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RESEARCH ARTICLE

CYTOTOXICITY AND GENOTOXICITY EFFECTS OF A NEEM BASED PESTICIDE, NEEMASTRA ON MERISTEMIC CELLS OF ALLIUM CEPA

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ABSTRACT

The cytotoxic and genotoxic effect of neem based pesticide Neemastra (90 % neem oil extract and 10 % other inert compounds) was studied using *Allium cepa* test model. Based on EC50 curve, different concentrations of Neemastra were taken for conducting the experiment. It was found that the biopesticide inhibits the growth of the root length of the onion roots and it is concentration as well as time dependent. Cytological assayed on the root tips showed a decrease in the mitotic index with increased in interphase stage of the cells along with increased abnormalities. Bridges and fragments were numerous indicating clastogenic effects and laggard chromosomes indicating spindle poisoning.

INTRODUCTION

Neem plant (*Azadirachta indica* A Juss, family: Meliaceae) have proved to be a potential biopesticide, where the leaves and seed extract of this plant have been showing deleterious effects on insects (Senthil-Nathan *et al.*, 2009). The leaf extract contains a large proportion of Azadirachtin ($C_{35}H_{44}O_{16}$), a steroid akin to tetranortriterpinoid, as well as other isoforms and compounds such as Azadirachtanin, Azadirachtol, Isoazadirone, Isoazadirolide, Epoxyazadiracdione, Nimocinolide, Isonimolide, Epinimbin, Nimbiene, Nimocinol, Nimbandiole, Melianin A and B, Melianone and many other unidentified constituents (Rastogi and Mehrotra, 1993; 1995). The active principle alone is seldom as effective as the whole extract and therefore commercial preparations of neem-leaf extract are used rather than the isolated Azadirachtin. Pesticides based on neem extracts have many effects on insects and pests and it has been used, particularly as an anti-feedant, anti-attractant, or repellant (Sharma and Dhiman, 1993), as an ecdysone inhibitor (Warbic *et al.*, 1993) and as oviposition deterrent and sterilant (Schmidt and Pesel, 1987). Moreover, the products of neem in different formulations are being extensively used for their supposedly non pollutant and environmentally friendly nature, and are also being used for the treatment of a large number of diseases (Van Der Nat *et al.*, 1991). However, it was reported that this biopesticide of indigeneous medicinal importance has been found to be genotoxic in both somatic (Awasthy et al., 1995) and germinal (Awasthy and Chaurasia, 1995) cels in murine in vivo systems.

The *Allium cepa* assay is an efficient test for chemical screening and *in situ* monitoring for genotoxicity of environmental contaminants. The test has been widely used to study genotoxicity of many pesticides revealing that these compounds can induce chromosomal aberrations as chromosomal breaks and exchanges in root meristems of *A. cepa* (Rank and Nielsen, 1997; Feretti *et al.*, 2007). Since neem oil is use as herbal pesticide under the product name Neemastra in India, it becomes important to study the side effects of this pesticide in plants which are the non target sites of the pesticide.

Keeping the above information in mind, the present study was taken to assess the possibility of inhibition of cell division (toxicity) and chromosomal aberration (genotoxicity) by a neem based pesticide under the name Neemastra (90 % neem oil extract and other inert compounds) in Allium cepa root tips.

MATERIALS AND METHODS

Onion bulbs of nearly equal weight (10-20g) were used and adventitious roots were obtained by placing the base of the bulbs in separate conical flasks filled with filtered water kept at a temperature of 25 ± 0.5 ºC. Control bulbs were incubated in filtered water. A Commercial neem oil pesticide (Neemastra containing 90 % neem oil extract and 10 % inert compounds, Swaroop Agrochemical Industries) was purchased from a local dealer. The pesticide was diluted with filtered water at different concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 % for experiments. In order to determine the concentrations necessary for the genotoxicity test, root growth was monitored over 96 h. Between 20 and 30 roots were used at each concentration of Neemastra, and the average of root lengths were measured and used for calculation of percentage growth of exposed roots with respect to the control. These values were used to produce a dose–response curve. The length of the roots of both controlled and treated bulbs were measured periodically for 24 h, 48 h, 72 h and 96 h. After exposure, the roots of each of the controlled and treated bulbs were cut and fixed in ethanol–acetic acid (3:1 v ⁄ v) at 4 ºC for 24 h, then stained with acetocarmine (Sisco Research Laboratories Pvt Ltd, Mumbai) and placed on clean slides. Individually, the

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darkly stained meristem portion was cut with a razor blade and squash preparations of eight to ten roots were examined using 100 X magnification Olympus Image analyser, for each concentration. The frequency of aberrant cells was determined on the basis of the total number of computed cells and the number of dividing cells. The mitotic index (MI) was 1) the mitotic index (MI) was evaluated from 1000 cells per root, using the formula:

MI = (total cells in mitosis) / (total cells counted) x 100

Statistical analysis

The data obtained from different experiments in replicates, were analyzed using suitable statistical methods.

