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Assessment of cytogenotoxicity of the insecticide indoxacarb by *Allium cepa* assay

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Abstract
Pesticides, especially insecticides, are sprayed on crop plants to prevent their destruction by insects and other pests. Indoxacarb is one such insecticide that is regarded as a low-risk pesticide and used on cultivated plants including many fruits and vegetables. In this study indoxacarb was evaluated for cytotoxicity and genotoxicity by the *Allium cepa* assay. Four dilutions of the recommended dose of indoxacarb were tested on three sets of *A. cepa* bulbs which were allowed to undergo rooting in the solution of indoxacarb. Lowering of mitotic index of the cells of the root apical meristem relative to control was the parameter used to assess cytotoxicity while induction of chromosomal aberrations was used as an indicator of genotoxicity of indoxacarb. Various types of chromosomal aberrations were noticed in the cells of the roots treated with indoxacarb such as disturbed prophase, sticky chromosomes, c-metaphase, irregular metaphase, irregular anaphase, multipolar anaphase, chromosomal bridge, and vagrant chromosomes. The study clearly demonstrates that all the tested dilutions of indoxacarb are cytogenotoxic and thus, its use on plants poses a grave risk.

Keywords: *Allium cepa* roots, indoxacarb, mitotic index, chromosomal aberrations, cytotoxicity, genotoxicity

Introduction
Crop plants are susceptible to damage caused by pests and pathogens which can lead to substantial loss of yield. To protect the plants and increase their productivity several pesticides are routinely used in agriculture. Insecticides are the most commonly used pesticides which are usually sprayed on the foliage and aerial parts of plants to destroy insect pests. Besides this, they are also employed in the control of domestic pests such as mice, rats and mosquitoes (Wojciechowska 2016) [1]. However, the insecticides due to their bioaccumulation property can be potentially harmful to humans and other organisms. They persist in the environment in the soil, and via volatilization, spray drift, leaching and agricultural runoff, they can be transferred to non-target plants (Tudi *et al* 2021) [2]. Pesticide residue found in vegetables and fruits poses a great risk to the health of humans (Ferreti *et al* 2007) [3].

The carcinogenic/mutagenic effect of several pesticides in animal models is well documented (Ferreti *et al* 2007, IARC 1991) [3, 4]. Prenatal exposure to pesticides has been reported to be carcinogenic while continuous low level pesticide exposure can lead to birth defects (Ferreti *et al* 2007) [3]. Many pesticides are known to cause neurodegenerative diseases (Singh & Gautam 2021) [5]. In this study, the effect of indoxacarb, an insecticide that is readily available commercially at the local seed outlets, was studied through the use of the *Allium cepa* assay. Indoxacarb is a chemical belonging to the oxadiazine family and is utilized to control the larvae of lepidopteran insect pests. The insecticide enters through the stomach and contact routes, and acts by blocking the sodium channels in the nervous system of the insect (US EPA - Pesticides - Fact Sheet for Indoxacarb, 2000). Indoxacarb is designated by the EPA to be a “reduced-risk” pesticide that can be used as an organophosphate substitute and “is stated to have low acute and chronic toxicity and does not cause mutagenic, carcinogenic, developmental, or reproductive effects” (US EPA - Pesticides - Fact Sheet for Indoxacarb, 2000) [6]. The *A. cepa* assay was devised by Levan [7] and is an inexpensive, sensitive and rapid test which gives reliable results that are comparable to those obtained in mammalian and other eukaryotic models (Leme & Martin-Morales 2009, Macar 2020, Öztürk *et al* 2020, Srivastava & Singh 2020) [8-11]. The assay is used for screening chemical compounds for their toxic effect on mitotic division and chromosomes in meristematic cells of *A. cepa* roots (Grant 1982, Fiskesjö & Levan 1993, Rank 2003, Trushin *et al* 2013, Timothy *et al* 2014, Palmieri *et al* 2016, Macar *et al* 2019) [12-18]. Substances which cause chromosomal aberrations in plant assays can likely cause damage to chromosomes of other organisms too (Camilo-Cotrim *et al* 2022) [19]. The *A. cepa* assay has been used to investigate the cytogenotoxicity of several pesticides/insecticides including deltamethrin (Chauhan *et al* 1986) [20], malathion (Gogoi *et al* 2016) [21], imidacloprid (Fioresi *et al* 2020) [22], pyriproxyfen (Karaismailoğlu 2016) [23] and methiocarb (Tütüncü *et al* 2018, Viteri & Linares-Ramírez 2022) [24, 25].

