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Toshiki Asao\*, Hiroaki Kitazawa, Takuya Ban, M. H. R. Pramanik,  
Yoshihisa Matsui and Takashi Hosoki

*Faculty of Life and Environmental Science, Shimane University, Kamihonjo, Shimane 690 - 1102*

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## Summary

The following autotoxic organic acids: lactic, benzoic, *m*-hydroxybenzoic, *p*-hydroxybenzoic, vanillic, adipic, and succinic in eight leaf vegetables, cultured hydroponically, were analyzed by GC-MS method. These substances that are adsorbed by activated charcoal are effective growth inhibitors were bioassayed, using the seedlings of their origin as test materials. Strong inhibitors, such as adipic acid in parsley, lactic acid in celery, benzoic, *p*-hydroxybenzoic and succinic acid in mitsuba, vanillic acid in curly-leaf lettuce, succinic acid in edible burdock, benzoic, *m*-hydroxybenzoic and succinic acid in garland chrysanthemum, benzoic and *p*-hydroxybenzoic acid in pak-choi, benzoic, *p*-hydroxybenzoic and adipic acid in kale were found.

**Key Words:** autotoxicity, autotoxic substances, bioassay, leaf vegetables.

## Introduction

Young (1984) reported that the root exudates of asparagus reduced the growth of asparagus seedlings. Yu and Matsui (1993) identified the phytotoxic substances that accumulated in the nutrient solution used for hydroponic culture of tomato. Furthermore, we found that the root exudates of cucumber (Asao et al., 1999b) and taro (Asao et al., 2003) were autotoxic. Thus, autotoxication would be one cause of the inhibition on the successive culture of vegetables.

Leaf vegetables, which demonstrated autotoxicity when they were cultured hydroponically, were grown with and without the addition of activated charcoal (AC) to the nutrient solutions (Asao et al., 2001). The growth and yield of pak-choi, kale, curly-leaf lettuce, head lettuce, edible burdock, garland chrysanthemum, celery, parsley and mitsuba were reduced in the absence of AC, while growth of komatsuna, chinese cabbage, radish, takana, welsh onion, perilla and spinach were unaffected. Among the affected vegetables, the growth of parsley was most severely inhibited. However, autotoxic substances absorbed on AC and the degree of growth inhibition have not been clarified.

In this study, the autotoxic substances from eight leaf vegetables absorbed on AC were identified, and the most effective inhibitors were evaluated by bioassay.

## Materials and Methods

Eight leaf vegetables, analyzed for autotoxic substances were: parsley (*Petroselinum crispum* Nym.) cv. Curl-paramount, celery (*Apium graveolens* L.) cv.

Mini-white, mitsuba (*Cryptotaenia japonica* Hassk.) cv. Yanagawa-suikou, curly-leaf lettuce (*Lactuca sativus* L.) cv. Banchu-red-fire, edible burdock (*Arctium lappa* L.) cv. Hagobo, garland chrysanthemum (*Chrysanthemum coronarium* L.) cv. Ohba-shingiku, pak-choi (*Brassica campestris* L.) cv. Choyo and kale (*Brassica oleracea* L.).

## Identification of autotoxic substances

The activated charcoal (AC) that was used to trap organics exuded from roots of leaf vegetables grown hydroponically (Asao et al., 2001) was desorbed three times using a mixture of methanol (100 ml) and 0.4 M aqueous NaOH (100 ml). Thus, each batch of AC (200 g) was gently shaken with the alkaline methanolic solution for 12 hr at room temperature, using an electric shaker. The three extracts were combined and filtered. And the filtrates, neutralized and concentrated to 25 ml by a rotary vacuum evaporator at 40°C. Organic compounds in the concentrate were extracted as described by Yu and Matsui (1993). The concentrated solution was adjusted to pH 2.0 with 4 M HCl, extracted three times with 35 ml of refined diethyl ether (DE2), and another three times with 35 ml of ethyl acetate (EA2). The DE2 and EA2 fractions were dried over anhydrous CaSO<sub>4</sub> and concentrated to 5 ml each in a rotary evaporator at 40°C.

