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Screening for Plants Sensitive to Heavy Metals Using Cytotoxic and Genotoxic Biomarkers

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ABSTRACT

Cytotoxic and genotoxic sensitivity to heavy metals were investigated in five plant species using a mitotic index (MI) and mitotic aberration (MA) as biomarkers. The results showed that *Eucrosia bicolor* and *Allium cepa* was more sensitive to cadmium toxicity at concentrations ranging from 0.02 to 0.09 mg.L^{-1} of $CdCl_2$ than *A. cepa* var. ascolonicum, *Zephyranthes rosea* and *Wedelia triloba*. A decrease in the ‰MI and an increase in the ‰MA were found in *E. bicolor* as early as 24 hr after Cd treatment. Testing with lead (Pb), copper (Cu) and arsenic (As) showed that *E. bicolor* and *A. cepa*, to be sensitive to toxicity by Pb at the lowest dose of 0.2 mg.L^{-1} of $Pb(NO_3)_2$. Cyto-genotoxicity from Pb was also dose dependent. *E. bicolor* showed greater sensitivity than *A. cepa* in testing for Cu sensitivity. The lowest dose (1.00 mg.L^{-1}) of $CuCl_2$ caused a significant decrease in the ‰MI only in *E. bicolor*. The highest frequency of chromosome aberration type induced by Cu was different from that of Pb. *E. bicolor* was less sensitive to As cyto-genotoxicity compared with *A. cepa*. Only at 10.00 mg.L^{-1} of $NaAsO_2$ could a significant decline in the ‰MI and an increase in the ‰MA be observed in *E. bicolor*, whereas the lowest dose which affected the ‰MI and the ‰MA of *A. cepa* was 0.25 mg.L^{-1}. This is the first report which has studied and explored the potential of *E. bicolor* for use as a heavy metal cytotoxic and genotoxic bioindicator.

Keywords: *Allium cepa* test, *Eucrosia bicolor,* genotoxicity, heavy metals, mitotic index

INTRODUCTION

The widespread contamination of heavy metals in the environment has become a serious problem in many countries. Pollution from toxic metals has progressively affected living organisms in ecosystem including the top consumers, human beings. Bioaccumulation at higher doses of heavy metals, such as cadmium (Cd), lead (Pb), copper (Cu) and arsenic (As), through the food chain carries risks in many forms to humans including neurotoxicity, hepatotoxicity, nephrotoxicity, mutagenicity and carcinogenicity (Filipic and Hei, 2004; Graham-Evans *et al.*, 2004; Benavides *et al*., 2005; Depault *et al.*, 2006; Islam *et al.*, 2007). Emphasis on heavy metals toxicity has been directed to their abilities to induce free radical formation and biomethylation as well as altering gene regulation (Durham and Snow, 2006). High Cd toxicity at low concentrations was a result of its ability to dissolve easily in water (An, 2004). Toxicity of Cd to animals and other organisms has been shown by a number of investigations and it has been shown to reduce plant growth,

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photosynthesis and chlorophyll content as well as to induce oxidative stress (Degraeve, 1981; Li and Xiong, 2004; Wang and Zhou, 2005; Mishra *et al.*, 2007). In addition to the phyto-toxic effects, Cd was also shown to inhibit plant cell proliferation, to alter RNA synthesis and to induce chromosomal aberrations similar to those observed in animal cells (Rosas *et al.*, 1984; Toppi and Gabbrielli, 1999; Unyayar *et al.*, 2006). Lead is known to be toxic to various systems, for instance, the reproductive system, the liver and kidney system and also the immune system (Silbergeld *et al.*, 2000). Pb toxicity stems from its ability to bind strongly to different types of biomolecules such as amino acids, DNA and RNA, and several enzymes, thus interfering with many metabolic pathways. Arsenic shows the same trend as lead. It is an effective mitotic poison due to its ability to induce spindle disturbances (Patra *et al.*, 2004). Exposure of human leukemia cells to As led to an increase in the number of DNA single-strand breaks in a dose-dependent manner (Yedjou and Tchounwou, 2007). Cytotoxicity and genotoxicity of copper in human cells have been reported as the result of reactive oxygen species generation, oxidative DNA base modifications and DNA strand breaks (Schwerdtle *et al.*, 2007; Kashanian *et al*., 2011).

