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CAPROLACTAM, AN INHIBITORY ALLELOCHEMICAL EXUDED FROM GERMINATING BUCKWHEAT (*FAGOPYRUM ESCULENTUM*) SEEDS

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Abstract – An allelochemical, which showed an inhibiting activity against the radicle growth of cress seeds, was isolated from the exudates of germinating buckwheat seeds. It was identified as caprolactam based on its ^{1}H and ^{13}C NMR spectra. The occurrence of caprolactam in the exudates from seeds of various plant species was studied on the basis of HPLC retention time and its UV spectrum. Caprolactam was detected in the exudates from seeds of sunflower as well as buckwheat, but not in those from seeds of cress, rice, pea, lettuce and radish. The mode of exudation of caprolactam from buckwheat seeds into the culture solution, was studied in relation to seed germination. About 0.8 μg of caprolactam was found in a dry seed. Upon water imbibition for 1 day about 15 % of caprolactam was exuded into the culture solution. The content of caprolactam in an imbibed seed was about 0.69 μg. These results suggest that caprolactam, already presents in dry seeds, was exuded from the seeds into the environment during seed germination stage. Caprolactam inhibited the radicle growth of cress seeds at the concentrations higher than 3 mg/L. All these results suggest that caprolactam may play as an allelochemical in the inhibitory allelopathy of buckwheat seeds during seed germination stage.

INTRODUCTION

The term allelopathy was coined by Molisch in 1937.[1] Allelopathy is derived from Greek, "allelon" of each other and "pathos" to suffer – the injurious effect of one upon another. Presently, the term generally refers to both detrimental and beneficial biochemical interactions among all classes of plants, including

microorganisms. The study of allelopathy has a long history. According to Rice,[2] Lee and Monsi found a report by Banzan Kumazawa in a Japanese document some 300 years old that rain or dew washing the leaves of red pine was harmful to crops growing under the pine.[3] Historically, this is considered to be the first report on allelopathy. The allelopathic properties of plants can be exploited successfully as a tool for pest and weed control. In fact, several compounds extracted from higher plants have been used in agriculture.[4] Several crops inhibit weeds – a very promising trend for sustainable weed management. Buckwheat (*Fagopyrum* spp.) is not only an important crop in many countries, but is also useful for soil improvement and reduction of pests and weeds.[5] In upland fields, buckwheat markedly suppressed growth of quackgrass (*Agropyron sepens* L.),[6] the biomass of *Digitaria ciliarris*, *Galinsogaciliata*, *Echinochloa crus–galli*, *Portulaca oleracea*, *Chenopodium album* and *Amaranthus lividus*.[7] The allelochemicals in the Japanese cultivars of buckwheat have been isolated and identified as some phenolic compounds, ferulic, caffeic and chlorogenic acids,[8] long chain fatty acids,[9] fagomine, 4–piperidone and 2–piperidinemethanol,[10] and gallic acid and (+)–catechin.[11] Palmitic acid methylester, vanillic acid, rutin, gallic acid derivatives and 4–hydroxy–acetophenone derivatives were isolated as allelochemicals from root exudates of buckwheat.[12] However, these allelochemicals have been isolated and identified from different adult plant organs and root exudates. Information on allelopathy during seed germination, which occurs in the very early stages of development, is limited. The objectives of this study were to isolate and identify allelochemical(s) exuded from buckwheat seeds during seed germination.

RESULTS AND DISCUSSION

Figure 1 shows interaction of buckwheat and cress seeds. Radicle growth of cress seeds was significantly inhibited by buckwheat seeds, whereas radicle growth of buckwheat seeds was not affected with cress seeds. These results suggest that allelochemical(s) inhibiting the radicle growth of cress seeds are exuded from germinating buckwheat seeds into the culture solution. An allelochemical, which showed inhibitory activity for the radicle growth of cress seeds, was isolated from the exudates of germinating buckwheat seeds. The ^{1}H and ^{13}C NMR spectra of the isolated compound were absolutely identical with those of authentic caprolactam, hexahydro–2H–azepin–2–one (Figure 2). Caprolactam, a precursor in the fabrication of 6–nylon (perlon L),[13] had

