

Genotoxicity of Selected Insecticides in *Glycine Max* Root Cells by Single Cell Gel Electrophoresis (Comet) Assay

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Abstract:-

- **Objective:-** The objective of this research work is to do a comparative study on the genotoxicity of chemical insecticides and bio insecticides in plants by keeping apical root cells of *Glycine max* as model.
- **Methods:-** Soybean seeds bought from the market located in south Kerala were divided into four sets so that each set contain four seeds. Set 1 and Set 2 treated with same amount of chemical insecticide solutions, Chlorpyrifos and LARA-909 respectively while set 3 treated with Bio insecticide OCTO Plant protector and set 4 was maintained as control. Comet assay was performed in root tip meristems of soybean after seeds germination. Chemical Insecticide solutions were prepared so that it contained their active ingredient at a concentration of 50 ppm, 100 ppm, 150 ppm and 200 ppm.
- **Result:-** The respective study revealed that 200ppm of insecticides treated seeds were unable to germinate. Both bio and chemical insecticides showed increase in genotoxicity with respect to increase in its concentrations. LARA- 909 contain Cypermethrin showed more genotoxicity in this study and Bio insecticide showed DNA damage than chlorpyrifos contain Chlorpyrifos at all concentrations.
- **Conclusion:-** From above results it can be concluded that over use of both chemical and bio insecticides is highly genotoxic to plants.

Keywords:- *Glycine max*, Genotoxicity, Cypermethrin, Chlorpyrifos, Comet assay.

I. INTRODUCTION

Insecticides are substances widely used in agriculture field to get rid from destructive insect or other animals known as pests that attack crops. Kerala is a state in India where agriculture is considered as its backbone. Soybean is a protein rich crop that widely cultivate in almost all over the world. According to studies, some pesticides banned in many foreign countries, are still being blindly used in Kerala. It is found that there is an increase of 21-30% for Cypermethrin and Chlorpyrifos at 7.5% in Kerala Agriculture field [Article; "Wide use of toxic pesticides in State", THE HINDU 2010].

DDT also known as Di chloro diphenyl trichloro ethane is an organochlorine compound developed in 1874 and then it became popular in its insecticide property by 1939 [Anfossi et al., 2004]. As its high genotoxicity effect has been identified, its use strictly prohibited by U.S Government in 1972[Lear, Linda et al., 2009]. But still it is found to be used by many countries in various brand names. India is one of those countries and is the largest consumer of DDT [van den Berg H et al., 2008]. In Kerala Chlorpyrifos is a powder form insecticide that is sold in pesticide shops in the name of DDT. It is used for many household and agricultural purposes in order to kill pests. Chlorpyrifos is one of the main chemical constituents of Chlorpyrifos. Genotoxicity of chlorpyrifos from chlorpyrifos in soybean root cells has been tested in this study. Studies show that although chlorpyrifos is not mutagenic, it is moderately toxic to humans and poisons to Central nervous system, Respiratory system, CVS [Pesticide Information Profile, 1993]. Previous works on chlorpyrifos tells that it may be toxic to some plants such as Head Lettuce [McEwen, F. L. and G. R. Stephenson et al., 1979].

Cypermethrin is a synthetic chemical insecticide used to control many pests in vegetable crops. LARA 909 is an insecticide with 5% cypermethrin and 50% of chlorpyrifos. Cypermethrin is coming under pyrethroids classification of chemical insecticides. It acts as a neurotoxin in insects[Anand Kumar Singh, Manindra Nath Tiwari et al., 2012]. Cypermethrin is moderately toxic material by dermal absorption or ingestion [Pesticide Information Profile, 1993]. Even though its degradation on soil and plant is easy it can be effective for weeks when applied to indoor inert surfaces[Jaya Raj, Mohineesh, Ruma Ray et al., 2013]. It is found to be highly toxic to fish, bees and aquatic insects according to the reports of National Pesticides Telecommunications Network (NPTN).

Single cell gel electrophoresis (comet) assay is one of the simplest and efficient technique to detect DNA damages in eukaryotic cells[S. Nandhakumar, S. Parasuraman et al., 2011] . This assay measure genotoxicity of chemicals in cells occurring as a result of their DNA strand breaks. After assay is performed, damaged DNAs will be appeared as comets under fluorescence microscope. There is many free software available for comet analysis. Parameters commonly measured in comets includes Comet length, Tail length, Head diameter, Percentage of DNA in head, Percentage of DNA in tail and Tail moment [T. S. Kumaravel et al., 2009]. In the present study we have used an open software called

Open Comet [Benjamin M. Gyori et al.,2014] to analyze percentage DNA in comet tail for determining genotoxicity of chemicals. This parameter is found to be one of the good measures for DNA damage.

A case study done by a team from the Kerala Agricultural University (KAU) published in The NEWS Minute in 2017 revealed the presence of banned pesticides such as Cypermethrin, Chlorpyrifos in crops. The respective study evaluates, compare and discuss genotoxicity of Cypermethrin and Chlorpyrifos with that of a bio insecticide to give awareness about the extensive use of pesticides in agriculture industry.

