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Clastrogenic instrict of Sodium Bispyribac and Pinoxaden Herbicides on *Drosophila melanogaster*

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Abstract

The present research is attributed to evaluate the genotoxic potential of two herbicides namely Sodium Bispyribac and Pinoxaden, at sublethal level, LC_{20} using larval salivary gland polytene chromosomes of *Drosophila melanogaster*. Standardization of exposure concentration was achieved by exposing second instars larvae of *Drosophila* to serial dilution concentration of the selected herbicides, for continuum of 24 hours. Thereafter, on the basic of observed mortality in treated and control groups, exact value of LC_{20} was calculated by applying probit analysis and the corresponding values are 4.4 pl/ml for Sodium Bispyribac and 7.1pl/ml for Pinaxoden. Subsequently, exposure of standarized concentration was provided to second instar larvae, by mixing specified concentration in culture medium and, simultaneously negative control were also maintained. Third instar larvae were sacrificed for temporary squash preparation of polytene chromosomes and chromosomal complement were scrutinized for various types of structural chromosomal abnormities, in herbicide exposure group and natural population. Procured data indicated that the selected herbicides affected the structural integrity of polytene chromosomes, by inducing intrachromosomal, interchromosomal and telomeric fusions, paracentric inversions, inversion complexes, intrachromosomal and interchromosomal ectopic pairings due to induced homozygocity, chromatid breaks, chromosomal translocations and homologous chromosomal asynapses with respective value with respective number 45.66 ± 6.65 and 21 ± 3.60 in Sodium Bispyribac treated individuals and control, whereas in Pinoxaden treated and controls value 48.64 ± 8.6 and 25.32 ± 3.66 in control group. Subsequently, analysis of procured data, indicated that selected herbicides induced statistical significant genotoxicity in treated individuals in comparison to control groups ($p<0.05$).

Keywords: Genotoxicity, Bispyribac, Pinoxaden, Polytene, *Drosophila*

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Introduction

With implementation of synthetic agro-formulations, although food production has escalated, but the detrimental consequences of concerned chemicals cannot be ignored on biotic and abiotic components of environment. Numerous genotoxicity evaluation reports are procurable in scientific literature, specifying that such synthetic chemicals: induced significant destruction of structural and functional integrity of genetic material, influence the exchange of sister chromatids and micronuclei frequency, interrupt the normal synthesis and functionality of hormones, cause respiratory problems, affect structural and function integrity of reproductive organs, induce birth defects, increase cases of cancer and genetic abnormalities in living organisms and additionally, produce adverse effects on environment[1-5]. Therefore genotoxicity evaluation of different agro-chemicals, by applying innovative methodologies with appropriate biomarkers of different test models, has become very essential, before excessive use of such chemicals in agricultural fields.

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Motivated by particular cognition, present research work has been executed, that deals with mutagenicity assessment of two herbicides namely Sodium Bispyribac and Pinoxaden, using *Drosophila* larvae polytene chromosomes. Very limited information is available about cytotoxicity and clastrogenic properties of Sodium Bispyribac and Pinoxaden. The herbicides, Sodium Bispyribac (sodium;2,6-bis[(4,6-dimethoxypyrimidin-2-yl)oxy]benzoate) and Pinoxaden ([8-(2,6-diethyl-4-methylphenyl)-7-oxo-1,2,4,5-tetrahydropyrazolo[1,2-d][1,4,5] oxadiazepin-9-yl] 2,2-dimethylpropanoate) were registered in 2001 and 2005 respectively, by EPA for commercial use, to control weeds including grasses, broadleaf plants, on wheat and barley. Half life period of Sodium Bispyribac is 42 days[6] whereas for Pinoxaden is 16 days[7]. Sodium Bispyribac is a contact herbicide that inhibits plant enzyme acetolactate synthetase (ALS), and therefore affects the synthesis of amino acids, whereas Pinoxaden is a systemic herbicide that affects the action of ACCase, thereby preventing fatty acid synthesis. Both of selected chemicals *viz*: Sodium Bispyribac and Pinoxaden, are postemergence herbicides. Pinoxaden belongs to the phenylpyrazolin group of chemicals, used to control a broad range of grass species including *Alopecurus myosuroides, Apera spica-venti, Avena ludoviciana, Avena sterilis, Avena fatua, Lolium multiflorum, Lolium rigidum, Phalaris minor, Phalaris paradoxa and Poa trivialis* etc.