RESULTS

Root growth measurement

The effect of different concentrations of neem based pesticide on longitudinal growth roots was analysed (fig 1 and table 1). 1% neem based pesticide arrested growth after 24 h, without apparent root death. At concentrations of 0.4, 0.6, 0.8 and 1.0, %, the root growth was reduced compared to control roots. These findings indicate that neem based pesticide causes inhibition of root growth in a concentration dependent manner. Further, inhibition of root growth by neem based pesticide is time dependent, because longer the roots were exposed to different concentrations of pesticide more is the inhibition (fig 1 and table 1). Complete inhibition was observed after 72 h of exposure to the pesticide.

Effect on the Mitotic Index (MI)

Results revealed that there was a decrease in the MI of exposed roots and the decrease was significantly dependent on the concentration and the time of treatment. Fig 2 showing a graph

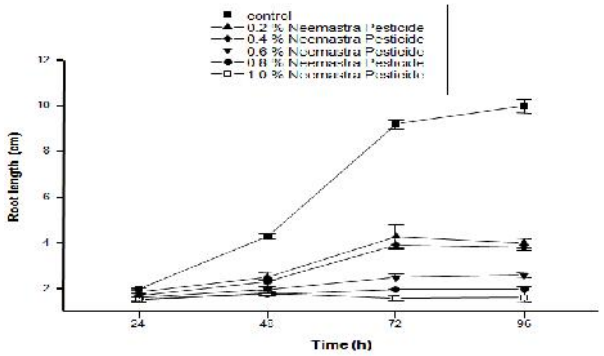

Fig 1 Effect of Neemastra pesticide in the length (cm) of the roots of *Allium cepa* kept in different concentrations for 24 h, 48 h,72 h and 96 h. Data represent mean values ± S.E.M; n=20.

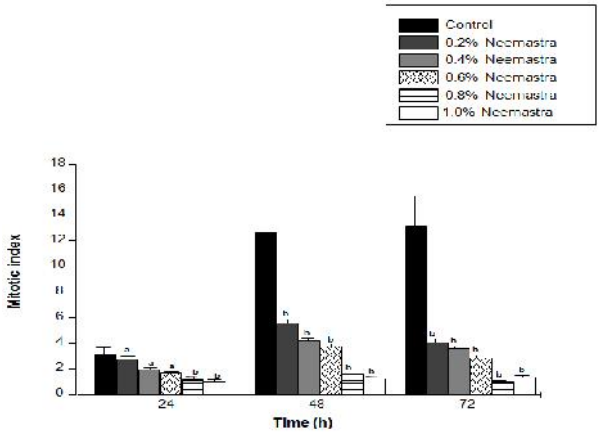

Fig 2 Histogram showing the effect of Neemastra pesticide on mitotic index of the meristem cells of *Allium cepa* kept in different concentrations for 24 h, 48 h and 72 h with respect to the controls. Data represent mean values ± S.E.M; n=10.
[a, b] *P* values significant at < 0.05, 0.01 levels respectively, compared to controls (Student's t test).

Table 1 Changes in the length (cm) of the roots of *Allium cepa* kept in different concentrations (%) of neem based pesticide, Neemastra for 24 h , 48 h ,72 and 96 h .Data represent mean values ± S.E.M; n=20.

