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Allelopathic substances in *Pueraria thunbergiana*

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Abstract

Leaves of *Pueraria thunbergiana* possess allelopathic activity and the putative compounds causing this growth inhibitory effect were isolated from their aqueous methanol extract. The chemical structures of these growth inhibitors were determined by high-resolution MS and ^{1}H NMR spectral data as *cis*,*trans*-xanthoxin and *trans*,*trans*-xanthoxin. *cis*,*trans*-Xanthoxin and *trans*,*trans*-xanthoxin inhibited the root growth of cress (*Lepidium sativum* L.) seedlings at concentrations greater than 0.3 and 3 μM, respectively. The doses required for 50% inhibition on the cress roots were 1.1 and 14 μM for *cis*,*trans*- and *trans*,*trans*-xanthoxin, respectively. The concentrations of *cis*,*trans*- and *trans*,*trans*-xanthoxin in *P. thunbergiana* leaves were 51.4 and 72.5 ng g^{-1} fresh weight, respectively. The effectiveness of *cis*,*trans*- and *trans*,*trans*-xanthoxin on the growth inhibition and the occurrence of both xanthoxins in *P. thunbergiana* suggest that xanthoxins may contribute to the growth inhibitory effect of *P. thunbergiana*, and may play an important role in the allelopathy of *P. thunbergiana* after being released into the soil.

Keywords: *Pueraria thunbergiana*; Leguminosae; Allelopathy; Cress; Growth inhibitor; Phytotoxicity; Xanthoxin

1. Introduction

A variety of secondary plant metabolites are released into the soil, either as exudates from living plant tissues or by decomposition of plant residues (Rice, 1984; Putnam and Tang, 1986). Some of these substances may be associated with allelopathy, and play an important role in chemical interactions in natural plant communities (Einhelling, 1996; Seigler, 1996; Dayan et al., 2000). In fact, a number of plants have been reported to possess inhibitory effects on the growth and population of neighboring or successional plants by releasing allelopathic substances into the soil (Rice, 1984; Putnam and Tang, 1986; Inderjit, 1996; Narwal, 1999).

The perennial legume *Pueraria thunbergiana* is widely used in agricultural systems in tropical regions as a forage or cover crop to reduce soil erosion by wind and water (Tian et al., 1999; Schroth et al., 2000; Chikoye et al., 2001). This legume is also useful for weed management because its large leaves reduce the quantity of light reaching weeds (Ekeleme et al., 2000). In addition, aqueous extracts of *P. thunbergiana* leaves suppressed the germination and growth of lettuce (Fujii, 1994), indicating that this plant may produce allelopathic substances. However, no information is available on the identification of allelopathic substances in this plant. In this study, two putative growth inhibitors causing allelopathic effects were isolated from an aqueous methanol extract of *P. thunbergiana* leaves and concentrations of these growth inhibitors were determined.

2. Results and discussion

The growth inhibitory substances present in the active fractions from the HPLC elution were characterized as follows. The molecular formulas of the substances were determined to be $C_{15}H_{22}O_3$ (*m*/*z* 250.1563; calculated for 250.1569) and $C_{15}H_{22}O_3$ (*m*/*z* 250.1573; calculated for 250.1569) for inhibitor **1** and **2**, respectively, based on their high-resolution mass spectra. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$, TMS as internal standard) showed 1.00 (3H, *s*), 1.19 (3H, *s*), 1.21 (3H, *s*), 1.25–1.28 (1H, *m*), 1.58–1.65 (1H, *m*), 1.66 (1H, *dd*, 8.9 and 14.4 Hz), 2.13 (3H, *bs*), 2.40 (1H, *ddd*, 1.7, 5.1 and 14.4 Hz), 3.87–3.96 (1H, *m*), 5.86 (1H, *d*, 8.2 Hz), 6.37 (1H, *d*, 15.2 Hz), 7.21 (1H, *d*, 15.0 Hz), 10.20 (1H, *d*, 8.2 Hz) for