The aim of this study was to investigate the effect of indoxacarb on *A. cepa* by studying root length as a growth parameter and, mitotic index (MI) and chromosomal aberration frequency (CA) as indicators of cytotoxicity and genotoxicity, respectively.

Materials and Methods
Preparation of indoxacarb solutions
A 7 mL/10 L stock solution (aqueous) of indoxacarb 14.5% SC (suspension concentrate) was prepared as per the recommended dose for chickpea pest management (Pulses

Research Station Portal JAU) [26]. The stock solution was diluted with distilled water to obtain 1:1, 1:2, 1:4 and 1:8 dilutions of indoxacarb volume/volume, respectively.

Experimental setup

Healthy medium sized bulbs of *Allium cepa* L. were taken and their root primordia exposed by removing the older roots and basal tissue with the help of a blade. For rooting, the *A. cepa* bulbs were placed on 100 mL beakers containing different dilutions of insecticide solution such that the base of the onion was in contact with the solution. The beakers with the onion bulbs were kept in a plant growth chamber maintained at 26ºC, 60% RH and a daily cycle of 16h light and 8h dark periods. The solution was replenished in the beakers every day to compensate for evaporation. Three replicates of the experiment were set up. Roots from the unexposed bulbs (kept on beakers containing distilled water) served as the control in all the sets. On the sixth day, randomly selected roots from each bulb were excised and the lengths of 10 roots from each bulb for each treatment were measured. Thereafter, roots were fixed for 24 h in acetic alcohol (45% acetic acid: ethanol, 1:3) fixative. After fixation the root tips were transferred into vials containing 70% ethanol and kept in the refrigerator for cytogenetic studies.

Cytogenotoxicity studies

Root tips were softened by treating with a solution containing 1N HCl: 45% acetic acid (3:1). Temporary squash preparations of root tips were then made on microslides by staining either with 2% acetocarmine or 2% acetoorcein. The slides were scanned under the microscope at 400X magnification. Ten random optical fields were observed for each treatment to score MI and CA, the parameters used to evaluate the cytogenotoxicity of indoxacarb. MI and CA were calculated as given below:

$$\text{MI} = \frac{\text{number of dividing cells}}{\text{total number of cells}}$$

$$\text{CA} = \frac{\text{number of cells with chromosomal aberrations}}{\text{number of dividing cells}}$$

Statistical analysis

One-way analysis of variance (ANOVA) was performed with Tukey's post hoc multiple comparison tests at significance level $p < 0.05$ to compare the means for MI and CA values. Statistical analysis was carried out with IBM SPSS Statistics-21 and the graphs were plotted with the help of MS Excel software.

Results

In the present investigation root length, mitotic index and chromosomal aberration frequency were the parameters used to study inhibition of root growth, cytotoxicity and genotoxicity, respectively, caused by indoxacarb treatment in *A. cepa*. Following treatment with indoxacarb, the root length was found to decrease with increase in concentration of the insecticide (Table 1, Fig. 1).

Table 1: Average values of root length, and mitotic index and chromosomal frequency of root tip cells in *A. cepa* after treatment with different dilutions of indoxacarb.