Among different innovative methodologies, implementable for genotoxicity evaluation, the larval salivary gland polytene chromosomes had been considered as appropriate assessing tool for genotoxicity evaluation, as genetic damage induced by environmental agents can be easily observed in the form of structural chromosome abnormalities[8-14]. During present study, enhanced percentage frequency of chromosomal aberrations in treated stocks in comparison to control stocks has been considered as basic criterion, to evaluate genotoxic instrict of selected herbicides.

Material And Methods

Test organism: *Drosophila melanogaster* (fruit fly) is an ideal test organisms, as it possess short life span, easy adaptability to laboratory conditions, small diploid number (2n=8) and presence of four polytene chromosomes due to homologous pairing and endoreduplication of chromosomes. *Drosophila melanogaster,* an holometabolous insect, consisting of egg, larva, pupa and adult or imago stages, had been used as test model for present research execution. Initial population of *Drosophila melanogaster* was received from *Drosophila* breeding laboratory, Department of Biotechnology, Punjab Agriculture University, Ludhiana. The *Drosophila melanogaster* culture, for the present experimental execution is maintained on agar powder, brewer's yeast, sucrose, dextrose and corn meal along with fungicides i.e. phosphoric and propionic acid in cultural vials at 25^{0}C. The medium was kept hydrated for easy movement of larvae and kept away from dirt, direct sunlight and other heat sources.

Herbicidal Profile: Two herbicides were selected for the concerned genotoxicity evaluation including Sodium Bispyribac with IUPAC Name, Sodium2,6-bis[(4,6-dimethoxy-2-pyrimidinyl)oxy) benzoate, Molecular formula $C_{19}H_{17}N_4NaO_8$ and Pinoxaden with IUPAC name 8-(2,6-diethyl-4-methylphenyl)-1,2,3,4,tetrahydro-7-oxo-7-H-pyrazolo[1,2 d] [1,4,5] oxadiazepin-p-9-9-yl 2,2-dimethylpropanoate, Molecular formula C_{23} H_{32} N_2 O_4 . Locally available packets of concerned herbicides *viz* Sodium Bispyribac and Pinoxaden, available under respective trade names Adora and Axial, had been procured, and were used as such because basic motive of research execution is to assess the cytotoxicity and clastrogenic potential of concerned synthetic formulations, which are actually going to be used in the agricultural fields.

Exposure concentration standardization: For concentration standardization purpose, second instar larvae were exposed to serial dilution for 24 hours, and subsequently mortality was assessed in treated samples and control stocks. All experiment sets was repeated in triplicates for treated samples along with respective control. On the basic of observed mortality, the exact value LC_{20} was calculated separately for both selected herbicides, by probit analysis[15]. The value corresponds to 4.4 pl/ml for Sodium Bispyribac and 7.1pl/ml for Pinaxoden (Figure 1,2). Exposure of selected herbicides was provided to second instar *Drosophila* larvae, by mixing the LC_{20} value in culture medium and keeping larvae, in culture medium for 24 hours and subsequently, shifting to fresh culture medium for further growth.

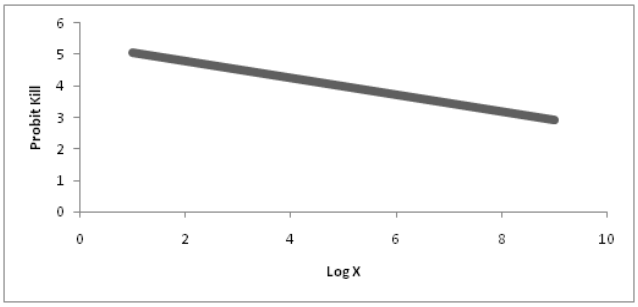


Figure 1. Regression line between Probit kill and concentration of Sodium bispyribac.

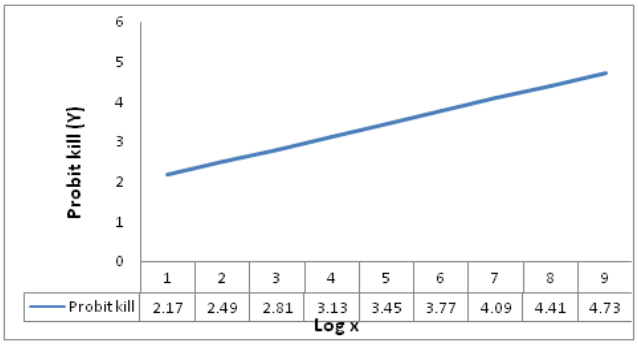


Figure 2. Regression line between Probit kill and concentration of Pinoxaden.

