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Isolation and identification of an allelopathic substance from peel of *Citrus junos*

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Abstract

The inhibitory effect of *Citrus junos* peel on plant growth using lettuce (*Lactuca sativa* L.) as a bioassay material was investigated, since the powder of the peel had been found to inhibit growth of weeds. Basic, neutral and acidic fractions were separated from the aqueous fraction obtained from the methanol extract of *C. junos* peel. All fractions inhibited the growth of lettuce seedlings, but by far the greatest inhibition was observed with the neutral fraction. Thus, the latter was further purified and an allelopathically active substance was isolated. The structure of the substance was determined from high-resolution MS and ^{1}H and ^{13}C NMR spectral data as abscisic acid-β-D-glucopyranosyl ester (ABA-GE). ABA-GE inhibited hypocotyl and root growth of lettuce seedlings at concentrations greater than 0.3 μM, and the concentrations for 50% inhibition of hypocotyl and root growth were 2.3 and 1.4 μM, respectively. The effectiveness of ABA-GE on inhibition of growth and the occurrence of ABA-GE in the peel itself suggested that ABA-GE may play an important role in the allelopathic potential of *C. junos* peel. The peel may be potentially useful for weed management in a field setting.

Keywords: *Citrus junos*; Rutaceae; Abscisic acid-β-D-glucopyranosyl ester; Allelopathy; Growth inhibitor; Lettuce; Phytotoxicity

1. Introduction

Citrus junos is cultivated in the southern part of Japan, whose fruit is processed into juice and is often preferred to vinegar as an ingredient in sauces and salad dressings for its special flavor. After juice extraction, the fruit pulp is generally discarded as waste. In a series of trials for useful substances in food processing wastes, it was found that *C. junos* peel possessed potent allelopathic activity and the powder of the peel was also effective as a weed suppressive agent (Fujihara and Simizu, 1999).

Chemicals with allelopathic activity are present in many plants and in various organs, including leaves and fruits (Rice, 1984; Putnam and Tang, 1986; Inderjit, 1996), and have potential as either herbicides or templates for new herbicide classes (Duke, 1986; Einhellig and Leather, 1988; Putnam, 1988; Gross and Parthier, 1994; Seigler, 1996; Duke et al., 2000). Over many years, various types of allelochemicals have been isolated and characterized from hundreds of plants (Rice, 1984; Dodge, 1987; Rizvi and Rizvi, 1992; Narwal, 1999). However, the information about allelochemicals in citrus fruits is limited. One exception was the recent study on the allelopathic activity of *C. junos* (Fujihara and Simizu, 1999), but the substances responsible for the allelopathic potential were not identified. In this study, the main allelopathic substance present in *C. junos* was isolated from the peel, and its chemical identity and biological activity were determined.

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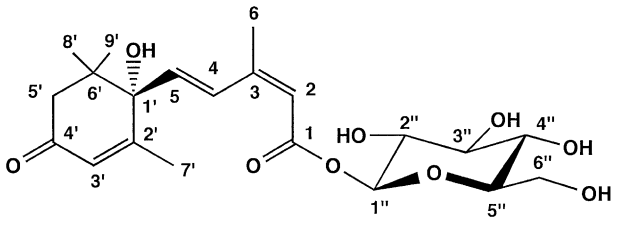

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2. Results and discussion

2.1. Allelopathic potential of C. junos peel extract

Since the powder of *C. junos* peel had been found to possess potent allelopathic activity (Fujihara and Simizu, 1999), the peel was extracted with aqueous methanol and the extract was partitioned with dichloromethane. Both the aqueous and dichloromethane fractions inhibited hypocotyl and root growth of lettuce seedlings (Fig. 1). However, the inhibitory activity in the aqueous fraction was much greater than that of the dichloromethane fraction. At a concentration of 10 mg dry weight peel equivalent ml^{-1}, the aqueous and dichloromethane fractions caused 72 and 38% inhibition of the lettuce hypocotyls, and 86 and 35% inhibition of the lettuce roots, respectively, compared to the length of control seedlings.

