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COUMARINS FROM *HALDINA CORDIFOLIA* LEAD TO PROGRAMMED CELL DEATH IN GIANT MIMOSA: POTENTIAL BIO-HERBICIDES

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Abstract

Phytotoxicity of isoscopoletin and umbelliferone, isolated from bark and wood of *Haldina cordifolia*, on the germination and growth of *Mimosa pigra*was investigated. When compared to the control treatment, 100µ Misoscopoletin delayed germination for 3 days, whereas 100 µM umbelliferone, which proved to be more effective, delayed germination for 4 days. Both coumarins caused stunted root growth, but only umbelliferone caused swollen roots. In contrast to roots treated with umbelliferone, cross-sections of roots treated with isoscopoletin showed smaller root diameter and fewer cortical cells. Moreover, the vascular bundles of roots treated with umbelliferone were more developed than those treated with isoscopoletin. Transmission electron microscopy revealed that the cells of the root tip and maturation zone exposed to either coumarin showed thickened cell walls; disruption of cell membranes; increased number of disorganized mitochondria, Golgi apparatus, and endoplasmic reticulum; as well as increased number of plastids and plastoglobules. However, vacuolization and autophagosomes were found in the root maturation zone of roots treated with umbelliferonein a greater extent than those treated with isoscopoletin. These results suggest that isoscopoletin and umbelliferone might be involved in accelerating senescence or programmed cell death in giant Mimosa, resulting in reduced growth. Therefore, they could be considered potential for a development of bio-herbicides for giant mimosa control.

Key words: Isoscopoletin, Umbelliferone, *Haldina cordifolia*, *Mimosa pigra*, Program cell death.

Introduction

Mimosa pigra, commonly known as giant mimosa, is a dicot weed belonging to the family Fabaceae. It is widespread in the tropics, particularly in Africa, Australia, India, Southeast Asia, and some Pacific Islands. The IUCN Invasive Species Specialist Group has listed it as one of the World's 100 worst invasive species (Wilson, 2006) due to its invasiveness, potential for spreading, and economic and environmental impacts (Sakai *et al*., 2001; Simberloff *et al*., 2005). Many attempts, including mechanical and chemical management orintegrated approaches using several weed management techniques, have been used to reduce its infestation (Paynter & Flanagan, 2004). Although the application of various types of chemical herbicides such as glyphosate, metsulfuron methyl, paraquatand diuron have proved effective, its increased application has led to environmental pollution and human health risks. Moreover, herbicide resistance may emerge as a problem of the continual use of some particular herbicides (Singh *et al*., 2006). Consequently, natural herbicides are an alternative weed control strategy that is safer and more environment-friendly. Recently, naturally derived compounds have gained popularity because they are easily degradable, and lead to less environmental degradation in terms of residues and toxicity. In fact, there is a variety of plant secondary metabolites with allelopathic effects, many of which have demonstrated promising activity on weed control. For example, *trans*-ferulic, *p*-hydroxybenzoic and vanillic acids, isolated from roots and shoots of 17 days old wheat, were found to inhibit root elongation of weeds (Wu *et al*., 2002). *Trans*-cinnamic acid, coumarin, coumaric acid, and chlorogenicacid, obtained from aqueous and methanol extracts of 16 wild Asteraceae plants, were shown to exhibit allelopathic effects on lucerne (*Medicago sativa* cv. Vernal, Fabaceae) and *Echinochloacrus-galli* (Poaceae) (Chon *et al*., 2003). Salicylic acid and sinapicacids, isolated from the methanol extract of the underground parts of *Ophiopogonjaponicus* (L.f.) Ker Gawl, also showed significant inhibitory activity on germination of *E. crus-galli* (L.) Beauv (Lin *et al*., 2004), while 4'-methoxyapigenin and 3,7-dimethoxykaempferol, isolated from exudates of *Cistusladanifer* L. (Cistaceae), significantly inhibited root length of curled dock (*Rµmexcrispus* L.) (Chaves *et al*., 2001). interestingly, sesquiterpene lactones, isolated from *Magnolia grandifolia*, were found to inhibit germination and growth of some mono- and dicotyledon species in a similar manner to that of commercial herbicide Logran (Marcias *et al*., 2000). Volatile terpenes and essential oils, such as 1,8-cineole, also showed inhibitory effects on the growth of *E. crus-galli*and *Cassia obtusifolia* L. (Romagni *et al*., 2000). Even though plant secondary metabolites continue to be a potential source for the development of natural herbicides, recently, much attention has been paid to microbial secondary metabolites. Example of these is bialaphoas, which isa tripeptide produced by fermentation of *Streptomyces viridochromeogenes* and *Streptomyces hygroscopicus* (Murasaki *et al*., 1986). Therefore, searching for potential plant constituents that can be used as natural herbicide continues to be a priority since they can reduce production costs, human health risks, and pollution to ecosystems.

Our preliminary study has shown that the methanol extract of *H. cordifolia* was able to inhibit the germination and growth of giant mimosa. In order to verify the compounds responsible for these activities, we have proceeded with the isolation of the major constituents of this plant to evaluate their effects. Now we report the isolation of isoscopoletin and umbelliferone from the methanol extracts of bark and wood of *H. cordifolia*, as well as the evaluation of the effects of both compounds on the germination, growth, and anatomical changes of giant mimosa.

Materials and Methods

Plant material: *Haldina cordifolia* (Roxb.) Ridsdale was collected from Chainat province, central Thailand, in September 2012. The plant material was identified by Srunya Vajrodaya a taxonomist of Department of Botany of Kasetsart University, Bangkok, Thailand, and the voucher specimens (voucher no. Rungcharn 001) were deposited in the Comparative and Ecological Photochemistry Unit, Department of Botany, Kasetsart University.

Extraction and isolation: Dried powdered wood (500 g) and bark (500 g) of *H. cordifolia* were extracted with MeOH (3 x 1,500 mL) at room temperature until exhaustion. The methanol solutions were evaporated under reduced pressure to give the crude methanol extracts of wood (15.1g) and bark (24.72 g), which were then extracted with $CHCl_3$ (3 x 300 mL) with the aid of unltrasound bath. The $CHCl_3$ solutions were combined and evaporated the give the $CHCl_3$ extract of wood (7.23 g) and bark (13.22 g). The $CHCl_3$ extract of wood was applied over a 0.2-0.5 mm Merck Si Gel column of (60 g, 1.5 cm x 100 cm), and eluted with mixtures of *n*-hexane-Et_2O and Et_2O-MeOH, wherein 50 mL fractions were collected as follows: Frs 1-2 (*n*-hexane-Et_2O, 19:1), 3-4 (*n*-hexane-Et_2O, 9:1), 5-6 (*n*-hexane-Et_2O, 3:1), 7-8 (*n*-hexane-Et_2O, 1:1), 9-10 (Et_2O), 11-12 (Et_2O-MeOH, 3:1), 13-14 (Et_2O-MeOH, 1:1), 15-16 (Et_2O-MeOH, 1:3), 17-18 (MeOH). Fractions 7-8 (45 mg) were combined and crystalized in a mixture of n-hexane to give 33.1 mg of isoscopoletin (**1**). The $CHCl_3$ extract of bark was applied over a 0.2-0.5 mm Merck Si Gel column of (60 g, 1.5 cm x 100 cm), and eluted with mixtures of *n*-hexane-Et_2O and Et_2O-MeOH, wherein 50 mL fractions were collected as follows: Frs 1-2 (*n*-hexane-Et_2O, 19:1), 3-4 (*n*-hexane-Et_2O, 9:1), 5-6 (*n*-hexane-Et_2O, 3:1), 7-8 (*n*-hexane-Et_2O, 1:1), 9-10 (Et_2O), 11-12 (Et_2O-MeOH, 3:1), 13-14 (Et_2O-MeOH, 1:1), 15-16 (Et_2O-MeOH, 1:3), 17-18 (MeOH).Frs 8 was precipitated to give 32.9 mg of umbelliferone (**2**).

