https://doi.org/10.26651/allelo.j/2019-46-2-1209 0971-4693/94 Euro 20.00
Allelopathy Journal 46 (2): 205-214 (March, 2019) *International Allelopathy Foundation* 2019
Table: 1, Figs : 6

Cytogenetic and genotoxic effects of *Ipomoea cairica* (L.) Sweet leaf aqueous extract on root growth of *Allium cepa* var. *agrogarum* (L.)

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(Received in revised form: January 12, 2019)

ABSTRACT

We examined the allelopathic potential of leaf aqueous extract of invasive plant *Ipomoea cairica* on growth and mitosis of roots of *Allium cepa var. agrogarum (L.)*. Root growth was inhibited by leaf aqueous extract in dosage-dependent manner. Chromosomal aberrations (C-mitosis, chromosome bridge, chromosome stickiness and micronucleus) were observed and the mitotic index decreased with increasing concentrations of extracts. The extracts induced the accumulation of reactive oxygen species (ROS). The plant root system is most sensitive organ to environmental stresses. Results obtained provide valuable information about the allelopathy and suggested that *I. cairica* is potential source to develop environmentally safe bioherbicides.

Key words: *Allium cepa*, allelopathy, bioherbicides, cell division, chromosomal aberrations, cytogenetic, DNA damage, genotoxic effects, invasive plant, *Ipomoea cairica*, leaf aqueous, Mitosis, reactive oxygen species, root growth.

INTRODUCTION

Allelopathy is a mechanism that enables plants to promote or inhibit the germination, growth, development and reproduction of other organisms by releasing secondary metabolites into their common environment (12). Allelopathic effects play an important role in the invasive success of exotic plants (37). This is known as the "novel weapons hypothesis" theory, which says that some exotic plants owe their success to their producing novel chemicals with harmful effects (29). During the past decades, many studies have been conducted on their effects on physiology, population dynamics and reproductive ecology, but little is known about the cytological effects. Plants growth depends on the cell division in stem cells in the shoot and root apical meristem, followed by cell elongation (7). There is increasing evidence that allelochemicals significantly affects the cell membrane, microtubule organization, the structure of nuclei, cell division and cell differentiation (4,28). Many allelochemicals are easily and rapidly degradable due to short half-life with no unnatural, toxic ring structures and low halogen substitution (13). The allelopathy has great potential to offer novel environmental friendly compounds for weed management, which reduces the harmful impacts on the environment and provide great benefits to agricultural ecosystems (6).

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Ipomoea cairica (L.) Sweet (family Convolvulaceae) is very fast-growing perennial creeper plant native of tropical Africa or South America (32). In China, it first invaded the Hong Kong as an exotic specie in 1912, but now it has spread to most provinces in South China (10). This weed has invaded the natural landscapes, abandoned farmland, roadsides, residential areas and even the planted forests and has caused severe damage to ecosystems (20). It is listed as one of the worst invasive alien species in China (3).

Allium cepa var. *agrogarum* (L.) (2n=16) is good indicator to monitor the genotoxic effects of environmental substances (24). *A. cepa* is convenient to store and handle, give quick responses to genotoxic compounds, possesses stable chromosome number and clearly distinguishable mitotic phases, exhibits diversity of chromosomal morphologies with rare occurrences of spontaneous chromosomal damage and responds to treatments similar to other plants and thus shows good correlation with other test systems (8,26). The United Nations Environment Programme and the International Program on Chemical Safety validated the *A. cepa* root chromosomal aberration assay as a reliable plant bioassay (21).

This research aimed: (i) to evaluate the effects of *I. cairica* leaf aqueous extracts on the root growth of *A. cepa* var. *agrogarum*, (ii) to examine the chromosomal and cytological aberrations and size of meristematic root tip cells of *A. cepa* var. *agrogarum* and (iii) to examine genotoxicity in nuclei of root cells by detecting the induction of DNA damage.