Plant bioassays have been recommended by the United Nations Environment Program (UNEP), the World Health Organization (WHO) and the US Environment Protection Agency (US-EPA), as a highly efficient system for screening of cytotoxic and genotoxic chemicals (Grant, 1999; Ma, 1999). The exploitation of plants as cyto-genotoxic bioindicators has been well recognized, such as in the *Allium* genotoxicity test or the *Allium* test, due to the use of *Allium cepa* (Levan, 1938; Kristen, 1997). It is also recognized that plants species which are easy to culture and handle, have large chromosomes and are sensitive to a wide range of toxicants, are reliable plants for using as cytotoxic and genotoxic bioindicators. This is in line with Grant (1982), who reported that *Allium cepa* (2n=16) gave a positive response to some 76% of the chemicals that were tested. Assays with *Lycopersicum esculentum* (2n=24) showed positive responses to 19 out of 21 tested chemicals (Grant and Owens, 2002). Chromosome aberrations in *Zea mays* (2n=20) were induced by at least 78 tested chemicals (Grant and Owens, 2006). In addition to the sensitivity, simplicity and efficiency in using higher plants for bioassays, there was also an excellent correlation between the plant testing system and other test systems, including the mammalian cell system (Grant, 1978; Fiskesjo, 1985; Kristen, 1997; Saxena *et al.*, 2005; Dimitrov *et al.*, 2006). Many reports showed that toxicity from heavy metals, air pollutants, many chemical compounds and physical and chemical mutagens can be assessed with micronucleus assay, chromosome aberration assay and gene mutation assay (Kristen, 1997; Kim *et al.*, 2003; Yi and Meng, 2003; Grant and Owen, 2006). In addition, different forms of anomalies can occur in plants, such as mitotic abnormality, chromosome aberration and the appearance of micronuclei, which can all be used as cytotoxic and genotoxic biomarkers. (Cotelle *et al.*, 1999; Grant and Owen, 2001; Uhl *et al.*, 2003; Fusconi *et al.*, 2006; Hala *et al*., 2007; Yildiz *et al.*, 2009).

The current study was conducted to screen for plants sensitive to heavy metals using cytotoxic and genotoxic biomarkers. In addition to the common onion (*Alllium cepa*) and shallot (*Allium cepa* var. ascolonicum), screening used three other plant species—namely, Peruvian lily (*Eucrosia bicolor*), rain flower (*Zephyranthes rosea*), and climbing wedelia (*Wedelia triloba*)—by treating the plants with various types of heavy metals. The frequency of mitotic cell division and chromosome aberration were subsequently examined. The sensitive plants selected from this experiment were explored for their potential as bioindicators for assessing heavy metals contamination in an ecosystem.

MATERIALS AND METHODS

Plant materials

Heavy metal sensitivity was investigated in five plant species—namely, common onion (*Allium cepa*) which has been well-accepted as a common testing plant for genotoxic assay, shallot (*Allium cepa* var. ascolonicum), Peruvian lily (*Eucrosia bicolor*), rain flower (*Zephyranthes rosea*), and shoots of climbing wedelia (*Wedelia triloba*). All plants were obtained from the Silpakorn University greenhouse.

Testing chemicals

Four types of heavy metals in the form of solutions of cadmium chloride ($CdCl_2$. $2.5H_2O$, Sigma Aldrich Chemical Co.) at 0.00, 0.02, 0.03, 0.06 and 0.09 $mg.L^{-1}$, sodium arsenite ($NaAsO_2$, Fluka Chemical) at 0.00, 0.25, 1.00 and 10.00 $mg.L^{-1}$, lead nitrate ($Pb(NO_3)_2$, Sigma Aldrich Chemical Co.) at 0.00, 0.20, 25.00 and 200.00 $mg.L^{-1}$, and copper chloride ($CuCl_2$, Univar USA Inc.) at 0.00, 1.00, 2.00 and 3.00 $mg.L^{-1}$, were prepared in distilled water. The doses of the testing chemicals were designed to cover the range from low permissible concentrations to high nonpermissible concentrations. These high nonpermissible concentrations were based on the industrial standards for effluent (Thai Ministry of Science, Technology and Environment, 1996).

Screening for heavy-metal-sensitive plants using mitotic and chromosome aberration assay

The heavy-metal-sensitive plant species were initially screened using a strong heavy metal mutagen, Cd (Gomes *et al.*, 2008). Decreases in mitotic cell division and increases in chromosome aberration were examined and used as cytotoxic and genotoxic biomarkers. At least five healthy bulbs, without older outer scales and existing roots, of *A. cepa*, *A. cepa* var. ascolonicum, *E. bicolor*, *Z. rosea* , and five shoots of *W. triloba* were placed in small bottles with their basal ends dipped in distilled water and kept at room temperature. When the newly emerged roots were 1-2 cm in length, they were treated with different concentrations of cadmium (0, 0.02, 0.03, 0.06 and 0.09 $mg.L^{-1}$) for 24 hr, followed by leaving in water for 48 hr to recover.

After recovery, the treated roots were fixed in Carnoy I (3:1 ethanol/glacial acetic acid; v/v) and stored overnight in a refrigerator (4 ^oC). A squash technique was used in the preparation of specimens for microscopic examination. Initially, fixed root tips were hydrolyzed in 1 N HCl at 60 ^oC for 5 min, washed with distilled water and stained with aceto-orcein or aceto-carmine for 5 min. For each treatment, 5,000 cells (1,000 cells/slide) from five bulbs were examined under a light microscope at 600× magnification. The mitotic index (MI) and mitotic aberration (MA) were calculated from the proportion of dividing cells and abnormal cell per 1,000 examined cells, respectively. (Grant, 1982; Fiskesjo, 1985; Fiskesjo, 1997; Soliman, 2001; Hala *et al.*, 2007).