Figure 1. Interaction of buckwheat and cress seeds. Left: buckwheat seeds, Center: buckwheat seeds (upper) were cultured with cress seeds (below), Right: cress seeds. Ten seeds of the same or different species were cultured in a Petri dish in the dark for 1 day. Bar; 5mm

been already found in sunflower seedlings as a natural growth–inhibiting substance.[14] However, it has not been reported so far that caprolactam plays a role in allelopathy of buckwheat during seed germination. On the basis of HPLC retention time and UV spectrum of caprolactam, its occurrence in dry seeds of buckwheat (*Fagopyrum esculentum*), sunflower (*Helianthus annuus*), cress (*Lepidium sativum*), rice (*Oryza sativa*), pea (*Pisum sativum*), lettuce (*Lactuca sativa*) and radish (*Raphanus sativus*) and in seed exudates and imbibed seeds of them 1 day after the start of imbibition was measured. Caprolactam occurred in the dry and imbibed seeds, and the seed exudates of sunflower as well as buckwheat. However, no caprolactam was detected in the other plant species tested (Table 1).

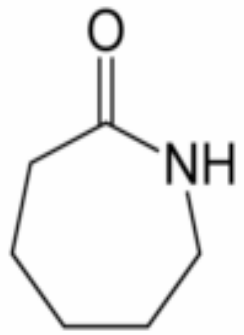

Figure 2. Chemical structure of caprolactam

About 0.80 μg of caprolactam was found in one dry seed of buckwheat. Upon water imbibition for 1 day about 15% of caprolactam was exuded into the culture solution. The content of caprolactam in one imbibed seed was about 0.69 μg. These results suggest that caprolactam, already presents in dry seeds, was exuded into the environment during seed germination stage. Biological activity of caprolactam was tested on the radicle growth of germinating cress seeds. Caprolactam inhibited the cress radicle elongation at concentrations higher than 3 mg/L (Figure 3). In conclusion, the results of this study suggest that caprolactam exuded from germinating buckwheat seeds plays important roles in the inhibitory allelopathy of buckwheat seeds.

Table 1. Caprolactam content in the dry seeds of buckwheat, sunflower and other plant species and in their wet seeds and exudates, 1 day after the start of water imbibition. Data are means of 3 experiments ± SE.

Material	Caprolactam content (μg/one seed eq.)	
	Buckwheat (*Fagopyrum esculentum*)	Sunflower (*Helianthus annuus*)
Dry seeds	0.80 ± 0.07	0.06 ± 0.01
Imbibed seeds	0.69 ± 0.06	0.12 ± 0.01
Seed exudates	0.12 ± 0.01	0.10 ± 0.01

Other plant species in which the caprolactam was not detected: *Lepidium sativum*; *Oryza sativa*; *Pisum sativum*; *Lactuca sativa*; *Raphanus sativus*.

EXPERIMENTAL

Mix–culture of buckwheat and cress seeds

Buckwheat (*Fagopyrum esculentum*) and cress (*Lepidium sativum*) seeds were sterilized with 1% sodium hypochlorite for 30 min and rinsed with distilled water, respectively. Ten wet seeds of buckwheat were incubated together with 10 wet seeds of cress in a Petri dish (3.5 cm) containing 1 mL of distilled water. The dishes were incubated at 25 ˚C in the dark. After 1 day, the lengths of radicles of each seeds were measured. Ten seeds of each species alone were also incubated as a control. Experiments were repeated three times.

Isolation of inhibitory allelochemical(s)

About 2,000 seeds of buckwheat which were sterilized with 1% sodium hypochlorite for 30 min and rinsed with distilled water were allowed to be put on a stainless steel net (3 mm mesh) in a stainless steel tray (40 x 40 x 5 cm^3) containing 2 L of distilled water. The seeds on the net, in contact with the water, were cultured at 25 ˚C in the dark for 3 days. The culture solution was collected every day and replaced with fresh distilled water. The culture solutions were filtered through a sheet of filter paper and evaporated to dryness *in vacuo* at 35 ˚C. The concentrate (6.24 g) was dissolved in 100 mL of methanol and filtered. The residue was dissolved in 100 mL of distilled water. The methanol–soluble fraction and the methanol–insoluble and water–soluble fraction thus obtained were evaporated to dryness *in vacuo* at 35 ˚C, respectively. The inhibitory activity was detected in the methanol–soluble fraction. The concentrate (3.2 g) was centrifuged by Viva spin (M.W. 3000, VIVASCIENCE) at 3000 rpm for 4 hr. The concentrate (1.12 g) of the M.W. < 3000, which showed growth–inhibiting activity, was applied to a C_{18} Sep–Pak cartridge column (Waters). The column was gradually eluted with 40 mL of 0, 25, 50, 75% and finally 100% MeOH in distilled water. The strong inhibitory activities were found in the 25% and 50% MeOH eluates. The latter eluate was concentrated *in vacuo* at 35 ˚C and gave 25.2 mg. The concentrate was subjected to HPLC (ODS–120A, TOSOH, Japan, 7.8 x 300 mm, 0–10 min; 0% MeCN in H_2O, 10–25 min; linear