MATERIALS AND METHODS

Preparation of aqueous extracts

Fresh leaves (about 5 Kg) of *Ipomoea cairica* were collected from the Botanical Garden, South China Normal University, Guangzhou (GPS coordinate: 23.13887°N, 113.35127°E) in August 2017. The leaves were washed with distilled water, air-dried and were powdered using an electric grinder and sieved through 1 mm dia mesh. Eight g powder was soaked in 100 ml distilled water in glass beaker and stirred for 24 h, then the extract was filtered through a Millipore membrane (0.22 μm) to remove solid materials. This stock solution was stored in refrigerator at 4°C until use. We further diluted it with distilled water to obtain three concentrations: 0, 0.5, 1% (w/v) for allelopathy experiments.

Culture conditions and cytological studies

Healthy *A. cepa* var. *agrogarum* bulbs of uniform size (2.5 cm dia) were selected for this study. The outer dry epidermis of each bulb was removed and care was taken not to damage the root primordia before use. The bulbs were set in plastic containers by immersing the root primordia in distilled water (Figure 1) (pH 5.8) and grown at 25±2°C until the root length reached approx 2.0 cms. Then the roots of *A. cepa* var. *agrogarum* were treated with distilled water (control group), 0.5% or 1% of extract concentrations. After exposure, the root length was measured every 12 h. In each treatment group, five seedings were examined for exposure experiments. All solutions were replaced with fresh

every 12 h. Ten root tips from each treatment were cut and fixed in absolute ethanol and glacial acetic acid [3:2 (v/v)] for 1 h at room temperature. After rinsing thrice with distilled water , the sample was hydrolyzed with 1 M hydrochloric acid, 95 % ethanol and 99.8 % acetic acid [5:3:2 (v/v)] for 5-7 min at 60 °C at end of each time interval (12h). The tips were gently squashed and stained with Carbol Fuchsin reagent for 30 min at room temperature (36). A microscope (ECLIPSE 50i Nikon, Japan) was used to observe cell divisions.

Figure 1. The *Allium cepa* var. *agrogarum* were exposed to different concentrations of leaf aqueous extracts.

***In situ* cell death**

The root cells were examined using the Evans blue staining assay. Evans blue can leak through ruptured membranes and stain dead cells, thus indicating the cell death. The roots were submerged in aqueous Evans blue solution [0.25% (w/v)] for 10 min, washed with distilled water for 1 h and then transferred to N, N-dimethylformamide (DMF, Tianjin Damao Chemicals, Analytical Grade) for 1h. The absorbance of Evans blue was measured at 600 nm using a spectrophotometer (DU730 UV/Vis Spectrophotometer, Beckman Coulter Inc., Fullerton, USA) (Gaff, Okongoog. O 1971).

Single-cell gel electrophoresis

Single-cell gel electrophoresis was done as per the method of Jiang *et al*. (14) and Qin *et al*. (24) with minor modifications. Exposed and control roots tips were cut into pieces gently with 500 µl pre-cooled Galbraith's buffer (45 mM $MgCl_2 \cdot 6H_2O$, 30mM $C_6H_5Na_3O_7 \cdot 2H_2O$, 20 mM MOPS, 0.1% Triton X-100, pH 7.0) (9) in petri dish kept on the ice and then filtered through 50 µm nylon filter to acquire the nuclear suspension. Subsequently, 50 µl nuclear suspension was dropped on a glass microscopic slide pre-coated with 1 % normal melting point agarose, mixed with same volume of 1 % low melting point agarose at 37 °C. A coverslip was placed on the mixture and left in dark at 4 °C for 30 min. After the removal of coverslips, the slides were incubated in pre-cooled lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl and 1% Triton X-100, pH 10.0) for 15 min in dark at 4 °C to dissolve cellular membranes and remove attached proteins. Then the slides were subjected to electrophoresis at 0.72 V cm^{-1} (19 V, 300 mA) for 30 min at 4°C. After electrophoresis, the slides were rinsed thrice with 400 mM Tris buffer (pH 7.5), fixed with 95 % ethanol and air dried. The slides were stained with SYBR

Gold for 15 min and dipped in pre-cooled water to remove the excess stain. The specimens were viewed in epifluorescence with Nikon ECLIPSE 50i microscope. For each slide, 30 randomly chosen nuclei were analyzed using CASP software 1.2.3 (15). The Olive Tail Moment values were calculated as a measure of DNA damage (17).