Dilution of indoxacarb	Average root length (cm)	Mean MI ± SE	Mean CA ± SE
0 (Control)	8.210	0.04340 ± 0.005887	0.00000
1:1	3.395	0.01860 ± 0.004285[a]	0.55000 ± 0.059161[b]
1:2	5.055	0.01740 ± 0.002561[a]	0.58600 ± 0.062976[b]
1:4	5.990	0.01800 ± 0.003391[a]	0.40400 ± 0.073932[b]
1:8	6.505	0.02540 ± 0.003530[a]	0.23600 ± 0.049457[b]

[a, b] Values are significantly different from control at $p < 0.05$

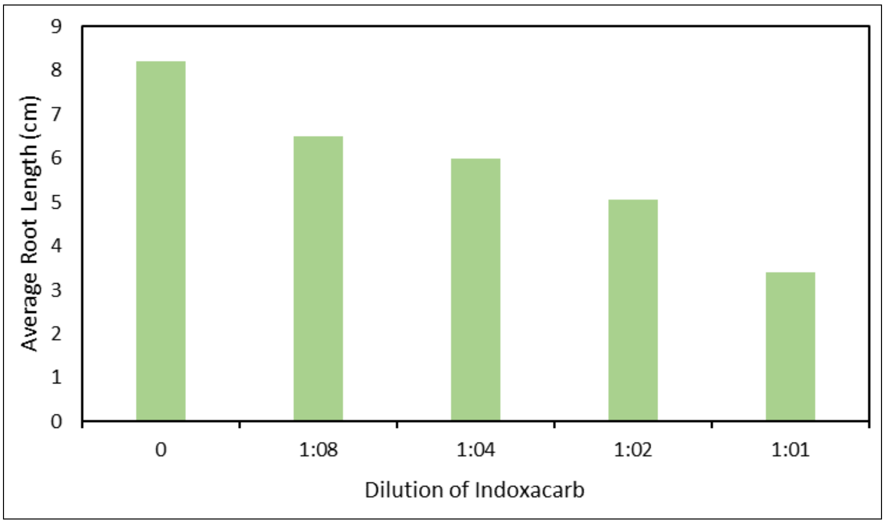

Fig 1: Effect of different dilutions of indoxacarb on average root length of *A. cepa*.

The mean MI values recorded for root tip cells of *A. cepa* are written in Table 1 as the mean MI ± Standard Error (SE). The mean MI values for 1:1, 1:2, 1:4 and 1:8 dilution of indoxacarb were 0.019, 0.017, 0.018 and 0.025, respectively as compared to the value for untreated control which was 0.043 (Table 1). Thus, the mean MI was found to be much lower than control for the dilutions of indoxacarb used. The difference between mean MI for all indoxacarb dilutions and control was significant at $P< 0.05$ (Fig. 2). Though a decrease in mean MI was observed relative to that for control, the difference between means for different dilutions of indoxacarb was not significant.

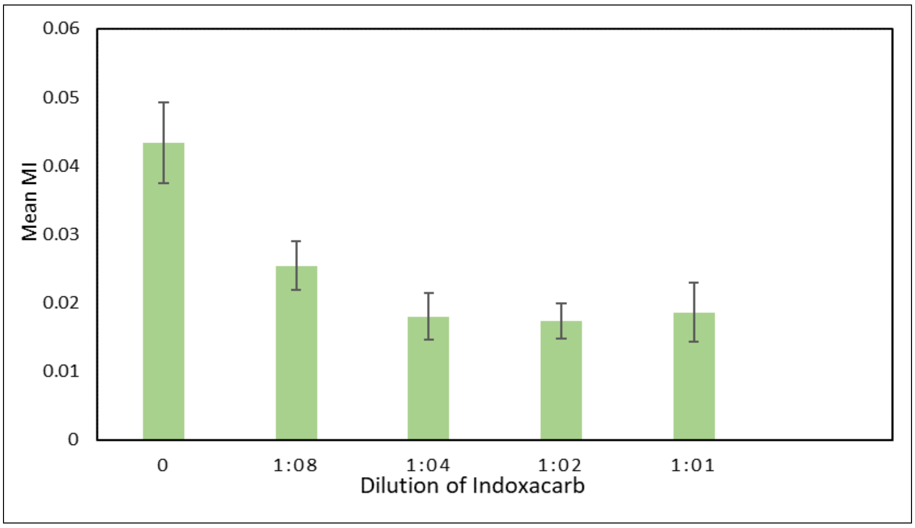

Fig 2: Effect of different dilutions of indoxacarb on MI of root tip cells of *A. cepa*. Standard error of the means is denoted by error bars.