Bioassay on seed germination and seedling growth: The effects of scopoletin (**1**) and umbelliferone (**2**), isolated from wood and bark of *H. cordifolia*, were studied using Petri dishes (id 9 cm) lined with two layers of filter paper. The concentrations of each compound used were 0, 10, 100, 500, and 1,000 µM. The commercial herbicide acetochlor, at 100 µM, was used as a control. Five ml of each treatment was applied to the filter paper. Twenty seeds of *Mimosa pigra* were placed in each Petri dish. Three Petri dishes of each treatment were maintained as replicates. Seeds were incubated in a growth chamber (GS-2430B, Korea) at 27 °C, in the dark, with a relative humidity of approximately 75% for 7 days. The percentage of seed germination was measured daily, while shoot and root growth were measured on the 3rd, 5th, and 7th day after treatment.

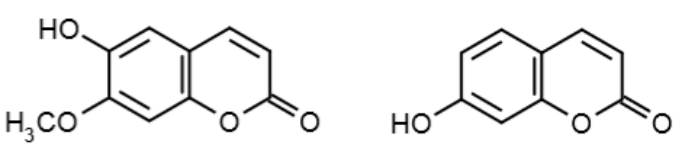
Transmission Electron Microscopy (TEM): For histological examination, shoot and root tips were collected after 7 days of incubation following a coumarin treatment. Tissue samples were divided into 3 zones (i) root tip zone (ii) maturation zone of root, and (iii) maturation zone of shoot. Fragments were immediately fixed in 2.5% glutaraldehyde (Becthai, USA) in 0.1 M sodium phosphate buffer pH 7.2 at 4 °C for 12 hours, and subsequently postfixed in 2% osmium tetroxide (Becthai, USA) for 2 hours. After washing with distilled water, rapid dehydration was done in acetone series (30–100%), followed by infiltration, embedding in Spurr's Resin and polymerization at 70°C for 9 h

Blocks were cut into thick sections (1 µm) with glass knife of ultra-microtome (Leica, UCT) for light microscopy. Thick sections were mounted onto glass slides and stained with 1% Toluidine blue (Becthai, USA) at 85°C for 5 min and examined in a light microscope (Axiostar plus, Cark Zeiss, Aalen, Germany). Thin sections (60 nm) were placed on film-coated grids, then stained with uranyl acetate (Becthai, USA) for 30 min and followed by lead citrate (Becthai, USA) for 30 min. Examination was conducted using a transmission electron microscope (Hitashi, HT7700, Japan) at 120 kV accelerating voltage.

Statistical analysis: All experimental treatments had three independent replicates. Significance differences for all statistical tests were evaluated at the level of $p \leq 0.05$ with ANOVA and LSD tests. All data analyses were conducted using the R program.

Result and Discussion

Isolation and Purification of Coumarins: Column chromatography of the crude extracts of wood and bark of *Haldina cordifolia* afforded, respectively, isoscopoletin (**1**) and umbelliferone (**2**) whose structure (Fig. 1) was elucidated by analysis of their ^1H and ^{13}C NMR spectra, as well as comparison of their ^1H and ^{13}C NMR data with those previously reported for isoscopoletin (Kim *et al*., 2006) and umbelliferone (Nath *et al*., 2005).



Isoscopoletin Umbelliferone

Fig. 1. Structure of isolated coumarins; isoscopoletin and umbelliferone from *Haldina cordifolia.*

Germination: The phytotoxic effects of isoscopoletin (**1**) and umbelliferone (**2**) at different concentrations on the seed emergence of giant mimosais shown in Fig. 2. The commercial herbicide acetochlor was used as a control. Germination of giant mimosa seeds treated with both coumarins did not differ from that of the control at day 7 because by that time they had completely germinated in all treatments. However, it clearly shows that both coumarins retard giant mimosa germination rate in the first few days. Not only does isoscopoletin(**1**) and umbelliferone(**2**) affect germination rate of giant mimosa differently but the phytotoxic activity of each coumarin was found to be dose dependent also. At day2, 100% and 68.87% of the giant mimosa seeds in the control treatment and commercial herbicide treatment, respectively, germinated, whereas 77.8%, 44.47%, and 0% of the seeds germinated when treated with 10 µm, 100 µm, 500 µm, and 1,000 µm of isoscopoletin (**1**), respectively. However, when compared to the control, the germination rate was found to be 60%, 24.47%, 4.47%, and 0% when treated with umbelliferone (**2**) at the concentrations of 10 µm, 100 µm, 500 µm, and 1,000 µm. At day 5, seeds that received isoscopoletin (**1**) treatments were found to completely germinate, while seeds that received umbelliferone (**2**) treatments at 500 µM and 1,000 µM still showed delayed germination, with 93.33% and 68.87% of the germinating seeds, respectively. By day 6, there was no difference between the isoscopoletin (**1**), umbelliferone (**2**), and control treatments as all the seeds germinated completely. In summary, when compared to the control, isoscopoletin (**1**) could delay germination on average for 3 days, whereas umbelliferone (**2**), which proved more effective, could delay germination on average for 4 days.

Seedling growth: Inhibitory effects of each coumarin on seedling growth of giant mimosa germination were observed daily during the 7-days incubation period. In the growth assay, it was found that shoot and root length of giant mimosa decreased remarkably with increasing concentrations of both coumarins (Fig. 3 and 4). The results showed that isoscopoletin (**1**) and umbelliferone (**2**) significantly inhibited shoot and root elongation. The degree of inhibition was dependent on the concentration of the coumarins: the higher the concentration, the higher the inhibition. The maximum reduction on seedling growth was observed at 1,000 µm of each compound. The growth of seedlings at day 7 in the isoscopoletin (**1**) treatment ranged from 14.6% to 91.09%, when compared to the control for shoots, and from 45.83 to 84.9%, when compared to the control for roots, respectively (Fig. 4). On the other hand, umbelliferone (**2**) was found to have less effect on the seedling, showing the growth from 13.37 to 20.8%, when compared to the control for shoots, and from 27.6 to 74.48%, when compared to the control for roots (Fig. 4). Overall, it can be concluded that the allelopathic effects of isoscopoletin (**1**) and umbelliferone (**2**) on giant mimosa seeds had more of an impact on germination speed and seedling length than on the final germination rate. Moreover, it was also found that umbelliferone (**1**) was a stronger inhibitor than isoscopoletin (**2**). These results were consistent with previous reports done by Abenavoli *et al*., 2004; 2008. They found that roots of *Arabidopsis* and maize which were exposed to coumarin showed stunt primary root and increased number of lateral root. Lupini *et al*. (2010) suggested that this inhibitory effect may mediated by auxin. Lupini *et al*. (2014) used *Arabidopsis* auxin mutants (auxin transport/redistribution inhibitors) to investigate possible interaction between coumarin and auxin in root development. The results confirmed root development in *Arabidopsis* was influenced by a functional interaction between coumarin and auxin polar transport.