Estimation and visualization of ROS in root tips

Analysis of ROS production was evaluated by staining the roots with 2,7-dichlorofluorescein diacetate (DCFH-DA) (11). Ten root tips from each treatment were incubated in 0.25 μM DCFH-DA dye for 15 min, then washed and observed under fluorescence microscope (Nikon ECLIPSE 50i). The fluorescence intensity was quantified using the image processing and analysis software Image J 1.8.0 (National Institutes of Health, http://rsb. info.nih.gov/ij, USA).

Statistical analysis

Analysis of variance data was performed with SPSS 21.0 software (SPSS Inc., Chicago, USA). For statistical analysis, one-way analysis of variance (ANOVA) was used to determine the significance at $P < 0.05$.

RESULTS AND DISCUSSION

Toxicity of leaf aqueous extracts on root growth

The influences of *I. cairica* leaf aqueous extract on root growth of *A. cepa* var. *agrogarum* changed with test concentrations (Figure 2). Roots of control group grew well during the whole test, while the root growth was almost completely stopped by 1.0 % leaf aqueous extract . In first 12 h, there were significant differences between the 0.5 % and 1.0 % leaf aqueous extract on the root growth of *A. cepa* var. *agrogarum* displaying dose-dependent effects. With increasing concentration and incubation time significant reductions in plant growth occurred in treatments and a range of morphological changes occurred. At 0.5 %, the root morphology of *A. cepa* var. *agrogarum* was similar to control 12 h after treatment, but swelling was seen after 24 h exposure. After 24 h at 1.0 %, root tips were swollen and became yellow in colour. Allelochemicals usually cause deleterious effects in physiological processes [photosynthesis, respiration, water relations and nutrient transport (33)]. Plant roots act as structural anchors to support the plant in the rooting substrate and

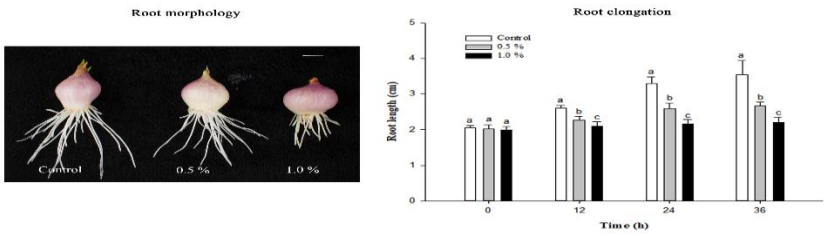

Figure 2. Effects of leaf aqueous extract on root growth of *Allium cepa* var. *agrogarum* at different concentrations (36 h after exposure). Scale bars = 1 cm.

uptake of both water and nutrients (18). Root length is important to exploit the natural resources than other plant organs and is good indicator of the effects of allelochemicals (5). In this study, the leaf aqueous extract significant reduced the root growth compared to control. A number of similar studies report the reduction in plant growth after treatment with allelochemicals (30). This suggests that some substance in the extract possibly plays vital role in mediating the ecological interactions between the *I. cairica* and other species during the process of invasion.