The effects of different exposure durations

Cadmium-sensitive plants screened by the first experiment were subsequently used to evaluate the effects of different exposure durations to heavy metals. Healthy bulbs with root tips were treated with 0.06 $mg.L^{-1}$ $CdCl_2$ for 24 hr and 48 hr. The frequencies of MI (‰MI) and MA (‰MA) were subsequently determined.

Sensitivities of selected-plants to Pb, Cu and As

Heavy-metal-sensitive plant species selected from the screening test were used as cyto-genotoxic bioindicators for assessing other heavy metal contamination. Healthy root tips of selected plants were exposed to 0.00, 0.20, 25.00 and 200.00 $mg.L^{-1}$ of $Pb(NO_3)_2$, 0.00, 1.00, 2.00 and 3.00 $mg.L^{-1}$ of $CuCl_2$ and 0.00, 0.25, 1.00 and 10.00 $mg.L^{-1}$ of $NaAsO_2$, for 24 hr. Susceptibility to the testing chemicals was evaluated based on the

frequency of mitotic inhibition and chromosome aberration induction, using the same microscopic method as in the screening experiment.

Statistical analysis

The data from the experiments with each tested plant species were statistically analyzed using one-way ANOVA and Duncan's multiple range test to compare differences among treatments. The significant difference was set at the level of $P < 0.05$.

RESULTS

Screening for heavy-metal-sensitive plants using mitotic and chromosome aberration assay

According to the cytotoxic-indicative parameter (‰MI), mitotic depression was observed in all Cd-treated groups. Cadmium clearly inhibited mitotic cell division in the root tip cells of three plant species—namely, *E. bicolor*, *Z. rosea* and *A. cepa*. Responses to Cd toxicity at concentrations ranging between 0.02 and 0.09 $mg.L^{-1}$ $CdCl_2$ occurred in a dose dependent manner as depicted in Figure 1. Susceptibility to Cd toxicity as examined by cyto-genotoxic biomarkers showed significant differences only with *E. bicolor* and *A. cepa* (Figure 1). The higher Cd concentrations promoted greater ‰MA values. On the other hand, the frequency of aberration induced by Cd in *Z. rosea* was not different from the control. Fluctuations in the response to Cd, as well as low sensitivity were found in two plant species—*A. cepa* var. ascolonicum and *W. triloba*.

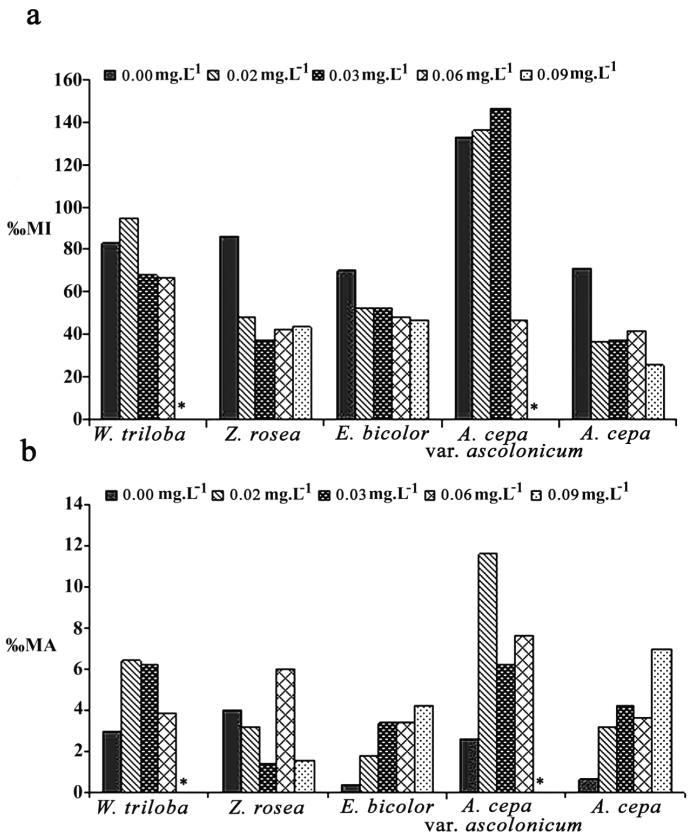

Figure 1 (a) Mitotic index (‰ MI); and (b) Mitotic aberration index (‰ MA) of root tip cells from five plants species treated with 0.00, 0.02, 0.03, 0.06 and 0.09 $mg.L^{-1}$ $CdCl_2$ for 24 hr. The asterisk (*) represents cases of no data due to the death and collapse of root tip cells.

Therefore, only two tested plants (*E. bicolor* and *A. cepa*) were selected based on the sensitivity to Cd as illustrated by the lower ‰MI values and the higher ‰MA values.

