	2.44±0.01	2.33±0.04	2.39±0.12
	Male	2±0.001	2.08±0.01	2.08±0.01	1.9±0.03	1.98±0.07
Arista length (µm)	Female	274±0.5	294±2.3	294±2.3	265±2.6	285±4.9
	Male	251±1.6	260±0.8	260±0.8	244±1.4	253±6.2
Life history traits						
Fecundity (in no.)		23.83±3.7	15.54±3.5	18.75±1.8	20.72±1.1	15.54±3.5
Pupation time (day)		4.94±0.2	6±0.2	6.9±0.04	3.89±0.18	5.93±0.02
Adult emergence time (day)		8.93±0.2	9.9±0.3	10.94±0.01	7.84±0.08	9.85±0.02
Pupation height (cm)		4±0.04	0.4±0.2	2.04±0.3	3.01±0.3	0.45±0.1
Survival rate (%)		81±3.2	68±2.7	72±3.1	78±3.1	68±2.9

Values represent mean ± SD.

Effects of temperature on adversity of Alar

Result of the experiment to study temperature dependent Alar effect (200 ppm) exhibited interesting observations. For all morphological traits studied an increase in size occurred at a temperare lower than 23 °C and higher than 28 °C (Table 2). Similarly, for life history traits much delay was recorded in pupation time and adult emergence time in temperatures lower than 23 °C compared to temperature higher than 28 °C. On the contrary, fecundity and survival rate scored lower value at higher temperature.
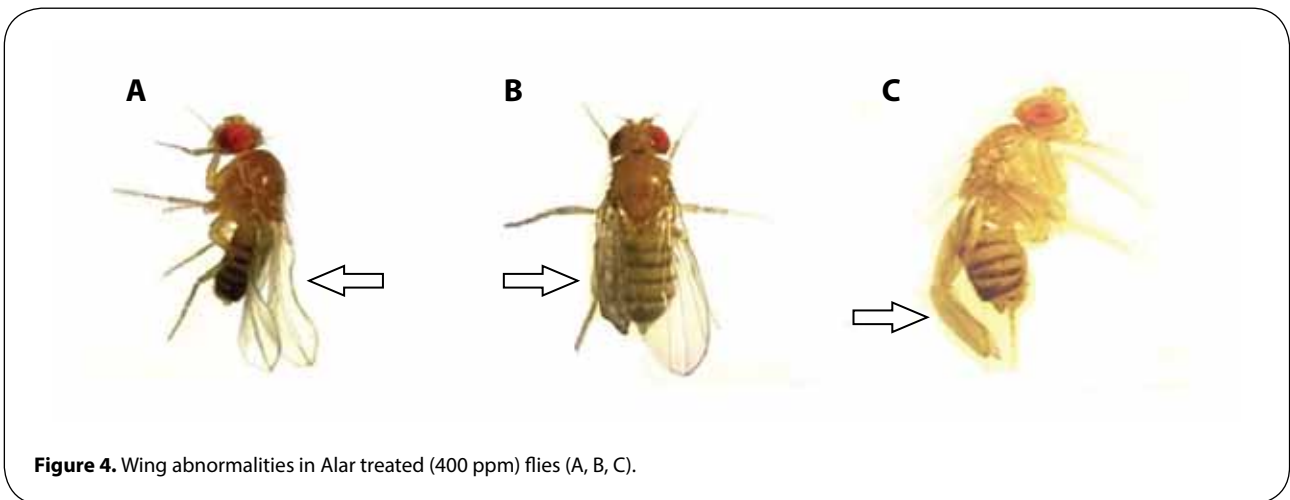
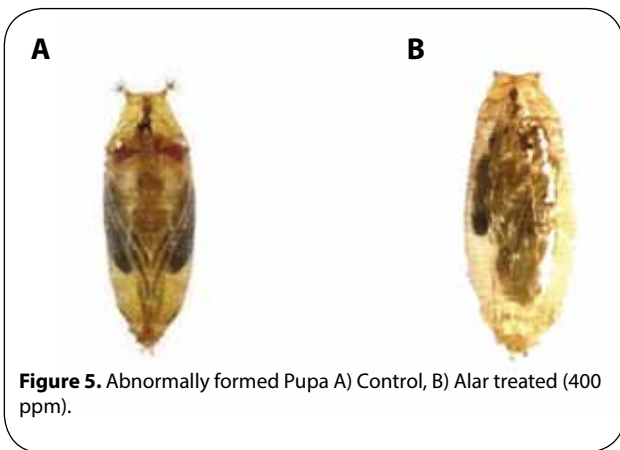**Figure 4.** Wing abnormalities in Alar treated (400 ppm) flies (A, B, C).

