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ISOLATION AND IDENTIFICATION OF POTENT STIMULATORY ALLELOPATHIC SUBSTANCES EXUDED FROM GERMINATING BURDOCK (*Arctium lappa*) SEEDS

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Abstract –Two stimulatory allelopathic substances, which have a significant promotive activity for the shoot growth of cockscomb (*Celosia cristata*), were isolated from the exudates of germinating burdock (*Arctium lappa*) seeds. They were identified to be arctigenic acid and arctigenin by ^{1}H and ^{13}C NMR and EIMS spectra of them, respectively. Both substances showed growth-promoting activity at the concentrations higher than 10 mg/L for the cockscomb growth test. The contents of arctigenic acid and arctigenin were 58.5 and 39.0 mg/L in the exudates of germinating burdock seeds, respectively. These results suggest that arctigenic acid and arctigenin play important roles in the stimulatory allelopathy of burdock seeds during seed germination stage.

INTRODUCTION

Molisch[1] coined the term "allelopathy" to refer to biochemical interactions between all types of plants including microorganisms. His discussion indicated that he meant the term to cover both inhibitory and stimulatory reciprocal biochemical interactions.[2] Since then, various chemical agents involved in allelopathy have been isolated and identified from different adult plant organs and the volatiles, exudates and leaches released from them. However, information on allelopathy during seed germination, which occurs in the very early stages of development, is limited. Using various species of seeds, we have hitherto studied chemical communication between plant-plant interactions during seed germination stage. As inhibitory allelopathic substances, vanillic acid,[3] *p*- hydroxymandelic acid,[4] sundiversifolide[5] and pisatin[6] were isolated and identified from the exudates of germinating seeds of watermelon (*Citrulus*

vulgaris), barnyard grass (*Echinochloa crus-galli*), sunflower (*Helianthus annuus*) and pea (*Pisum sativum*), respectively. On the other hand, we have also reported on the stimulatory allelopathy during the seed germination stage: when cress (*Lepidium sativum*),[7] burdock (*Arctium lappa*), or oat (*Avena sativum*) seeds[8] were mixed cultured with several plant species, they promoted significantly the shoot growth of neighboring plants, especially that of cockscomb (*Celosia cristata*), suggesting that stimulatory allelopathic substance(s) are exuded from their germinating seeds into the culture solution. The allelopathic substance, which significantly promoted the shoot growth of cockscomb, was isolated from the exudates of germinating cress seeds and determined to be 4-deoxy-β-L-*threo*-hex-4-enopyramunosyl-(1→2)-L-rhamnopyranose sodium salt (named lepidimoide) by spectral analyses and total synthesis.[7,9,10] Lepidimoide is widespread in the exudates of germinating seeds of various plants species.[11,12] It promoted, not only shoot growth, but also leaf development, flowering, and seed production in *Arabidopsis thaliana*[13] and light-induced chlorophyll accumulation in cotyledons of sunflower seedlings.[14] Furthermore, lepidimoide inhibited leaf senescence in *Avena*[15] and abscission of bean petiole explants.[16] It was therefore suggested that lepidimoide acts as a phytohormone-like substance as well as an allelopathic substance. On the other hand, the chemical structure of bioactive substance(s) involved in the stimulatory allelopathy of burdock and oat seeds, has not yet been clarified.

In the present paper, we report on the isolation and identification of stimulatory allelopathic substances exuded from germinating seeds of burdock, and their roles in the stimulatory allelopathy of burdock seeds during seed germination stage.