Effects of different exposure durations

A longer exposure time (48 hr) to Cd resulted in higher cyto-genotoxicity than the 24 hr treatment, as judged from the decrease in the ‰MI and the increase in the ‰MA (Table 1). Relative mitotic inhibitions were 30% and 41.9% in the root tip cells of *E. bicolor* treated with 0.06 $mg.L^{-1}$ of $CdCl_2$ for 24hr and 48 hr, respectively. Mitotic suppression was clearly seen in *A. cepa* in which cell division was inhibited by up to 65% in the 48 hr treated group.

Sensitivities of *E. bicolor* and *A. cepa* to Pb, Cu and As

Two cadmium-sensitive tested plants, *E. bicolor* and *A. cepa*, were used to assess cytotoxic and genotoxic sensitivity to Pb, Cu and As. It was found that *E. bicolor* was susceptible to toxicity from Pb, like in *A. cepa* (Table 2), but was more sensitive to Cu cyto-genotoxicity than the onion (Table 3). However, *E. bicolor* was slightly less sensitive to the As treatments (Table 4).

Dose-dependent cytotoxicity and genotoxicity of Pb was observed in both plant species, as expressed by the decrease in the ‰MI and the increase in the ‰MA when the $Pb(NO_3)_2$ concentration increased (Table 2). A significant decrease in the ‰MI was found, even in the treated groups exposed to the lowest dose of $Pb(NO_3)_2$. The ‰MI declined from 110.00 to 66.00 and from 103.80 to 94.20 in the case of *A. cepa* and *E. bicolor*, respectively. Six types of aberration were induced by Pb, the highest frequency of aberration was for micronucleus (MN) in both *A. cepa* and *E. bicolor*.

E. bicolor was more sensitive to Cu than *A. cepa*, with a significant decrease in the ‰MI from 114.80 to 104.80, even though it was

Table 1 Mitotic index and mitotic aberration index of *Allium cepa* and *Eucrosia bicolor* root tip cells treated with 0.0 $mg.L^{-1}$ and 0.06 $mg.L^{-1}$ of $CdCl_2$ for 24 hr and 48 hr.

Plant species	Test concentration ($mg.L^{-1}$)	Mitotic index[a]		Mitotic aberration index[a]	
		24 hr	48 hr	24 hr	48 hr
A. cepa	0.0	71.0± 3.09	104.4±3.09	0.6±0.40	2.8±0.66
	0.06	41.4± 4.41	36.2±2.03	3.6±0.87	4.4±1.20
	Relative mitotic inhibition (%)	41.69	65.33		
E. bicolor	0.0	69.0±3.33	79.2±3.92	0.4 ± 0.24	0.0±0.00
	0.06	48.2±3.12	35.0±2.58	3.4 ± 0.92	4.4±0.87
	Relative mitotic inhibition (%)	30.0	41.9		

[a] =Values expressed as mean ± SD.

Table 2 Mitotic index, chromosome aberrations and mitotic aberration index of *Allium cepa* and *Eucrosia bicolor* root tip cells treated with various concentrations of $Pb(NO_3)_2$ for 24 hr.

Plant species	Test concentration ($mg.L^{-1}$)	Mitotic index	Chromosome aberration (‰)						Mitotic aberration index
			Bridge	Disturbed	MN	Laggard	Fragment	Sticky	
A. cepa	0.0	110.0±3.52^{c}	0.4±0.25^{a}	0.0±0.00^{a}	0.6±0.40^{a}	0.2±0.20^{a}	0.0±0.00^{a}	0.0±0.00^{a}	1.2±0.58^{a}
	0.2	66.0±5.26^{b}	0.0±0.00^{a}	0.2±0.20^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.2±0.20^{a}	0.4±0.25^{a}
	25.0	58.0±4.47^{b}	0.2±0.20^{a}	0.6±0.40^{a}	6.4±2.44^{a}	0.0±0.00^{a}	0.2±0.20^{a}	0.2±0.20^{a}	7.6±2.32^{ab}
	200.0	27.6±11.42^{a}	0.2±0.20^{a}	0.6±0.25^{a}	12.2±4.99^{b}	0.2±0.20^{a}	0.2±0.20^{a}	1.2±0.49^{b}	14.6±5.34^{b}
E. bicolor	0.0	103.8±4.63^{c}	0.0±0.00^{a}	0.2±0.20^{a}	0.2±0.20^{a}	0.0± 0.00^{a}	0.0± 0.00^{a}	0.2±0.20^{a}	0.6±0.25^{a}
	0.2	94.2±2.60^{b}	0.2±0.20^{a}	0.6±0.40^{a}	0.4±0.40^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.0±0.00^{a}	1.2±0.49^{ab}
	25.0	90.2±1.88^{b}	0.0±0.00^{a}	0.4±0.25^{a}	1.4±0.75^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.0±0.00^{a}	1.8±0.86^{ab}
	200.0	79.0±1.30^{a}	0.4±0.25^{a}	0.8±0.37^{a}	1.2±0.49^{a}	0.0±0.00^{a}	0.2±0.20^{a}	0.4±0.40^{a}	3.0±1.05^{b}

MN = Micronucleated cells; Values expressed as mean ± SD.

