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Cytotoxicity and Genotoxicity of Imidacloprid, Spinosad and Bifenthrin - Myclobutanil Combination to *Allium cepa* Root Tip Meristematic Cells

Asita Okorie Asita[1], Relebohile Rebecca Mohale[1] & Sibusisiwe Magama[1]

[1]Department of Biology, National University of Lesotho, P.O. Roma 180, Maseru 100, Lesotho, Southern Africa

Correspondence: Asita Okorie Asita, Department of Biology, National University of Lesotho, P.O. Roma 180, Maseru 100, Lesotho, Southern Africa. Tel: 266-522-13-292. E-mail: aoasita@yahoo.co.uk

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Abstract

Pesticides use boosts agricultural yield by reducing crop losses. However, some pesticides are mutagens and while technical grade active ingredients may produce mixed results in cytotoxicity and mutagenicity tests in *in vitro* and *in vivo* assays, synergistic interaction of pesticides, their metabolites or impurities in pesticide formulations often produce cytotoxic and genotoxic effects. This study assessed three concentrations (mg mL^{-1}) (0.04, 0.08 and 0.16) each of Aphicide Plus® (AP) (imidacloprid at 20g L^{-1}); Eco Fruit-fly Bait GF120® (EF) (spinosad at 0.24 g L^{-1}) and Rosecare 3® (RC3) (combination of bifenthrin at 2.0 g L^{-1} and myclobutanil at 7.5 g L^{-1}) for their effect on the *(P+M)/ (A+T) Ratio*, cytotoxicity and genotoxicity using the *Allium cepa* assay. *A. cepa* seedlings were treated for 24 hours, root tip squashes were prepared and the slides were examined under the microscope. For each pesticide treatment and the negative control, 6000 cells were examined and the cells were classified into interphase, normal (N) or aberrant (ABN) mitotic division stage. The cytotoxicity and genotoxicity induced by each pesticide concentration was compared with the value for the negative control using t-test. The 0.08 mg mL^{-1} of AP, 0.04 mg mL^{-1} of EF, and 0.08 and 0.16 mg mL^{-1} of RC3 induced significant change in the (P+M)/(A+T) ratio, ($p > 0.05$). All three concentrations of each pesticide significantly depressed the mitotic index (MI) and were adjudged cytotoxic ($P < 0.05$). Genotoxicity (GT) was expressed as the number of aberrant mitotic cells (AMC) per 100 mitotic cells scored. The three concentrations of each pesticide induced genotoxicity ($P < 0.05$).

Keywords: insecticide, fungicide, chromosome aberration

1. Introduction

In the field and during storage, agricultural products are threatened by insects, rodents, birds and other pests (Hayma, 2003). It was suggested by Raja et al. (2001), that insect damage in stored grains and other durable commodities may amount to 10-40% in developing countries, where modern storage technologies have not been introduced.

The control of pests that compete for food and fiber will continue to be an issue as the world population increases. According to Robson (2019) there are 7.7 billion people on the planet today and this number will grow to 9.7 billion in 2050. The need for food and fiber will continue and the need to control pests, using many means including agricultural chemicals will increase (Robson, 2019).

In order to cut down losses in agriculture, pesticides have been integrated into modern agricultural process to control weeds, diseases and insect pests that can markedly reduce the amount of harvestable produce and thereby increase outputs and productivity (Aktar et al., 2009). Pimentel et al. (1993) argued that, on average, the economic benefits from pesticide use are about four times their direct cost to the users. About one-third of the agricultural products are produced by using pesticides (Liu et al., 2002). Without pesticide application the loss of fruits, vegetables and cereals from pest injury would reach 78%, 54% and 32% respectively (Cai, 2008).

Pesticides are different from other agricultural inputs in that they do not directly boost yields in the way that fertilizers do; instead they reduce crop losses caused by pests (Jha and Regmi, 2009).

Though pesticides use reduces crop losses and so increases agricultural output, pesticides are known to produce a wide spectrum of adverse health and environmental effects. The same pesticides that are effective in controlling serious harmful insects like the rice weevil (*Sitophilus oryzae*) and the European corn borer (*Ostrinia nubilalis*) are also lethal to beneficial insects like the honey bee (*Apis mellifera*) and pollinators, including the honey bee, are responsible for seventy-five percent of the world's food production Robson (2019). According to a report of WHO and UNEP, worldwide there are more than 26 million human pesticide poisonings with about 220,000 deaths per year (Richter, 2002).

In a review of the literature on the health effects of pesticides, Mansour (2004) concluded that there is strong scientific evidence that pesticides, as a whole, can induce severe effects to human health ranging from myelotoxicity to cytogenetic damage and carcinogenicity. Other health effects of pesticides include acute and persistent damage in the nervous system (Kamel et al., 2007), lung and respiratory disorders (Hoppin et al., 2008), alterations in the reproductive organs (Hileman, 1994) birth defects (Rojas et al., 2000).

Most of the adverse effects of pesticides to health, according to Norppa (2004) are the result of the genetic damage induced by genotoxic agents in somatic as well as in germinal cells and any genetic activity of chemicals, it has been suggested, is most likely to result from cell division abnormalities (Parry et al., 1999).

Pesticides residues are known to persist in soil (Subbarao, 1999), water (Medina et al.. 1999) and in fruits and vegetables (Girotti et al., 2009) and represent a risk for human health. Genotoxicity and mutagenicity of pesticides for non-target organisms and their influence on ecosystems are of worldwide concern (Pimentel et al., 1998).