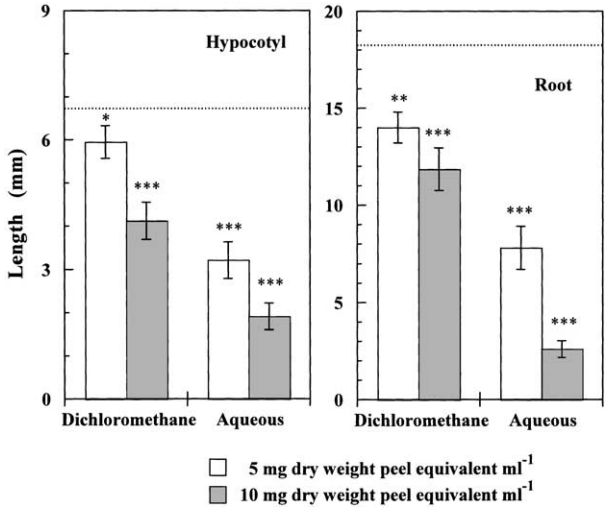

Fig. 1. Effects of dichloromethane and aqueous fractions obtained from *C. junos* peel on hypocotyl and root growth of lettuce seedlings. Root and hypocotyls length of the lettuce seedlings was determined after 60 h of incubation in the dark at 25 °C. Means±s.e. from five independent experiments with 10 plants for each determination are shown. Root and hypocotyl length of control plants were 18.2±1.1 mm and 6.7±0.4 mm, respectively, which were indicated by horizontal broken lines. Asterisks indicate significant differences between control and treatments as determined by Student's *t*-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

The aqueous fraction was separated by ion exchange columns to neutral, acidic and basic fractions, and their biological activities were determined. All three fractions suppressed hypocotyl and root growth of the lettuce seedlings, with the most marked inhibition being achieved by the neutral fraction (Fig. 2). Thus, purification and isolation of allelopathic substances proceeded using only the neutral fraction.

2.2. Isolation and identification of allelochemical

The neutral fraction was purified by columns of Diaion HP20 and Sephadex LH-20, C_{18} Sep-Pak cartridge and HPLC. An allelopathically active substance was isolated as a colorless residue. The molecular formula of the substance was determined to be $C_{21}H_{31}O_9$ (m/z 427.1987; calculated for 427.1968) based on its high-resolution mass spectrum. The ^1H NMR and ^{13}C NMR spectra of the substance are shown in Table 1. From a comparison of these data with those reported in the literature (Koshimizu et al., 1968; Loveys and Milborrow, 1981), the substance was identified as abscisic acid-β-D-glucopyranosyl ester (ABA-GE; **1**).

2.3. Biological activity

The biological activities of ABA-GE **1** and (+)-ABA (Sigma) were determined with lettuce seedlings (Fig. 3). At concentrations greater than 0.3 and 0.1 μM, respectively, ABA-GE **1** and (+)-ABA inhibited the growth