Figure 5. Abnormally formed Pupa A) Control, B) Alar treated (400 ppm).

Table 3. Effect of Alar (400 ppm) on selected life history traits of *Drosophila melanogaster* analysed by one-way ANOVA (Sex ignored).

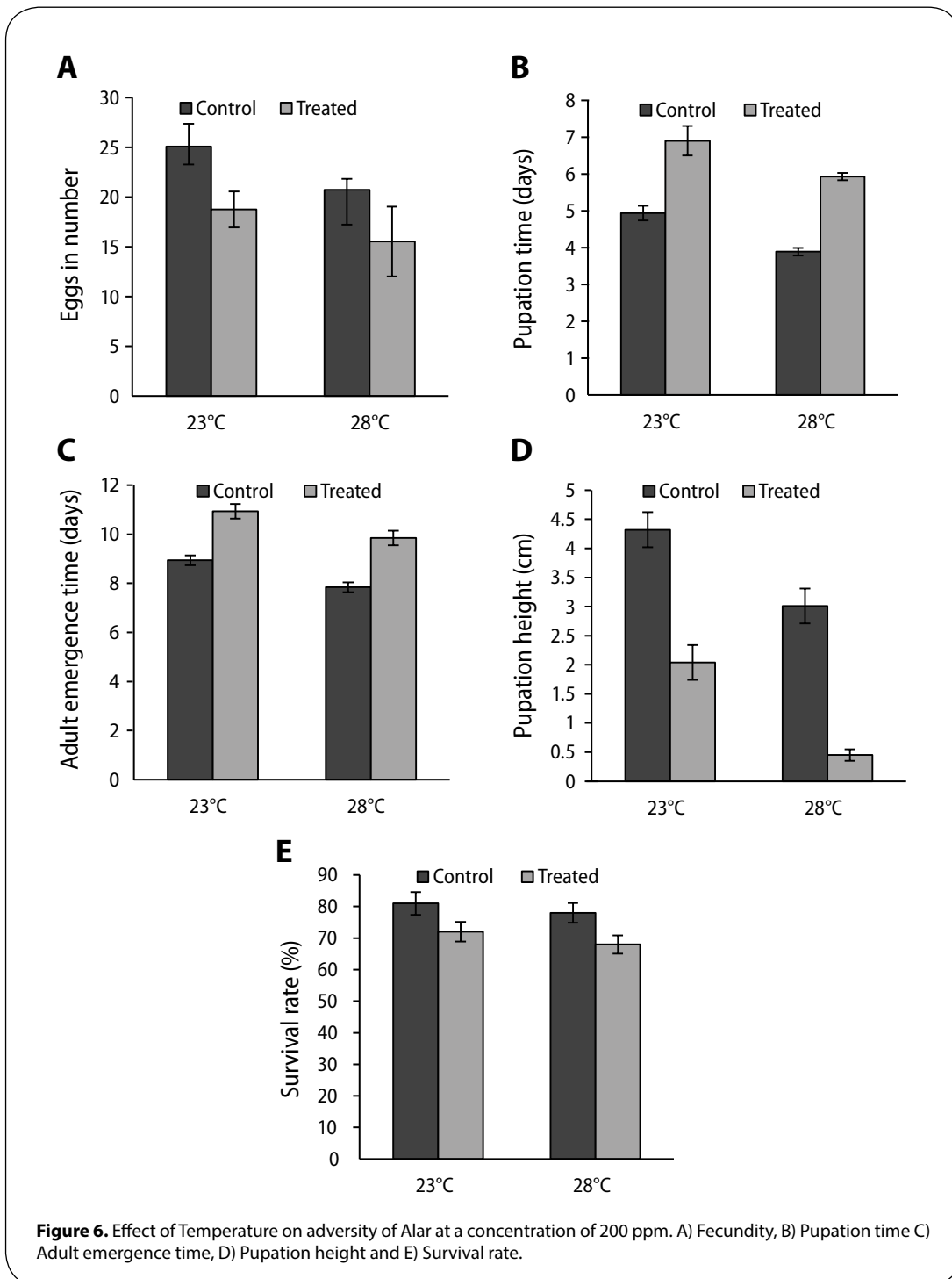| Life history traits	Sum of squares (SS)	df	Mean square (MS)	F	*p*-value
Fecundity (in no.)	687.24	1	687.24	12.77	0.0009
Pupation time (day)	22.71	1	22.71	860.82	<0.0001
Adult emergence time (day)	18.52	1	18.52	488.78	<0.0001
Pupation height (cm)	301.35	1	301.35	663.37	<0.0001
Survival rate (%)	3366	1	3366	12.02	0.0008

