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ISOLATION AND IDENTIFICATION OF AN ALLELOCHEMICAL EXUDED FROM GERMINATING PEA (*Pisum sativum*) SEEDS

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Abstract –When pea seeds were cultured in a Petri dish together with cress seeds, the hypocotyl and root growth of cress was significantly inhibited, whereas the epicotyl growth of pea was promoted. These results suggest that the growth of each seeds is either inhibited or promoted with allelochemical(s) exuded from each seeds during seed germination. An allelochemical, which showed a growth-inhibiting activity against cress seeds, was isolated from the exudates of germinating pea seeds. It was identified as 6a-hydroxy-3-methoxy-8,9-methylenedioxy peterocarpan (pisatin) based on its ^{1}H NMR spectrum. The endogenous pisatin level (22 mg/L) in the exudates from germinating pea seeds seems to be a good agreement with the exogenous concentration (>3 mg/L) of pisatin required to inhibit the hypocotyl and root growth of cress. These results suggest that pisatin may play an important role in the allelopathy of pea seeds during germination.

Chemical substances releasing from plant organs into neighboring environment as a biological information give stimulating or inhibiting onto development and/or growth of other plants: this chemical communication is called as an allelopathy.[1-3] Recently, we have reported that seed exudates of several plant species during seed germination showed stimulating or inhibiting activity for growth of other plant species.[4] Pea generally showed a growth-inhibiting effect on the growth of other plant species. In this paper, we report the isolation and identification of allelochemical(s), which showed inhibitory activity against the growth of cress seedlings, from the exudates of germinating pea seeds, and its role in the allelopathy.

Pea (*Pisum sativum*) seeds were sterilized with sodium hypochlorite solution (1% of active chlorine) for 30 min and rinsed with distilled water. Five seeds were alternately placed together with ten seeds of cress (*Lepidium sativum*) on a filter paper (No. 1, Toyo, Ltd., Tokyo) moistened with 2.5 mL of distilled water in a 4.5-cm Petri dish. The dishes were incubated at 25°C in the dark. After 3 days, the lengths of shoots and roots of pea or cress were measured. Five seeds of pea or ten seeds of cress alone were also incubated as control. The experiments were repeated five times. When pea seeds were cultured together with cress seeds, the hypocotyl and root growth of cress was significantly inhibited by pea seeds, whereas the epicotyl growth was promoted (Figure 1). These results suggest that allelochemical(s) showing either growth-inhibiting or -promoting activity for each plant species are exuded from each germinating seeds into neighboring environment.

For the purpose of identifying the allelochemical(s), eight hundred seeds of pea were sterilized with sodium hypochlorite solution (1% of active chlorine) for 30 min and rinsed with distilled water, and allowed to put on a stainless steel net (3 mm mesh) in a stainless steel tray (40 × 40 × 5 cm^3) containing 1.3 L of distilled water. The seeds on the net, in contact with the water, were culture 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 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, water-soluble fraction were evaporated to dryness *in vacuo* at 35°C, respectively. Biological activity was determined using the cress root growth test. Ten seeds of cress were placed on a filter paper moistened with 500 mL of test solution in a 3.3-cm Petri dish and kept for 3 days at 25°C in the dark, after which the lengths of their hypocotyls and roots were measured. The inhibitory activity was detected