The DE2 and EA2 extracts were analyzed by gas chromatography coupled to a mass spectrometer (GC-MS, Hitachi M-80B) before or after methylation. Fraction DE2 yielded a number of peaks on gas chromatogram, whereas EA2 yielded only a few detectable peaks. An aliquot of the concentrated DE2 fraction (1 or 2 ml) was diluted in 50 ml of ether solution, treated with diazomethane, and concentrated in a rotary evaporator

before being bubbled with an N<sub>2</sub> stream in a water bath at 35°C. One- $\mu$ l of the concentrated sample was injected into a GC-MS unit coupled with a capillary column (GL Science, TC-5, 60 m). Helium was used as the carrier gas at a pressure of 0.8 kg·cm<sup>-2</sup>. The initial column temperature was held at 100°C for 2 min, then raised at 5°C·min<sup>-1</sup> to a final temperature of 260°C with isotherm for 10 min. The injector temperature was held at 270°C. The ionization voltage and temperature in the electron impact (EI) mode were 70 eV and 250°C, respectively.

#### Bioassay with vegetable seedlings

The autotoxic effects by the identified compounds were evaluated by using seedlings of plants from which they originated. Aqueous solutions of the identified acids at concentrations of 0 (control), 50, 100, 200 and 400  $\mu$ M were prepared with a 75% Enshi nutrient solution (EC 2.0 dS·m<sup>-1</sup>) (Pramanik et al., 2000). The

test solutions were added as per treatment to 400 ml conical flasks, which were wrapped with black polyethylene to avoid direct light to the roots (Asao et al., 1999a, b). One seedling was transplanted to each flask with urethane foam as a support. Plants were placed in a growth chamber at 25°C and a 16-hr photoperiod under a fluorescent light having a light intensity of 74 - 81  $\mu$ mol·m<sup>-2</sup>·s<sup>-1</sup>. To minimize the effect of microbial degradation in the bioassay (Sundin et al., 1990), the test solutions were renewed at 3- or 4-day intervals. The seedlings were grown for two weeks and the dry weights of shoots were recorded. Each treatment was replicated 10 times.

### Results and Discussion

Analyses of root exudates released from AC by GC-MS after methylation revealed the presence of the following acids: lactic, benzoic, *p*-hydroxybenzoic, adipic and succinic in parsley, edible burdock, and kale;

**Table 1.** Effects of identified compounds at different concentrations on the dry weight (mg) of shoot in eight leaf vegetables.

Compounds <sup>z</sup> (Concentration, $\mu$ M)		Parsley	Celery	Mitsuba	Curlyleaf lettuce	Edible burdock	Garland chrysan- themum	Pakchoi	Kale
Control (No compound) <sup>y</sup>	0	29a <sup>x</sup>	23a	34a	54a	142a	17a	71a	80a
Lactic acid	50	22a	21a	35a	43a	150a	17a	69a	79a
	100	22a	19a	35a	43a	131a	18a	71a	63b
	200	22a	16b	36a	47a	126a	19a	44b	63b
	400	16b	16b	36a	50a	110b	19a	41b	48c
Benzoic acid	50	22a	18a	32a	58a	124a	11b	61a	64b
	100	16b	18a	32a	64a	121a	12b	39b	52c
	200	16b	17a	28b	53a	82b	13b	35b	45c
	400	12c	19a	24c	40b	72b	12b	30c	35c
<i>m</i> -hydroxy- benzoic acid	50	- <sup>w</sup>	-	37a	-	-	13b	-	-
	100	-	-	37a	-	-	13b	-	-
	200	-	-	34a	-	-	11c	-	-
	400	-	-	23b	-	-	12c	-	-
<i>p</i> -hydroxy- benzoic acid	50	22a	20a	33a	63a	116a	17a	91a	59b
	100	19b	24a	34a	55a	106a	18a	49b	52b
	200	18b	17a	26b	52a	103b	16b	34c	46c
	400	13c	17a	23c	33b	90b	12c	29c	34c
Vanillic acid	50	-	-	-	32b	-	16a	-	-
	100	-	-	-	33b	-	15a	-	-
	200	-	-	-	33b	-	14b	-	-
	400	-	-	-	33b	-	11c	-	-
Adipic acid	50	18b	-	37a	58a	135a	17a	-	56b
	100	17b	-	36a	58a	130a	17a	-	66b
	200	17b	-	36a	52a	130a	18a	-	46c
	400	13c	-	36a	46a	110a	16a	-	37c
Succinic acid	50	22a	-	33a	50a	116a	14b	-	69a
	100	23a	-	31a	51a	94b	13b	-	66a
	200	18b	-	28b	43b	85b	11c	-	51b
	400	16b	-	21c	41b	83b	10c	-	37c

<sup>z</sup> Compounds identified in the root exudates (adsorbed on the activated charcoal) of eight leaf vegetables grown hydroponically.