Table 4. Effect of Alar (400 ppm) on selected morphological traits of *Drosophila melanogaster* analysed by two-way ANOVA.

Traits	Source of variation	Sum of squares (SS)	df	Mean square (MS)	F	*p*-value
Wing length (mm)	Alar Sex Interaction Error	0.16 5.86 0.02 0.03	1 1 1 156	0.16 5.86 0.02 0	848.64 30451 84.24	<0.0001 <0.0001 <0.0001
Body length (mm)	Alar Sex Interaction Error	0.09 5.27 0.01 1.11	1 1 1 156	0.09 5.27 0.01 0.01	12.75 741.27 1.7	0.0005 <0.0001 0.1942
Arista length (μm)	Alar Sex Interaction Error	9030 33235 1199 358.5	1 1 1 156	9030 33235 1199 2.3	3929 14462 521.75	<0.0001 <0.0001 <0.0001

The effects of temperature and exposure to Alar were found to be significant for all the selected life history traits. However, no two-way interaction between temperature condition and exposure to Alar was found (Table 5; Figure 6). As there are sex specific differences in morphometric traits, we performed two-way ANOVA separately for males and females. The individual effects of temperature condition and exposure to Alar were found to be significant for both sexes. Their interaction, however was not significant (Table 5).

***In situ* molecular stress assessment by Hsp70 expression level**
Figure 7 (A–D) shows β-galactosidase activity following X-gal staining as a signature of Hsp70 expression level in third instar larvae of Bg[9] stock of *Drosophila melanogaster* exposed to different concentrations of Alar (200, 400, 10 000 ppm Alar). The intensity of blue coloration is proportional to the level of Hsp70 expression and molecular stress and apparent in different parts of the digestive tract, especially in the midgut (MG) and hindgut (HG) (Figure 7: B–D) of the Alar exposed larvae. The untreated

Figure 6. Effect of Temperature on adversity of Alar at a concentration of 200 ppm. A) Fecundity, B) Pupation time C) Adult emergence time, D) Pupation height and E) Survival rate.

larvae (control), on the other hand, expressed much less or no blue coloration in their digestive tract (Figure 7A).

Genotoxicity and neurotoxicity assessment by alkaline comet assay

Larvae exposed to H_2O_2 (positive control) and to different concentrations of Alar (400, 10 000, 20 000 ppm) showed a significant increase in DNA damage in the cells of cerebral ganglia (Table 6). Figure 8A represents images of brain cells of third instar larvae after 60 hours of treatment with different concentrations of Alar. Figure 8B is

the graphical representation of the data of three selected DNA damage parameters (Tail Length, % Tail DNA, and Tail Moment) obtained from comet assay.

Assessment of the potential of Alar for inducing X-linked mutation

We isolated 1734 F1 living flies from the 50 experimental vials and sorted them carefully under microscope for sexing. We found 1087 female flies in contrast to 647 male flies and tested by chi-square method for the deviation from 1:1 female:male ratio. A significant deviation

Table 5. Effect of Temperature on adversity of Alar (200 ppm) on selected morphological and life history traits of *Drosophila melanogaster* analysed by two-way ANOVA.