^{a,b,c} = Values in the same column, in each plant species, followed by the same letter are not significantly different ($P < 0.05$).

Table 3 Mitotic index, chromosome aberrations and mitotic aberration index of *Allium cepa* and *Eucrosia bicolor* root tip cells treated with various concentrations of $CuCl_2$ for 24 hr.

Plant species	Test concentration ($mg.L^{-1}$)	Mitotic index	Chromosome aberration (‰)					Mitotic aberration index
			Bridge	Disturbed	MN	Laggard	Sticky	
A. cepa	0.0	117.8±2.84^{c}	0.0±0.00^{a}	0.0±0.00^{a}	0.2±0.20^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.2±0.20^{a}
	1	111.6±3.49^{c}	0.0±0.00^{a}	0.2±0.20^{a}	0.8±0.37^{a}	0.2±0.20^{a}	0.2±0.20^{a}	1.4±0.25^{ab}
	2	94.0±1.18^{b}	0.4±0.25^{a}	0.8±0.80^{a}	1.6±0.87^{a}	0.4±0.25^{a}	0.0±0.00^{a}	3.2±0.97^{bc}
	3	80.4±2.34^{a}	0.4±0.25^{a}	12.0±0.49^{b}	1.6±1.12^{a}	0.0±0.00^{a}	1.0±0.45^{b}	4.2±1.24^{c}
E. bicolor	0.0	114.8±2.06^{c}	0.0±0.00^{a}	0.2±0.20^{a}	0.2±0.20^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.4±0.25^{a}
	1	104.8±2.54^{b}	0.4±0.40^{a}	0.4±0.25^{ab}	0.4±0.25^{a}	0.0±0.00^{a}	0.0±0.00^{a}	1.2±0.37^{a}
	2	85.0±2.05^{a}	0.0±0.00^{a}	0.8±0.37^{ab}	0.8±0.37^{a}	0.2±0.20^{a}	0.0±0.00^{a}	1.8±0.49^{ab}
	3	82.0±3.45^{a}	0.8±0.58^{a}	1.6±0.68^{b}	0.4±0.25^{a}	0.0±0.00^{a}	0.0±0.00^{a}	2.8±0.80^{b}

MN = Micronucleated cells; Values expressed as mean ± SD.

^{a,b,c} = Values in the same column, in each plant species, followed by the same letter are not significantly different ($P < 0.05$).

exposed to the lowest dose, as shown in Table 3. In contrast, *A. cepa* was judged to be copper-cytotoxic tolerant from the significant decrease in the ‰MI at only the higher tested doses (2 and 3 $mg.L^{-1}$). An increase in the Cu concentrations was found to be associated with an increase in the ‰MA in both plants. The highest frequency of chromosome aberration type induced by Cu was different from Pb, being disturbed mitotic aberration in the former and MN in the latter.

Slightly less sensitivity to As toxicity was shown in the *E. bicolor* root tip cells. Unlike *A. cepa*, the cytotoxic and genotoxic effect of As significantly reduced the ‰MI and increased the ‰MA in *E. bicolor* only at the highest dose of 10 $mg.L^{-1}$ $NaAsO_2$ (Table 4). The types of aberration induced by As were different between *E. bicolor* and *A. cepa*. In *E. bicolor*, two aberration types—namely, disturbed (1.20-1.40‰) and sticky (1.40‰)—were significantly induced by 1.00 and 10.00 $mg.L^{-1}$ of $NaAsO_2$ compared with the control. In the case of *A. cepa*, only the sticky type was significantly induced by As (Table 4).

Table 4 Mitotic index, chromosome aberrations and mitotic aberration index of *Allium cepa* and *Eucrosia bicolor* root tip cells treated with various concentrations of $NaAsO_2$ for 24 hr.

Plant species	Test concentration ($mg.L^{-1}$)	Mitotic index	Chromosome aberration (‰)					Mitotic aberration index
			Bridge	Disturbed	MN	Laggard	Sticky	
A. cepa	0.0	110.0±3.52^{c}	0.4±0.25^{ab}	0.0±0.00^{a}	0.6±0.40^{a}	0.2±0.20^{a}	0.0±0.00^{a}	1.2±0.58^{a}
	0.25	91.2±1.59^{b}	0.8±0.20^{b}	0.4±0.25^{a}	1.6±0.75^{a}	1.2±0.20^{b}	0.0±0.00^{a}	4.4±0.81^{b}
	1	95.8±1.99^{b}	0.4±0.25^{ab}	1.4±0.93^{a}	1.4±0.75^{a}	0.8±0.49^{ab}	0.0±0.00^{a}	4.4±1.21^{b}
	10	80.6±2.87^{a}	0.0±0.00^{a}	0.8±0.58^{b}	1.0±0.55^{a}	0.4±0.25^{ab}	2.2±0.97^{b}	4.8±0.49^{b}
E. bicolor	0.0	103.8±4.63^{b}	0.0±0.00^{a}	0.2±0.20^{a}	0.2±0.20^{a}	0.0±0.00^{a}	0.2±0.20^{a}	0.6±0.25^{a}
	0.25	92.2±2.82^{ab}	0.0±0.00^{a}	0.8±0.37^{ab}	0.2±0.20^{a}	0.2±0.20^{a}	0.0±0.00^{a}	1.2±0.20^{a}
	1	90.0±7.13^{ab}	0.0±0.00^{a}	1.4±0.25^{b}	0.2±0.20^{a}	0.0±0.00^{a}	0.0±0.00^{a}	1.6±0.40^{ab}
	10	80.4±3.27^{a}	0.0±0.00^{a}	1.2±0.37^{b}	0.2±0.20^{a}	0.0±0.00^{a}	1.4±0.51^{b}	2.8±0.80^{b}