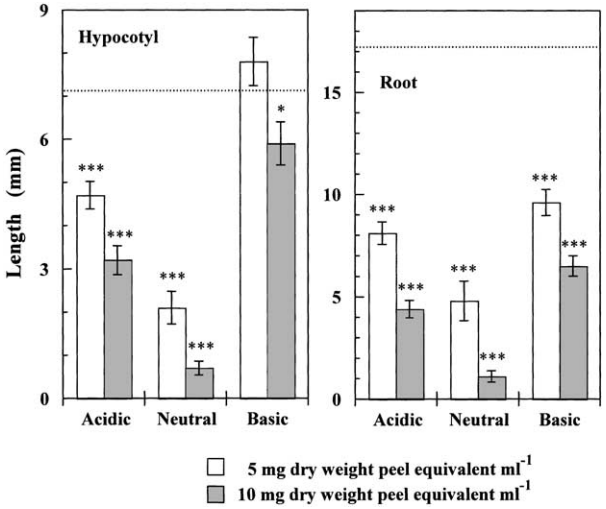

Fig. 2. Effects of acidic, neutral and basic fractions isolated from an aqueous extract of *C. junos* peel on hypocotyl and root growth of lettuce seedlings. Root and hypocotyls length was determined after 60 h of incubation in the dark at 25 °C. Means±s.e. from five independent experiments with 10 plants for each determination are shown. Root and hypocotyl length of control plants were 18.2±1.1 mm and 7.1±0.5 mm, respectively, which were indicated by horizontal broken lines. Asterisks indicate significant differences between control and treatments as determined by Student's *t*-test: *, $P<0.05$; ***, $P<0.001$.

Table 1
^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic chemical shifts and multiplicities of the active substance (in CD_3OD, TMS as internal standard)

Position	^{13}C δ (ppm)	^{1}H δ (ppm)	Multiplicity	J (Hz)	Integration
Aglycone					
1	165.9				
2	118.1	5.84	*s*		1H
3	153.6				
4	129.2	7.81	*d*	16.1	1H
5	139.3	6.32	*d*	16.1	1H
6	21.3	2.04	*d*	1.5	3H
1′	80.6				
2′	166.3 or 165.9				
3′	127.7	5.91	*s*		1H
4′	201.0				
5′	50.6	2.19	*d*	16.9	1H
		2.54	*d*	16.9	1H
6′	42.9				
7′	19.6	1.92	*d*	1.5	3H
8′	23.6	1.06	s		3H
9′	24.7	1.02	s		3H
Sugar					
1″	95.4	5.49	*d*	8.0	1H
2″	74.0	3.32	*t*	8.0	1H
3″	78.1	3.36	*t*	8.0	1H
4″	71.1	3.41	*t*	8.0	1H
5″	78.8	3.34	*m*		1H
6″	62.4	3.66	*dd*	6.1, 11.0	1H
		3.84	*dd*	2.1, 11.0	1H

of the lettuce hypocotyls and roots. 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 concentrations required for 50% inhibition of the lettuce hypocotyls in the assay (defined as I_{50}), as interpolated from the concentration–response curves, were 2.3 and 0.84 μM for ABA-GE **1** and (+)-ABA, respectively, and those of the lettuce roots were 1.4 and 0.48 μM for ABA-GE **1** and (+)-ABA, respectively. Comparing I_{50} values, the inhibitory activity of ABA on the hypocotyls and roots, respectively, was 2.7- and 2.9-fold greater than that of ABA-GE **1**. Thus, the activity of ABA-GE **1** on the hypocotyls and roots of the lettuce seedlings was about one third of that of (+)-ABA.

The level of ABA-GE **1** in the peel was found to be 0.3 nmol mg^{-1} dry weight (0.13 μg mg^{-1} dry weight) by HPLC analysis. Thus, 10 mg dry weight peel, of which extract was tested for its inhibitory activity by lettuce seedlings (Figs. 1 and 2), contains 3 nmol ABA-GE **1**. When lettuce seedlings were incubated in a Petri dish containing 1 ml medium with 3 nmol ABA-GE **1**, their hypocotyls and roots were inhibited to 42 and 25% those of control seedlings, respectively (Fig. 3). Considering the recovery of ABA-GE **1** through the quantification process, ABA-GE **1** may be responsible for most of the inhibition caused by the neutral fraction in the assay (Fig. 2).