Traits	Sex	Source of variation	Sum of squares (SS)	df	Mean square (MS)	F	*p*-value
Body length (mm)	Female	Temperature	0.1	1	0.1	22.34	<0.0001
		Alar	0.18	1	0.18	37.94	<0.0001
		Interaction	0.00	1	0.00	0.02	0.8877
		Error	0.73	156	0.00		
	Male	Temperature	0.46	1	0.46	39.07	<0.0001
		Alar	0.07	1	0.07	6.34	0.0128
		Interaction	0.00	1	0.00	0.01	0.9205
		Error	1.82	156	0.01		
Wing length (mm)	Female	Temperature	0.16	1	0.16	33.85	<0.0001
		Alar	0.1	1	0.1	21.67	<0.0001
		Interaction	0.00	1	0.00	0.34	0.5607
		Error	0.72	156	0.00		
	Male	Temperature	0.39	1	0.39	232.26	<0.0001
		Alar	0.24	1	0.24	146.48	<0.0001
		Interaction	0.00	1	0.00	0.83	0.3637
		Error	0.26	156	0.00		
Arista length (µm)	Female	Temperature	3506.26	1	3506.26	374.22	<0.0001
		Alar	16060.06	1	16060.06	1714.09	<0.0001
		Interaction	8.56	1	8.56	0.91	0.3416
		Error	1461.63	156	9.37		
	Male	Temperature	1890.63	1	1890.63	167.48	<0.0001
		Alar	3724.9	1	3724.9	329.96	<0.0001
		Interaction	0.4	1	0.4	0.04	0.8417
		Error	1761.05	156	11.29		
Fecundity (in no.)		Temperature	286.9	1	286.9	50.61	<0.0001
		Alar	661.83	1	661.83	116.76	<0.0001
		Interaction	6.56	1	6.56	1.16	0.2849
		Error	430.8	76	5.67		
Pupation time (day)		Temperature	40.8	1	40.8	1839.72	<0.0001
		Alar	160.4	1	160.4	7231.92	<0.0001
		Interaction	0.06	1	0.06	2.89	0.0911
		Error	3.46	156	0.02		
Adult emergence time (day)		Temperature	48.11	1	48.11	2493.64	<0.0001
		Alar	161.6	1	161.6	8375.49	<0.0001
		Interaction	0.00	1	0.00	0.02	0.8877
		Error	3.01	156	0.02		
Pupation height (cm)		Temperature	84.33	1	84.33	195.51	<0.0001
		Alar	234.45	1	234.45	543.53	<0.0001
		Interaction	0.8	1	0.8	1.84	0.1769
		Error	67.29	156	0.43		
Survival rate (%)		Temperature	576.6	1	576.6	6.14	0.0143
		Alar	3765.91	1	3765.91	40.11	<0.0001
		Interaction	0.03	1	0.03	0.00	1
		Error	14645.52	156	93.88		

(χ^2=56.7; p<0.05) was recorded which suggests mortality of male resulting from probable induction of X lined lethal mutation in treated male by Alar.