II. MATERIALS AND METHODS

➤ Soybean seeds

Soybean seeds were bought from a market located in South Kerala, South India in 2017. Seeds of almost uniform size, colour and weight were selected for the experimental purpose. These seeds were divided into five sets so that each set contain four seeds. In order to confirm the quality of seeds one set of seeds allowed to grow in a petri plate containing cotton by using water. The seeds showed good and normal growth within seven days. It confirmed the qualities of seeds are good.

➤ Insecticide solutions

Solutions of Chlorydust containing 1.5% chlorpyrifos and LARA 909 contain 5% cypermethrin is prepared in four tubes so that each tube contains their respective major constituent at a concentration of 50ppm, 100ppm, 150ppm and 200ppm. Similarly, bio insecticide is made into solutions of 50ppm, 100ppm, 150ppm and 200ppm.

➤ Treatment of seeds with insecticides

Set two, set three and set four seeds were soaked in 2ml of different concentrations i.e.; 50 ppm, 100ppm, 150ppm and 200ppm of insecticide solution in an Eppendorf's tubes. Set five seeds soaked in 2ml water that was maintained as the control. Seeds kept in insecticide solution and water for 24 hrs. After 24 hrs seeds were transferred to a petri dish containing cotton. Transferred seeds were allowed to germinate.

➤ Single Cell Gel DNA Electrophoresis (Comet) Assay

The germinated root apical meristem is excised from both insecticides treated and control seeds. The root tip squashes were made by grinding with PBS in Eppendorf's tube using small pestle. The cell suspension centrifuged for 5 minutes at 1000rpm. The cell debris centrifuged down, and the supernatant contain the root cells for assay. The supernatant is collected in separate tubes which are used as samples for performing comet assay.

The procedure used is basically that described by S. Nandhakumar and S. Parasuraman in 2011. Required number of glass slides were cleaned and sterilized. Slides were dipped in 0.75% Normal Melting Point Agar up to one third area of slide and allowed to dry for 20 minutes. 1% of Low Melting Point Agar was added to samples and mixed well in their Eppendorf's tubes. After proper mixing it is loaded above the first layer of normal melting point agar and places the cover slip and put slide on a cold condition for fixing for 20 min. At last a layer of Normal Melting Point Agar is added to the sample by removing cover slip. Then the cover slip placed again, and slides were placed in cold condition for 10 minutes. These slides were kept in lysis solution for 2 hours and cover slips are removed. After that these slides dipped into the electrophoresis buffer for 20min. Then electrophoresis can be carried out at 24 Volt for 30min.

➤ Microscopic examination of slides

After electrophoresis, slides were treated with neutralization buffer and stained with 1X Ethidium bromide. Slides were photographed using inverted epi florescent microscope Olympus CXX41 attached with opit pro5CCD camera. Comets were scored using open comet scoring software.

III. RESULTS

➤ After treating with insecticides

It is found that seeds which are treated with 200ppm of both chemical and biological insecticides didn't germinate till three days and after that they started to decay in the petri dishes. Meanwhile seeds treated with 50ppm, 100ppm and 150 ppm concentrations of insecticides germinated slowly as compared to the control.

Insecticides	% DNA in tail		
	50ppm	100ppm	150ppm
Control	0	0	0
Chlorydust (chlorpyrifos)	51%	54%	60%
LARA 909 (cypermethrin)	56%	61%	66%
OCTA Plant protector (Bio- insecticide)	55%	60%	65%

Table 1:- Results of comet assay performed on control, seeds treated with bioinsecticides and seeds treated with chemical insecticides.

➤ Comet Assay Results

Comet assay slides containing insecticide treated cells observed under fluorescent microscope after staining showed comets in the system monitor while the control cells appeared round. Images obtained from each slide saved for further reading and analysis.

➤ Measuring comets

There is much free software available for scoring comet images obtained from fluorescent microscopy. Software measures comet parameters such as Comet length, Tail length, Head diameter, Percentage of DNA in head, Percentage of DNA in tail and Tail moment. Among those parameters percentage of DNA in tail is found to be more significant and reflect the DNA break frequency. The result obtained from Open comet software is as in the table.