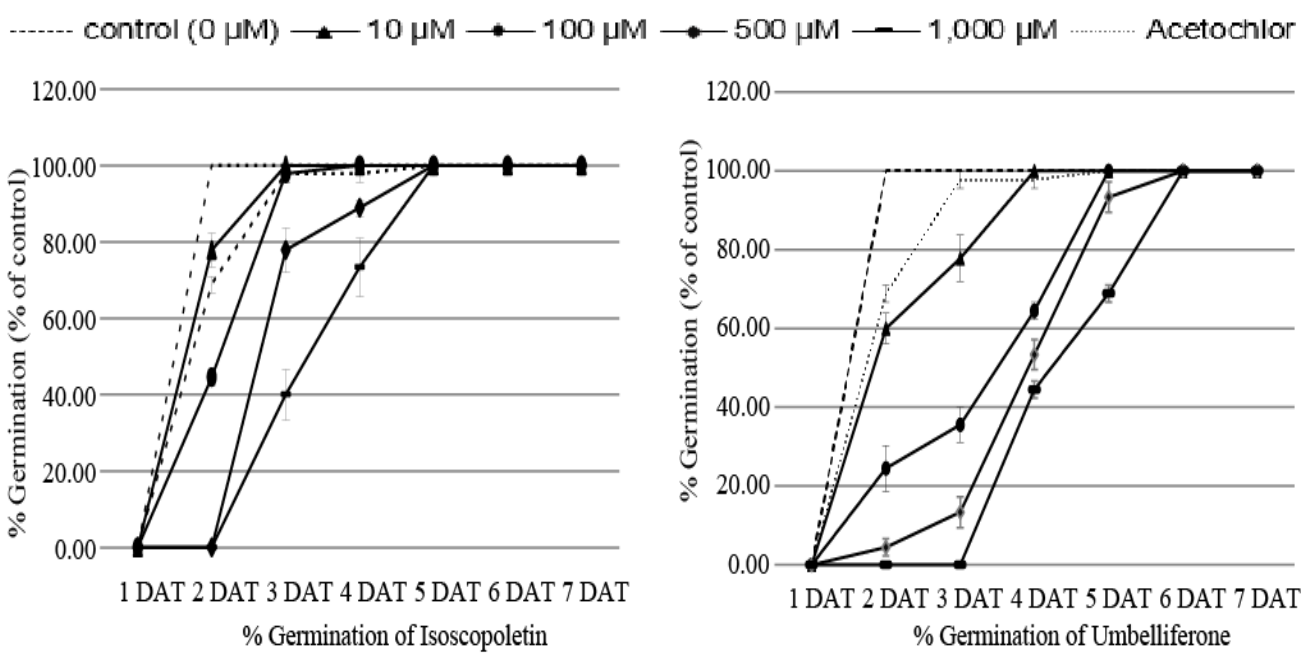


Fig. 2. Effect of isoscopoletin (a) and umbelliferone (b) on germination of giant mimosa over the time expressed as the percentage of the control. Acetochlor, a commercial herbicide, was used as internal control.

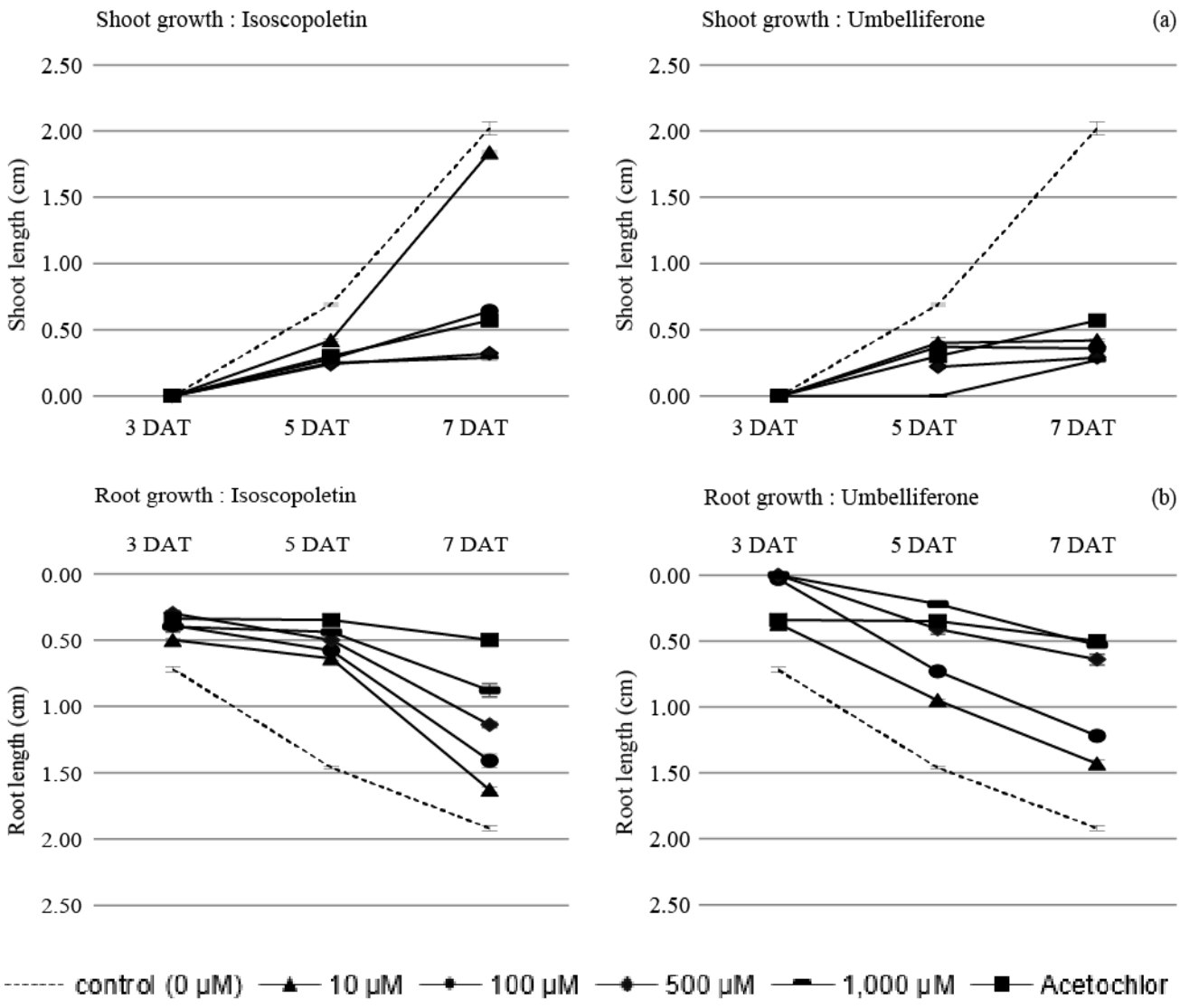


Fig. 3. Effect of isoscopoletin and umbelliferone on shoot and root growth of giant mimosa over the time. Acetochlor, a commercial herbicide, was used as internal control. (a) comparing growth rate of shoot after treated with isoscopoletin (a; left) and umbelliferone (a; right) (b) comparing growth rate of root after treated with isoscopoletin (b; left) and umbelliferone (b; right)