Exposure Period	Neemastra pesticide					
	Control	0.2%	0.4%	0.6%	0.8%	1.0%
24h	2.0 ± 0.04	1.7± 0.12 [b]	1.6±0.10 [b]	1.6 ± 0.05	1.5 ± 0.06 [b]	1.5 ± 0.06 [b]
48h	4.2± 0.15	2.2 ± 0.11[b]	2.0± 0.12 [b]	1.7 ± 0.05[c]	1.75 ± 0.12 [c]	1.75 ± 0.20[c]
72h	9.3 ± 1.7	4.2 ± 0.15[c]	3.9± 0.21 [b]	2.2 ± 0.15[c]	1.9 ± 0.01 [c]	1.5 ± 0.10[c]
96h	10.1± 1.2	3.9 ± 0.12[b]	3.75±0.10 [b]	2.3 ± 0.12 [c]	1.95 ± 0.02 [b]	1.5 ± 0.08 [b]

[b, c]: *P* values significant at < 0.01 and < 0.001 levels respectively, compared to controls (Two-way ANOVA test).

Table.2 Frequency of chromosomal abnormalities in *Allium cepa* root meristem cells.

Neemastra pesticide		Mitotic index	Total abnormalities (%)	Chromosomal abnormalities (%)		
				Lagging and Fragments	Abnormal metaphase Anaphase	sticky bridges in
Control	24h	3.2 ± 0.5	0.9 ± 0.1	0.3 ± 0.01	0.2 ± 0.003	0.4 ± 0.01
	48h	12.7 ± 1.0	0.75 ± 0.1	0.2 ± 0.01	0.23 ± 0.01	0.32 ± 0.01
	72h	13.2 ± 2.3	0.96 ± 0.1	0.4 ± 0.01	0.3 ± 0.002	0.26 ± 0.002
0.2(%)	24h	2.8 ± 0.2[a]	1.76 ± 0.2[a]	0.81± 0.02[a]	0.45 ± 0.02[a]	0.5 ± 0.01[a]
	48h	5.6 ± 0.3[b]	6.9 ± 0.05[b]	1.70 ±.0.01[a]	1.3 ± 0.02[a]	0.39 ± 0.5[a]
	72h	4.1 ± 0.3[b]	8.31 ± 2.7[b]	2.05± 0.02[b]	1.6 ± 0.03[a]	0. 45 ± 0.2[a]
0.4 %	24h	2.0 ± 0.12[a]	1.90 ± 0.2[a]	1.07± 0.01[a]	0.53 ± 0.02[a]	0.54 ± 0.01[a]
	48h	4.2± 0.23[b]	7.2 ± 0.3[b]	1.87 ±.0.01[a]	1.38 ± 0.01[a]	0.49 ± 0.05[a]
	72h	3.6 ± 0.20[b]	9.2 ± 1.7[b]	1.51± 0.02[b]	1.0 ± 0.03[a]	0.51 ± 0.02[a]
0.6 %	24h	1.73 ±0.10[a]	1.50 ± 0.10[a]	1.14± 0.01[a]	0.66 ± 0.02[a]	0.48± 0.01[a]
	48h	3.8 ± 0.13[b]	7.7 ± 0.2[b]	1.96 ±.0.02[a]	1.43 ± 0.01[a]	0.53 ± 0.05[a]
	72h	2.9± 0.15[b]	9.64 ± 1.0[b]	1.98± 0.02[b]	1.41 ± 0.03[a]	0.57 ± 0.02[a]
0. 8%	24h	1.2 ± 0.2[b]	1.33 ± 0.3[b]	2.13± 0.02[a]	1.43 ± 0.05[a]	0.7 ± 0.03[a]
	48h	1.6 ± 0.1[b]	7.67 ± 0.8[b]	4.97 ± 0.04[a]	2.17 ± 0.04[a]	2.8 ± 0.04[a]
	72h	1.0 ± 0.1[b]	9.4 ± 1.2[a]	3.9 ± 0.03[a]	2.2 ± 0.06[a]	1.7 ± 0.03[a]
1.0 %	24h	1.0 ± 0.2[b]	1.0 ± 0.2[b]	2.65± 0.03[a]	1.95 ± 0.05[a]	0.7 ± 0.03[a]
	48h	1.3 ± 0.1[b]	4.8 ± 0.4[b]	3.85 ± 0.04[a]	1.98 ± 0.04[a]	1.87 ± 0.04[a]
	72h	1.38 ± 0.1[b]	4.4 ± 1.2[a]	4.03 ± 0.02[a]	2.32 ± 0.01[a]	1.71 ± 0.03[a]

[a, b] *P* values significant at < 0.05, 0.01 levels respectively, compared to controls (Student's t test). Data represent mean values ± S.E.M; n=10