Methodology of slide preparation: Salivary glands (bilobed glands) of health and active *Drosophila* larvae were dissected out in 0.67% saline solution, by splitting the thorax region subsequently, hydrolyzed in 1N HCl and stained in 2% acetoorcein[15].The slides with well spread chromosomes were immediately studied and photographed under suitable magnification Structural chromosomal aberrations were identified with the help of the standard *Drosophila melanogaster* map. Thereafter, the statistical analysis of the aberrations in chromosome in treated stocks was compared to that of control stocks.

Result analysis : Procured data was analyzed for various types of chromosomal aberrations including fusions inversions, ectopic pairings, asynapses, translocations and breaks in Sodium bispyrabac and Pinaxoden treated groups as well as their controls and subsequently mean, standard deviation was calculated and furthermore, statistical significance of data was calculate by applying students 't' test (Table 1,2).

Result

The present study is attributed to genotoxicity instrict evaluation of selected herbicides namely Sodium bispyrabac and Pinaxoden, at LC_{20} value, using larval salivary polytene chromosomes, exposed for continuum of 24 hours. Herbicide exposure limit was calculated by probit analysis, after observing mortality of second instar larvae treated with serial dilution of selected herbicides. Subsequently, third instar larvae were sacrificed for temporary squash preparation for treated samples and control stocks. Thereafter chromosomal complements with proper banding pattern were photographed and scrutinized for various types of structural malformations. In Sodium Bispyribac treated stocks intra and interchromosomal chromosomal fusions (3), due to induced stickiness, paracentric inversions,

Table 1. Statistical analysis of chromosomal aberrations in Sodium bipyrbac exposed stocks and control groups

Type	Fusions Mean ± S.D.	Inversions Mean ± S.D.	Inversion complex Mean ± S.D.	Breaks Mean ± S.D.	Translocations Mean±S.D.	Asynapses Mean ± S.D.	Ectopic pairings Mean ± S.D.	Total Mean±S.D.	t value
Treated	23.66±3.5	10.66±4.6	3.33±1.52	0.66±1.15	0.33±0.5	2.6±1.15	4.33±3.2	45.66±.6.65	2.7*
Control	10.33±0.57	8.33±2.081	--	--	--	1.66±1.15	0.66±0.5	21.00±3.605	

S.D (Standard Deviation); Degree of freedom = 4; *p<0.05

Table 2. Statistical analysis of chromosomal aberration in Pinoxaden exposed group and control stocks

Type	Fusion (Mean± S.D)	Inversion (Mean± S.D)	Inversion complex (Mean±S.D)	Break (Mean±S.D)	Translocation (Mean± S.D)	Asynapsis (Mean±S.D)	Ectopic pairing (Mean±S.D)	Total	t value
Treated	22.66±2.06	11±1.73	6.33±1.15	1.00±1.00	1.33±0.57	3.66±0.57	2.66±1.52	48.64±8.60	4.56*
Control	13.33±0.59	6.66±0.58	___	___	___	2±1.41	3.33±1.08	25.32±3.66	

Degree of freedom= 4; S.D (Standard Deviation); *p<0.05

intra and interchromosomal ectopic pairings, inversion complexes, homologous asynapses, chromatid breaks and translocations with respective value 71,32,13,10,8,2 and 1 were reported whereas in control, various types of aberrations like chromosomal fusions, paracentric inversions, asynapses and ectopic pairings with respective values 31,25,5 and 2 were observed. In Pinoxaden treated stocks, fusions, inversions, inversion complexs, asynapses (Figure,4), ectopic pairings, translocations and breaks with respective number 68,33,19, 11,8,4 and 3, were reported and in control stocks chromosomal fusions, inversions, ectopic pairings and asynapses with respective number 40,20,10 and 6 had been reported (Figure 5, 6). It had been observed that both of selected herbicides possess maximum tendency to induce stickiness in chromosomes that had resulted in chromosomal fusion including intrachromosomal, interchromosomal and telomeric fusions. Additionally, formation of inversion complexes had also been reported. Comparatively more incidences of asynapses, ectopic pairings were reported in herbicides exposed stocks than the control groups, whereas translocations and breaks had been noticed exclusively in herbicide exposed groups only. Furthermore, obtained data indicated statistically significant, induced genotoxicity by both of herbicides *viz* Sodium bispyribac and Pinoxaden, in comparison to control population when scrutinised with student't' test.



Figure 3. Interchromosomal fusion in polytene chromosomes of Sodium bispyribac exposed larva.