Discussion

Indiscriminate use of food additives and preservatives poses severe health problems in human and model organisms. Unfortunately, characterization of adversity of many such food additives and fruit preservatives remains outside the scientific research interest. Alar, *i.e.* Daminozide, is such a chemical which is almost ubiquitous for its use in apple preservation and finds its route of entry into the human body and that of other species who are natural consumers of fruits through apple slices and juice. We determined the LC_{50} value of Alar for both
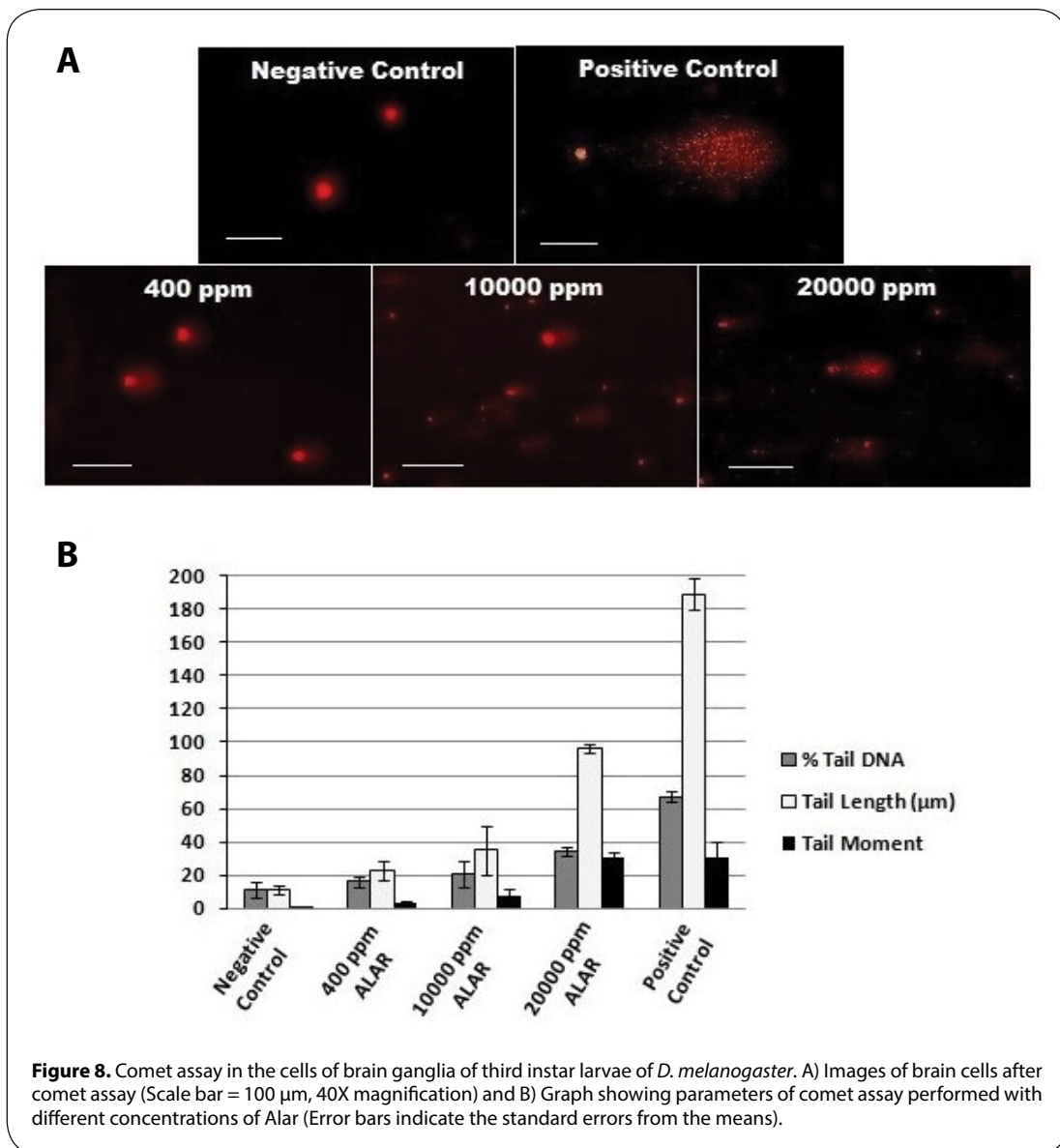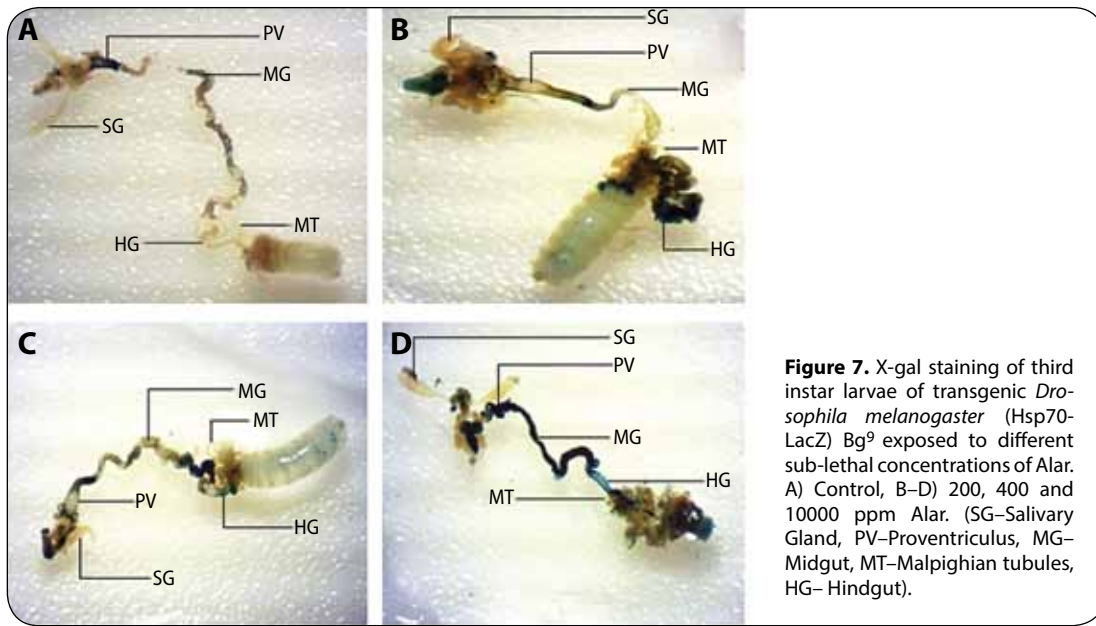
Table 6. Effect of Alar on comet parameters in cells of brain ganglia of *D. melanogaster*. Values are median ± SE of three experiments.
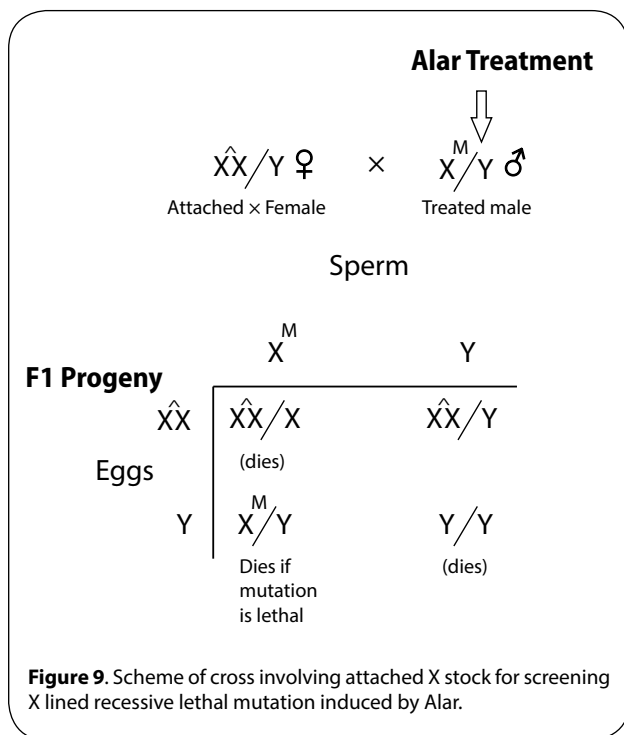
Group	Tail DNA (%)	Tail Length (µm)	Tail Moment (arbitrary units)
Negative Control	11.107±0.65	11±0.54	1.221±0.09
400 ppm Alar	16.112±0.58	23±1.11	3.705±0.19
10 000 ppm Alar	20.48±1.55	35±3	7.167±0.94
20 000 ppm Alar	34.07±0.53	96±0.54	31.038±0.54
Positive Control	67.34±2.9	189±9.35	125.23±8.7