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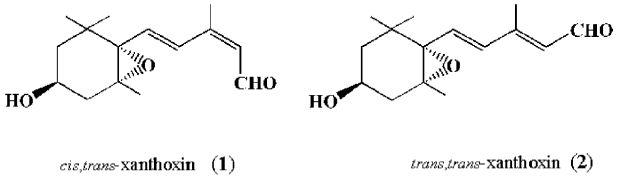
inhibitor **1**, and 1.00 (3H, *s*), 1.19 (3H, *s*), 1.21 (3H, *s*), 1.25–1.28 (1H, *m*), 1.58–1.65 (1H, *m*), 1.66 (1H, *dd*, 8.9 and 14.4 Hz), 2.30 (3H, *bs*), 2.40 (1H, *ddd*, 1.7, 5.1 and 14.4 Hz), 3.87-3.96 (1H, *m*), 5.98 (1H, *d*, 8.1 Hz), 7.24 (1H, *d*, 15.4 Hz), 7.27 (1H, *d*, 15.6 Hz), 10.12 (1H, *d*, 8.2 Hz) for inhibitor **2**. From the comparison of these data with those reported in the literature (Burden and Taylor, 1970; Taylor and Burden, 1972; Sakai et al., 1992), these inhibitors were identified as *cis*,*trans*-xanthoxin (**1**) and *trans*,*trans*-xanthoxin (**2**), respectively. The endogenous level of *trans*,*trans*-xanthoxin (**2**) was 1.4-fold greater than that of *cis*,*trans*-xanthoxin (**1**) in *P. thunbergiana* leaves (Table 1). Xanthoxin was found in several plant species (e.g. Firn et al., 1972; Taylor and Burden, 1972). Nevertheless this is the first report of its presence in *P. thunbergiana*.



cis,trans-xanthoxin (**1**) *trans,trans*-xanthoxin (**2**)

cis,*trans*-Xanthoxin (**1**) and *trans*,*trans*-xanthoxin (**2**) inhibited the root growth of cress at concentrations greater than 0.3 and 3 μM, respectively (Fig. 1). Increasing the concentrations increased the inhibition of the growth in bioassay. When percentage length of test plants was plotted against logarithm of the concentrations, both concentration–response curves were linear between 20 and 80% inhibition. The doses required for 50% inhibition on cress roots, interpolated from the concentration-response curves, were 1.1 and 14 μM for *cis*,*trans*-xanthoxin (**1**) and *trans*,*trans*-xanthoxin (**2**), respectively.

Xanthoxin is considered to be a precursor of abscisic acid in higher plants, since *cis*,*trans*-xanthoxin (**1**) was converted into abscisic acid in plants (Nonhebel and Milborrow, 1987; Sindhu et al., 1990) and in cell-free systems (Cowan and Richardson, 1997). But, *trans*, *trans*-xanthoxin (**2**), which is more abundant than *cis*, *trans*-xanthoxin (**1**) in plants (Table 1; Firn et al., 1972; Parry et al., 1990), was not converted into abscisic acid (Parry et al., 1988). Thus, its significance as an endogenous intermediate in the pathway of abscisic acid biosynthesis is uncertain (Parry et al., 1988; Zeevaart et al., 1989). Xanthoxin has also been implicated as an inhibitor in several developmental processes, such as seed germination (Taylor and Burden, 1972), stem extension (Anstis et al., 1975), phototropism (Shen-Miller et al., 1982), and gravitropism (Feldman et al., 1985). In addition, both of *cis*,*trans*- and *trans*,*trans*-xanthoxin (**1**, **2**) inhibited the germination and growth of several plant species (Taylor and Burden, 1972). Thus, xanthoxin probably plays a role in plants as a growth inhibitor.

Table 1
Concentrations of xanthoxin in *P. thunbergiana* leaves

Concentration	(ng g^{-1} fresh weight)
cis,*trans*-xanthoxin (**1**)	51.4±4.6
trans,*trans*-xanthoxin (**2**)	72.5±6.3

Means±S.E. from three independent experiments with four assays for each determination ($n=12$) are shown.

In the present experiment, the putative compounds causing allelopathic active substances were isolated from an aqueous methanol extract of *P. thunbergiana* leaves, and the chemical structures of the substances were determined from their spectral data as *cis*,*trans*- and *trans*,*trans*-xanthoxin **1** and **2**. The effectiveness of *cis*,*trans*- and *trans*,*trans*-xanthoxin **1** and **2** on the growth inhibition in several plant species (Fig. 1; Taylor and Burden, 1972) and the occurrence of these compounds in *P. thunbergiana* (Table 1) suggests that xanthoxins may contribute to the growth inhibitory effect of *P. thunbergiana*, and may play an important role in the allelopathy of *P. thunbergiana* after being released into the soil by decomposition of plant residues.

