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Isolation and identification of an allelopathic substance in *Pisum sativum*

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Abstract

The residue of peas (*Pisum sativum* L.) has allelopathic activity and the putative compound causing this inhibitory effect was isolated from a methanol extract of pea shoots. Chemical structure of this compound was determined by high-resolution MS, IR and ^{1}H NMR spectral data as pisatin. Pisatin inhibited growth of cress (*Lepidium sativum* L.) and lettuce (*Lactuca sativa* L.) seedlings at concentrations greater than 10 and 30 μM, respectively. The doses required for 50% growth inhibition of roots and hypocotyls of cress were 61 and 91 μM, respectively, and those of lettuce were 78 and 115 μM, respectively. The concentration of pisatin in the pea shoots was 32.7 nmol g^{-1} fresh weight. The effectiveness of pisatin on growth inhibition in cress and lettuce, and its occurrence in pea shoots suggest that it may contribute to the growth inhibitory effect of pea residue, and may play an important role in pea allelopathy.

Keywords: *Pisum sativum*; Leguminosae; Allelopathy; Cress; Growth inhibitor; Lettuce; Phytotoxicity; Pisatin

1. Introduction

Allelopathy is one expression of the general phenomenon of chemical interaction and is probably of widespread significance in the functioning of natural communities (Einhelling, 1996; Seigler, 1996; Dayan et al., 2000). In fact, a number of plants have inhibitory effects on the growth of neighboring or successional plants by releasing allelopathic chemicals into the soil, either as exudates from living tissues or by decomposition of plant residues (Rice, 1984; Putnam and Tang, 1986; Inderjit, 1996; Narwal, 1999).

In field experiments, residues and extracts of pea plants suppressed the growth and population of several plant species (Cochran et al., 1977; Purvis, 1990; Schenk and Werner, 1991; Tsuchiya and Ohno, 1992; Akemo et al., 2000), indicating that pea may have chemicals involved in allelopathy. However, little information is available on the identification of allelopathic active substances in pea plants. In this paper, a growth inhibitor causing an allelopathic effect was isolated from a methanol extract of pea shoots and the biological activity and concentration of the growth inhibitor were determined.

2. Results and discussion

2.1. Identification of allelochemical

The growth inhibitory substance present in the active fraction from the HPLC elution was characterized as follows. The molecular formula of the substance was determined to be $C_{17}H_{14}O_6$ (*m*/*z* 314.0783; calc. for 314.0790) based on its high-resolution mass spectrum. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$, TMS as internal standard) showed 3.74 (3H, *s*), 4.00 (1H, *d*, 11.3 Hz), 4.18 (1H, *dd*, 11.3 and 0.9 Hz), 5.27 (1H, *s*), 5.92 (1H, *d*, 1.2 Hz), 5.95 (1H, *d*, 1.2 Hz), 6.38 (1H, *d*, 2.4 Hz), 6.42 (1H, *s*), 6.44 (1H, *dd*, 8.3 and 2.4 Hz), 6.82 (1H, *s*) and 7.32 ppm (1H, *d*, 8.4 Hz). The IR spectrum showed ν_{max} (Nujol) 3610, 1625, 1490, 1160, 1125, 1030, 930 cm^{-1}. From the comparison of these data with those

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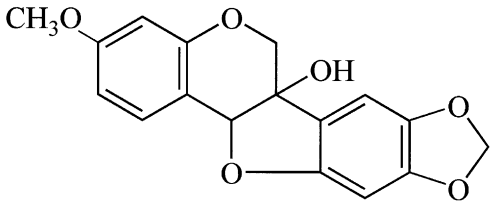
reported in the literature (Perrin and Bottomley, 1962; Perrin and Perrin, 1962), the substance was identified as 6a-hydroxy-3-methoxy-8,9-methylenedioxypterocarpan (pisatin; **1**). The concentration of pisatin **1** in pea shoots was 32.7 nmol g^{-1} fresh weight, which was determined by HPLC analysis.

Pisatin **1** was originally isolated from pea as a phytoalexin by Cruickshank and Perrin (1961) and its biosynthesis has become one of the targets of recent studies of the regulation of gene expression in plants, including reports of accumulation of pisatin **1** in pea being preceded by increases in activities of several biosynthetic enzymes and mRNA (Ruan and Straney, 1994; Borejsza-Wysocki et al., 1997; Delserone et al., 1999). However, there have been few reports of pisatin **1** as a growth inhibitor.