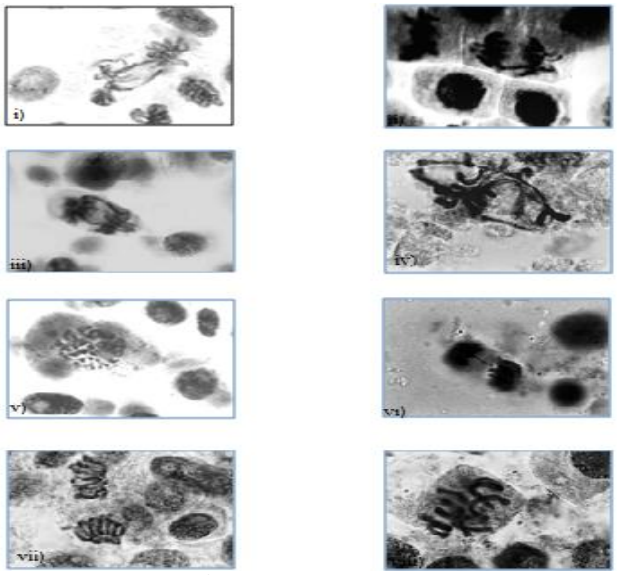

Fig 3 Development of various chromosomal aberrations in the root meristem stem cells of *A. cepa* after treatment with different concentrations of neem based biopesticide, Neemastra. Chromosomal aberrations like sticky bridges in anaphase, distorted spindle apparatus, loss of chromosomal segment during metaphase were observed using 100x magnification olympus image analyser.

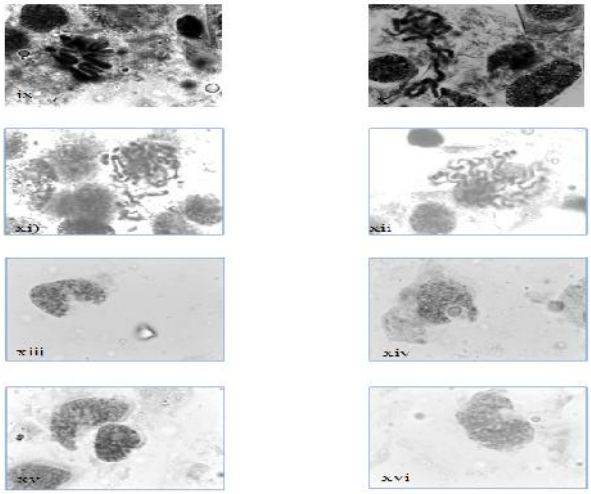

Fig 4 Development of various chromosomal aberrations in the root meristem stem cells of *A. cepa* after treatment with different concentrations of of neem based biopesticide, Neemastra. Chromosomal aberrations like fragmentation, dispersed metaphase and cell rupture were observed using 100x magnification olympus image analyser.

plotted between mitotic index of exposed roots at different concentrations and time clearly revealed the effect of Neemastra on the mitotic index. With respect to the time of exposure of 24 h, 48 h, and 72 h, the MI of control roots were 3.2, 12.7 and13.2. As shown in table 2, there was a significant reduction of MI in the roots treated with different concentrations (0.2%, 0.4%, 0.6%, 0.8% and 1%) of the biopesticide with respect to time of exposure of 24 h, 48 h, and 72 h. It was further observed that at a concentration of 1% of the biopesticide, the cell division was found to be arrested immediately after 24 h as there was no change in the MI after 48 h and 72 h of exposure.

Chromosomal aberrations

Results of the microscopic analysis of the treated *Allium cepa* root tips are summarised in table 2. Figure 3 and 4 show that chromosomal aberrations were induced at all the tested concentrations and were statistically significant (fig 2). Various types of chromosomal aberrations, such as chromosomal fragments, bridges, stickiness and dispersed metaphase were recorded indicating the genotoxicity of the Neemastra pesticide. Chromosomes with disturbed spindles and fragments were also present in appreciable amounts. Bridging of the chromosomes was quite common at anaphase stage (fig 3 and table 2). Vacuolization of the cytoplasm and rupture of cell membranes were seen in many cells following treatment at higher concentrations (1% Neemastra) as shown in fig 4.