MN = Micronucleated cells, Values expressed as mean ± SD.

^{a,b,c} = Values in the same column, in each plant species, followed by the same letter are not significantly different ($P < 0.05$).

DISCUSSION

This is the first report which has investigated and explored the potential use of *E. bicolor* as a heavy-metal cytotoxic and genotoxic bioindicator. Sensitivity to heavy metals was shown to be species dependent. In the case of Cd, only *E. bicolor* and *A. cepa* were significantly affected by a cadmium chloride solution in a dose dependent manner, while the responses to Cd fluctuated in the other three plant species. Sensitivity to Cd toxicity has been reported to be different among plant species (An, 2004; Unyayar *et al.*, 2006). An (2004) suggested that *Sorghum bicolor* was more sensitive to Cd toxicity than *Z. mays, Triticum aestivum* and *Cucumis sativas* in acute toxicity testing as indicated by the root growth endpoint. Cadmium nitrate ($CdNO_3$) induced higher percentages of micronucleate cells (%MN) in *Vicia faba* than *Allium sativum* and thus

V. faba could be considered as a Cd-sensitive plant (Unyayar *et al.*, 2006). Consequently, it can be assumed that sensitivity and tolerance to Cd are controlled by genetic factors, as Cd-tolerant plants appear to be able to prevent the excess absorption of Cd, and also to detoxify the Cd after it has penetrated plant cells (Das *et al.*,1997; Hocking and McLaughlin, 2000). Different mechanisms have been proposed to deal with the tolerance to heavy metal stress in plants, for instance, heavy metal immobilization, compartmentalization and synthesis of stress proteins (Li and Xiong, 2004).

In the present studies, the cytotoxicity and genotoxicity of Cd was investigated by observing the decrease in the ‰MI and the increase in the ‰MA in root tip cells. The current findings agreed well with other reports which showed cytotoxicity and also genotoxicity of Cd in plant cells (Unyayar *et al*., 2006; Mishra *et al.*, 2007). Seth *et al.* (2008) suggested that exposure to Cd prevented cells entering cell division phases which then resulted in a decrease in the MI. Additionally, the primary action of Cd on the mitotic spindle promoted spindle-related abnormalities such as laggard chromosomes and bridges during cell division. The mitotic depression caused by heavy metals has been claimed to prevent a number of cells entering the prophase and thus blocking the mitotic phase of the cell cycle (Yildiz *et al.*, 2009). Considering the three sub-phases of interphase, there are a number of reports showing that either the synthetic phase (S) or the gap2 phase (G2) were frequently disturbed by cyto-genotoxic substances (Van't Hoff, 1968; Webster and Davidson, 1969; Salehzadeh *et al.*, 2003). According to Macleod (1969) and Brunori (1971), disturbances of the interphase stage at S or G2 could occur through inhibition of the sub-phases or an increase in the phase duration. Significant differences in mitotic cell division and mitotic aberration at the lowest investigated dose of Cd (0.02 $mg.L^{-1}$) in *E. bicolor* showed that the sensitivity to Cd cytotoxicity was similar to that found with *A. cepa*. Even though the results showed that *A. cepa* var. ascolonicum, *Z. rosea*, and *W. triloba*, had higher percentages of dividing cells (132.40, 86.20 and 83.0%, respectively) as shown in Figure 2, but they all had less sensitivity to Cd. Consequently, these plant species were omitted from further experiments.