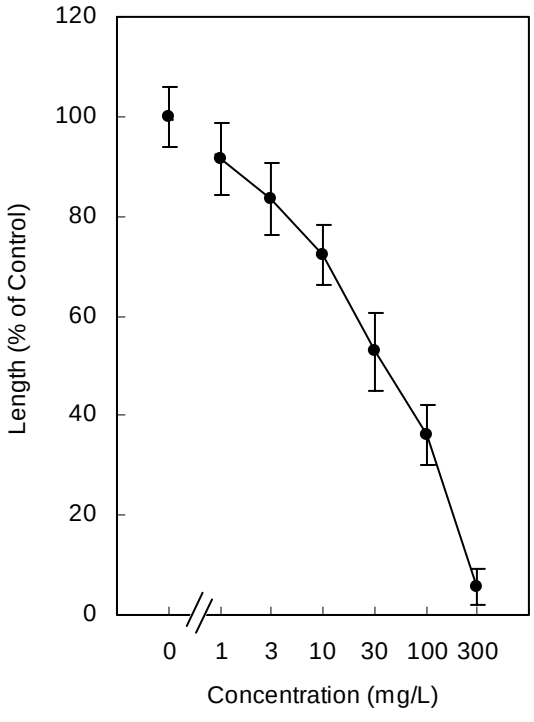

Figure 3. Effect of caprolactam on the radicle growth of cress seeds. Mean ± SE of results from 3 replicates of 10 plants.

gradient from 0% to 70% MeCN; 25–30 min; linear gradient from 70% to 100% MeCN, 1.5 mL/min, detector at 195 and 205 nm). The inhibitory activity was found in fraction with the retention time of 15.8 to 16.0 min. The eluates were concentrated *in vacuo* at 35 ˚C and gave 0.9 mg.

Spectrometric analysis

The ^{1}H and ^{13}C NMR spectrum was taken on a INOVA–500 AS NMR spectrometer (Varian).

Caprolactam (**1**); The ^{1}H NMR spectral data δ (500 MHz, D_2O) were as follows: 1.47 (2H, m, H–5), 1.51 (2H, m, H–3), 1.62 (2H, m, H–4), 2.34 (2H, m, H–2) and 3.11 (2H, m, H–6). The ^{13}C NMR spectral data δ (125 MHz, D_2O) were as follows: 22.7 (C–3), 28.5 (C–5), 29.9 (C–4), 35.6 (C–2), 42.5 (C–6) and 182.9 (C–1).

Bioassay

Ten seeds of cress were placed on a filter paper moistened with 500 μL of test solution in a 2.7–cm Petri dish and kept for 24 hr at 25 ˚C in the dark, after which the lengths of their radicles were measured.

Determination of caprolactam

The occurrence of caprolactam in dry seeds and in imbibed seeds and seed exudates of buckwheat (*Fagopyrum esculentum*), sunflower (*Helianthus annuus*), cress (*Lepidium sativum*), rice (*Oryza sativa*), pea (*Pisum sativum*), lettuce (*Lactuca sativa*) and radish (*Raphanus sativus*) was studied. Each sixty seeds were sterilized with sodium hypochlorite for 30 min and rinsed with distilled water. The wet seeds were put on a filter paper moistened with 27 mL in a 9–cm Petri dish and incubated in the dark at 25 ˚C for 1 day. The culture solution was filtered through a filter paper and evaporated to dryness *in vacuo* at 35 ˚C. Sixty dry seeds or seeds imbibed for 1 day were homogenized with 90 mL of 80% acetone and filtered through a filter paper. The filtrate was evaporated to dryness *in vacuo* at 35 ˚C. These concentrates or the concentrates of the seed exudates were dissolved in methanol and analyzed by HPLC (ODS–120A, TOSOH, 4.6 x 250 mm, 0–20 min; linear gradient from 0% to 50% MeCN in H_2O, 0.8 mL/min, detector at 195 nm, caprolactam eluted at the retention time of 17.5 min), respectively. Co–chromatography was used to determine whether sample peak and caprolactam standard were equivalent. After the area of the sample peak was determined, the content of endogenous caprolactam was calculated from standard curve. The UV spectrum of the peak was compared with that of authentic caprolactam. The experiments were repeated three times.

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