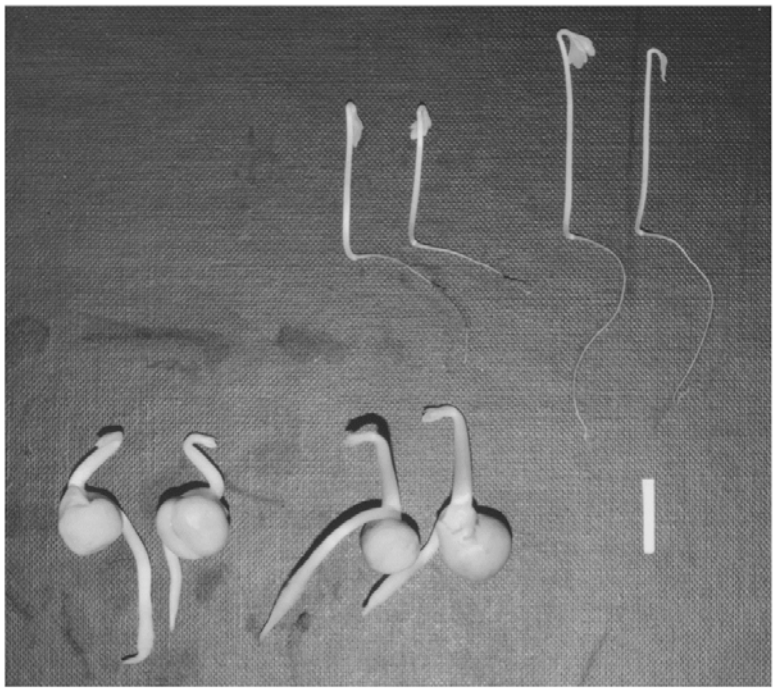

Figure 1. Interaction of pea and cress seeds. Left: pea seedlings, Center: pea seeds (below) were cultured with cress seeds (upper), Right: cress seedlings. Five seeds of the same or different species were cultured in a Petri dish in the dark for 3 days. Bar; 1 cm.

in the methanol-soluble fraction. The concentrate (131 mg) was applied to a C_{18} Sep-pack cartridge column (Waters). The column was eluted with 40 mL of 0, 30, 60% and finally 100% methanol in water. The strongest inhibitory activity was found in the 100% methanol eluate. The eluate was concentrated *in vacuo* at 35°C and gave 11 mg. The concentrate was subjected to HPLC (Develosil C30-UG-5, Nomura Chemical Co., Ltd., Japan, 10 × 250 mm, 0-20 min; linear gradient from 0% to 100% CH_3CN, 20-30 min; 100% CH_3CN, 2 mL/min, detector at 205 nm). The biological activity was found in fraction with the retention time of 21 to 22 min. The eluates were concentrated *in vacuo* at 35°C and gave 2.5 mg. The ^{1}H NMR spectrum was taken on a Bruker AVANCE-500 spectrometer. ^{1}H NMR spectral data (500 MHz, $CDCl_3$) were as follows: δ 7.38 (1H, d, *J* = 8.6 Hz, H-1), 6.81 (1H, s, H-7), 6.66 (1H, dd, *J* = 8.6 and 2.5 Hz, H-2), 6.46 (1H, d, *J* = 2.5 Hz, H-4), 6.40 (1H, s, H-10), 5.94 (1H, d, *J* = 1.2 Hz, O-CH_3-O), 5.91 (1H, d, *J* = 1.2 Hz, O-CH_3-O), 5.29 (1H, s, H-11a), 4.19 (1H, d, *J* = 11.7 and 0.7 Hz, H-6eq), 4.01 (1H, d, *J* = 11.7 Hz, H-6ax), and 3.78 (3H, s, OMe). From the comparison of the data with that of reported in the literature (^{1}H NMR spectral data (250 MHz, $CDCl_3$): δ 7.37 (1H, d, *J* = 8.5 Hz, H-1), 6.80 (1H, s, H-7), 6.65 (1H, dd, *J* = 8.5 and 2.4 Hz, H-2), 6.45 (1H, d, *J* = 2.4 Hz, H-4), 6.39 (1H, s, H-10), 5.94 (1H, d, *J* = 1.2 Hz, O-CH_3-O), 5.90 (1H, d, *J* = 1.2 Hz, O-CH_3-O), 5.28 (1H, brs, H-11a), 4.18 (1H, d, *J* = 11.6 and 0.6 Hz, H-6eq), 4.01 (1H, d, *J* = 11.6 Hz, H-6ax), and 3.77 (3H, s, OMe)),[5] isolated substance was identified as 6a-hydroxy-3-methoxy-8,9-methylenedioxy peterocarpan (pisatin) (Figure 2). Pisatin has originally been isolated from pods of pea (*Pisum sativum*) as an antifungal principle.[6, 7] However, it has not yet been reported that pisatin plays a role in allelopathy of pea during seed germination.