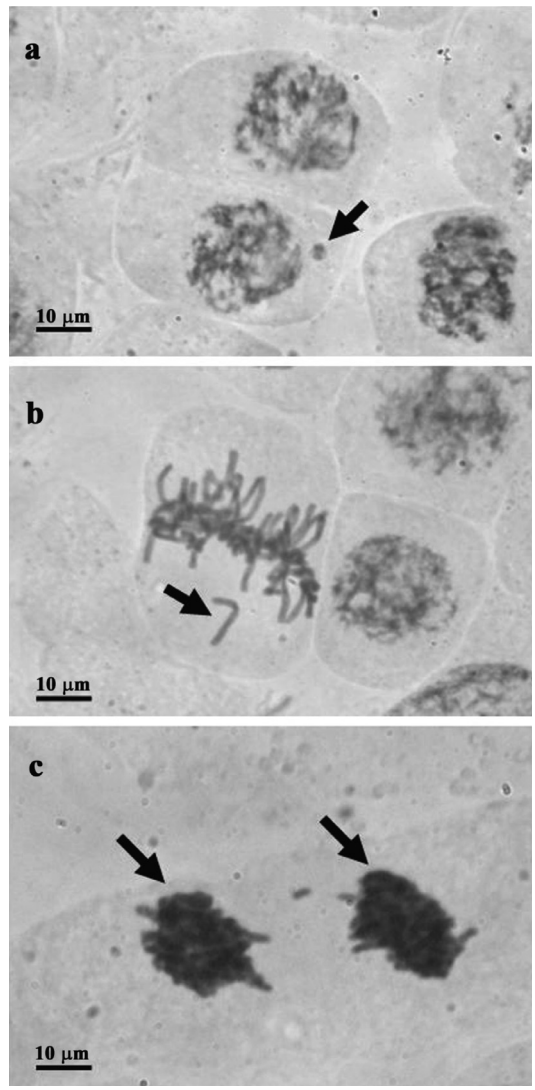

Figure 2 Aberration of interphase nucleus and mitotic cells in heavy-metal-treated root tips from *Eucrosia bicolor*. Abnormalities were observed (arrowheads) as: (a) micronucleus, (b) disturbed metaphase, and (c) nuclei sticky at telophase.

The results of the exposure to Cd for different periods of time showed consistent effects of cyto-genotoxicity measured by the two biomarkers namely, the decrease in the ‰MI and the increase in the ‰MA. Even though the higher indices of toxicity as depicted by the two biomarkers, would have to be subjected to a longer period of treatment, the shorter period of treatment still clearly showed cyto-genotoxicity caused by heavy metals (Table 1). In order to save time in testing, it was proposed that the shorter exposure time of 24 hr was sufficient and reliable. The current results also support the work of Das *et al.* (1997) who showed that cadmium could penetrate into plant cells and consequently induced genetic damage within an exposure time of just 24 hr.

The potential of *E. bicolor* as a cyto-genotoxic bioindicator for assessing other heavy metals contamination was investigated in the current study in experiments carried out in parallel with the *A. cepa* tests. The results showed that susceptibility to heavy metal toxicity was influenced by plant species as well as the concentration of the test chemicals. The response by *E. bicolor* to Pb toxicity was similar to that of *A. cepa* as represented by the decrease in the ‰MI and the increase in the ‰MA in the Pb-treated groups. A subsequent focus on the cytotoxicity of Pb which indicated an effect at the lowest dose, as was observed with *A. cepa*, implies that *E. bicolor* should be recognized equally with *A. cepa* as a Pb- sensitive plant. Furthermore, the results also showed that the cytotoxic endpoint (‰MI) recorded from the root tip cells of both plant species could be used as a biomarker for the assessment of Pb contamination at the permissible dose (0.2 $mg.L^{-1}$) of Pb contamination in waste water set by government (Thai Ministry of Science, Technology and Environment, 1996). Pb has been reported as an effective mitotic poison as well as a turbagen due to its ability to induce different types of spindle disturbance (Patra *et al.*, 2004; Kumar and Tripathi, 2008). Abnormality of mitotic spindle formation and function has been shown to be the cause of laggard chromosomes, disturbed and MN formation, with the latter two being found in the current investigation and representing the two highest frequencies of aberration type. Notably, MN was induced at high frequencies by $Pb(NO_3)_2$ in both plant species. It is most likely that the levels of lead nitrate toxicity are similar in these two plants.

Even though the results showed a greater sensitivity by *E. bicolor* to Cu toxicity than by *A. cepa* at the lowest investigated doses (1.00 $mg.L^{-1}$) of $CuCl_2$, the same type of aberration (mitotic disturbance) was induced in both plant species. The cytotoxicity and genotoxicity of Cu presented in this study were consistent with the report of Yildiz *et al.* (2009) who showed the effects of Cu on inducing chromosome aberration, DNA damage and root growth retardation in *A. cepa*. The results also support the classification of heavy metals with respect to the effect on cell division where copper showed marked effects like chromium (Cr), cobalt (Co) and Cd (Patra *et al.*, 2004). It is interesting to observe that the root tip cells of *E. bicolor* responded to $CuCl_2$ at concentrations as low as 1.00 $mg.L^{-1}$. This concentration is lower than the permissible level (2.00 $mg.L^{-1}$) of Cu in waste water (Thai Ministry of Science, Technology and Environment, 1996).