According to Zhang et al. (2011), worldwide, there are currently about 500 pesticides used widely and in large quantities. In China alone, more than 400 companies are manufacturers of over 300 varieties of original pesticides and 3,000 formulations or commercial names. It was estimated that globally there were about 5.6 billion pounds of pesticide used annually with some 25 million agricultural workers poisoned (Jeyaratnam, 1990). These pesticides are manufactured as insecticides, fungicides, herbicides, rodenticides and antimicrobials to protect crops. In the southern African sub-region, a number of commercial formulations of pesticides are being marked by South African Companies, three of which are Aphicide Plus® (AP) Eco Fruitfly Bait GF-120® (EF) and Rosecare 3® (RC3) all manufactured by Efekto.

Aphicide Plus® (AP) is a systemic suspension concentrate contact and stomach insecticide for use in the home garden to control sucking insects, as indicated on the label, on conifers, roses and other ornamentals. It contains imidacloprid (chloro-nicotinyl) a neonicotinoid insecticide as the active ingredient. Imidacloprid acts on the central nervous system of insects as an agonist of the nicotinic acetylcholine receptor (Chao and Casida, 1997) stimulating the neurons and causing a subsequent fatigue and interference in the transmission of nerve impulses (Zhang et al., 2000; Schulz-Jander and Casida, 2002). It is considered as an insecticide of low toxicity to mammals and other vertebtrates due to its poor penetration of the blood-brain barrier of these organisms (Sheets, 2001 a, b). The U.S. Environmental Protection Agency (U.S. EPA) has classified imidacloprid into Group E, no evidence of carcinogenicity, based on studies with rats and mice. (Gervais, et al., 2010; Thyssen and Machemer, 1999; Fed. Regist, 2005). However, following metabolic activation *in vitro*, imidacloprid produced calf thymus DNA adducts (Shah et al., 1997) and was mutagenic in TA98 and TA100 *Salmonella typhimurium* strains, with or without S9 metabolic activation (Karabay and Oguz, 2005). Imidacloprid also induced significant increases in the frequency of sister chromatid exchange and micronuclei formation in human peripheral blood lymphocytes (Feng et al., 2005; Costa et al., 2009), mice and rat bone-marrow cells (Karabay and Oguz, 2005; Demsia et al., 2007), and *Vicia faba* roots (Zang et al., 2000).

Eco Fruit fly Bait GF-120® (EF), is a selective concentrate bait for control of fruit fly species infesting various fruit and cucurbits. Eco Fruit fly Bait GF-120 contains the active ingredient spinosad (Naturalyte). Spinosad (SPN) is a naturally-occurring insecticide (larvicide) derived from the fermentation of a naturally occurring soil actinomycetes, *Saccharopolyspora spinosa* (Mertz and Yao, 1990; Mendonça et al., 2019). SPN acts on different receptors of insects, causing toxicological effects after ingestion or cuticle absorption. The mechanism of action of SPN is mainly through the nicotinic acetylcholine receptors and in a secondary pathway through the γ-amino-butyric receptors (GABA). As a consequence, SPN leads to hyper excitation, paralysis and death of the insect in the larval phase (Mendonça et al., 2019). According to the World Health Organisation, the pesticide SPN has low acute toxicity and presents a low toxicological risk potential (WHO, 2005). It is used to control a wide variety of pests. These include thrips, leaf miners, spider mites, mosquitoes, ants, fruit flies and others and has been registered for use in pesticides by the U.S. EPA since 1997 (Bunch et al., 2014). Spinosad was not carcinogenic to Fischer 344 rats at dose levels up to 0.05% (Yano et al., 2002). There are conflicting reports on the mutagenicity of spinosad. Spinosad has been reported to lack a mutagenic effect by U.S. EPA (1997) while Mansour *et al*. (2008)

found that spinosad was genotoxic to rat bone marrow cells. Spinosad lacked genotoxic activity in the *in vivo* somatic mutation and recombination test (SMART) in *D. melanogaster* (Akmoutsou et al., 2011). According to Marrs (2012), none of the genotoxicity studies showed mutagenic activity associated with spinosad. Chronic exposure studies failed to induce tumor formation in rats and mice; mice given up to 51 mg/kg/day for 18 months resulted in no tumor formation (Stebbins, 2002).

Rosecare 3® (RC3), is a contact insecticide plus a systemic fungicide formulated as a micro emulsion for the control of diseases including aphids, red spider mite, thrips, black spot, rust and powdery mildew on roses and other ornamentals. (https://www.hadeco.co.za/efekto/efekto-rosecare-3-rtu-750ml/). Rosecare 3® contains bifenthrin and myclobutanil as the active ingredients. Bifenthrin is a synthetic pyrethroid insecticide in the pyrethroid family constituting man-made versions of pyrethrins, produced naturally by chrysanthemum flowers (Johnson et al., 2010) and myclobutanil is a triazole fungicide. The insecticide bifenthrin is designed to be effective by contact or ingestion and affects the central and peripheral nervous systems of invertebrates thereby killing them (Tomlin, 2000). The U.S. EPA classified bifenthrin as a Category C, possible human carcinogen, based on studies in mice. Other studies indicate that bifenthrin does not cause cancer or birth defects when fed to rats or rabbits that ate bifenthrin when pregnant (Johnson et al., 2010). A "FAO/WHO Joint Meeting on Pesticide Residues" (JMPR) examined the test battery for the assessment of mutagenicity of bifenthrin and concluded that the results were mixed (WHO, 2017). The JMPR concluded that, whereas some tests were clearly negative (such as the Ames test on different strains of *Salmonella typhimurium* with and without metabolic activation), other tests, such as the Mouse Lymphoma Mutagenesis Assay or the unscheduled DNA synthesis test with rat hepatocytes showed weak positive response or yielded inconclusive results with technical bifenthrin (WHO, 2017).