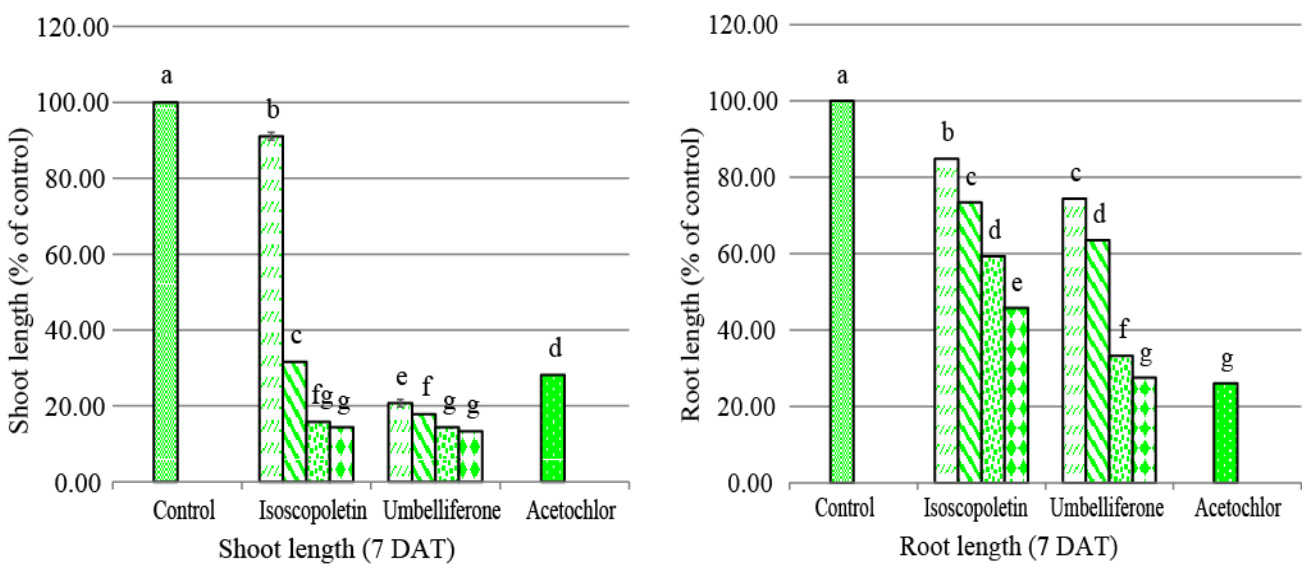


Fig. 4. Comparing shoot (a) and root (b) length at day 7 after treated with iscopoletin and umbelliferone at different concentrations. Acetochlor, a commercial herbicide, was used as internal control.

Table 1. NMR data of compound 1 and 2 (^{1}H (500Hz) and ^{13}C in chloroform - d6) and Isoscopoletin and Umbelliferone.

Position	Compound 1 Isoscopoletin		Reference (Bong Gyu Kim *et al.*, 2006)		Compound 2 Umbelliferone		Reference (Mala Nath *et al.*,2005)	
	^{1}H (mult., J Hz)	^{13}C	^{1}H (mult., J Hz)	^{13}C	^{1}H (mult., J Hz)	^{13}C	^{1}H (mult., J Hz)	^{13}C
1	-	161.47	-	160.7	-	161.9	-	161.2
2	6.26 (d, 9.5)	113.36	6.28 (d, 9.4)	112.6	6.15 (d, 9.6)	112.8	6.22 (d,9.5)	111.2
3	7.59 (d, 9.5)	143.31	7.94 (d, 9.4)	144.3	7.85 (d, 9.3)	144.7	7.95 (d,9.5)	144.3
4	-	111.48	-	111.5	-	112.9	-	111.2
5	6.84 (s)	107.50	7.05 (s)	112.0	7.50 (d, 8.7)	130.4	7.55 (d,9.0)	129.5
6	-	149.72	-	143.6	6.83 (dd, 2.4 8.4)	113.7	6.82 (d,9.0)	113.0
7	-	144.03	-	151.8	-	161.0	-	160.3
8	6.91 (s)	103.18	7.06 (s)	100.0	6.74 (d, 2.4)	103.26	6.75 (s)	102.1
9	-	150.24	-	148.4	-	157.0	-	155.4
7 – OCH3	3.95 (s)	56.41	3.91 (s)	56.41	-		-	

Changes in morphology, anatomy, and ultrastructure of giant mimosa root: Both isoscopoletin (1) and umbelliferone (**2**) were found to cause severe visible damage in giant mimosa seedlings, resulting in reduction of root and shoot growth (Fig. 5). Based on the dwarfed growth of giant mimosa seedlings, it was speculated that treatment with these coumarins might have caused anatomical malformations. Fig. 6 shows the alterations in tissue organization of the shoot, maturation zone of the root and the root tip of giant mimosa after treatment with isoscopoletin (**1**) or umbelliferone (**2**). Roots treated with isoscopoletin (**1**) decreased markedly in diameter, whereas those treated with umbelliferone (**2**) became much thicker when compared with those of the control, especially in the maturation zone (Fig. 6D-I, Table 2). The number of layers in the cortex of the root tip in the umbelliferone (**2**) treatment also differed significantly from those of the control and of the isoscopoletin (**1**) treatment. However, in the umbelliferone (**2**) treatment, the cortical layers in the maturation zone were much higher than those in other treatments (Table 2). When the shapes of the cortical cells between each treatment were compared, it was found that the cortical cells in each of the coumarin treatments became rounder and had thicker cell walls, when compared with those in the control. However, in the root tip and the maturation zones, the cortical cells in the isoscopoletin (**1**) treatment were comparatively smaller than those in the other treatments (Fig. 6 E, H). It was found also that the cortical cells in roots treated with umbelliferone (**2**) became rounder and had thicker cell walls, but their cell sizes were larger than those in the control and in the isoscopoletin treatments. Not only did we find differences in the number of cell layers, cell shape and size, but there was also a significant change in the exodermis which seemed to accumulate some substances inside the cells, resulting in black circles in the exodermis layer around the root in both the isoscopoletin (**1**) and umbelliferone (**2**) treatments (Fig. 6E, H, I). Vascular tissues in these treatments seemed to develop faster than that of the control (Fig. 6E, F, H, I). Furthermore, the diameter of the stele in the umbelliferone (**2**) treatment was much wider than that found in the control (Table 2). Although, the vascular tissues had a smaller stele diameter in the isoscopoletin (**1**) treatment, their development were found to be as rapid as those in the umbelliferone (**2**) treatment (Fig. 6B, E).

Different degrees of deterioration in the ultrastructure of root cells in each treatment were also observed by transmission electron microscopy (TEM) as shown in Figs. 7-8. In the control, the root tips showed active Golgi apparatus, endoplasmic reticulum, and mitochondria (Fig. 7A-B), however, they were found to have increased number of mitochondria with disrupted cristae, Golgi apparatus, fragmented ER, and increased distorted plastid when treated with both coumarins (Fig. 7C-L). The plastids had increased formation of plastoglobules (Fig. 7K, L), and electron-dense deposits were found in the cell wall, intercellular space, vacuole, cytoplasm, mitochondria and Golgi apparatus (Fig. 7C, D, F). Thickening cell wall, shrunk protoplast, membrane blabbing, and disrupted plasma membrane were also observed (Fig. 7C, F, I), especially in the root tip of the isoscopoletin (**1**) treatment, where the plasma membrane showed the greatest deterioration. Mitochondria in root tips treated with isoscopoletin (**1**) showed a regular shape but with large and fewer dilated cristae, and contained a dark matrix (Fig. 7C). However, umbelliferone (**2**) caused the most deleterious effects on the disruption of the Golgi apparatus and distortion of mitochondria shape with fewer cristae (Fig. 7I, J). Umbelliferone (**2**) also caused active vacuolization as numerous small vacuoles were found near the central vacuole (Fig. 7H).

In the root maturation zone, the major alterations induced by both coumarins were autophagy in root cells, disruption of Golgi apparatus, fragmentation of ER, and increased number of plastids with plastoglobules inside. Electron-dense deposits were found in vacuoles, cytoplasm, thickened cell walls and intercellular space (Fig. 8B, C, D and J). In the presence of isoscopoletin (**1**), active vacuolization was observed all over the cells, along with obvious disruption of organelles (Fig. 8C, D). Although the root cells of maturation zone treated with umbelliferone (**2**) did not show much disruption of organelles, it was fully occupied with a big vacuole due to vacuolization. Additionally, in the umbelliferone (**2**) treatment, the cells had decreased cytoplasm volume with more but distorted mitochondria and many fragments of ER cisternae and ribosomes (Fig. 8G-L).