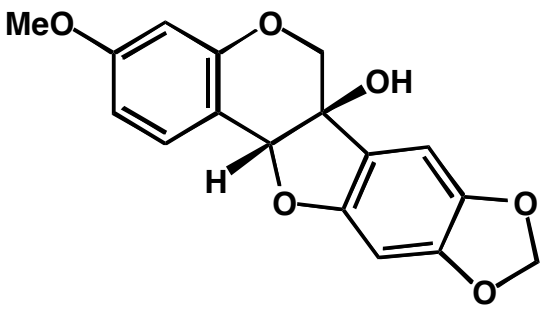

Figure 2. Chemical structure of pisatin.

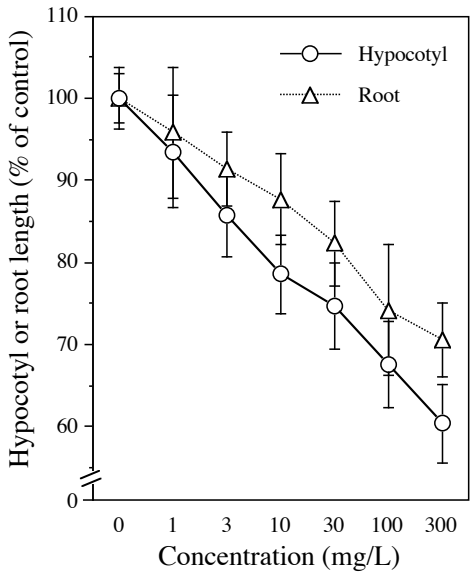

Figure 3. Effect of isolated pisatin on the hypocotyl and root growth of cress. Means ± SE of results from 3 replicates of 10 plants.

To determine the pisatin level in the exudates of germinating pea seeds, ten pea seeds were sterilized with sodium hypochlorite solution (1% of active chlorine) for 30 min and rinsed with distilled water. The wet seeds were put on a filter paper moistened with 4 mL of distilled water in a 9-cm Petri dish and incubated in the dark at 25°C for 3 days. The culture solution was collected and filtered through a filter paper. The filtrate was evaporated to dryness *in vacuo* at 35°C. The concentrate dissolved in methanol was divided

into two lots. One lot was assayed for the cress hypocotyl growth test. The biological activity was determined after 3 days and calculated as pisatin-equivalents from the dose-response curves of pisatin (Figure 3). Another was analyzed by HPLC (Develosil C30-UG-5, Nomura Chemical Co., Ltd., Japan, 4.6 × 250 mm, 0-20 min; linear gradient from 0% to 100% CH_3CN in water, 0.8 mL/min, detector at 205 nm, pisatin eluted at the retention time of 19.1 min). Quantification was performed by measuring the area of peak and calibrating. The experiments were repeated three times. Biological activity of isolated pisatin was determined using the cress root growth test. As shown in Figure 3, pisatin inhibited the hypocotyl and root growth of cress at the concentrations higher than 3 mg/L. The concentration of pisatin in the exudates of five seeds was estimated to be about 22 mg/L by measuring the peak area of HPLC (Table 1). In addition, biological activity of the exudates was studied on the hypocotyl and root growth of cress seedlings. From the dose-response curves of pisatin (Figure 3), it was calculated in pisatin-equivalents, that the content of pisatin was about 24 mg/L (Table 1). The results reasonably agree with that obtained by physicochemical analysis. All these results suggest that pisatin exuded from germinating pea seeds may play an important role in the allelopathy of pea seeds. This is the first report for the allelochemical involves in allelopathy during germinating stage. On the other hand, epicotyl growth of pea was promoted with cress seeds (Figure 1). We previously reported that germinating cress seeds exuded a potent growth-promoting substance lepidimoide during seed germination,[8,9] suggesting that epicotyl growth of pea may be promoted by lepidimoide. In conclusion, the results of this study suggest that pisatin exuded from germinating pea seeds plays an important roles in the inhibitory allelopathy of pea seeds.

Table 1. The content of pisatin in the exudates of five pea seeds for 3 days in the dark. The content of pisatin was determined by physicochemical assay and by biological assay. Average data from three independent experiments.

	Physicochemical assay	Biological assay
Pisatin content (mg/l)	22±2	24±3

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