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2.2. Biological activity

Pisatin **1** inhibited the root and hypocotyl growth of cress at concentrations greater than 10 μM, and those of lettuce at concentrations greater than 30 μM (Fig. 1). Increasing the concentration increased the inhibition of the growth in both bioassays. When percentage length of test plants was plotted against logarithm of the concentrations, all concentration–response curves were linear between 20 and 80% inhibition. The doses required for 50% inhibition on roots and hypocotyls of cress, interpolated from the concentration–response curves, were 61 and 91 μM, respectively, and on those of lettuce were 78 and 115 μM, respectively.

2.3. Allelopathy of pea

In an attempt to exploit allelopathy as a weed control strategy in place of man-made chemical herbicides, mulch of killed pea plants was suppressed by the growth of several weed species and decreased the weed population (Akemo et al., 2000). Pea residue also inhibited the growth of lettuce, wheat and sorghum (Cochran et al., 1977; Purvis, 1990; Tsuchiya and Ohno, 1992).

In the present experiment, an allelopathically active substance was isolated from a methanol extract of pea shoots and its chemical structure was determined as

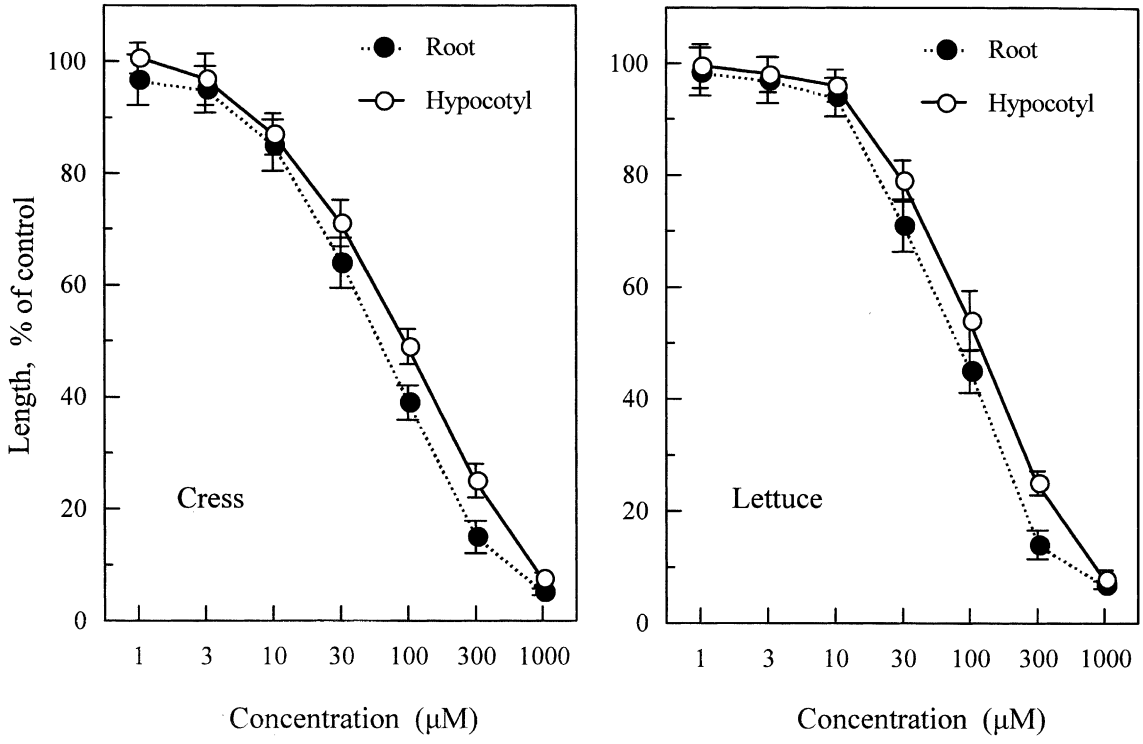

Fig. 1. Effects of pisatin **1** on growth of hypocotyls and roots of cress and lettuce seedlings. Means±SE from three independent experiments with 10 plants for each determination are shown. Root and shoot length of control plants were 13.1±1.3 and 7.4±0.8 mm for cress, respectively, and 16.3±1.5 and 7.1±0.7 mm for lettuce, respectively.

pisatin **1**. It inhibited the growth of cress and lettuce seedlings at concentrations greater than 10 and 30 μM, respectively (Fig. 1). The concentration of pisatin in pea shoots was 32.7 nmol g^{-1} fresh weight. Thus, 1 kg pea shoots may be able to release 32.7 μmol of pisatin **1** into the soil by decomposition of the residue, which may inhibit the growth of the neighboring or successional plants. In addition pisatin **1** concentration in pea plants was markedly increased by fungal infection (Morandi, 1996; Morandi et al., 2002). The effectiveness of pisatin **1** on the growth inhibition in cress and lettuce and its occurrence in pea shoots suggest that it may contribute to the growth inhibitory effect of pea residue, indicating that it may have an important role in the allelopathy of pea.