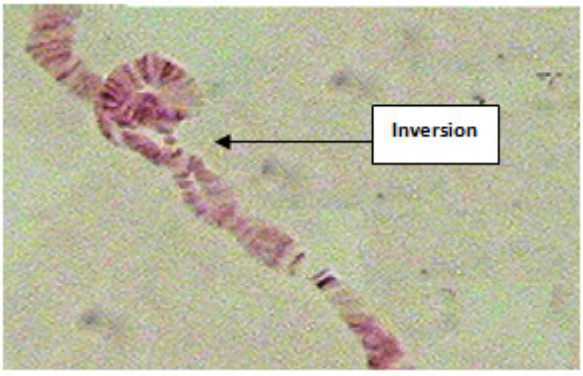


Figure 4. Asynapsis of homologue in polytene chromosomes of Pinoxaden exposed larva.

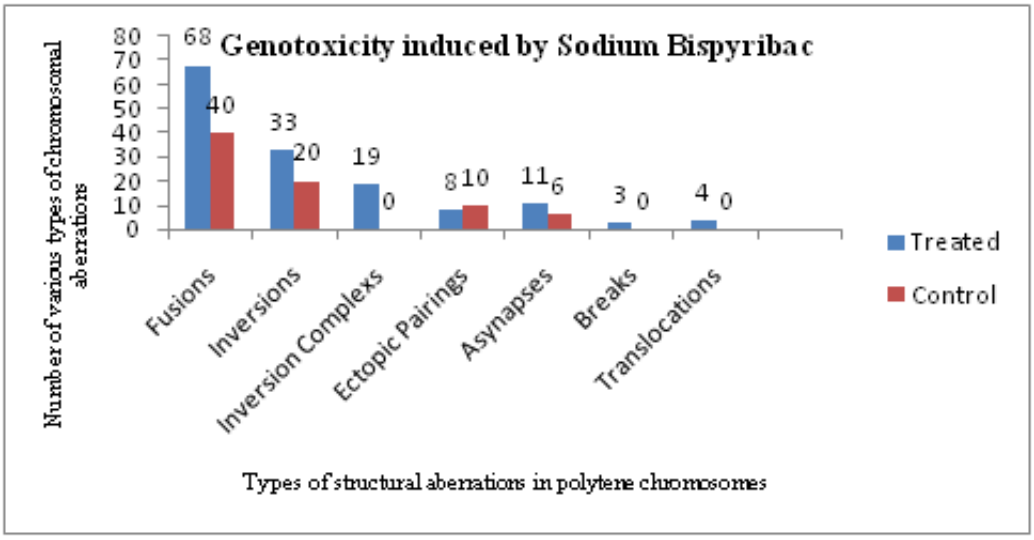


Figure 5. Graphical depiction of number and types of various structural chromosomal aberrations in Sodium Pispyribac exposed larvae and natural population. In herbicide exposed samples, maximum incidences of chromosomal fusions were reported, followed by paracentric inversions, ectopic pairings, inversion complexes and asynapses. Not even a single incidence of chromatid breaks and translocations was reported in natural population.

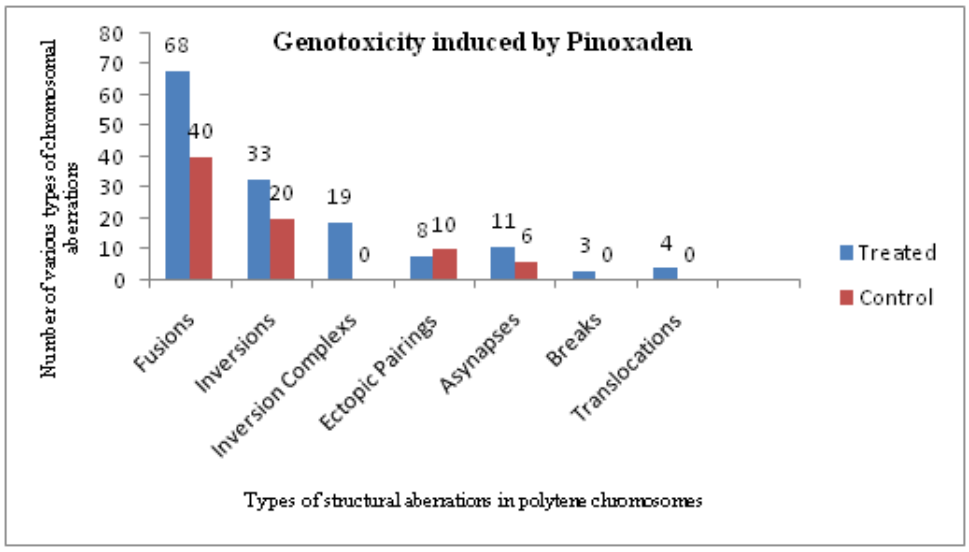


Figure 6. Graphical presentation between number and types of structural chromosomal aberrations in Pinoxaden exposed individual and natural population. Genotoxicity of concerned herbicide was observed in the form of enhanced incidences of chromosomal fusion, followed by paracentric inversions, inversion complexes, ectopic pairings, asynapses. Breaks and translocations were reported in herbicide exposed groups only.