In our previous study, the lipophilic extracts from bark and wood of *H. cordifolia* were found to exhibit inhibitory effects on germination of some weeds such as giant mimosa and sandbur (Suksungworn *et al*., 2016). In order to verify the secondary metabolites in the extracts, which are responsible for this phytotoxicity, we have proceeded with isolation and identification of the constituents of these extracts. Fractionation and purification of the major constituents from the methanol extract of wood and bark of this plant led to isolation of isoscopoletin (**1**) and umbelliferone (**2**). Although umbelliferone (**2**) has been previously isolated from the wood of *H. cordifolia* (Kasinadhuni *et al*., 1999), to the best of our knowledge, this is the first report of isoscopoletin (**1**) from this plant.

The results from the bioassays revealed that both coumarins were able to inhibit germination and growth of giant mimosa. Although they did not completely inhibit germination, they did retard it for 3 days (isoscopoletin) and 4 days (umbelliferone), respectively (Figs. 2-4). These results are in accordance with previous studies, which reported allelopathic effects of coumarins. For example, it was observed that coumarins had the ability to inhibit germination of several plants such as seeds of *E. crus-galli*, *Amaranthushypochondriacus* L., durum wheat (*Triticumturgidum*), goosegrass (*Eleusineindica* L.), and lettuce (Abenavoil *et al*., 2006; Khan *et al*., 2006; Anya *et al*., 2005). However, this is the first report of the effect of coumarins on giant mimosa.

Several mechanisms of inhibition of germination and growth by coumarins have been proposed. Serrato *et al*. (2006) have found that coumarins, isolated from *Tageteslucida* Cav., affected the respiration of dicot seeds of *L. sativa*, *T. pretense*, and *P. ixocarpa*, and led to reduced growth. The presence of coumarins in root exudates of sweet vernal grass (*Anthoxanthumodoratum* L., Poaceae) interfered with phosphorus uptake of nearby plants, and led to reduced growth (Yamamoto, 2009). It was also found that respiration and photosynthesis of wheat were affected by coumarins. They caused changes in nitrogen uptake and metabolism, and subsequently led to reduced root formation and root growth (Abenavoli *et al*., 2006). Moreover, morphological changes in *Zea mays* seedling were observed due to coumarin exposure (Abenavoli *et al*., 2004).

Both isoscopoletin (**1**) and umbelliferone (**2**) caused stress to roots of giant mimosa, resulting in stunted growth (Figs. 5 and 6). Well-developed vascular tissue is one signal of programmed cell death as shown by Palavan-Unsal *et al*. (2005) in carrots treated with cadmium (Toppi *et al*., 2012). Thickening of cell walls,as observed in isoscopoletin (**1**) and umbelliferone (**2**)treatments, is a response to stress which has been also found in soybean exposed to aluminum (Yu *et al*., 2011).

Intercellular space formation was found to increase in a response to both coumarins. It was suggested that a thickened cell wall and increased intercellular space would provide an extra compartment and play a role in coumarin depositim mobilization. It is considered as an acclimation response mechanism to toxicity exposure, and could allow for the detoxification of coumarin out of the cell as has been shown in soybean exposed to cadmium (Kevresan *et al.*, 2003). Moreover, the various patterns of electron-dense deposits found in cytoplasm, vacuole, middle lamella, and the cell wall suggest that coumarins were transported in both symplastic and apoplastic pathways. It was also suggested that sequestration of this toxin in the cell wall or compartmentalization in vacuoles could help plants to detoxify toxins out of the cytoplasm. Similar results were reported in lead transportation in *L. chinensis* and *L. davidii* (Zheng *et al*., 2012), and tobacco By-2 cells exposed tomicrocystin (Huang *et al*., 2009). However, increased intercellular space also caused less cell-to-cell adhesion, which might disturb inter-cell or inter-tissue communication for regular root growth and development.

Mitochondria are the centers of respiration in the cell, and coumarins might increase mitochondrial reactive species that subsequently damage mitochondrial membranes, resulting in swelling and damaged mitochondria (Vacca *et al*., 2006). Increased number of mitochondria reflects the compensation of the cell to maintain energy production with dysfunctional mitochondria.

An increased number of plastoglobules and starch grains in plastids was observed in giant mimosa in response to isoscopoletin (**1**) and umbelliferone (**2**) treatments. Our finding coincides with previous reports which have shown that many plant species exposed to biotic and abiotic stresses have increased number of plastids and plastoglobules as well (Zhang *et al*., 2010). This increased starch grain might be associated with cell wall thickening of mimosa as was shown in *Allium cepa* (Wierzbicka, 1998). Although the role of plastoglobule formation under stress is not yet fully understood, there is some evidence demonstrating that increased plastoglobule formation was related to increased production of antioxidants, which help plants to cope with stress by protecting the membrane from peroxidation by ROS (Brehelin *et al*., 2007).

Vacuolization and autophagy were frequently observed in the root maturation zone of giant mimosa after coumarin exposure. Similar results were obtained from plants exposed to heavy metals such as Pband Cd (Hall, 2002; Jabeen *et al*., 2009). Vacuolization may be an adaptive response to exclude coumarins out of the cytoplasm and sequester them in a vacuole. It may also function to reduce transfer of coumarin to the shoot. Autophagy is known as another adaptive stress avoidance mechanism, which increases the probability of survival in unfavorable environments (Toppi *et al*., 2012). Autophagy can degrade macromolecules and recycle damaged and unwanted materials occurring in cells due to stress. *L. chinensis* and *L. davidii* have been shown to use this mechanism to remove excess heavy metals from cells (Huang *et al*., 2009). Therefore, the autophagy found in giant mimosa might also provide tolerance and detoxification of coumarins in giant mimosa. The observed ultrastructural changes in this study are characteristics of programmed cell death that can be interpreted as an acclimatory response in order to survive under coumarin exposure. Therefore, isoscopoletin (**1**) and umbelliferone (**2**) from *H. cordifolia* can be considered as potential candidates for allelochemicals that may be used as future bio-herbicides.

Fig. 5. Morphology changes of giant mimosa seedlings after treated with isoscopoletin and umbelliferone compared with the control and acetochlor, a commercial herbicide.