Myclobutanil, according to the European Food Safety Authority (EFSA) is a systemic fungicide with preventive, curative and eradicant properties that belongs to the class of conazole fungicides approved for use in the EU and many other countries (EFSA, 2010). Conazoles are a diverse group of commercially important fungicides with clinical and agricultural applications for cereals' treatment, vegetables, fruits and flowers and also as medical products (Ross et al., 2009; Hester et al., 2012). Myclobutanil works by inhibiting the biosynthesis of ergosterol, a critical component of fungal cell membranes. If these membranes are unable to grow, the growth of the fungus is inhibited as well (domyown.com/myclobutinil-c-114_468.html). Myclobutanil is moderately toxic to birds, fish, aquatic invertebrates, algae, honeybees and earthworms (Lewis et al., 2016) It has been shown that the toxicity of triazole fungicides passes through oxidative stress, causing DNA damage or apoptosis (Ross et al., 2012; Hester et al., 2006). However, Myclobutanil did not show any genotoxic potential in both *in vitro* and *in vivo* genotoxicity tests (EFSA, 2010).

Examination of the literature on the genotoxicity and mutagenicity of the technical grade active ingredients (TGAIs) of the three pesticides (imidacloprid, spinosad, bifenthrin, and myclobutanil) reveal conflicting reports, but we could not find reports of tests using the *Allium cepa* chromosome aberration assay for some. The present study aimed therefore to investigate the cytotoxicity and genotoxicity of different concentrations of three pesticide formulations (Aphicide Plus®, Eco Fruit-fly Bait GF-120® and Rosecare 3®), containing these active ingredients using the *Allium cepa*, chromosome aberration assay because synergistic interaction or potentiation between or among pesticides that are mixed together is a well-known phenomenon (Cloyd et al., 2007).

The *Allium cepa* L assay is one of the established plant bioassays, validated by the international programme on chemical safety (IPCS, WHO), as an efficient and standard test for chemicals screening, *in situ* monitoring of the genotoxicity of environmental substances (Leme and Marin-Morales, 2009). The *Allium cepa* L assay is an *in vivo* assay that tests genotoxicity using chromosomes and therefore detects chromosome structural and numerical alterations (Tedesco and Laughinghouse, 2012; Bonciu et al., 2018). The results obtained, using the *Allium cepa* assay have been shown to be similar with those of mammalian and non-mammalian test systems (Constantin and Owen, 1982; Fiskesjö, 1985; Cauhan et al., 1999; Aydemir et al., 2008). *Allium cepa* root cells, just like many plant cells, have the monooxygenase enzyme systems (MFO-system) and therefore the ability to activate promutagens (Plewa and Gentile, 1982; Higashi, 1988; Fiskesjö, 1985).

2. Materials and Methods

2.1 Test Organism

Seeds of *Allium cepa* variety, Texas Grano 502 P.R.R®, obtained from Sakata seeds, Lanseria 1748, South Africa were used for the study.

2.2 Pesticides

Pesticides used in the study were products of Efekto; Aphicide Plus® Reg. No. L8780 Act No. 36 of 1947 N-AR1162 (contained imidacloprid at 20 g L^{-1} in the formulation; Manufacturer's recommendation, 10 – 20 mL L^{-1} of water = 101 – 51 x dilution); Eco Fruitfly Bait GF-120® model number 6001379103341 (contained spinosad (naturalyte) at 0.24 g L^{-1}; Manufacturer's recommendation, 1.2 ml in 9 to 30L of water = 8.5 – 26 x dilution); Rosecare 3® registration number L7599 N-AR 0726 Act No. 36 of 1947 (contained bifenthrin at 2.0 g L^{-1} and myclobutanil at 7.5 g L^{-1} in the formulation; Manufacturer's recommendation, 10ml per 1L of water = 101 x dilution). All the pesticides were products of Agro-Serve (Pty) Ltd, South Africa. The pesticides were purchased from the Maseru Garden Centre, Lesotho, Southern Africa.

2.3 Chemicals

Methanol (Absolute) was a product of Associated Chemical Enterprises (PTY) LTD of The Republic of South Africa; Hydrochloric acid and Glacial acetic acid were products of UNILAB of The Republic of South Africa; Aceto-carmine stain from Carolina Biological Supply Company, USA.

2.4 Genotoxicity Experiments

The preliminary assay to select concentrations of pesticides to use for the Genotoxicity (GT) assay (including the treatment of *Allium cepa* seedlings with pesticides, root harvest, slide preparation and scoring of slides) were conducted according to the methods of Asita et al. (2017). From the results of the preliminary assays to select the concentrations of pesticides to use, the following concentrations of pesticides (in $mgmL^{-1}$) AP (0.04, 0.08 and 0.16); EF (0.04, 0.08 and 0.16); and RC3 (0.04, 0.08 and 0.16) were assessed for cytotoxicity that is, mitotic index (MI) and genotoxicity (GT) following 24 hours of treatment of the *A. cepa* seedlings. When diluted in accordance with the manufacturers' recommendation, the concentrations of the individual active ingredient in the applied pesticide will be: imidacloprid at 20 g L^{-1} in formulation and at 101 – 51 x dilution = 20/101 – 20/51 = 0.198 – 0.392 mg mL^{-1} Spinosad at 0.24 g L^{-1} in the formulation and at 8.5 – 26 x dilution = 0.24/8.5 – 0.24/26 = 0.028 – 0.00923 mg mL^{-1}; Bifenthrin at 2 g L^{-1} in the formulation and at 101 x dilution = 2/101 = 0.02 mg mL^{-1}; myclobutanil at 7.5 g L^{-1} in the formulation and at 101 x dilution = 7.5/101 = 0.074 mg mL^{-1}. The tested concentrations (0.04, 0.08 and 0.16) are lower than the applied concentration of imidacloprid, a bit higher than the recommended concentration for use for spinosad and bifenthrin but similar to the recommended concentration for use for myclobutanil. In each assay, three root tips (triplicate) were assessed at each concentration. On each of three slides (n = 3) per treatment, a total of 2000 cells, classified into interphase or dividing cell, that is, prophase (normal, N or aberrant, ABN), metaphase (N or ABN), anaphase (N or ABN) or telophase (N or ABN) were scored. The aberrant (ABN) category in each cell division stage comprised of cells having their mitotic division apparatus (Chromosomes, spindle fibres, kinetochore or centrosomes) damaged. Therefore, a total of 6000 cells each were examined and scored for the negative control (water) and treatment (pesticide concentration) groups.