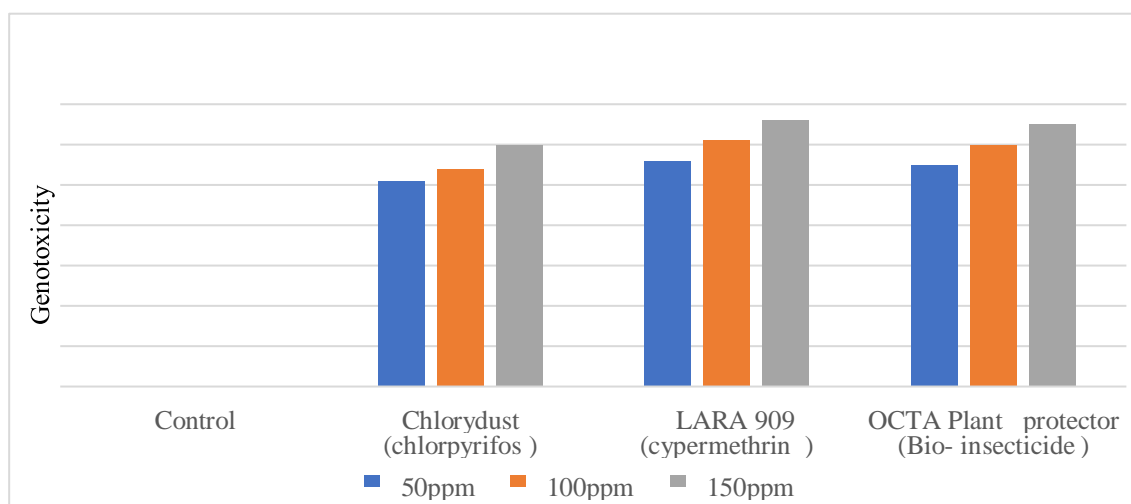


Fig 1:- Genotoxic effect of insecticides on cells at different concentrations

IV. DISCUSSION

Agricultural industry uses some chemicals for better maintenance of crops and to get maximum yield. These chemicals are known as pesticides or insecticides. There are many pesticides identified till now causing environmental hazards. Most of them are genotoxic to plant, animals and water bodies. In comet assay percentage of DNA in comet tail is one of most significant parameters that depicts the amount of genotoxicity in cells. All pesticides used in this study showed highest genotoxicity in plants at a concentration of 200ppm that prevented seeds from their germination and led to its decay. This indicates that very high concentrations like 200ppm is highly toxic to plants. Among three pesticides treated cells observed under microscope after comet assay, LARA 909 contains cypermethrin showed high percentage of DNA in its comet tail at all concentrations of 50ppm, 100ppm, and 150ppm. Chlorydust containing chlorpyrifos was the pesticide with less genotoxicity in this study and OCTA Plant protector (Bio-insecticide) was the average one. Toxicity of cypermethrin is characterized by its effect on hyper-excitation of the Central Nervous System of exposed organism [Anand Kumar Singh, Manindra Nath Tiwari et al., 2012]. It's already proven that a pesticide called Vila containing Cypermethrin as its active ingredient is genotoxic in the onion root tip meristematic cells [Asita Okorie Asita and Matebesi L.P et al., 2010]. Genotoxicity of the synthetic pyrethroid cypermethrin in brain ganglia and anterior mid gut of *Drosophila melanogaster* has been reported through in vivo studies [Mukhopadhyay I et al., 2004]. Work of Amer, S.M.; Ibrahim, A.A. and El-Sherbeny, K.M. in 1993 showed the Cypermethrin cause genotoxicity by induction

of chromosomal aberration and sister chromatid exchange. The invasive effect of Cypermethrin in ecosystem is characterized by its extreme toxicity to fishes and invertebrates.

Chlorpyrifos is an organophosphate introduced in 1965 against pests including insects and worms [Pesticide Information Profile et al., 1993]. It is characterized by its toxic effect on Central Nervous System of insects that make WHO to put chlorpyrifos under class 2; moderately hazardous chemical. It has been already proven that the wide range of genotoxic effect induced by chlorpyrifos in onion root tip cells is indicative of the wide range targets in the cell that are susceptible to its effect inducing on spindle fibers, chromosomes, kinetochore, centrioles and enzymes [Asita AO, Makhalemele R et al., 2008]. Pesticide Information Profile reported that Chlorpyrifos is moderately to very highly toxic to birds and is very highly toxic to freshwater fish, aquatic invertebrates and estuarine and marine organisms. In this study chlorpyrifos showed less genotoxicity as compared to other two.

Apart from chemical pesticides there are other natural control measures of pests and insects with minimum or no harmful effects to other animals and environments. They are derived from biological elements such as extracts of plants like neem or from microorganisms. Hence it is called 'Bio pesticides. Octo plant protector is a widely used bio pesticide in Kerala that enables crops to grow fast and healthy lead to quality and higher yield. In the present study, Octo showed less genotoxicity than Cypermethrin and more toxicity than Chlorpyrifos at all concentration. Similarly, the research work of "An examination of the potential

Genotoxic carcinogenicity of a biopesticide derived from the neem tree” by Rosenkranz, H. S., and Klopman in 1995 showed that a natural bio insecticide extracted from the neem called Azadirachtin found to be genotoxic and able to cause cancer in mouse. This proved that even bio pesticides can cause genotoxicity in plants and animals at some stages.

The mechanism of how these pesticides cause genotoxicity in plants with increase in concentration and side effects in human body on consuming crops treated with these pesticides must be considered in future research to find out a minimum critical concentration above which it should not be used. A comparative research study such as described in this paper is one basic approach to give awareness about genotoxic effect on plants by both chemical and bio pesticides.

V. CONCLUSION

The result of present study suggested that over use of both bio pesticides and chemical pesticides are very genotoxic to plants and may responsible for harmful ecological effects and various human diseases by consumption of pesticides treated crops.

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