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Sunlight decreased genotoxicity of azadirachtin on root tip cells of *Allium cepa* and *Eucrosia bicolor*

W. Kwankua [a,*], S. Sengsai [a], C. Kuleung [b], N. Euawong [a]

[a] Department of Biology, Faculty of Science, Silpakorn University, Nakhon Pathom 73000, Thailand
[b] Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

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ABSTRACT

Utilization of neem plant (*Azadirachta indica* A. Juss) extract for pest control in agriculture has raised concerns over contamination by the residues to the environment. Such residues, particularly azadirachtin (Aza), may cause deleterious effect to non-target organisms. This investigation was conducted to find out if Aza could be inactivated through exposures to sunlight. Activity of Aza was assessed as its ability to cause cytotoxic and genotoxic effects in the forms of nuclei abnormality and chromosome aberration as measured by mitotic index (MI) and mitotic aberration (MA). Varying concentrations of Aza were tested on *Allium cepa* and *Eucrosia bicolor*. It was found that the MI of all root tip meristematic cells of *A. cepa* and *E. bicolor* treated with 0.00005%, 0.00010%, 0.00015%, and 0.00020% (w/v) Aza-containing neem extract for 24 h, were significantly lower than the controls. Complementary to the lower levels of MI, the Aza-treated groups showed higher MA levels in all cases investigated. Furthermore, the decreasing levels of MI and the increasing levels of MA related well with the increasing concentration of Aza. Microscopic examination of root tip meristematic cells revealed that the anomaly found most often were mitotic disturbances and chromosomal bridges. Exposures of 0.00020% (w/v) Aza to sunlight for 3 days and 7 days decreased Aza ability to induce cytotoxicity and genotoxicity, both in terms of MI and MA, to root tip meristematic cells in *A. cepa* and *E. bicolor*. Photodegradation of Aza upon exposure to direct sunlight was confirmed by HPLC. The study implicates that Aza would unlikely cause long term deleterious effects to the environment since it would be inactivated by sunlight.

1. Introduction

Contamination by toxic agents in the environment, which is a consequence of overuses of synthetic pesticides and herbicides, has become a serious problem in many agricultural countries. Replacing those chemicals with nature friendly substances to reduce harm on environments, would presumably be an effective solution to the problem. Biochemical extracts from numerous plant species have been investigated for their efficacies against pests both in the field and on seed stock (Boeke et al., 2004). The extract from different parts of one plant species, the neem plant (*Azadirachta indica* A. Juss.), has become a widely used biopesticide. Neem extract contains more than 100 active chemicals, notably azadirachtin (Aza), salannin and meliantriol, which have been demonstrated to have insecticidal properties against a broad spectrum of insects (Vietmeyer, 1992). The most active secondary metabolite in neem is the triterpenoid Aza, which has a chemical structure similar to ecdysone, an insect steroid hormone involved in metamorphosis (Singh et al., 1993).

Although a neem extract has a potential to be used as an alternative biopesticide, the results of many studies showed that they also caused both positive and negative biological effects in human and non-target animals. For examples, taking unprocessed neem leaves at a given dose could render an anti-diabetic effect, while taking neem at another dose caused severe renal failure (Alam et al., 1990; Kadiri et al., 1999). Sinniah et al. (1982) reported that people who took 12 ml of neem oil for 2 days showed severe toxic symptoms. Neem kernel powder (75 and 100 mg/kg of body weight) fed to sheep showed a good anthelmintic effect against intestinal nematodes. However, sheep were adversely affected when fed with 100 g/cap of leaves (Ali and Salih, 1982; Ahmed et al., 1994). In addition, experiments in murine showed that neem leaves extract induced many deleterious effects such as abnormality of bone marrow cells, altered chromosome structure and chromosome numbers in spermatocytes, failure of chromosome synapsis during metaphase I, deformation of sperm's head, and reduction of sperm numbers (Khan and Awasthy, 2001, 2003). These undesirable effects have

* Corresponding author. Fax: +66 34 273046.
E-mail address: wimol@su.ac.th (W. Kwankua).