RESULTS AND DISCUSSION

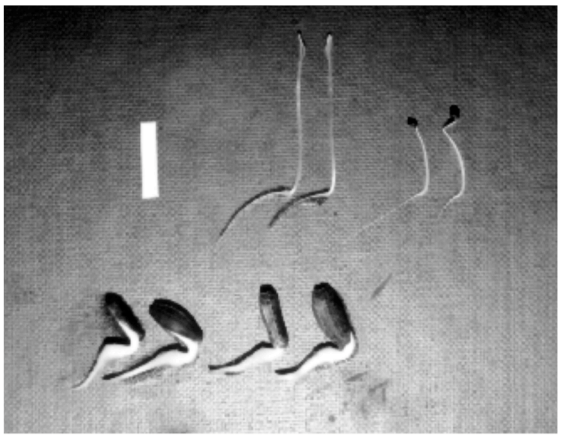
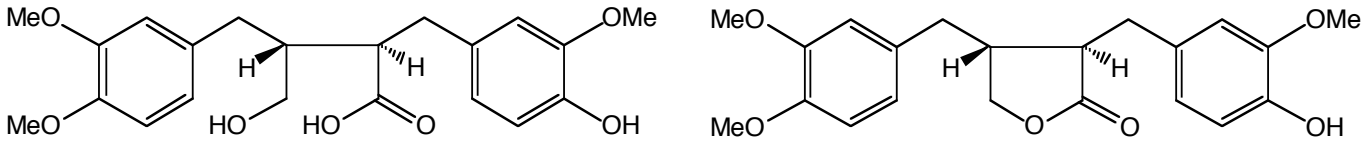


Figure 1. Interaction of burdock and cockscomb seedlings. Left; burdock seedlings, Center; burdock seeds (below) were cultured with cockscomb seeds (upper), Right; cockscomb seedlings. Ten seeds of the same or different species were cultured in a Petri dish in the dark for 4 days. Bar; 1 cm.

Figure 1 shows interaction of burdock and cockscomb seeds. Hypocotyl growth of cockscomb seedlings was significantly promoted by burdock seeds, whereas growth of burdock seedlings was not influenced with cockscomb seeds. These results suggest that allelopathic potential(s) promoting the hypocotyl growth of cockscomb seedlings are exuded from germinating burdock seeds into the culture solution. Two allelopathic substance(s), which showed promotive activity for the hypocotyl growth of cockscomb seedlings, were isolated from the exudates of germinating burdock seeds. Compound **(1)** was analyzed by ^{1}H and ^{13}C NMR spectra and identified as arctigenic acid (Figure 2).[17] The finding of arctigenic acid was the

first time. Compound **(2)** was also analyzed by ^{1}H and ^{13}C NMR spectra and identified as arctigenin (Figure 2).[18] Arctigenin has originally been isolated from burdock seeds as an antifungal substance.[19] Arctigenic acid was first found as a naturally occurring substance, although it was synthesized from arctigenin by Nishibe *et al*..[17] However, it has not yet been reported that both arctigenic acid and



Arctigenic acid (Compound **(1)**) Arctigenin (Compound **(2)**)

Figure 2. Chemical structures of compounds **(1)** and **(2)**.

arctigenin have a potent plant growth-promoting activity.

Biological activities of isolated arctigenic acid and arctigenin were measured using the bioassay of cockscomb hypocotyl growth. As shown in Figure 3, both substances promoted the hypocotyl growth of cockscomb seedlings at the concentrations higher than 10 mg/L. Arctigenic acid showed higher activity than arctigenin at the concentrations of 10 to 100 mg/L, but its activity decreased at the concentration of 300 mg/L. Dose-response curve of arctigenin was linear at the concentrations used, and its activity showed higher than arctigenic acid at the concentration of 300 mg/L.

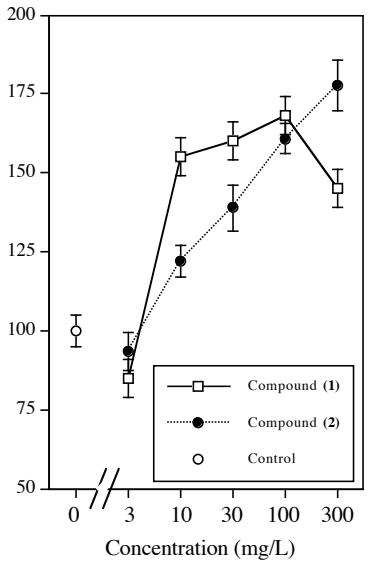


Figure 3. Effects of compounds **(1)** (arctigenic acid) and **(2)** (arctigenin) on the growth of cockscomb hypocotyls. Data replesent the means ± SE.

To establish whether arctigenic acid and arctigenin contribute to stimulatory allelopathy of burdock seeds, the contents of arctigenic acid and arctigenin in the exudates of germinating burdock seeds were compared to biological activity of the exudates. The contents of arctigenic acid and arctigenin were estimated to be 58.5 and 39.0 mg/L by measuring the peaks area of HPLC (Table 1). From dose-response curves of them, their contents were

Table 1. The contents of arctigenic acid and arctigenin in the exudates of germinating burdock seeds for 4 days in the dark.