Mitotic index

The changes in mitotic index and chromosomal aberrations of root meristem cells of control and treated roots are presented in Table 1. After 72 h, the mitotic index was 0 % in 1.0 % treatment group. During the mitosis normal chromosome behavior (Mitosis Prophase Figure 3A, Metaphase Figure 3B, Anaphase Figure 3C, Telophase Figure 3D and Intermitosis Figure 3E) was observed in control. As shown in Table 1, the chromosomal aberrations (%) increased with concentration and exposure time. The extracts caused C-mitosis (Figure 3F) in the root tip cells in all treated groups. Chromosome bridges involving one or more chromosomes (Figure 3 I-K) were found in the leaf aqueous extracts treated groups. At 24 h and 48 h the frequency of sticky chromosomes (Figure 3G, H)

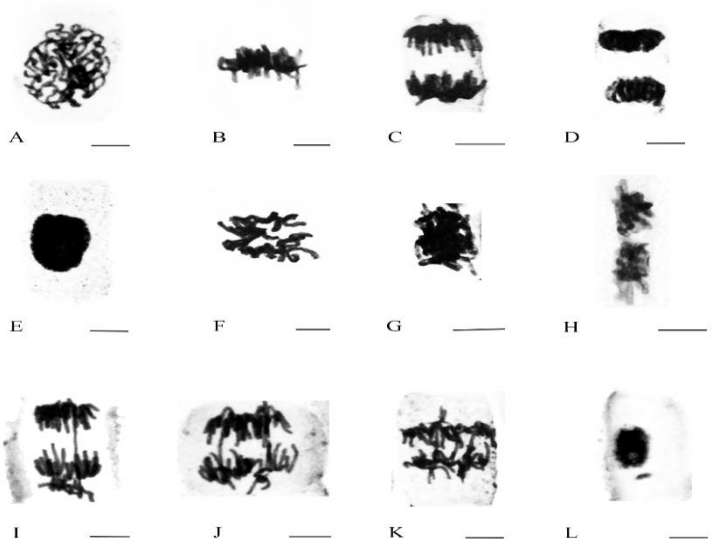

Figure 3. Effects of leaf aqueous extracts on the root meristem cell mitosis in *Allium cepa* var. *agrogarum*.
(A-E) Normal mitotic stages: (A) Prophase, (B) Metaphase, (C) Anaphase, (D) Telophase, (E) Interphase; (F) C-mitosis after treatment with 0.5% for 24 h; (G-H) Chromosome stickiness after treatment with 1% for 24 h (G) and 48 h (H); (I-K) Chromosome bridges after treatment with 0.5 % for 24 h (I) and with 1% for 24 h (J) , 48 h (K) ; (L) Micronucleus after treatment with 1 % for 48 h. Scale bars = 5 μm.

increased in metaphase and anaphase in 1.0 % treatment group. Chromosome stickiness indicates very toxic effects, probably causing cell death. Besides the above aberrations, the increase in Micronuclei (Figure 3L) was also observed in the cytoplasm. Root growth is driven by the proliferation of meristematic cells in the root apex and the subsequent cell elongation in the proximal side of the meristem (22). Our data clearly demonstrated that the leaf aqueous extracts were detrimental to cell mitosis, including the reduction in the mitotic index and the induction of chromosomal aberrations. Such reduction in mitotic index indicates that allelochemicals from the extract might arrest the mitotic cycle in cells and decrease the cell number entering mitotic division. Similar results were also obtained by Soltys *et al*. (28). They suggested that the allelopathic compound might influence the mitotic spindle organization (28). Since most forms of chromosome aberrations (bridge, stickiness and anomalous mitoses) are irreversible, they commonly determine cell death (23). Sanchez-Moreiras *et al.* (27) reported that such observations are rare and not all allelochemicals cause chromosomal aberrations. Andrade *et al*. (1) speculated that chromosomal abnormalities could be the effects of depolymerization of microtubules.

Table 1. Mitotic index and chromosomal aberrations (%) in root meristem cells of *Allium cepa* var. *agrogarum* exposed to leaf aqueous extract.