The untreated roots showed completely normal mitotic division stages (Fig. 3). But the meristematic cells of the roots treated with indoxacarb showed several types of chromosomal aberrations. CA was used as a measure of genotoxicity of indoxacarb. The mean CA value for control was zero while the values for 1:1, 1:2, 1:4 and 1:8 dilution of indoxacarb were 0.55, 0.586, 0.404 and 0.236, respectively. These values were significant at P< 0.05 (Fig. 4).

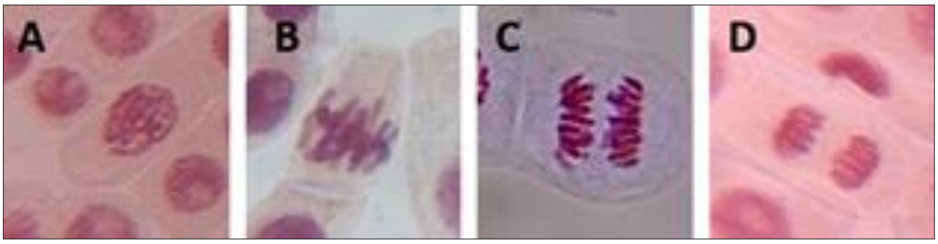

Fig 3: Stages of normal mitosis seen in the meristematic cells of untreated root tips of *A. cepa.* A. Prophase, B. Metaphase, C. Anaphase and D. Telophase (images at 400X).

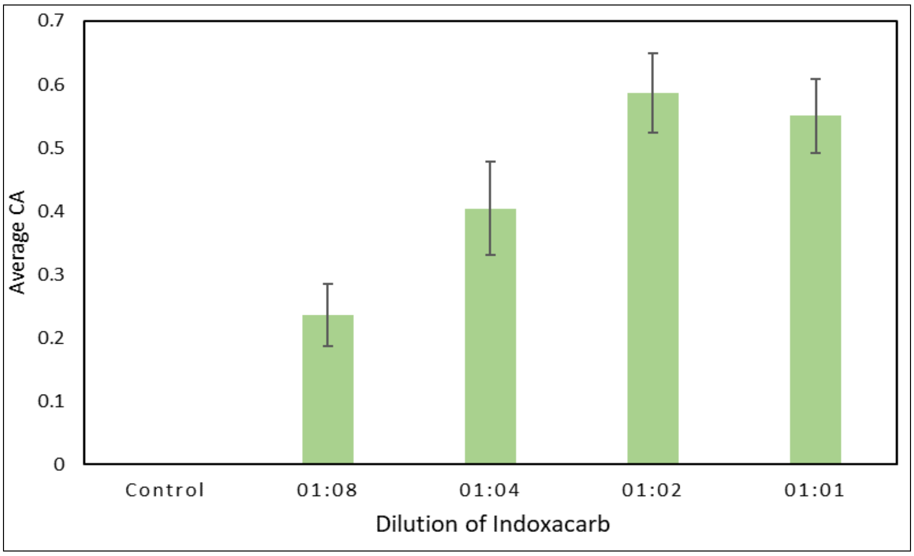

Fig 4: Effect of different dilutions of indoxacarb on CA of root tip cells of *A. cepa*.

Standard error of the means is denoted by error bars.

The type of chromosomal aberrations induced by indoxacarb included disturbed prophase, stickiness of chromosomes, c-metaphase, irregular metaphase and anaphase, multipolar anaphase and vagrant chromosomes (Fig. 5). The higher dilutions of indoxacarb (1:8, 1:4) resulted in disturbed prophase, sticky chromosomes, c-metaphase and irregular metaphase. The more concentrated solution of indoxacarb, 1:2 dilution, also caused irregular anaphase and multipolar anaphase while following treatment with the least dilution of indoxacarb, 1:1, vagrant chromosomes and chromosomal bridge were observed besides the above-mentioned chromosomal aberrations. The various chromosomal aberrations seen in different dilutions of indoxacarb are summarised in Table 2.