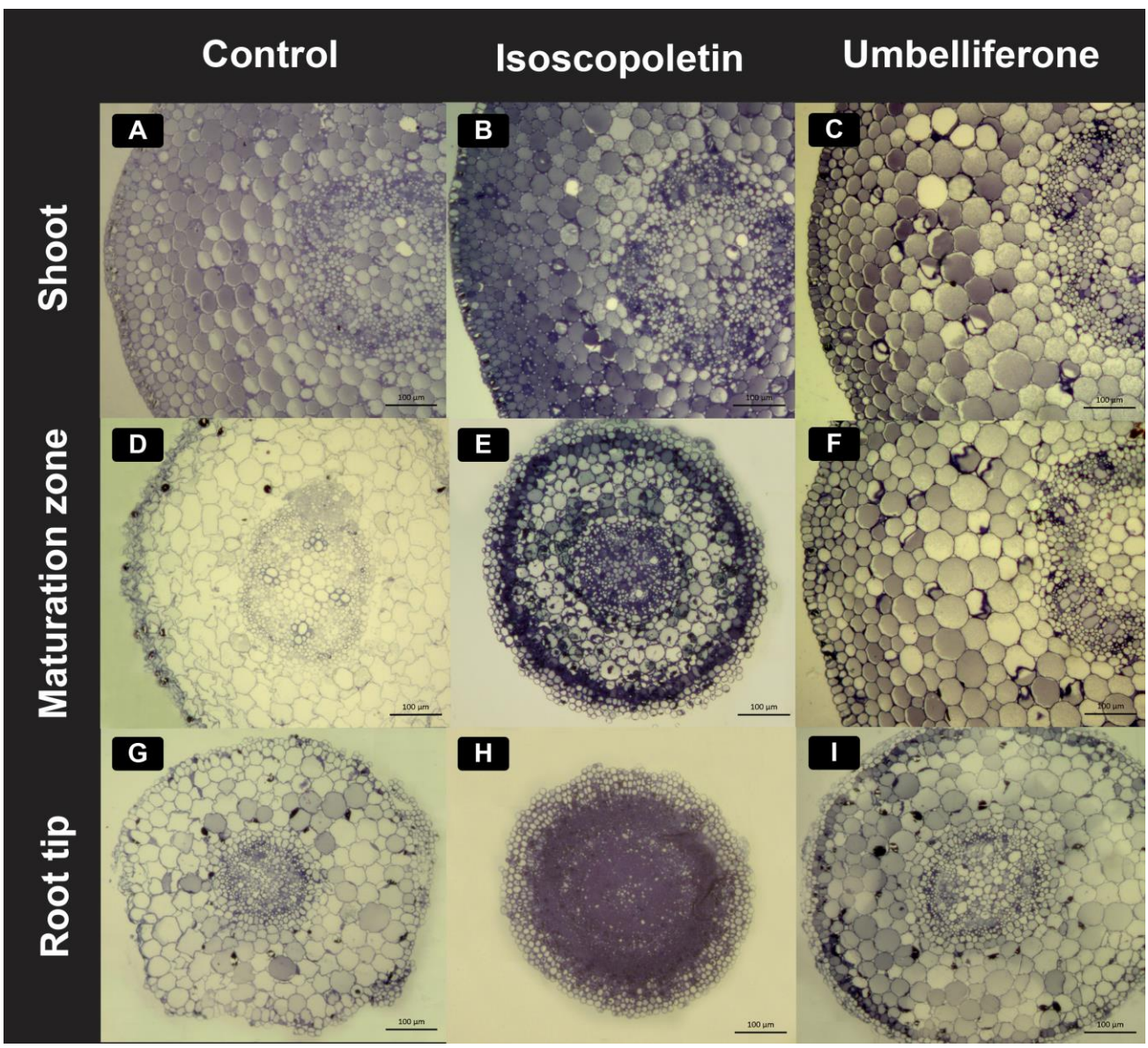


Fig. 6. Histolgical images of giant mimosa shoot (A,B,C) and root maturation zone (D,E,F) and root tip (G,H,Ii) exposed to isoscopoletin (B,E,H) and umbelliferone (C,H,I) compared with the control (A,D,G). Sections stained with toluidine blue.

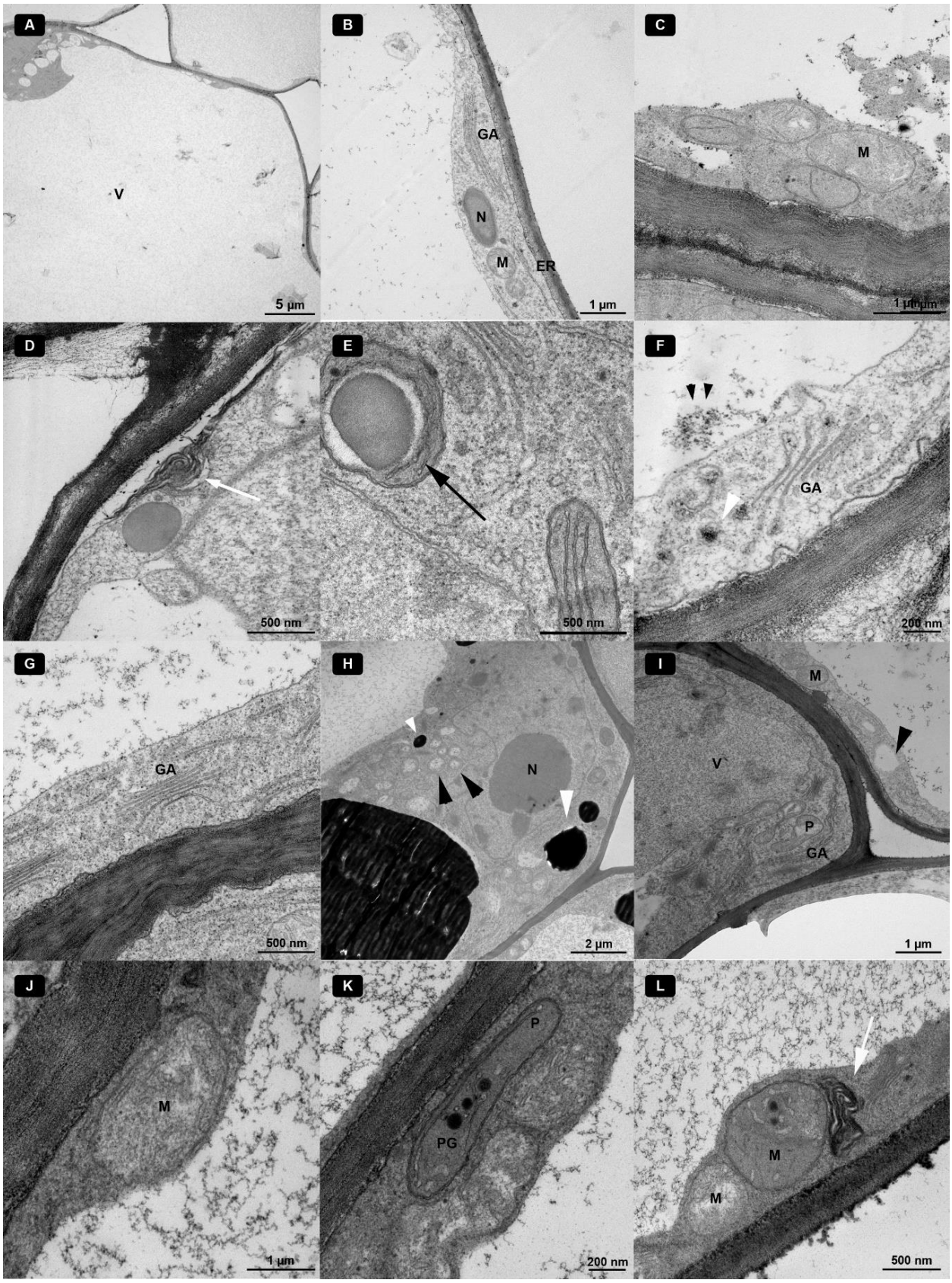


Fig. 7. Transmission electron micrographs (TEM) of giant mimosa root tip exposed to isoscopoletin (C-G) and umbelliferone (H-L) for 7 days. C. showed mitochondria with disrupted cristae, D. white arrow indicated the disrupted membrane, E. black arrow indicated autophagosome, F. electron-dense deposits found in vacuole (black arrow head) and in golgi apparatus(white arrow head), G. showed increased number of golgi apparatus and disrupted endoplasmic reticulum, H. white arrow head represented electron-dense deposits in cytoplasm whereas black arrow head represented vacuolization, I. showed increased number of golgi apparatus and distorted plastids, J. showed mitochondria with dilated and disrupted cristae, K. show plastoglobules in plastids and disorganized plastid and mitochondria, L. showed disrupted golgi apparatus as shown in white arrow.