Mitotic stage cells containing the following types of damages were classified as aberrant (ABN) (Asita et al, 2017):

- Chromosome fragments (F) – piece of chromosome broken from whole chromosome as a result of pesticide treatment and lacking centromere.
- Anaphase or Telophase bridge (A.B) – Dicentric chromosomes that form a bridge between both poles at anaphase or telophase. Often it indicates paracentric inversions or other possibilities that include breakage and fusion of chromosomes and sister chromatid reunion.
- Laggard (L) – whole chromosomes that fail to migrate to either pole at anaphase because of damage to the kinetochore,
- C-Mitosis (C-Mit) – Mitotic cells that lack spindle fibres so that the chromosomes lie scattered throughout the cell. The effect is usually produced in cells treated with the spindle poisons, colchicines or colcemid, hence C-Mitosis.
- Sticky chromosomes (S) - sticky chromosomes fail to condense completely so that at metaphase, the chromosomes are still long like prophase chromosomes and remain entangled with each other. In extreme cases, chromatin masses, undistinguishable as chromosomes is seen as a clump. If such a damage occurs in interphase cells, they are referred to as pyknotic cells.

2.5 Analysis of Slide Preparations

2.5.1 Cytotoxicity

The mitotic index (MI) was expressed as the number of dividing cells per 100 cells scored according to the formula:

MI = Number of dividing cells/Total number of cells scored x 100. (1)

The MI was used as a measure of cytotoxicity (CT). The MI of each treatment group was compared with that of the negative control group using t-test at a probability level of 0.05, using the SPSS for windows, version 11.0 software.

2.5.2 Genotoxicity

Genotoxicity (GT) was expressed as the number of aberrant mitotic cells (AMC) per 100 mitotic cells [*i.e* AMC + normal mitotic cells (NMC)] scored according to the formula:

Frequency of GT = AMC/ (AMC + NMC) x 100 (2)

The mean GT of each group of three slides per concentration of test agent was compared with that of the negative control group using t-test. P values less than 0.05 ($P < 0.05$) were considered as indicative of significance.

3. Data analysis

Data were expressed as mean ± SD of three values. For the determination of cytotoxicity and genotoxicity, the mean value of each group of three slides per concentration of test pesticide, was compared with that of the negative control group using student's t-test. P-values less than 0.05 ($p < 0.05$) were considered as indicative of significance. Analysis was performed using the SPSS for windows version 11.0 software.

4. Results

4.1 Cytotoxicity (CT) and Genotoxicity (GT) of the Pesticides in the A. Cepa Root Tip Chromosome Aberration Assay

Photographs of the most representative pictures of normal mitotic cells and cells containing the different types of chromosome aberrations that were observed and scored are presented in Figure 1.