Time (h)	Extract Concentration (%)	Mitotic index (%)	Number of dividing cells	Normal dividing cells (%)		Anomalously dividing cells (%)			Anomalous mitoses
				Metaphase	Anaphase	C-mitosis	Chromosome bridge	Chromosome stickiness	
24	Control	176	1000	58.1	40.7	0.2	0.4	0.6	1.2
	0.5	74	500	46.8	38.6	4.2	3.4	7	14.6
	1.0	41	200	39.5	26.5	11.5	5.5	17	34
48	Control	181	1000	56.2	41.9	0.5	0.9	0.5	1.9
	0.5	37	200	43.5	38.5	4	4.5	9.5	18
	1.0	5	50	28	22	10	8	32	50
72	Control	165	1000	55.1	43.8	0.3	0.7	0.1	1.1
	0.5	6	50	26	22	11	4	37	52
	1.0	0	0	0	0	0	0	0	0

DNA damage

DNA damage at the individual cell level was estimated by the Single-cell gel electrophoresis. The images and data of the comet assay are presented in Figure 4. The comet tail was missing in control group, indicating that the nuclear DNA was unharmed. Comet tail lengths were greater with increasing concentrations and prolonged exposure time. These results indicated that leaf aqueous extract induced the DNA strands to break in root tip cells. The Single-cell gel electrophoresis is a reliable and sensitive method to monitor the DNA damage (35). The Single-cell gel electrophoresis using *Nicotiana tabacum*, *Solanum tuberosumand* and *Talinum triangulare* have been successfully used to

monitor the genotoxicity of soils highly contaminated with heavy metals, but few studies used this method to detect the allelochemical-induced DNA damage in plants (16). In all exposures, there was progressive increase of DNA damage, which was particularly significant at higher concentrations. This DNA damage may be explained by an increase in activities of both free radicals and reactive oxygen species, leading to DNA strand breaks (34). Rodriguez *et al*. speculated that DNA damage had negative effects on the cell mitosis and might contribute to genomic instability (25).

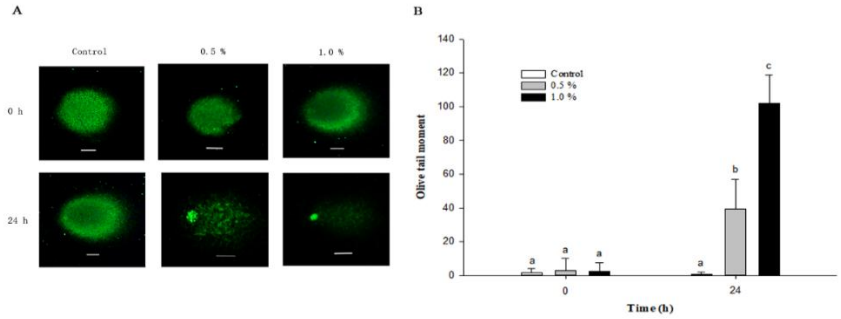

Figure 4. Leaf aqueous extract induced DNA damage in root tip cells of *Allium cepa* var. *agrogarum*. (A) Fluorescence images of Single-cell gel electrophoresis, scale bar = 50 μm; (B) The olive tail moment was calculated as a measure of DNA damage and values with different letters differ significantly from each other within each time group.

Cell viability and ROS accumulation

Evans blue dye was used as marker of membrane integrity that living cells have the ability to exclude the dye at the plasma membrane, while the damaged cell is stained blue. A 4-5 folds high-level uptake of Evans blue was observed in 1.0 % group compared to control (Figure 5). The results indicated that the higher concentration of leaf aqueous extract used, induced higher cellular damage. ROS accumulation in root tips was estimated using the DCFH-DA marker (Figure 6). The fluorescence signal intensity in control group was low, but it increased significantly with increasing extract concentrations. The fluorescence intensity significantly at both 0.5 % and 1.0 % concentrations increased over the controls. Reactive oxygen species (ROS) are natural byproducts of cellular oxidative metabolism and play important role in plant defence mechanisms (2). A transient increase in ROS in plant cells in response to elicitation by allelopathic chemicals has been elucidated recently (4). Some allelochemicals can depolarize the cell membrane, increase the membrane permeability, induce the lipid peroxidation and damage the DNA and proteins (19). This is in agreement with the results of Evans blue test for cell death in the present work. Similar observations were also reported by others, indicating that reorganization of microtubule and disruption of microfilament network might be associated with ROS leading to the damage of microtubulins and actins (31).