p<0.0001 in comparison with control.

the larval and pupal stages of *Drosophila* and the result showed that LC_{50} value for pupae was lower than the LC_{50} value for larvae. The reason may be that the pupal stage

Figure 7. X-gal staining of third instar larvae of transgenic *Drosophila melanogaster* (Hsp70-LacZ) Bg[9] exposed to different sub-lethal concentrations of Alar. A) Control, B–D) 200, 400 and 10000 ppm Alar. (SG–Salivary Gland, PV–Proventriculus, MG–Midgut, MT–Malpighian tubules, HG– Hindgut).

Figure 8. Comet assay in the cells of brain ganglia of third instar larvae of *D. melanogaster*. A) Images of brain cells after comet assay (Scale bar = 100 μm, 40X magnification) and B) Graph showing parameters of comet assay performed with different concentrations of Alar (Error bars indicate the standard errors from the means).

Alar Treatment

⇓

$\hat{X}X/Y$ ♀ × X^M/Y ♂

Attached × Female   Treated male

Sperm

X^M Y

F1 Progeny

$\hat{X}X$ | $\hat{X}X/X$ | $\hat{X}X/Y$
           | (dies) |

Eggs

Y | X^M/Y | Y/Y
  | Dies if mutation is lethal | (dies)

Figure 9. Scheme of cross involving attached X stock for screening X lined recessive lethal mutation induced by Alar.

is the metabolically active developmental stage devoid of feeding where maximum tissue rearrangements are taking place. Thus, food already ingested in the larval life happens to manifest its effect on pupae (Dad *et al.*, 2011). Moreover, the pupal stage is non-excretory and hence chance of removal of Alar from the body is nil and thus comparatively less concentration of Alar exerts lethality in the pupal stage.