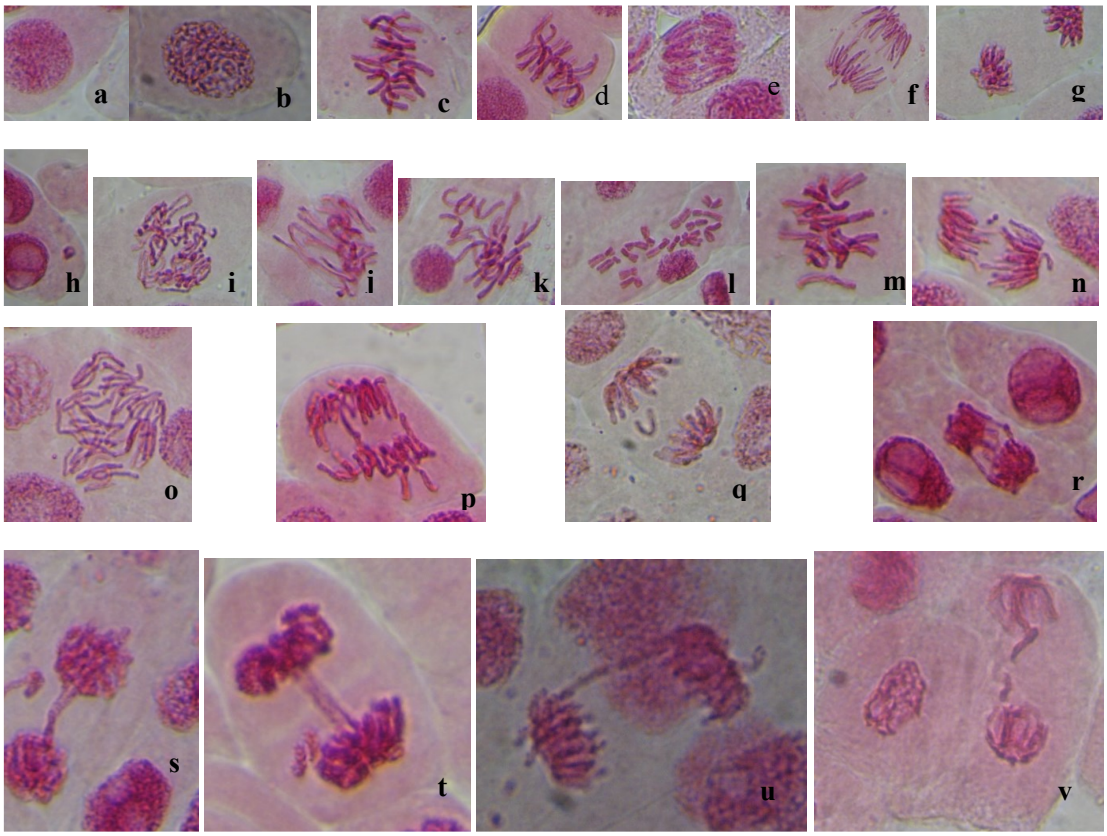

Fig. 1. Photomicrographs of cells of *Allium cepa* showing untreated cells in normal division stages (First row) and Chromosomal aberrations (arrowed) in cells treated with pesticides. (a) Interphase (b) Normal Prophase (c) Normal metaphase (d) Normal metaphase (e) Early anaphase (f) Late anaphase (g) Telophase (h) Pyknotic interphase nuclei with micronucleus (i) Prophase with sticky chromosomes (j) Metaphase with sticky chromosomes (k) Metaphase with sticky chromosomes (l) C-metaphase (m) Metaphase with dislocated chromosome (n) Late anaphase with dislocated chromosome (o) Anaphase with sticky and scattered chromosomes (p) Late anaphase with chromosome bridge (q) Telophase with lagging chromosome (r) Telophase with sticky chromosomes and

bridge (s) telophase with chromosome bridge and lagging (t) telophase with chromosome bridge and fragment (u) Telophase with chromosome bridge (v) Telophase with chromosome fragment and lagging. Magnification is 1000 X.

4.2 Cytotoxicity and Genotoxicity of the Pesticides

The results of the cytotoxicity (effects on the mitotic index) and genotoxicity (damage to mitotic apparatus) of the treatment of onion root tip meristem cells with different concentrations of the pesticides are presented in Table 1.

Table 1. Toxic and Genotoxic Effects of Aphicide Plus®, Eco Fruit-fly Bait GF-120® and Rosecare 3® on Onion Root Tip Meristem Cells Following 24h Exposure to Three Different Concentrations of Each Pesticide

				Cells in division stages per 2000 cells scored												Prophase + Metaphase and Anaphase + Telophase Analysis				
	Conc. (mg/mL)			Proph		Metaph		Anaph		Teloph		Total						(P+M) /		
TC		Statistics	Interph	N	ABN	N	ABN	N	ABN	N	ABN	N	ABN	N + ABN	Total Cells Scored	P+M	A+T	(A+T)	MI	GENOTOXICITY
Water	Water			12								20								
		Mean	1790.00	4	1	26	1	6	0	52	0	8	2	210	2000	152.0	58.0	2.6	10.67	1.05
		S.D	75.54	63	1	8	1	1	0	21	0	76	0	76	0	55.6	20.0	0.1	3.51	0.41
Aphicide Plus	0.04	Mean	1920.33	38	4	15	2	3	0	15	1	72	8	80	2000	59.7	20.0	2.9	3.67*	10.38*
		S.D	13.65	2	2	24	2	6	0	1	1	14	2	14	0	24.5	4.4	0.5	0.58	3.55
	0.08	Mean	1978.67	2	12	1	2	0	0	1	3	4	18	21	2000	17.7	3.7	8.1*	1.00*	86.90*
		S.D	5.86	4	3	1	1	0	0	1	2	6	1	6	0	2.9	3.1	6.9	0.00	22.68
	0.16	Mean	1962.00	13	8	6	3	1	0	6	2	25	13	38	2000	29.0	9.0	3.3	2.00*	33.35*
		S.D	14.00	1	1	1	1	1	0	0	1	2	1	2	0	0.0	2.0	0.8	0.00	0.86
Eco Fruit-fly Bait,GF12 (	0.04	Mean	1900.67	55	13	8	2	1	1	11	8	76	23	99	2000	78.7	20.7	3.8*	5.00*	23.49*
		S.D	7.37	2	4	2	1	1	1	4	3	6	3	7	0	5.1	2.3	0.2	0.00	2.63
	0.08	Mean	1972.33	9	7	3	2	0	0	6	4	14	13	28	2000	21.7	9.7	2.5	1.33*	48.62*
		S.D	6.51	2	3	1	1	0	1	2	3	4	3	7	0	5.7	5.5	0.6	0.58	3.27
	0.16	Mean	1986.67	2	6	2	0	0	0	2	1	6	8	13	2000	10.3	3.0	4.6	1.00*	57.90*
		S.D	2.52	2	3	1	1	0	1	1	1	3	3	3	0	4.5	1.7	3.2	0.00	17.59
Rosecare3	0.04	Mean	1926.33	36	7	13	3	0	1	12	2	60	13	74	2000	59.0	14.7	4.6	3.67*	18.01*
		S.D	13.87	8	2	4	2	0	1	6	1	12	5	14	0	7.0	7.0	2.0	0.58	5.78
	0.08	Mean	1944.33	20	10	5	2	0	1	11	7	36	20	56	2000	37.0	18.7	2.0*	2.67*	35.21*
		S.D	11.93	6	3	2	1	0	1	3	2	9	6	12	0	7.2	4.9	0.2	0.57	6.67
	0.16	Mean	1959.67	10	10	2	3	0	0	5	10	17	23	40	2000	25.3	15.0	1.7*	2.00*	57.64*
		S.D	5.51	5	5	1	4	0	0	3	1	5	6	6	0	6.1	2.0	0.3	0.00	11.09

TC = Test Compound; Amphicide Plus®; Eco Fruit-fly Bait, GF120®; Rosecare 3®; MI = Mitotic Index; Interph = Interphase; Proph = Prophase; Metaph = Metaphase; Anaph = Anaphase; Teloph = Telophase; A&T = (Anaphase & Telophase); S.D = Standard deviation; * Significant difference from control in the t-test at $P<0.05$ and 4 d.f.