ABA-GE **1** was considered to be a physiologically-inactive main form, conjugated ABA in plants, and an end-product of ABA metabolism rather than a storage or transport form (Milborrow, 1970, 1978; Neill et al., 1983; Zeevaart, 1983; Lehman and Vlasov, 1988). However, recently an ABA-GE-cleaving enzyme, apoplastic β-D-glucosidase was detected (Dietz et al., 2000). The enzyme releases free ABA from the ABA-GE **1**, which indicates ABA-GE **1** may have important physiological functions in plants. The effectiveness of ABA-GE on the inhibition of growth (Fig. 3) coupled to the discovery of this key enzyme (Dietz et al., 2000) suggest that exogenously applied ABA-GE **1** may be absorbed by plant roots and hydrolyzed by apoplastic β-D-glucosidase, with the subsequent release of ABA, which would potentially inhibit competing plant growth.

2.4. Allelopathy of *C. junos*

C. junos peel powder and its methanol extract inhibited the growth of lettuce, *Chenopodium album*, *Sonchus oleraceus* and *Digitaria ciliaris* (Fujihara and Simizu, 1999). The effectiveness of ABA-GE **1** on inhibiting growth and the occurrence of ABA-GE **1** in *C. junos* peel suggest ABA-GE **1** may play an important role in the allelopathic activity of the peel. Synthetic chemical herbicides may continue to be a key component in most integrated weed management systems, but controlling weeds through allelopathy is one strategy to reduce herbicide dependency (Duke, 1986; Putnam, 1988; Seigler, 1996; Narwal, 1999). It has also been shown that certain plant residues and extracts may function as weed suppressive agents (Bhowmik and Doll, 1982; Putnam and Tang, 1986; Einhellig, 1996). It may also be important to consider that ABA-GE **1** has been found in soils from agricultural fields in which several crop plants were produced (Hartung et al., 1991). The existence of ABA-GE **1** in the soils was attributed to its exudation from the plant roots (Sauter and Hartung, 2000). It is possible that the peel itself may be important as a weed suppressive residue or mulch.

3. Experimental

3.1. Extraction and separation

Freeze-dried peel (180 g dry wt) of *C. junos* Tanaka was crushed and extracted with 80% aq. cold MeOH (3.6 l) for three days at 4 °C. After filtration using filter paper (No. 2, Toyo, Tokyo, Japan), the filtrate was concentrated at 40 °C in vacuo to produce an aqueous extract. The latter was partitioned five times with an equal volume of dichloromethane and all five dichloromethane phases were combined. The biological activities

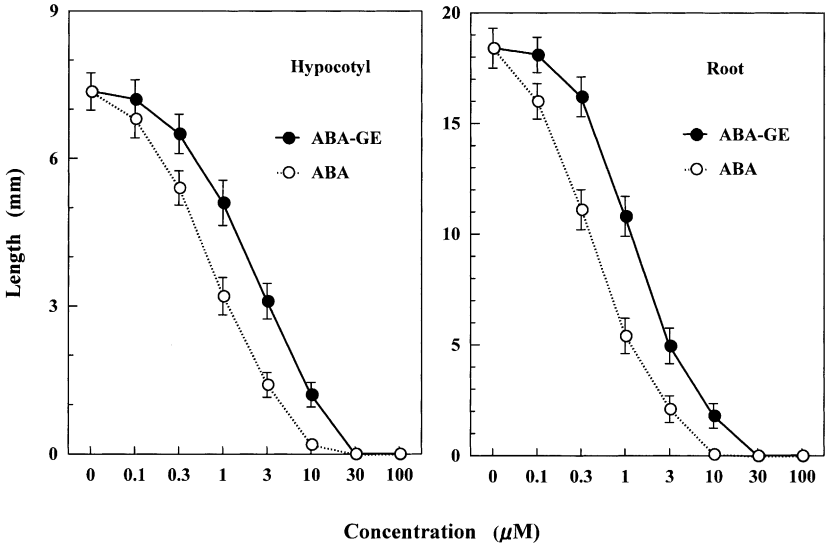

Fig. 3. Effects of ABA-GE **1** and (+)-ABA on hypocotyl and root growth of lettuce seedlings. Means±s.e. from five independent experiments with 10 plants for each determination are shown.

of the dichloromethane and aqueous extract were determined by a lettuce bioassay (see below).