Table 2: Types of chromosomal aberrations observed in root tip cells of *A. cepa* after treatment with different dilutions of indoxacarb.

S. No.	Dilution of indoxacarb	Chromosomal aberrations
1.	Control	None
2.	1:8	Disturbed prophase, sticky chromosomes, c- metaphase, irregular metaphase
3.	1:4	Disturbed prophase, sticky chromosomes, c- metaphase, irregular metaphase
4.	1:2	Disturbed prophase, sticky chromosomes, c- metaphase, irregular metaphase, irregular anaphase, multipolar anaphase
5.	1:1	Disturbed prophase, sticky chromosomes, c- metaphase, irregular metaphase, irregular anaphase, multipolar anaphase, vagrant chromosomes, chromosomal bridge

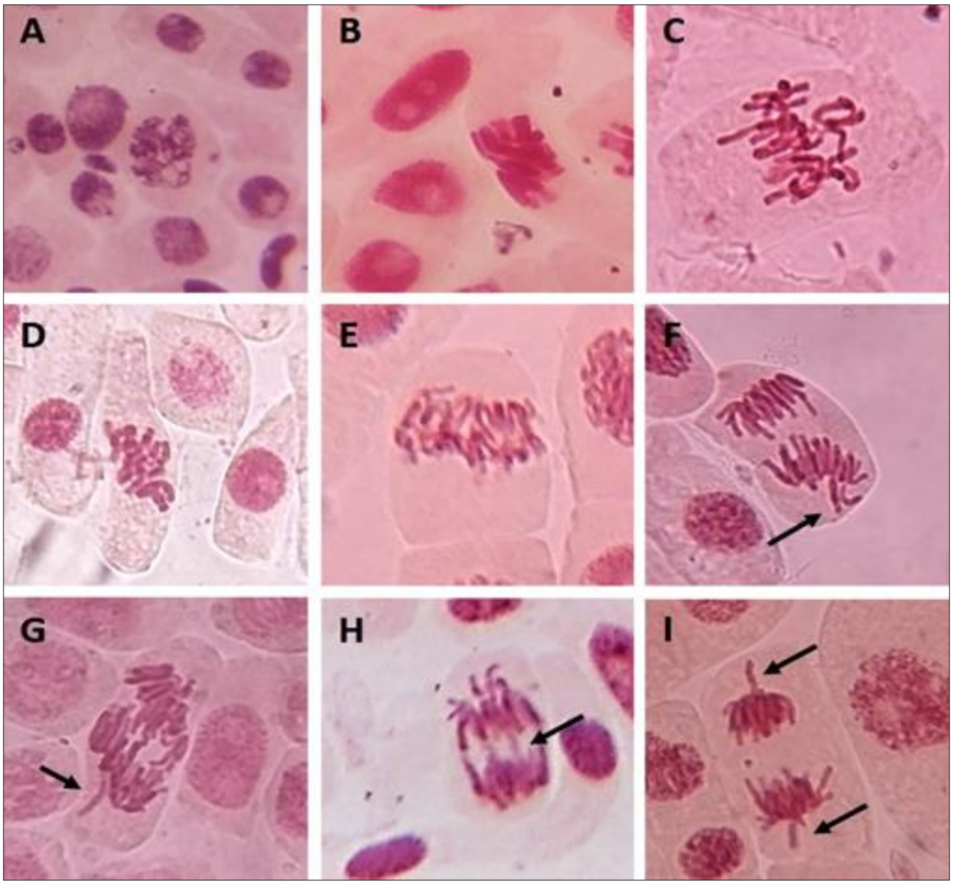

Fig 5: Meristematic cells of roots of *A. cepa* showing types of chromosomal aberrations seen after treatment of roots with different dilutions of indoxacarb. A. disturbed prophase, 1:1; B. sticky chromosomes, 1:8; C. irregular metaphase, 1:1; D. c-metaphase,1:2; E. irregular anaphase, 1:1; F and G. multipolar anaphase with vagrant chromosomes, 1:8 and 1:4; H. chromosomal bridge at anaphase, 1:4; and vagrant chromosomes at late anaphase, 1:2 (images at 400X).