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raised concerns whether the use of neem-derived products as pesticides is safe. Residues left behind after the application of neem extract to crops may cause harms to people as well as to other animals in the ecosystem.

To evaluate the cytotoxicity and genotoxicity of known and unknown toxic agents, more than 200 short-term assays have been developed (Grant, 1994; Soliman, 2001; Akinboro and Bakare, 2007). Among those assays, mitotic cell division inhibition and chromosome aberration induction have been widely used as indicators of cytotoxicity and genotoxicity. Consistent with many reports, *Allium cepa* assay, which is established by the international program on chemical safety and the World Health Organization (WHO), is one of the most effective and sensitive methods for mutagent testing (Rank and Nielsen, 1994). Thailand is a country which has been actively promoting the use of neem extract as pest control. Together with that campaign there has been an environmental concern over whether the use of Thai commercial neem extract at the manufacturer's recommended dose of 0.00015–0.00025% (w/v) is toxic to plants and animals. Farmers are also interested in knowing what factors would affect the toxicity of Aza, and how long would toxicity of Aza last in the normal circumstances of use. It was found that the persistence of active metabolites in neem extract depend on many factors such as temperature during storage or application, acidic or basic condition of solution and neem extract formulation (Yakkundi et al., 1995; Stark, 1996; Kongkathip and Sombutsiri, 1996). Additionally, Javed et al. (2007) reported that neem extract could persist in soil up to 4 months. It, therefore, became an objective of this study to assess genotoxicity of Aza after exposing the chemical to sunlight for certain periods of time. Apart from using *A. cepa* assay as a standard plant for testing cytotoxicity and genotoxicity we also aimed at exploring the use of *Eucrosia bicolor* as an alternative testing plant species.

2. Materials and methods

2.1. Testing solutions

Varying concentration of Aza testing solutions, namely, 0.00005%, 0.00010%, 0.00015%, and 0.00020% (w/v) Aza were prepared from commercial 0.1% (w/v) Aza-containing neem extract stock solution. Actual concentration of Aza in the 0.00005%, 0.00010%, 0.00015%, and 0.00020% (w/v) Aza testing solutions were estimated by HPLC and found to contained Aza at the levels of 8.30, 16.61, 24.91 and 33.21 mg/L, respectively. Controls were set up similarly except that distilled water was used instead of Aza.

The study on the effect of sunlight on the activity of Aza was carried out by first exposing 0.00020% (w/v) Aza in flat-shaped bottles to direct sunlight for 3 and 7 days before the solutions were used for the study. The sunlight-unexposed 0.00020% (w/v) Aza solutions were the solutions kept in dark before being used as controls (0 day sunlight exposed).

Table 1
Phase index (‰PI) and Mitotic index (‰MI) of root tip cells treated with 0.00000%, 0.00005%, 0.00010%, 0.00015% and 0.00020% (w/v) Aza-containing neem extract for 24 h.