In the present work, Alar was found to be a toxic agent for life history and morphological traits and probably acts as teratogenic agent in *Drosophila melanogaster*. The results showed delayed developmental regimen and significant increase in body, wing and arista length. This modulation of life history and morphological traits may be local adaptation of the insect against Alar. It may be possible that the larger body size is adaptive and a fly with such features somehow manages to survive in the stressful Alar containing medium. Similarly, extension of developmental phases beyond the normal schedule may be another survival strategy in unfavorable cultural conditions. Alternately, Alar may have toxic effects on the genes that regulate ontogenetic development. Intuitively, genes of TOR pathway or activating lifespan extending genes such as sir2 (Roy *et al.*, 2017) may be the target, though other genes may also get affected. We recorded a drop in fecundity which suggests strong effect of Alar on germinal tissue and shortening of pupation height which is the indicator of pre-adult fitness (Casares *et al.*, 1997). Alternatively, low mean puparium height among the treated larvae is indicative of neurotoxicity. It may be possible that neurotoxicity induced by Alar affected the mobility of larvae due to which they cannot move up to the upper part of the culture vial, the preferred site of

control larvae for pupation. It is possible that Alar might have created lethal conditions for some larvae. Surviving larvae might have adjusted or adapted to this toxicant by altering their morphology and life history traits.

The present study also revealed an effect of temperature on the adversity of Alar on morphological and life history traits of *Drosophila melanogaster*. The results are consistent with previous studies on the effect of the pesticide (Das *et al.*, 2010; Podder & Roy, 2015) and temperature condition (Schnebel & Grossfield,1992; Rezaei, 2012; Chen *et al.*, 2013; Chang *et al.*, 2014) on life history traits. We observed developmental delay among the flies treated at the lower temperature of 23 °C than the flies reared at the higher temperature at 28 °C. Additionally, a greater increase in body length, wing length and arista length was recorded at lower temperature than in flies reared at higher temperature. At this point it is very difficult to explain the cause behind the temperature effects on adversity of Alar. More incisive analyses are needed to unravel the exact etiology.

Alar was found to be highly toxic at higher concentrations, as revealed by Hsp70 expression in transgenic *Drosophila melanogaster* (hsp70-lacZ) strain Bg[9]. Usually, Hsp70 expression increases in response to subcellular molecular stress and means to maintain the cellular and molecular homeostasis (Hightower, 1991). Considerable numbers of studies have used Hsp70 as reporter gene in evaluating the toxic potential of some toxicants (Nazir *et al.*, 2001; Mukhopadhyay *et al.*, 2002; Siddique *et al.*, 2013). In the present study, we observed overexpression of Hsp70 in larval proventriculus, midgut and hindgut. Exposure of the larvae to Alar occurs through their feeding and hence the gut tissues express Hsp70 immediately to mitigate the imperilments induced by Alar following its consumption.

We recorded DNA damage through comet assay in the cerebral ganglionic cells of mature third instar larvae exposed to different concentrations of Alar. This observation unambiguously revealed that Alar is a neurotoxicant causing neurodegeneration in the cells of cerebral ganglia of treated larvae. Though we did not perform this experiment on the other tissues from different parts of larval body, but predictively we can say this kind of genotoxicity in form of DNA damage may have induced by Alar in other body tissues as well. We found X-lined recessive lethal mutations as we recorded selective excess mortality of male sex (Figure 9) in our genetic screening experiment involving cross using attached X stock. That suggests alar has mutagenic potential too.

In recent years, *Drosophila melanogaster* has been emerged as a popular model eukaryote for in vivo genotoxicity studies (Mukhopadhyay *et al.*, 2004; Siddique *et al.*, 2005). The fly has worldwide distributions in orchards and markets and therefore, it becomes an innocent victim of pesticides or preservatives used indiscriminately in those orchards and fruit processing units. These chemicals enter into human body through direct or indirect routes and also affect other non-target living animals like *Drosophila*. These facts justify the use of *Drosophila*

melanogaster in present study. In summary, the present work is the first ever systematic study on the adversity of fruit preservative Alar on living system and the results help realize that the effects of this chemical cannot be ignored and further research attempts are warrant to characterise its toxicity at molecular level.

Acknowledgements

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