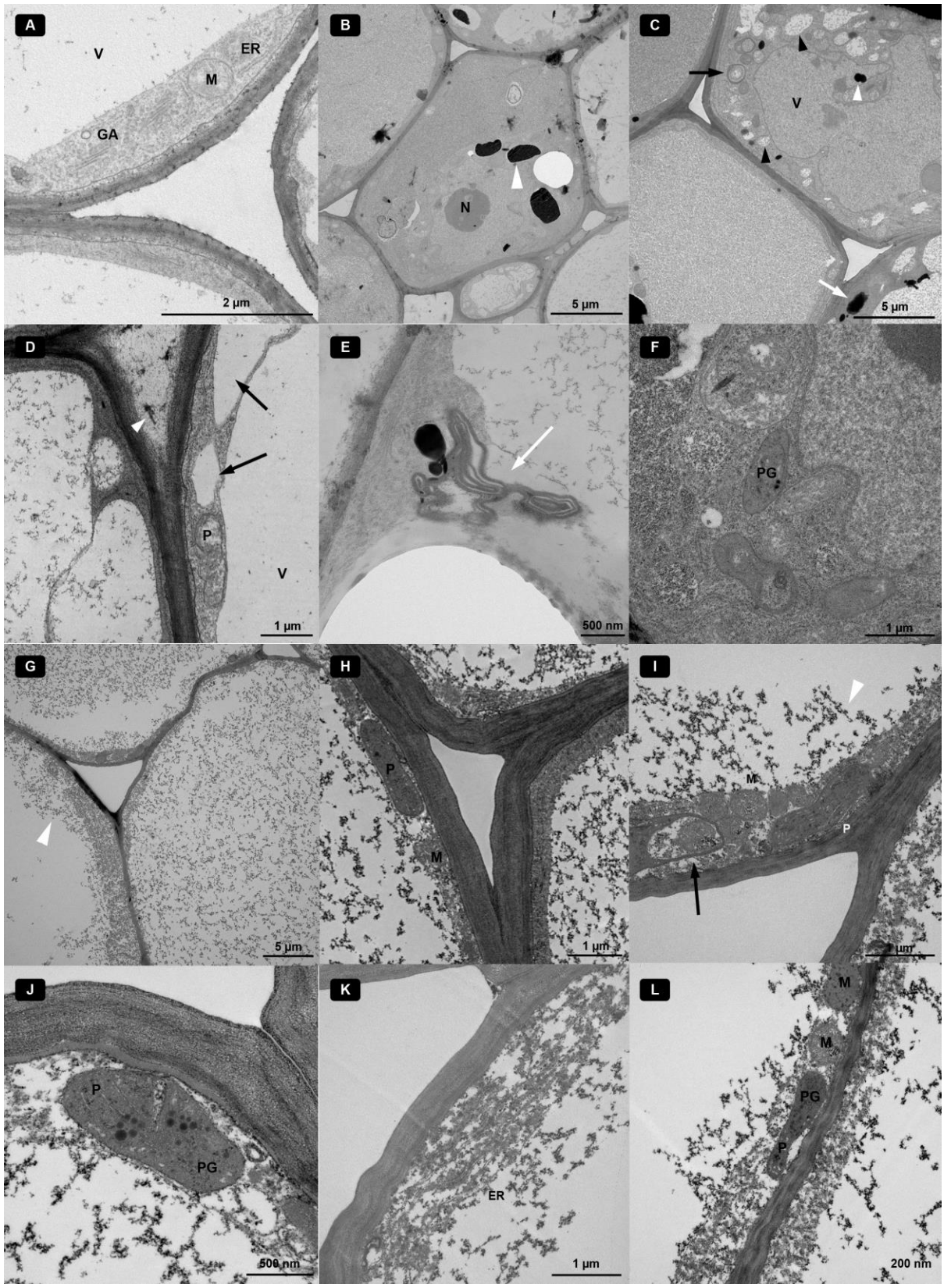


Fig. 8. Transmission electron micrographs (TEM) of giant mimosa root maturation zone exposed to isoscopoletin (B-F) and umbelliferone (G-L) for 7 days. B. white arrow head represented electron-dense deposits, C. black arrow represented autophagosome, white arrow head represented electron-dense deposit, black arrow head represented vacuolization, D. white arrow head was electron-dense deposits in intercellular space, black arrow was vacuolization, E. showed disrupted golgi apparatus, F. showed distorted shape of plastids with plastoglobules, G. white arrow head showed electron-dense deposits in vacuole, H. showed thickening cell wall and condense cytoplasm with disorganized mitochondria and plastid, I. showed increased number of disrupted plastid with plastoglobule and mitochondria with disrupted cristae, J. showed plastids with increased number of plastoglobules, K. showed fragment of ER, L. showed mitochondria with disrupted cristae and distorted plastids with plastoglobules.

Table 2. Anatomical characteristics of giant mimosa shoot and root treated with either isoscopoletin or umbelliferone, 7 days after treatment.

	Mean diameter of root (µm)	Mean diameter of stele (µm)	Number of cortical cells in tangential row
Shoot			
Control	1,197.92 ± 20.80b	397.92 ± 7.12b	11.75 ± 0.63ab
Isoscopuletin	1,183.33 ± 15.59b	393.75 ± 10.96b	11.25 ± 0.25b
Umbelliferone	1,600.00 ± 46.77a	493.75 ± 11.47a	13.25 ± 0.48a
Maturation zone of root			
Control	848.96 ± 31.66b	297.92 ± 13.77b	6.75 ± 0.25b
Isoscopuletin	610.42 ± 16.09c	217.71 ± 3.56c	7 ± 0.41b
Umbelliferone	1,487.50 ± 53.44a	472.92 ± 20.80a	12.25 ± 0.75a
Root tip			
Control	638.54 ± 9.06b	168.75 ± 1.20c	5 ± 0.41b
Isoscopuletin	500.00 ± 7.41c	186.46 ± 1.99b	4.25 ± 0.25b
Umbelliferone	747.92 ± 18.20a	227.08 ± 8.76a	7.75 ± 0.25a

Conclusion

Isoscopoletin and umbelliferone were isolated from *Haldina cordifolia*. They could delay germination and inhibit growth of *Mimosa pigra*. Stunted roots were commonly found in both treatments. Roots had more deleterious symptoms after treated with umbelliferone. Changed ultrastructures might involve in programmed cell death mechanism.

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References

Abenavoli, M.R., A. Nicolo, A. Lupini. S. Oliva and A. Sorgona. 2008. Effects of different allelochemicals on root morphology of *Arabidopsis thaliana*. *Allelopathy J*., 22: 245-252.

Abenavoli, M.R., A. Sorona, S. Albano and G. Cacco. 2004. Coumarin Differentially Affects The Morphology of Different Root Tyoes of Maize Seedlings. *J. Chem. Ecol*., 30: 1871-1883.

Abenavoli, M.R., G. Cacco, A. Sorgona, R. Marabottini, A.R. Paolacci, M. Ciaffi and M. Badiani. 2006. The inhibitory effects of coumarin on the germination of durum wheat (*Triticum turgidum* ssp. durum, cv. Simeto) seeds. *J. Chem. Ecol*., 32: 489-506.

Anya, A.L., M. Macias-Rubalcava, R. Cruz-Ortega, C. Garcia-Santana, P.N. Sanchez-Monterrubio, B.E. Hernandez-Bautista and R. Mata. 2005. Allelochemicals from *Stauranthus perforatus*, a Rutaceous tree of the Yucatan Peninsula, Mexico. *Phytochem*., 66: 487-494.

Brehelin, C., F. Kessler and K.J. Van Wijk. 2007. Plastoglobules: versatile lipoprotein particles in plastids. *Trends Plant. Sci*., 12: 260-266.

Chaves, N., T. Sosa and J.C. Escudero. 2001. Plant growth inhibiting flavonoids in exudate of *Cistusladanifer* and in associated soils. *J. Chem. Ecol*., 27: 623-631.