Plant species	Neem extract conc. (%)	Phase index (‰ PI)					Mitotic index (‰MI)
		PI-I	PI-P	PI-M	PI-A	PI-T	
A. cepa	0.00000	907 ± 2.5^{a}	46 ± 4.2^{c}	15 ± 1.8^{b}	9 ± 1.1^{b}	23 ± 2.3^{c}	92.6 ± 2.5^{c}
	0.00005	954 ± 8.2^{c}	25 ± 2.9^{ab}	6 ± 2.2^{a}	5 ± 1.4^{ab}	10 ± 3.3^{ab}	45.8 ± 8.2^{ab}
	0.00010	973 ± 13.2^{c}	14 ± 5.7^{a}	5 ± 2.7^{a}	2 ± 1.9^{a}	5 ± 2.9^{a}	26.8 ± 13.2^{a}
	0.00015	924 ± 20.0^{ab}	38 ± 12.5^{a}	13 ± 3.9^{ab}	7 ± 1.9^{ab}	18 ± 6.3^{bc}	76.2 ± 20.0^{ab}
	0.00020	951 ± 2.9^{bc}	23 ± 1.8^{a}	8 ± 1.6^{ab}	5 ± 0.9^{ab}	13 ± 1.2^{abc}	49.2 ± 2.9^{ab}
E. bicolor	0.00000	930 ± 3.3^{a}	24 ± 2.4^{b}	22 ± 0.5^{d}	13 ± 1.2^{b}	11 ± 0.6^{c}	69.8 ± 3.3^{b}
	0.00005	952 ± 4.9^{b}	15 ± 3.3^{a}	14 ± 1.9^{bc}	8 ± 1.2^{a}	11 ± 1.0^{bc}	47.2 ± 4.9^{a}
	0.00010	954 ± 2.9^{b}	16 ± 2.1^{a}	15 ± 0.8^{c}	9 ± 1.8^{a}	6 ± 0.9^{a}	45.8 ± 2.9^{a}
	0.00015	962 ± 4.7^{b}	14 ± 2.6^{a}	11 ± 1.4^{ab}	$6. \pm 1.2^{a}$	6 ± 1.0^{a}	37.4 ± 4.7^{a}
	0.00020	963 ± 0.4^{b}	12 ± 1.8^{a}	9 ± 1.2^{a}	7 ± 1.1^{a}	8 ± 0.8^{ab}	36.2 ± 0.4^{a}

PI = phase index of interphase (-I), prophase (-P), metaphase (-M), anaphase (-A) and telophase (-T).Values were expressed as ‰PI or ‰MI ± standard deviation.

[a,b,c] Values for each parameter in the same row, in each plant species, followed by the same letter are not significant different ($P < 0.05$.).

Table 2
Chromosome aberrations and Mitotic aberration index (‰MA) of root tip cells treated with 0.00000%, 0.00005%, 0.00010%, 0.00015% and 0.00020% (w/v) Aza-containing neem extract for 24 h.

Plant species	Neem extract conc. (%)	Chromosome aberration (‰)						Mitotic aberration index (‰MA)
		Bridge	Disturb	Laggard	Fragment	MN	Bi	
A. cepa	0.00000	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0^{a}
	0.00005	0.6 ± 0.4	0.2 ± 0.2^{a}	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.4	0.0 ± 0.0	1.4 ± 0.7^{a}
	0.00010	0.0 ± 0.0	0.6 ± 0.6^{a}	0.0 ± 0.0	0.2 ± 0.2	0.2 ± 0.2	0.0 ± 0.0	1.0 ± 0.4^{a}
	0.00015	0.8 ± 0.6	1.2 ± 0.6^{ab}	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2	0.2 ± 0.2	2.8 ± 1.0^{ab}
	0.00020	0.6 ± 0.4	2.8 ± 1.0^{b}	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.6	0.2 ± 0.2	4.4 ± 1.7^{b}
E. bicolor	0.00000	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.2 ± 0.2	0.4 ± 0.2^{a}
	0.00005	0.0 ± 0.0	2.8 ± 0.9^{b}	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.6 ± 0.4	3.6 ± 0.9^{b}
	0.00010	0.0 ± 0.0	3.6 ± 0.4^{b}	0.0 ± 0.0	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	4.2 ± 0.6^{b}
	0.00015	0.0 ± 0.0	2.8 ± 0.4^{b}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.58	4.0 ± 0.5^{b}
	0.00020	0.0 ± 0.0	3.0 ± 0.3^{b}	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	3.4 ± 0.5^{b}

MN = Micronucleated cells, Bi = Binucleated cells Values were expressed as ‰ (chromosome aberration) or ‰MA ± standard deviation.

[a,b,c] Values for each parameter in the same row, in each plant species, followed by the same letter are not significant different ($P < 0.05$).