E. bicolor showed slightly less cytotoxicity and genotoxicity response to As than did *A. cepa*, based on the decrease in the ‰MI and the increase in the ‰MA which were significantly different from the control only at high concentrations of $NaAsO_2$ (up to 10.00 $mg.L^{-1}$). Yi *et al.* (2007) reported on the sensitivity of *A. cepa* to As at a concentration ranging from 0.3 to 100.0 $mg.L^{-1}$ as assessed by a micronucleus test. The researchers suggested that the decrease in the ‰MI might have been the result of a slower progression through the synthetic (S) phase during mitotic cell division. The effects of As on the spindle apparatus and chromosomal peripheral proteins were shown by disturbed and chromosome stickiness formation in both *A. cepa* and *E. bicolor* in the current study.

These two types of abnormality usually lead to chromosome bridges and breaks which implies the potential of arsenic as clastogenic (Souguir *et al.*, 2008).

Kwaunkua *et al.* (2010) reported on the use of *Eucrosia bicolor* as a genotoxic bioindicator. However, its high potential at a level comparable to *A. cepa* could not be determined at the time until a more thorough study was carried out as in this report. Even though *E. bicolor* is native to Peru and Ecuador, it can be found growing throughout Thailand Kwaunkua *et al.* (2010). This tropical bulb species is very easy to propagate and is generally used as a pot plant for landscape design. The general appearance of *E. bicolor*, as shown in Figure 3, suggests that the plant could thrive in a normal tropical environment. A clone of *E. bicolor* used in this research has the somatic chromosome number of 2n=50. Even though *E. bicolor* has a relatively high number of chromosomes, it was found that chromosome aberrations could be observed clearly due to the large size of the chromosomes in this plant species.

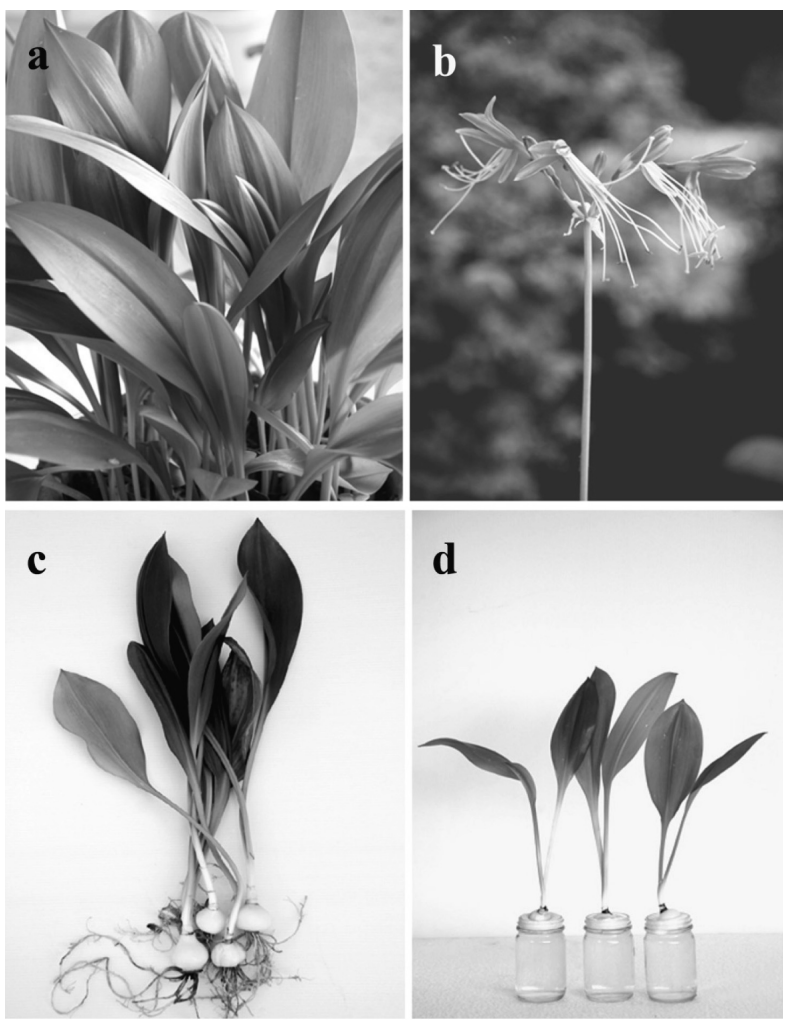

Figure 3 *Eucrosia bicolor*, grown in pots showing: (a) healthy plants; (b) flowers; (c) root bulbs; and (d) in use for assessing genotoxicity.

CONCLUSION

The degree of sensitivity to heavy metal cyto-genotoxicity in plants was shown to be species dependent. Of the five species tested, two (*E. bicolor* and *A. cepa*) were found to be Cd-sensitive plants. An exposure time of 24 hr was sufficient to produce a response that *E. bicolor* was a reliable cyto-genotoxic bioindicator for Cd toxicity. Testing with three other heavy metals—namely, Pb, Cu and As—showed that *E. bicolor* can be used as an alternative heavy-metal cyto-genotoxic bioindicator. This tropical bulb species was equally sensitive to Pb as *A. cepa*, but more sensitive than *A. cepa* in Cu genotoxicity testing.

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