4.3 The (P+M)/ (A+T) Ratio, Cytotoxicity and Genotoxicity Induced by the Pesticides

The following results were observed following the treatment of root tips of *A. cepa* seedlings with Aphicide Plus® (0.16 mg mL^{-1}, 0.08 mg mL^{-1} and 0.04 mg mL^{-1}), Eco Fruit-fly GF120® (0.16 mg mL^{-1}, 0.08 mg mL^{-1} and 0.04 mg mL^{-1}), Rosecare 3® (0.16 mg mL^{-1}, 0.08 mg mL^{-1} and 0.04 mg mL^{-1}) for 24 hours.

(P+M)/ (A+T) Ratio: Examination of the (P+M)/(A+T) ratio in column 19 of Table 1 shows that only the middle concentration (0.08 mg mL^{-1}) of Aphicide Plus®, the lowest concentration (0.04 mg mL^{-1}) of Eco Fruit-fly Bait, GF120® and the middle and highest concentrations (0.08 and 0.16 mg mL^{-1}) of Rosecare® 3 induced significant change in (P+M)/(A+T) ratio, when compared with the water treated negative control group ($p > 0.05$).

Cytotoxicity: Examination of the MI in column 20 of Table 1 shows that all three concentrations of each of the three pesticides tested induced a significant reduction of the MI when compared to the solvent (water) treated negative control ($P<0.05$) and were adjudged toxic to the root meristem cells of *A. cepa*.

Genotoxicity (GT): Examination of induction of genotoxicity in column 21 of Table 4 shows that all three concentrations of each of the three pesticides tested induced a significant increase in genotoxicity when compared to the solvent (water) treated negative control ($P<0.05$) and were adjudged genotoxic to the root meristem cells of *A. cepa*.

5. Discussion

In this study, three concentrations (mg mL^{-1}) each of three pesticides, Aphicide Plus® (0.16, 0.08 and 0.04), Eco Fruit-fly GF120® (0.16, 0.08 and 0.04), Rosecare 3® (0.16, 0.08 and 0.04) were accessed for their effect on the *(P+M)/ (A+T) Ratio*, cytotoxicity and genotoxicity using the onion (*Allium cepa* L.) root tip meristem chromosome aberration assay system. The results of the effects of the pesticides on the three endpoints following the treatment of onion root tip meristem cells with different concentrations of the pesticides are presented in Table 1.

The *(P+M)/ (A+T) Ratio*, cytotoxicity and genotoxicity results following treatment of *Allium cepa* L root tip meristem cells with Aphicide Plus® (imidacloprid (chloro-nicotinyl) at 20g L^{-1} in the formulation) are presented in Table 1, rows 6 to 11. Only the middle concentration (0.08 mg/mL) of Aphicide Plus®, induced significant increase in the relative proportions P+M and A+T ratio, when compared with the water treated negative control group ($p > 0.05$). The lowest and highest concentrations (0.04 and 0.16 mg mL^{-1} respectively) increased the (P+M)/(A+T) ratio also but the increases were not significant. A decrease in the proportion of dividing cells in A+T is an indication of metaphase arrest due to the poisoning of the spindle fibres, akin to the action of the well documented spindle poison, colcemid (Parry et al., 1999). The chemotherapeutic agents taxol, vincristine, vinblastine and nocodozole act similarly (Alberts et al., 2008). These compounds act by binding to and stabilizing microtubules, inhibiting their dynamic instability and causing various genetic disruptions, including the induction of cell cycle arrest (Alberts et al., 2008; Zhang et al,. 2015). Since only one concentration modified the (P+M)/(A+T) ratio, it is concluded that Aphicide Plus® could be a spindle fibres poison. All three concentrations of Aphicide Plus® tested reduced the mitotic index (MI) of treated root tip cell populations. Mitotic index is considered a parameter that allows one to estimate the frequency of cellular division (Marcano et al., 2004) and the reduction of mitotic activities has been used frequently to trace substances that are cytotoxic (Linnainmaa et al., 1978; Smaka-Kincl et al., 1996). Many investigators have recorded a depression of the mitotic index following the treatment of test organisms with pesticides (Panda and Sahu, 1985; Amer and Farah, 1974). In the present study, treatment of the *A. cepa* root tips with each of the three concentrations of Aphicide Plus® tested reduced the MI which indicated a reduction of cell division or proliferation, compared to the water treated negative control. It is concluded that the concentrations of Aphicide Plus® tested in the present study are cytotoxic since they modify normal MI, in this case, decreased the MI (Leme and Marin-Morales, 2009; Vieira and Silveira, 2018). Aqueous extracts of leaves and stems of the plant *P. barbatus*, increased the cell division of *A. cepa* roots (Iganci et al., 2006; Trapp et al., 2020). Similarly, all three concentrations of Aphicide Plus® tested induced genotoxicity. In studies performed by Karabay and Oguz (2005), they observed significant levels of chromosome aberration (CA) (breaks and chromosomal adherences) in erythrocytes of mice exposed to imidacloprid, the active ingredient in Aphicide Plus®, and also in the studies of Rodríguez et al. (2015), in which they observed genotoxicity for imidacloprid which induced different kinds of CA in *A. cepa* cells, including binucleated cells, losses, breaks, chromosomal bridges, and MN. Imidacloprid (0.036, 0.36 and 3.6 g L^{-1}) was also cytotoxic, reducing the MI and genotoxic in the *A. cepa* chromosome aberrations assay as it induced different types of CA, mainly bridges and chromosomal adherences (Bianchi et al., 2016). However, some studies which investigated the genotoxic effects of imidacloprid at several concentrations and different organisms have produced conflicting results, both positive and negative (Solecki, 2001; Feng et al., 2004,; Karabay and Oguz, 2005; Demsia et al., 2007; Jemec et al., 2007; Kreutzweiser et al., 2007; Rodríguez et al.,2015). Synergistic interaction or potentiation between or among pesticides that are mixed together is a well-known phenomenon (Cloyd et al., 2007). In *a* recent study (Ilyushina et al., 2020), using technical grade active ingredients (TGAIs), imidacloprid, imazalil and tebuconazole pesticides individually at doses of up to 120, 300 and 1000 mg kg^{-1} bwd^{-1}, respectively, did not induce micronucleus formation in PCE of mice bone marrow in CD-1 mice, whereas their combination in a ratio of 14.0/1.7/1.0 by weight demonstrated negative results in the Ames (*Salmonella typhimurium*) test but induced a statistically significant, dose-depended increase in micronuclei (MN) in polychromatic erythrocytes (PCEs) in mouse bone marrow at doses lower than those used separately but without suppressing erythropoiesis, meaning that it was not cytotoxic. They concluded that the observed effect may be mediated by the synergistic action of the tested pesticides, their metabolites or impurities. The synergism of endosulfan and chlorpyrifos was shown in cultured human peripheral blood lymphocytes by chromosomal aberration test and comet assay (Sultana Shaik et al., 2016). In the present study, the concentration of imidacloprid of 20g L^{-1} or 20 mg mL^{-1} in Aphicide Plus® formulation is much higher than the tested concentrations (0.16, 0.08 and 0.04 mg mL^{-1}). These very low concentrations still