3. Experimental

3.1. Plant material and isolation

Leaves of *P. thunbergiana* Benth. were washed thoroughly with tap water and rinsed with distilled water. After blotting dry with filter paper (No. 1; Toyo Ltd., Tokyo, Japan), the leaves (20 kg fresh weight) were homogenized in cold H_2O–MeOH (3:7, v/v, 60 l), with the homogenate filtered through filter paper (No. 2; Toyo Ltd.). The residue was homogenized again with cold H_2O–MeOH (1:1, 60 l), and filtered. The two fil-

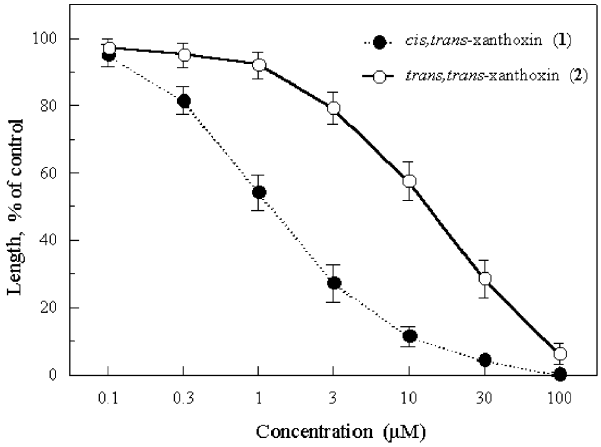


Fig. 1. Effects of *cis*,*trans*- and *trans*,*trans*-xanthoxin (**1** and **2**) on root growth of cress seedlings. Means±S.E. from three independent experiments with 10 plants for each determination are shown. Root length of control plants was 12.7±1.2 mm.

trates 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 (34.7 g) was subjected to silica gel CC (6 cm i.d.×80 cm; 500 g, silica gel 60, 70–230 mesh; Merck), eluted stepwise with benzene that contained increasing amounts of EtOAc (5% per step, v/v; 600 ml per step). The biological activity of the fractions was determined using a cress bioassay as described later, and activity was found in fractions obtained by elution with 85–95% EtOAc in benzene. After evaporation, the residue (1.5 g) was further purified by CC (2 cm i.d.×60 cm; 100 g, silica gel; benzene containing increasing amounts of acetone in 5% steps, v/v; 100 ml per step), yielding 65 mg of a yellowish active residue that was eluted by 30–40% acetone in benzene. After passing the active material through reversed-phase Sep-Pak cartridges (Waters) with H_2O–MeOH (1:1), the residue (38 mg) obtained was subjected to reversed-phase HPLC (0.8 cm i.d.×30 cm; TSK Gel ODS-120T; Toso, Tokyo; eluted at a flow rate of 2 ml min^{-1} with 40% MeOH, v/v; detected at 280 nm), yielding two active components, inhibitor **2** (0.8 mg) and inhibitor **1** (0.6 mg), which eluted with retention times of 37.5 and 41.8 min, respectively. The active substances **1** and **2** were characterized by analysis, high-resolution MS, and ^1H NMR spectra, and by comparison with literature data (Burden and Taylor, 1970; Taylor and Burden, 1972; Sakai et al., 1992).

3.2. Cress 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 seeds of cress (*Lepidium sativum* L.) were arranged on the filter paper and grown in the darkness at 25 °C. Control seedlings were treated with solution only contained Tween 20. The length of the roots of the cress seedlings was measured after 36 h and the percentage elongation of the roots was determined by reference to the elongation of control roots.

3.3. Quantification of xanthoxin 1 and 2

Leaves (20 g fresh weight) of *P. thunbergiana* were extracted with cold H_2O–MeOH (3:7, v/v, 200 ml) and filtered. The residue was homogenized again with cold H_2O–MeOH (1:1, 200 ml), and filtered. The two filtrates were combined and partitioned against EtOAc as described above, and the crude material was purified by a silica gel CC (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). After passing the active material through a reversed-phase Sep-Pak cartridge, the sample of xanthoxins **1** and **2** was injected onto a column for HPLC described above. Quantification was performed by interpolating the peak height on the chromatograms of HPLC to a standard curve constructed by the peak height of pure xanthoxins **1** and **2** isolated as described above. The overall recoveries of **1** and **2** through the entire quantification process were about 70%.

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