Compound	Content (mg/L)
Arctigenic acid **(1)**	58.5
Arctigenin **(2)**	39.0

calculated to show growth promotion by about 170% and about 150%, respectively. On the other hand, the exudates showed growth promotion by about 150%. Although biological activity of mixture of arctigenic acid and arctigenin was not studied, it was suggested that these substances play important roles in the stimulatory allelopathy of burdock seeds. Moreover, arctigenic acid and arctigenin were not detected in the exudates of germinating oat seeds (data not shown), which showed the same stimulatory allelopathy as burdock seeds, suggesting that stimulatory allelopathy of oat seeds is regulated by different substance(s) from arctigenic acid and arctigenin.

EXPERIMENTAL

Mix-culture of burdock and cockscomb seeds

Ten seeds of burdock (*Arctium lappa*) were placed together alternately with 10 seeds of cockscomb (*Celosia cristata*) at even intervals on filter paper (No.1; Toyo, Ltd., Tokyo, Japan), moistened with 2 mL of distilled water in a 9 cm Petri dish. The dishes were put into a tray with moistened filter paper and then covered with Saran Wrap (Asahi KASEI Co., Tokyo, Japan) to retain humidity and incubated at 23°C in the dark. After 4 days, the hypocotyls of each seedlings were measured. Ten seeds of each species alone were also incubated as a control. Experiments were repeated three times.

Isolation of plant growth-promoting substances

About 15,000 burdock seeds were sterilized in sodium hypochlorite solution (1% of active chlorine) for 30 min, and rinsed with distilled water. The wet seeds were sown on the stainless: netting (3 mm-mesh) in a tray (40 × 40 × 5 cm^3) contained with 1.3 L of distilled water and cultured at 23°C in the dark for 3 days. The culture solution was daily exchanged with fresh distilled water. All culture solutions were combined and filtered through filter paper. The filtrate was concentrated in dryness in vacuo at 40°C. The concentrate was fractionated into the methanol-soluble fraction and the methanol-insoluble, water-soluble one. Growth-promoting activity for the cockscomb hypocotyls was found in the latter fraction. The active fraction (399 mg) was separated into four fractions of 0, 30, 60 and 100% methanol in water (45 mL) by C_{18} cartridge chromatography (Sep-Pak Vac 35 mL (10 g), Waters). The 0% eluate showing promoting activity was concentrated in vacuo at 40°C. The concentrate (338 mg) was subjected to HPLC (Amide-80, Tosoh Ltd., 7.8 × 300 mm, 0-8 min; 85% CH_3CN in H_20, 8-13 min; linear gradient from 85% to 100% H_20, 13-25 min; 0% CH_3CN, 1.5 mL/min, detected at 205 nm). Growth-promoting activity was found in a fraction with a retention time of 5.8-8.6 min. The active fraction was further subjected to HPLC (Amide-80, Tosoh Ltd., 7.8 × 300 mm, 85% CH_3CN in H_20, 1.5 mL/min, detected at 205 nm). Promoting activities were found in the two peaks with retention times of 3.8-4.2 min and 5.9-6.2 min. Each active peak was evaporated to dryness in vacuo at 40°C, yielding 1.0 mg (compound **(1)**) and 1.3 mg (compound **(2)**), respectively.

Spectrometric analysis

The ESI-MS, the ^{1}H and ^{13}C NMR spectrum was taken on Platform (Waters), a JNMGX400 and a JNMGX100 NMR spectrometer (JEOL), respectively.