3. Experimental

3.1. Plant material

Seeds of pea (*Pisum sativum* L. cv. Progress No. 9) were soaked in running tap water for 6 h, sown in soil and grown in a greenhouse under natural day-light condition for 20 days. After harvesting, shoots of the pea plants were immediately frozen in liquid N_2 and stored at −80 °C. At the time of harvest, the seedlings were on average 4.9 cm tall with a fresh weight of 3.3 g per seedling, and had six internodes.

3.2. Extraction and isolation

Plant materials (1 kg fresh weight) were homogenized in cold MeOH (5 l) and the homogenate was filtered through filter paper (No 1; Toyo Ltd., Tokyo, Japan). The residue was homogenized again with 50% (v/v) cold aqueous MeOH (5 l), and filtered. The two filtrates were combined and concentrated at 35 °C in vacuo to give an aqueous residue. The residue was adjusted to pH 7.5 with 1 M phosphate buffer and the solution was partitioned three times against an equal volume of EtOAc. The EtOAc phase was evaporated to dryness after drying over anhydrous Na_2SO_4.

The crude material (4.8 g) applied to LC on a column (2 cm i.d. × 60 cm) of silica gel (100 g, silicagel 60, 70-230 mesh; Merck), eluted stepwise with benzene containing increasing amounts of EtOAc (5% per step, v/v; 200 ml per step). The biological activity of the fractions was determined using a lettuce bioassay as described later, and activity was found in fractions obtained by elution with 40–50% EtOAc in benzene. After evaporation, the residue (720 mg) was further purified on a silica gel column (2 cm i.d. × 40 cm, 60 g silica gel; *n*-hexane containing increasing amounts of EtOAc in 5% steps, v/v; 100 ml per step). Active fractions were eluted by 45–55% EtOAc in *n*-hexane. Then, these fractions were combined and evaporated to dryness in vacuo to 160 mg crude oil. The residue was dissolved in 20% aqueous MeOH (2 ml) and loaded onto reversed-phase C_{18} Sep-Pak cartridges (Waters). The cartridge was eluted with 20, 40, 60 and 80% (v/v) aqueous MeOH followed by MeOH (15 ml per step). The activity was found in fractions obtained by elution with 60% aqueous MeOH. After evaporation, the active material (40 mg) was finally purified by HPLC (0.8 cm i.d. × 30 cm, TSK gel ODS-120T; Toso, Tokyo; eluted at a flow rate of 1.5 ml min^{-1} with 60% aqueous MeOH, detected at 250 nm). Inhibitory activity was found in a peak fraction eluted between 31.5 and 32.5 min, yielding an active substance (5.8 mg) as a colorless powder. The active substance was characterized as pisatin **1** by high-resolution MS, IR and ^1H NMR spectra and comparison to pisatin data.

3.3. Cress and lettuce bioassays

Test samples were evaporated to dryness, dissolved in a small volume of MeOH, added to a sheet of filter paper (No. 2; Toyo Ltd.) in a 2.8-cm Petri dish and dried. Then, the filter paper in the Petri dishes was moistened with 0.8 ml of a 0.05% (v/v) aqueous solution of Tween 20, and 10 cress or lettuce seeds were arranged on the filter paper and grown in the dark at 25 °C. Control seedlings were treated with solution only contained Tween 20. The length of the hypocotyls and roots of their seedlings was measured after 36 and 60 h for cress and lettuce, respectively, and the percentage elongation of the seedlings was determined by reference to the elongation of control seedlings.

3.4. Quantification of pisatin

Frozen pea shoots were extracted with cold MeOH and the extract was purified by a silica gel column (2 cm i.d. × 40 cm, 60 g silica gel; *n*-hexane containing increasing amounts of EtOAc in 5% steps) and a C_{18} Sep-Pak cartridge. Then, a sample of pisatin **1** was injected onto a column for HPLC as described earlier. Quantification was performed by interpolating the peak height on the chromatograms of HPLC to a standard curve constructed by the peak height of pure pisatin **1** isolated from the pea shoots as described earlier. The overall recovery of pisatin **1** through the entire quantification process was about 78%.

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