<sup>y</sup> Control of all compounds.

<sup>x</sup> Mean separation within columns in each compound by Tukey's test at 5% level.

<sup>w</sup> Not detected.

lactic, benzoic, and *p*-hydroxybenzoic in celery and pakchoi. Also lactic, benzoic, *m*-hydroxybenzoic, *p*-hydroxybenzoic, adipic, and succinic were present in mitsuba; lactic, benzoic, *p*-hydroxybenzoic, vanillic, adipic, and succinic were detected in lettuce; and lactic, benzoic, *m*-hydroxybenzoic, *p*-hydroxybenzoic, vanillic, adipic, and succinic were found in garland chrysanthemum (Table 1).

Bioassays of the identified compounds in the root exudates of each vegetable revealed that adipic acid induced the retardation of the dry weight (DW) of parsley shoots even at 50  $\mu$ M (Table 1). Lactic acid in celery at 200  $\mu$ M reduced DW to 70% of control values, whereas that benzoic, *p*-hydroxybenzoic and succinic acid in mitsuba at 200  $\mu$ M decreased DW accumulation to 82, 76 and 82% of control values, respectively. Vanillic acid in curly-leaf lettuce and benzoic, *m*-hydroxybenzoic and succinic acids in garland chrysanthemum even at 50  $\mu$ M reduced DW to 59, 65, 76 and 82% of control, respectively; likewise, succinic acid at 100  $\mu$ M decreased DW in edible burdock to 66% of control. Benzoic and *p*-hydroxybenzoic acids in pakchoi at 100  $\mu$ M, and benzoic, *p*-hydroxybenzoic and adipic acids in kale at 50  $\mu$ M reduced DW to 55, 69, 80, 74 and 70% of control values, respectively.

In summary, adipic acid was the strongest inhibitor in parsley and lactic acid in celery. Benzoic, *p*-hydroxybenzoic and succinic acids induced strong inhibition in mitsuba. Vanillic acid caused the most severe growth inhibition in curly-leaf lettuce. Succinic acid in edible burdock, and benzoic, *m*-hydroxybenzoic and succinic acids in garland chrysanthemum induced strong inhibition. Benzoic and *p*-hydroxybenzoic acids strongly inhibited the growth of pak-choi while benzoic, *p*-hydroxybenzoic and adipic acids inhibited the growth of kale. Benzoic acid was the strongest overall inhibitor for all leaf vegetables among the substances tested. These results confirm that some unidentified compounds inhibited growth of leaf vegetables in hydroponic culture (Asao et al., 2001). In our experiments, some of these compounds absorbed on AC were identified as aromatic and aliphatic acids which have been cited as allelochemicals (Rice, 1984).

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#### 葉菜類の自家中毒物質の探索

浅尾俊樹・北澤裕明・伴 琢也・M. H. R. Pramanik・

松井佳久・細木高志

島根大学生物資源科学部 690-1102 松江市上本庄町

#### 摘 要

8種の葉菜類の自家中毒物質を探索するために水耕葉菜類に用いた活性炭に吸着された物質をGC-MS法で分析した。その物質は乳酸, 安息香酸, *m*-ヒドロキシ安息香酸, *p*-ヒドロキシ安息香酸, バニリン酸, アジピン酸およびコハク酸であった。同定された物質の中で, 顕著に生育抑制を引き起こす物質を探るため各葉菜類の苗を使ったバイオアッセイを行った。その結果, パセリではアジピン酸, セロリでは乳酸, ミツバでは安息香酸, *p*-ヒドロキシ安息香酸およびコハク酸, レタスではバニリン酸, 葉ゴボウではコハク酸, シュンギクでは安息香酸, *m*-ヒドロキシ安息香酸およびコハク酸, チンゲンサイでは安息香酸および*p*-ヒドロキシ安息香酸, ケールでは安息香酸, *p*-ヒドロキシ安息香酸およびアジピン酸が生育抑制を顕著に引き起こす物質として認められた。