DISCUSSION

Our findings suggest that neem oil inhibits longitudinal growth of the roots and the inhibition was time dependent as it was observed that with increase time exposure there was a delay in the growth of the roots. This probably indicates the cytotoxicity of the biopesticide on *Allium cepa* where cell division of the meristemic cells of the root tips was slowed down. Further, the result indicates that Neemastra causes inhibition of root growth in a concentration-dependent manner as it was observed that with an increased concentration of the pesticide, the growth rate of the root tips decreased significantly. In our study, the inhibitory effect of neem oil pesticide was correlated with the mitotic index (MI). It was noted that MI in the control was much higher than the treated ones. Thus, neem based pesticide mediated reduction in the MI may result in an inhibition in root growth with increased cell numbers in the interphase. It was reported that the cytotoxic effect of neem oil is believed to be affecting spindle protein or chromosome packaging (Awasthy *et al.*, 1995), which can be the same in this case. Chromosomal abnormalities increased about many folds in the cells of treated root tips (table 2) which could be partly responsible for the reduction in the MI and retardation in root growth. Chromosomal aberrations explained as genotoxicity are normally accompanied by some growth restrictions (Fiskesjo, 2007). In *A. cepa*, whenever chromosome aberrations occurred, there were always certain growth restrictions as was observed in this experiment. Also frequent were the bridges and fragments: such anomalies (i.e. the induction of chromosomal fragments and bridges at anaphase) give an indication of mutagenic events in the cell (Mishra, 1993).

It is clearly evident from our study that changes in the MI and chromosomal aberrations in the meristem are inversely related (table 2). The genotoxicity of neem oil has been reported in the testes of mice (Awasthy *et al.*, 1995) although chromosomal abnormalities have not been calculated. High incidence of synaptic disturbances and numerical variations in chromosomes confirms the cytotoxicity of the extract which is in full agreement with the observations made in mitotic chromosomes (Awasthy *et al.*, 1995; Awasthy *et al.*, 1999; Khan and Awasthy, 2001).

Results from our study showed at high concentration of the pesticide there were disruption of the cell membranes along with vacuolization and nuclear pycnosis. This may be due to the fact that the neem derived pesticide might cause cell death via the activation of apoptotic pathway following significant membrane damage as reported earlier (Ilio *et al.*, 2006; Bonincontro *et al.*, 2007).

It has been reported that neem and its derivatives work as systemic insecticides; it is absorbed into the plant and carried

throughout the tissues to be ingested by insects when they feed on the plant. This may make it effective against phloem feeders like *N. lugens* (Heyde *et al.*, 1984; Senthil-Nathan *et al.*, 2007). However, though neem derived pesticides proved effective against the insect pests our study proves it to be hazardous to plants where there is deteroriation in the growth of the plant due to the cytotoxic and genotoxic effects of neem derived pesticide.

Azadirachtin which is one of the main components of the neem plant, based upon its computer automated structure evaluation (Rosenkranz and Klopman, 1995a), is supposed to act as a genotoxic carcinogen due to the presence of furan moiety having the biophore O-CH= which incidentally occurs in many known genotoxic carcinogens including aflatoxins. Most of the toxic chemicals that produce genotoxic effects have been known to form reactive oxygen species as well as electrophilic free-radical metabolites that interact with DNA to cause disruptive changes.

It has been suggested that during metabolism of Azadirachtin, electrophillic ions and radicals are produced, interacting with nucleophillic sites in DNA and leading to breaks and other related damage in the latter (Klopman *et al.*, 1985).

Moreover, enzymatic biotransformation of the leaf extract of *Azadirachtica indica* has been been suspected to produce metabolites and free oxygen radicals (Sies, 1993) in a manner similar to other xenobiotics, including damage to spindle apparatus and to cause unequal distribution of the chromosomes, leading to mitosis disruptive changes.

Raizada *et al.*, 2001 on the other hand, have reported that Azadirachtin alone has not shown any adverse effects in rats. Further, it was reported that the pure compound Azadirachtin, the unprocessed materials of neem plant, the aqueous extracts and seed oil are safe to use as an insecticide. Conversely, the non-aqueous extracts turn out to be relatively toxic, suggesting that the other compounds than azadirachtin are responsible for the toxic effect (Boeke *et al.*, 2004).

In conclusion, although neem derived pesticides like Neemastra exhibit toxicity and genotoxicity as shown in our results, further research on the actual cause is yet to be identified. However, one cannot deny the potential danger of such pesticides and cannot be ignored in view of the long term genetic hazard on the agricultural plants as well as on man.

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