induced cytotoxicity and genotoxicity. The observed cytotoxic and genotoxic effects of the low concentrations of Aphicide Plus® may thus be attributed to the synergistic action of components of the Aphicide Plus®, their metabolites or impurities. The genotoxic effect of Aphicide Plus® observed in the present study demonstrated the genotoxic effect of imidacloprid containing pesticide formulation.

The *(P+M)/ (A+T) Ratio*, cytotoxicity and genotoxicity results following treatment of *Allium cepa* L root tip meristem cells with Eco Fruit-fly Bait GF-120® (Spinosad (Naturalyte) at 0.24 g L^{-1} in the formulation) are presented in Table 1, rows 12 to 17. Only the lowest concentration (0.04 mg mL^{-1}) of Eco Fruit-fly Bait GF120®, induced significant increase in the relative proportions P+M and A+T ratio, when compared with the water treated negative control group ($p > 0.05$). All the three concentrations (0.04, 0.08 and 0.16 mg mL^{-1}) of Eco Fruit-fly Bait GF-120® tested were cytotoxic and genotoxic.

There are conflicting reports on the genotoxicity/mutagenicity of spinosad in earlier studies.

Spinosad was reported to lack a mutagenic effect by U.S. EPA (1997) while Mansour et al. (2008) found that spinosad was genotoxic to rat bone marrow cells, but was not genotoxic in the *in vivo* somatic mutation and recombination test (SMART) in *D. melanogaster* (Akmoutsou et al., 2011). In a study that investigated the genotoxicity of dillapiol and spinosad (0.31, 0.96 and 1.6 µg mL^{-1}) in distilled water as solvent, using the somatic mutation and recombination test (SMART) in wings of *Drosophila melanogaster*, both compounds were genotoxic, inducing mainly mitotic recombination events (Aciole et al., 2014). In a recent study, spinosad (SPN) at the concentrations of 0.625; 1.25 and 2.5 g L^{-1} induced a high frequency of micronuclei in *Tradescantia pallida* (*T. pallida*) and was adjudged genotoxic, whereas, in the same study, 0.039; 0.078 and 0.156 µg mL^{-1} of spinosad were not mutagenic in the *in vivo* somatic mutation and recombination test (SMART) in *D. melanogaster* (Mendonça et al., 2019). The assessment of pure spinosad for genoxicity/mutagenicity using different test systems, *in vitro* and *in vivo*, as shown in the studies reported above, presents conflicting results. In the present study which used the *A. cepa in vivo* chromosome aberration test to evaluate Eco Fruit-fly Bait GF-120® (spinosad at 0.24 g L^{-1} or 0.24 mg mL^{-1} in the formulation) for cytotoxicity and genotoxicity, all the three concentrations (0.04, 0.08 and 0.16 mg mL^{-1}) tested were cytotoxic and genotoxic. The unequivocal genotoxic effect of Eco Fruit-fly Bait GF-120® observed in the present study demonstrates the genotoxic effect of spinosad containing pesticide formulation and suggests a possible effect of synergistic interaction of the contents of the pesticide formulation.