Arctigenic acid (1) $[\alpha]_D^{25}$ −3° (*c* 0.2, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 6.85 (1H, d, *J* = 8.1 Hz, H-5), 6.80 (1H, d, *J* = 1.9 Hz, H-2), 6.77 (1H, dd, *J* = 8.1 and 1.9 Hz, H-6), 6.77 (1H, t, *J* = 1.9 Hz, H-2'), 6.68 (1H, d, *J* = 7.9 Hz, H-5'), 6.65 (1H, dd, *J* = 7.9 and 1.9 Hz, H-6'), 3.83 (3H, s, MeO), 3.81 (6H, s, MeOx2), 3.52 (lH, dd, *J* = 11.4 and 4.2 Hz, H-9), 3.02 (1H, dd, *J* = 12.7 and 7.2 Hz, H-7'), 2.79 (1H, dd, *J* = 12.7 and 7.0 Hz, H-7'), 2.78 (1H, m, H-7), 2.75 (1H, m, H-8'), and 1.80 (1H, m, H-8); ^{13}C NMR (500 MHz, CD_3OD) δ 165.4 (C-9'), 148.5 (C-3), 146.0 (C-4 and C-3'), 145.0 (C-4'), 132.0 (C-1), 129.6 (C-1'), 122.7 (C-6'), 121.8 (C-6), 115. 8 (C-5'), 114.3 (C-5), 113.5 (C-2'), 113.0 (C-2), 63.6 (C-9), 56.6 (MeO), 56.4 (MeO), 56.4 (MeO), 44.8 (C-8'), 44.0 (C-8), 38.2 (C-7), and 36.8 (C-7'); ESI-MS (m/z) $[M+Na]^+$ = 413.

Arctigenin (2) $[\alpha]_D^{25}$ −15° (*c* 0.2, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 6.86 (1H, d, *J* = 8.1 Hz, H-5), 6.74 (1H, d, *J* = 8.0 Hz, H-5'), 6.71 (1H, d, *J* = 1.9 Hz, H-2'), 6.65 (1H, dd, *J* = 8.1 and 2.0, H-6), 6.64 (1H, s, H-2), 6.61 (1H, dd, *J* = 8.0 and 2.0 Hz, H-6'), 4.21 (1H, dd, *J* = 9.0 and 7.3 Hz, H-9), 3.99 (1H, dd, *J* = 9.0 and 7.5 Hz, H-9), 3.84 (3H, s, MeO), 3.82 (3H, s, MeO), 3.80 (3H, s, MeO), 2.94 (1H, dd, *J* = 13.8 and 5.2 Hz, H-7'), 2.85 (lH, dd, *J* = 13.8 and 7.0 Hz, H-7'). 2.71 (1H, ddd, *J* = 7.7, 7.0, and 5.2 Hz, H-8'), 2.60 (1H, m, H-7), and 2.56 (1H, ddd, *J* = 7.7, 7.0, and 7.0 Hz, H-8); ^{13}C NMR (500 MHz, CD_3OD) δ 182.5 (C-9'), 151.3 (C-3), 150.0 (C-4), 149.9 (C-3'), 147.6 (C-4'), 133.7 (C-1), 131.2 (C-1'), 123.8 (C-6'), 122.9 (C-6), 117.0 (C-5'), ll4.6 (C-5), 114.4 (C-2'), 113.8 (C-2), 73.7 (C-9), 57.3 (MeO), 57.2 (MeO), 57.1 (MeO), 48.6 (C-8'), 43.3 (C-8), 39.7 (C-7), and 36.2 (C-7'); ESI-MS (m/z) $[M+Na]^+$ = 395.

Bioassay

Ten seeds of cockscomb (*Celosia cristata*) was placed on a filter paper moistened with 500 μL of test solution in a 3.3 cm Petri dish and kept for 4 days at 23°C in the dark, after which the lengths of their hypocotyls were measured.

Determination of the contents of compounds (1) and (2) in the exudates of germinating burdock seeds

Twenty seeds of burdock were sterilized with sodium hypochlorite solution (1% of active chlorine) for 30 min, and rinsed with distilled water. The wet seeds were incubated in the 4.5 cm Petri dish containing with 4 mL of distilled water at 23°C in the dark for 2 days. The incubated seeds were removed from the dish and the residual solution was filtered through a sheet of filter paper. The filtrate was evaporated to dryness in vacuo at 40°C. The sample dissolved in distilled water was divided into two lots. One was assayed for the cockscomb hypocotyl growth test. Another was subjected to HPLC (Amide-80, Tosoh

Ltd., 7.8 □ 300 mm, 0-15 min; linear gradient from 85% CH_3CN in H_2O to 0% CH_3CN, 1.5 mL/min, detected at 205 nm, compound **(1)** eluted at Rt 3.9 min, compound **(2)** at Rt 6.1 min). Quantification of compounds **(1)** and **(2)** was performed by measuring the peak area and calibrating. Experiments were repeated three times.

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