Discussion

During present clastogenic and cytotoxicity evaluation study, genotoxic instrict of Sodium Bispyribac and Pinoxaden herbicides has been observed, in the form of enhanced percentage frequency of different structural malformations including intrachromosomal, interchromosomal and telomeric fusion, paracentric inversion, intrachromosomal and interchromosomal ectopic pairing, inversion complexes, asynapses of homologues, interstitial breaks and segmental translocation, in comparison to natural population. For the concerned study, salivary polytene chromosomes of third instar larvae were scrutinized for various structural chromosomal aberrations, both in treated and control population. Normal salivary polytene complement consists of X-chromosome, two longer autosomes of unequal size and one short chromosome. All chromosomes are attached at centromeric position to form chromocenter, and almost complete 4th polytene chromosomes is also, involved in formation of chromocentre.

In both Sodium bispyribac and Pinoxaden herbicides treated stocks, maximum aberrations were observed in the form of chromosomal fusion, which could be due to increased stickiness in chromosomes. Similarly elevated incidences of chromosomal ectopic pairings, inversions and inversion complexes were observed in herbicides treated stocks. Not even a single incidence of chromosomal translocation has been reported in control stocks.

During present investigation, elevated incidences of chromosomal fusions were encountered. Generally chromosomal fusion occur due to chromosomal stickiness and breakage[16], whereas, in a genotoxicity evaluation study, it had been concluded that chromosomal breaks induced due to various mutagens are repaired by certain proteins[17]. Furthermore, it had been observed that chromosomal fusion could also be initiated by the simultaneous breakage of two chromatids or by the loss of telomere capping[18]. After chromosomal fusion, numerous incidences of paracentric inversions were also reported in Sodium bispyribac and Pinoxaden exposed stocks, in comparison to natural population. Literature review specified that generally various environmental mutagens including drugs, preservatives, pesticides and radiations possess potential to cause, various type of aberrations in the genetic material by inducing chromtid breaks, and sometimes, the interstitially broken segment rotate by 180^0 and gets reinserted in the same place before the repair can take place, which ultimately produces inversions. In one of study, it was observed that copper affect the structural integrity of salivary gland polytene chromosomes of *Chironomus riparius*[19] and induced inversions at chromosomal weak points and most of the somatic

chromosome rearrangements occur more frequently in the specific regions of the chromosomes, composed of repetitive DNA loci. Similarly during present investigation also drastically increased percentage frequency of chromosomal inversion and inversion complex has been reported, in herbicides exposed samples, which could due to abnormal interaction of selected herbicides with genetic material. As compared to the elevated frequency of fusion, inversions, and ectopic pairings, herbicides treated individuals suffered from comparatively lower frequency of chromosomal translocations and breaks. During, present set of investigations, asynapsis of the somatically paired homologues of polytene chromosomes was also a predominant structural chromosomal malformation in the herbicide treated flies. Reports available in scientific literature suggest that asynapses generally occur due to denaturing of the binding proteins due to the action of chemicals[20-21]. Apart from asynapsis found in the present research work, chromosomal breaks also had an elevated frequency in herbicide treated stocks as compared to natural population. It was observed the mechanism of the repair of breaks in *D. melanogaster* in which, it was concluded that breaks generally occur due to inhibition of eukaryotic replication factors essential for repair of DNA double-strand breaks (DSBs) and lesions, caused by the action of certain mutagens[20].

Few reports related with toxicity and harmful effects of selected herbicides *viz:*Sodium Bispyribac and Pinoxaden, on living organisms are available[23-24]. Limited information is available about mutagenicity and clastrogenic properties of selected herbicides.

Concluding remarks: During present study also, It has been concluded that both of selected herbicides namely Sodium bispyribac and Pinoxaden, induced statistically significant genotoxicity in larval salivary polytene chromosomes at LC_{20} exposure limit by inducing various structural aberrations including intrachromosomal, interchromosomal and telomeric fusions, paracentric inversions, intrachromosomal and interchromosomal ectopic pairings, inversion complexes, asynapses of homologues, interstitial breaks and translocations, in comparison to natural population. It has been observed that selected herbicides induced stickiness in chromosomes that ultimately is responsible for induction of various types of chromosomal malformation. Further intensive evaluation for mutagenic assessment is recommended, before excessive application of selected herbicides in agricultural fields, additionally, alternative of pesticides should be implemented and use of natural predators should also be in plasticized.

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Conflict of Interest: None Declared

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