Chon, S.U., Y.M. Kim and J.C. Lee. 2003. Herbicidal potential and quantification of causative allelochemicals from several Compositae weeds. *Weed. Res*., 43: 333-450.

Hall, J.L. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot*., 53: 1-11.

Huang, W., W. Xing, D. Li and Y. Liu. 2009. Morphological and ultrastructural changes in tobacco BY-2 cells exposed to microcystin-RR. *Chemosphere*, 76: 1006-1012.

Jabeen, R., A. Ahmad and M. Iqbal. 2009. Phytoremediation of heavy metals: physiological and molecular mechanisms. *Bot. Rev*., 75: 339-364.

Kasinadhuni, V.R.R., G. Rajashekhar, R. Rajagopalan, V.M. Sharma, C. Vamsi Krishna, P. Sairam, G. Sai Prasad, S. Sadhukhan and G. Gangadhar Rao. 1999. Anti-ulcer potential of *Haldiniacordifolia*. *Fito*., 70: 93-95.

Kevresan, S., S. Kirsek, J. Kandrac, N. Petrovic and D.J. Kelemen. 2003. Dynamic of cadmium distribution in the intercellular space and inside cells in soybean roots, stems and leaves. *Biol. Plant*., 46: 85-88.

Khan, T.D., I.M. Chung, S. Tawata and T.D. Xuan. 2006. Weed suppression by *Passifloraedulis* and its potential allelochemical. *Weed. Res*., 46: 296-303.

Kim, B.G., Y. Lee, H.G. Hur, Y. Lim and J.H. Ahn. 2006. Production of three o-methylated Esculetins with *Escherichia coli* Expressing O-Methyltransferase from Poplar. *Biosci. Biotechnol. Biochem*., 70: 1269-1272.

Lin, D., E. Tsuzuki, Y. Sugimoto, Y. Dong, M. Matsuo and H. Terao. 2004. Elementary identification of phenolic allelochemicals from dwarf Lilytur plant (*Ophiopogon japonicas* K.) and their growth-inhibiting effects for two weeds in paddy rice field. *Plant. Prod. Sci*., 7: 260-265.

Lupini, A., A. Sorgona, A.J. Miller and M.R. Abenavoli. 2010. Short-time effects of coumarin along maize primary root axis. *Plant Signal Behav*., 8: e23156.

Lupini, A., F. Araniti, F. Sunseri and M.R. Abenavoli. 2014. Coumarin interacts with auxin polar transport to modify root system architecture in *Arabidopsis thaliana*. *Plant Growth Regul*., 74: 23-31.

Marcias, F.A., J.C. Galindo, D. Castellano and R.F. Velasco. 2000. Sesquiterpene lactones with potential use as natural herbicide models (I): trans, trans-germacranolides. *J. Agr. Food. Chem*., 48: 5288-5296.

Murasaki, T., H. Anzai, S. Imai, A. Satoh, N. Kozo and C.J. Thompson. 1986. The bialophos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterization of the gene cluster. *Mol. Gen. Genet*., 205: 42-53.

Nath, M., R. Jairath, G. Eng, X. Song and A. Kumar. 2005. Triorganotin (IV) derivatives of umbelliferone (7-hydroxycoumarin) and their adducts with 1, 10-phenanthroline: synthesis. *structural and biological studies, Spectrochim. Act. A*., 61: 3155-3161.

Palavan-Unsal, N., E.D. Buyuktuncer and M.A. Tufekci. 2005. Programmed cell death in plants. *Journal of Cell and Molecular Biology*, 4: 9-23.

Paynter, Q. and G.J. Flanagan. 2004. Integrating herbicide and mechanical control treatments with fire and biological control to manage an invasive wetland shrub. *Mimosa pigra, J. Appl. Ecol.*, 41: 615-629.

Romagni, J.G., S.N. Allen and F.E. Dayan. 2000. Allelopathic effects of volatile cineoles on two weedy plant species. *J. Chem. Ecol.*, 26: 303-313.

Sakai, A.K., F.W. Allendorf, J.S. Holt, D.M. Lodge, J. Molofsky. K.A. With, S. Baughman, R.J. Cabin, J.E. Cohen, N.C. Ellstrand, D.E. McCauley, P.O. Neil, I.M. Parker, J.N. Thompson and S.G. Weller. 2001. The population biology of the invasive species. *Ann. Rev. Ecol. Syst*., 32: 305-332.

Serrato, B., M. Vasquez, J.C. Calderon-Mugica, J.C. Marin and C.L. Cespedes. 2006. Antifungal and antibacterial activities of Mexican Tarragon (Tageteslucida). *J. Agri. Food Chem*., 54: 3521-3527.

Simberloff, D., I.M. Parker and P.N. Windle. 2005. Introduced species policy, management, and future research needs. *Front. Ecol. Envir*., 3: 12-20.

Singh, H.P, D.R. Batish and R.K. Kohli. 2006. *Handbook of sustainable weed management*, Haworth Press. New York

Suksungworn R., N. Sanevas, N. Wongkantrakorn, S.Vajrodaya and S. Duangsrisai. 2016. Phytotoxic effect of *Haldinacordifolia* on germination, seedling growth and root cell viability of weeds and crop plants. *NJAS - Wageningen Journal of Life Sciences*, 78: 175-181.

Toppi, L.S.D., E. Vurro, M. De Benedictis, G. Falasca, L. Zanella, R. Musetti, M.S. Lenucci, G. Dalessandro and M.M. Altamura. 2012. A bifasic response to cadmium stress in carrot: early acclimatory mechanisms give way to root collapse further to prolonged metal exposure. *Plant. Physiol. Bioch*., 58: 269-279.

Vacca, R.A., D. Valenti, A. Bobba, R.S. Merafina, S. Passarella and E. Marra. 2006. Cytochrome C is released in a reactive oxygen species-dependent manner and is degraded via caspase-like protease in tobacco BY-2 cells en route to heat-shock induced cell death. *Plant Physiol*., 141: 208-219.

Wierzbicka, M. 1998. Lead in the apoplast of *Allium cepa* L. roottips – ultrastructural studies. *Plant Science*, 133: 105-119.

Wilson, C. 2006. Parks and wildlife commission of the northern territory, Australia and Annie Lane, Northern Department of Primary Industry and Fisheries, Australia. 19 July. *Mimosa pigra* (shrub) GLOBAL INVASIVE SPECIES DATAABASE [online] Available: http://www.issg.org/database/species/ecology.asp?si=41&fr=1&sts=tss&lang=EN. [14 November 2014]

Wu, H., T. Haig, J. Pratley, D. Lemerle and M. An. 2002. Biochemical basis for wheat seedling allelopathy on the suppression of annual ryegrass (*Loliumrigidum*). *J. Agri. Food Chem*., 50: 4567-4571.

Yamamoto, Y. 2009. Movement of allelopathic compound coumarin from plant residue of sweet vernalgrass (*Anthoxanthumodoratum* L.) to soil. *Grassland Sci*., 55: 36-40.

Yu, H.N., P. Liu, Z.Y. Wang, W.R. Chen and G.D. Xu. 2011. The effect of aluminum treatments on the root growth and cell ultrastructure of two soybean genotypes. *Crop Prot*., 30: 323-328.

Zhang, R., R.R. Wise, K.R. Struck and T.D. Sharkey. 2010. Moderate heat stress of *Arabidopsis thaliana* leaves causes chloroplast swelling and plastoglobule formation. *Photosyn. Res*., 105: 123-134.

Zheng, L.J., T. Peer, V. Seybold and U. Lutz-Meindl. 2012. Pb-induced ultrastructural alterations and subcellular localization of Pb in two species of *Lespedeza* by TEM-coupled electron energy loss spectroscopy. *Envi. Exp. Bot*., 77: 196-206.

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