The aqueous phase (300 ml) was adjusted to pH 8.3 with 1 M KOH and passed through an anion exchange column (3 cm i.d. × 12 cm) of Dowex (1×8, Cl^-) and the column was washed with water (100 ml). The effluent and washings were directly passed through a cation exchange column (3 cm i.d. × 12 cm) of Dowex (50Wx8, H^+). Then, the effluent and washings were combined to form a neutral fraction. The anion exchange column was eluted with 2 M formic acid (500 ml) and eluate was collected as the acidic fraction. The cation exchange column was eluted with 2 M HCl (500 ml) and the eluate was collect as the basic fraction. The biological activities of neutral, acidic and basic fractions were determined using the standard lettuce bioassay.

3.2. Lettuce bioassay

Each fraction was added to a filter paper disc (No. 2, Toyo) in a 3.5-cm Petri dish and dried. The filter paper was then moistened with 0.05% (v/v) aq. solution of Tween 20 (0.8 ml), and 10 seeds of lettuce (*Lactuca sativa* cv. Grand Rapids L.) were sown on it. After 60 h incubation in the darkness at 25 °C, the lengths of roots and hypocotyls of the lettuce seedlings were determined. Control seedlings were sown on filter paper moistened with the aq. solution of Tween 20.

3.3. Purification of active component in the neutral fraction

The neutral fraction was loaded onto a column (3 cm i.d. × 30 cm) of Diaion HP20 (Mitsubishi Chemical, Tokyo, Japan), and eluted with water, 20, 40, 60 and 80% (v/v) aq. MeOH, and MeOH (300 ml per step). The biological activity of the fractions was determined by using the lettuce bioassay, and the inhibitory activity was found in fractions obtained by elution with 60 and 80% aq. MeOH. After evaporation, the residue was further purified by a column (2.5 cm i.d. × 20 cm) of Sephadex LH-20 (Amersham Pharmacia Biotech, Buckinghamshire, UK), and eluted with water, 20, 40, 60 and 80% (v/v) aq. MeOH, and MeOH (100 ml per step). The inhibitory activity was found in fractions obtained by elution with water and 20% aq. MeOH. The fractions were combined and evaporated, and the residue was dissolved in water (2 ml) and loaded onto a reversed-phase C_{18} Sep-Pak cartridge (Waters). The cartridge was eluted with water, 20, 40, 60 and 80% (v/v) aq. MeOH, and MeOH (15 ml per step). The activity was found in fractions obtained by elution with 20 and 40% aq. MeOH. After evaporation, the active component was finally purified by HPLC (10 mm i.d. × 50 cm, ODS AQ-325; YMC Ltd, Kyoto, Japan) eluted at a flow rate of 2 ml min^{-1} with 30% aq. MeOH, detected at 220 nm. Inhibitory activity was only found in a peak fraction eluted between 147 and 151 min, yielding an active component ABA-GE **1** (4.9 mg) as a colorless resin.

3.4. Quantification of ABA-GE (*1*)

Freeze-dried peel of *C. junos* was extracted with aq. cold MeOH and neutral fraction was purified using by ion exchange columns and a reversed-phase C_{18} Sep-Pak cartridge as described above. Then, the sample of ABA-GE 1 was injected onto a column for HPLC (4.6 mm i.d. × 15 cm, Hydrosphere C_{18}; YMC Ltd, Kyoto, Japan) eluted at a flow rate of 0.8 ml min^{-1} with 22% aq. MeOH, detected at 220 nm. The retention time of ABA-

GE **1** was 78.1 min under these conditions. Quantification of ABA-GE **1** was performed by interpolating the peak height on the chromatograms of HPLC to a standard curve constructed by the peak height of pure ABA-GE **1** isolated from the peel as described above. The overall recovery of ABA-GE **1** through the entire quantification process was about 75%.

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