Discussion

The inhibition of longitudinal growth of roots of *A. cepa* caused by different dilutions of indoxacarb tested is indicative of its toxic effect on the cells in the elongation zone of the roots. Root growth could possibly be inhibited by defective activity of the root apical meristem, slowing down of cell elongation or disappearance of cell wall during differentiation (Ciğerci *et al* 2023) [27].

MI and CA were the parameters used to screen different dilutions of indoxacarb for its cytotoxic and genotoxic effects. All dilutions of indoxacarb tested in the present investigation were found to lower the MI and induce chromosomal aberrations. Decrease in MI and induction of chromosomal aberrations has been considered to be an indicator of cytogenotoxicity of pesticides by several investigators (Chauhan *et al* 1986, Gogoi *et al* 2016, Fioresi *et al* 2020, Karaismailoğlu 2016, Tütüncü *et al* 2019, Ciğerci *et al* 2023, Ahmed 2014, Pandir 2018, Akyil 2021, Das *et al* 2017) [20-24, 27-31]. The reduction in MI could possibly be the outcome of disturbance in cell cycle such as arrest of cells in G1 or G2 stage, or inhibition of synthesis of DNA during S phase of the cell cycle (Sudhakar *et al* 2001, Gupta *et al* 2018) [32, 33]. The depression in the MI could also be due to unusual deviations in the duration of the mitotic phases (Ciğerci *et al* 2023) [27]. Lowered MI has also been suggested by de Oliveira *et al*. [34] to be due to c-metaphases since c-metaphases cause arrest of cell cycle at metaphase and thus development of meristematic tissue would be less because of lack of nuclear division.

In this study various types of chromosomal aberrations including stickiness of chromosomes, c-metaphase, irregular metaphase and anaphase, multipolar anaphase, chromosomal bridge and vagrant chromosomes were observed. The incidence of chromosomal aberrations, especially c-metaphase and multipolar anaphase, is suggestive of disruption of the spindle (Chauhan *et al* 1986, Ahmed 2014) [20, 28]. Multipolar anaphase may also be attributed to organization of multipolar spindle and in the following cell cycles it could eventually lead to aneuploidy and cell death (Vitre *et al* 2020) [35]. Spindle fibre dysfunction could be the reason for irregular anaphase or failure of chromosomes to move towards the poles which in turn reflects the disruption of microtubules by the insecticide (Singh & Roy 2017) [36]. The chromosome abnormality observed in all dilutions of indoxacarb was stickiness of chromosomes which might result from the failure of proper condensation of chromatin fibres causing their entanglement (McGill *et al* 1974, Klasterska *et al* 1976) [37, 38].

Breakage and fusion of chromosomes or chromatids could lead to the formation of multicentric chromosomes which become manifested as chromosome bridges during anaphase (Ahmed 2014, Fenech *et al* 2020) [28, 39]. Vagrant chromosomes, which move towards the pole of the cell ahead of the rest of the chromosomes during anaphase, are most likely the result of unequal distribution of chromosomes or non-disjunction of chromatids (Dutta *et al* 2018) [40].

Conclusion

Indoxacarb is an agricultural insecticide commonly used for protecting crop plants from their insect pests. This study clearly shows the cytotoxicity and genotoxicity of indoxacarb in the cells of the root apical meristem of *A. cepa*. Even 1:8 dilution of indoxacarb resulted in significant reduction of mitotic index in comparison to the control as well as induction of different types of chromosomal aberrations. Hence, the use of indoxacarb for protection of crops should be avoided.

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