The *(P+M)/ (A+T) Ratio*, cytotoxicity and genotoxicity results following treatment of *Allium cepa* L root tip meristem cells with Rosecare 3® (bifenthrin at 2.0 g L^{-1} and myclobutanil (a triazole) at 7.5 g L^{-1} in the formulation) are presented in Table 1, rows 18 to 23. The middle and highest concentration (0.08 and 0.16 mg mL^{-1}) of Rosecare 3®, induced significant increase in the relative proportions P+M and A+T ratio, when compared with the water treated negative control group ($p > 0.05$). All the three concentrations (0.04, 0.08 and 0.16 mg mL^{-1}) of Rosecare 3® tested were cytotoxic and genotoxic. Reports of mutagenicity and genotoxicity studies of bifenthrin using different assay test systems are mixed, some positive while others are negative. Bifenthrin was not mutagenic in mutagenicity assays including the Ames test, *in vivo* rat bone marrow cells, Chinese hamster ovary (CHO) cells, and unscheduled DNA synthesis (UDS) at concentrations of bifenthrin up to 2.5 µl mL^{-1}. However, bifenthrin showed mutagenic properties in a mouse lymphoma L5178 Y cells assay (U.S. Environmental Protection Agency, 1988; Johnson et al., 2010). Bifenthrin (technical grade-purity 95%) concentrations (0.016, 0.0347 and 0.0628 ppm) caused different types of chromosome aberrations in livers cells of the fish, *Channa punctatus* (Chaudhari and Saxena, 2015) and DNA damage in gill tissue of the fresh water fish, *Danio rerio* when assessed using the alkaline comet assay (Reddy et al., 2013). The Rosecare 3® evaluated in the present study is a formulation containing bifenthrin at 2.0 g L^{-1} (2 mg mL^{-1}) and was unequivocally cytotoxic and genotoxic in the *A. cepa* chromosome aberration assay. However, a 225 mg L^{-1} mixture of dichloran (0.24); dichlofluanid (0.03); iprodione (2.97); chlorothalonil (0.08) and bifenthrin (1.10) caused significant increase in the frequency of micronuclei, reduced mitotic index and increased anaphase chromosome aberrations in *Allium cepa* root (Feretti et al., 2007). Reports of mutagenicity and genotoxicity studies of myclobutanil using different assay test systems are mixed, some positive while others are negative. Myclobutanil was not genotoxic in the *in vitro* Ames Bacterial reverse mutation assay, did not induce chromosomal aberrations with and without metabolic activation up to 200 µg mL^{-1} in the *in vitro* cytogenetics structural Chromosomal Aberration Assay, was not genotoxic in the CHO/HGPRT assay nor in the *in vivo* Mouse Micronucleus test and did not induce an increase in unscheduled DNA synthesis up to toxic dose. 0.1-1000 g mL^{-1} tested (U.S. EPA, 2000; EFSA, 2010). Myclobutanil did not induce mutations in the Big Blue mouse liver *in vivo* assay system (Ross et al., 2009) and was not cytotoxicity nor genotoxic to four human cell lines (ACHN, SH-SY5Y, LS-174T, HepG2) when screened with the γH2AX In Cell Western (ICW) assay that simultaneously determines cytotoxicity and genotoxicity of xenobiotics on cells cultured in a 96-well plate format after 24 h of treatment (Graillot et al., 2012). The Rosecare 3® evaluated in the present study is a

formulation containing myclobutanil (7.5 g L^{-1} or 7.5 mg mL^{-1}) and was unequivocally cytotoxic and genotoxic in the *A. cepa* chromosome aberration assay. A 225 mg L^{-1} mixture of either, dimethoate (0.10); vinclozolin (0.92); procymidone (1.44); fenarimol (0.03); hexaconazole (0.12); myclobutanil (0.27) or parathion-ethyl (0.11); folpet (1.03); procymidone (0.14); acephate (0.05); myclobutanil (0.49) caused significant increase in the frequency of micronuclei, significant reduction in mitotic index and increase in anaphase chromosome aberrations in *Allium cepa* root (Feretti et al., 2007). The unequivocal genotoxic effects of Rosecare 3® observed in the present study demonstrate the genotoxic effect of bifenthrin and myclobutanil containing pesticide formulation and suggest a possible effect of synergistic interaction of the contents of pesticide formulation.

Treatment with each of all the three pesticides caused a dose related cytoxicity (reduction of the MI) without any real direct action on the spindle, with the exception of the treatment with 0.08 mg mL^{-1}. Aphicide Plus®, 0.04 mg mL^{-1} of Eco Fruit-fly Bait GF120® and 0.08 and 0.16 mg/mL of Rosecare 3®. The mitotic index decreased with increased dose and at the same time, the relative proportions of P+M and A+T types remained the same. This may be taken as a typical indicator of cytotoxic damage (Parry et al., 1999).

The abnormal dividing or mitotic cells that were induced by the pesticides and scored in the present study were prophase, metaphase, anaphase and telophase cells with sticky chromosomes, c-metaphase, cells with dislocated chromosomes, cells with chromosome bridge and or lagging chromosome and cells with chromosome fragments.

Stickiness has been shown to be as a result of DNA condensation (Österberg et al., 1984) and entanglement of inter-chromosomal chromatin fibers which led to subchromatid connections between chromosomes (Patil and Bhat, 1992). Levan (1938) described colchicine mitosis (c-metaphase or c-anaphase) as an inactivation of the spindle followed by a random scattering of the condensed chromosomes in the cell. According to Yildiz and Arikan (2008), a large number of laggard chromosomes and C-anaphases indicate a test compound acted as a potent spindle inhibitor. The induction of vagrant chromosomes according to Elghamery et al. (2003), leads to the separation of unequal number of chromosomes in the daughter nuclei and subsequently formation of daughter cells with unequal sized or irregularly shaped nuclei at interphase. The induction of anaphase/telophase bridges has been attributed to chromosome breaks, stickiness and breakage and reunion of the broken ends (Parry et al., 1985; Badr et al., 1992).

6. Conclusion

Of the three concentrations (mg mL^{-1}) (0.04, 0.08 and 0.16) of each pesticide tested, the 0.08 mg mL^{-1} of Aphicide Plus®, 0.04 mg/mL of Eco Fruit-fly Bait GF120® and 0.08 and 0.16 mg mL^{-1} of Rosecare 3® induced significant change in (P+M)/(A+T) ratio, ($p > 0.05$). All three concentrations of each pesticide significantly depressed the mitotic index (MI) and were adjudged cytotoxic ($P < 0.05$). Each concentration of the three pesticides tested, induced genotoxicity ($P < 0.05$). The aberrations induced were, sticky chromosomes, c-metaphase, dislocated chromosomes, Chromosome bridges, lagging chromosomes and chromosome fragments. Whereas genotoxicity tests of some of the pure compounds yielded mixed results in different test systems, the three concentrations of the three pesticide formulations were cytotoxic and genotoxic in the *A. cepa* toot tip meristem assay being reported. Because most adverse health effects by genotoxic agents are the result of genetic damage and any genetic activity of chemicals is most likely to result from cell division abnormalities, these genotoxic pesticides have potential to cause adverse environmental and health effects.

Conflict of interest

The authors declare that they have no conflict of interest

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