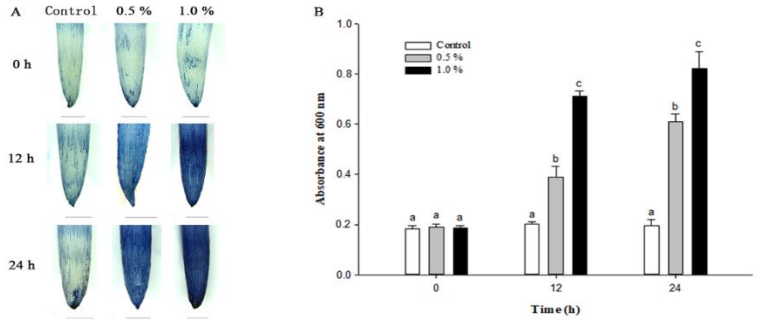

Figure 5. Effects of leaf aqueous extract on cell death of *Allium cepa* var. *agrogarum*.
(A) Root tips stained with Evans blue after exposure to different concentrations of leaf aqueous extract; (B) The degree of cell death in root tips estimated by Evans blue staining. Scale bars = 0.5 mm.

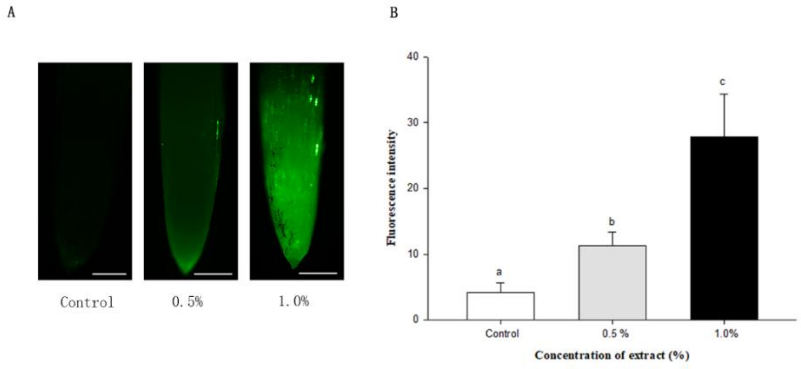

Figure 6. Effects of leaf aqueous extract on ROS generation after 3 hours treatment using the fluorescent dye DCFH-DA.
(A) Fluorescence images; (B) Fluorescence intensity of root tips of different treatment. Scale bars = 0.5 mm.

CONCLUSIONS

The leaf aqueous extracts from *I. cairica* caused root growth inhibition in *A. cepa* var. *agrogarum*. The root growth inhibition was associated with abnormal cell mitosis; accompanied by increased ROS production and cell death. These findings lead to better understanding of allelopathic effects of leaf aqueous extracts of *I. cairica* on *A. cepa.* These studies need to be further explored for the possible development of a natural herbicide for weeds control.

ACKNOWLEDGMENTS

We sincerely thank South China Normal University for funding the international joint training programme for top graduate students (to N.W. & S. L.). This research is financed by the Natural Science Foundation of China (NSFC, 31670266) and the Guangdong Pearl River Scholar Funded Scheme (2012). We are extremely grateful to Prof. Paul Giller, School of Biological, Earth and Environmental Sciences, University College Cork, to improve the manuscript English language.

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