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Enhanced Glucosinolates in Root Exudates of *Brassica rapa* ssp. *rapa* Mediated by Salicylic Acid and Methyl Jasmonate

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ABSTRACT: Elicitation studies with salicylic acid (SA) and methyl jasmonate (MJ) inducing a targeted rhizosecretion of high levels of anticarcinogenic glucosinolates in *Brassica rapa* ssp. *rapa* plants were conducted. Elicitor applications not only led to an accumulation of individual indole glucosinolates and the aromatic 2-phenylethyl glucosinolate in the turnip organs but also in turnip root exudates. This indicates an extended systemic response, which comprises the phyllosphere with all aboveground plant organs and the rhizosphere including the belowground root system and also root exudates. Both elicitor applications induced a doubling in 2-phenylethyl glucosinolate in root exudates, whereas application of MJ enhanced rhizosecreted indole glucosinolates up to 4-fold. In addition, the time course study revealed that maximal elicitation was observed on the 10th day of SA and MJ treatment. This study may provide an essential contribution using these glucosinolates as bioactive additives in functional foods and nutraceuticals.

KEYWORDS: Rhizosecretion, secondary plant metabolites, 2-phenylethyl glucosinolate, indole glucosinolate, signaling molecule

INTRODUCTION

Glucosinolates are a group of secondary plant metabolites found almost exclusively in plants of the order Brassicales. Glucosinolates consist of a sulfur-linked β-D-glucopyranose moiety and an amino acid-derived side chain.[1] The side-chain structure determines whether they are classified as aliphatic, aromatic, or indole glucosinolates.[2] Certain individual glucosinolates are known to confer health-promoting effects due to the anticarcinogenic properties of their isothiocyanates. Thus, these anticarcinogenic glucosinolates can be used as bioactive ingredients in functional foods and nutraceuticals.[3] Of note is that the hydrolysis products of indole 3-indolylmethyl glucosinolate and its derivatives[4,5] as well as aromatic isothiocyanates derived from 2-phenylethyl glucosinolate[6,7] have been reported to be effective against the development of some types of cancer.

In addition to the accumulation of plant compounds in the plant tissue, certain plant compounds such as primary plant metabolites, for example, recombinant proteins,[8] and also specific secondary plant metabolites such as phenolics[9−12] are known to be secreted as root exudates. Exploiting this secretion process would allow health-promoting metabolites to be more easily isolated compared, for example, to extraction from plant tissues by solvents. However, only limited information is available about how to elicit the production of secondary plant metabolites in root exudates.

Elicitation studies with salicylic acid (SA) and methyl jasmonate (MJ) were seen to induce glucosinolate accumulation in the plant, especially that of indole glucosinolates. This is because both SA and MJ activate the plant's defense response, which includes the synthesis of glucosinolates as plant defense compounds.[13−17] In addition, SA application is known to cause particularly an increase of 2-phenylethyl glucosinolate in plant tissue, also contributing to plant resistance.[18] From these studies, we propose that enhancing the glucosinolate concentration in plant tissues, especially indole glucosinolates and 2-phenylethyl glucosinolate, after the application of SA or MJ could also increase the concentration of these anticarcinogenic glucosinolates in root exudates.

Therefore, to develop an effective plant-based system by optimizing cultivation conditions for the production and isolation of functional glucosinolates in root exudates, our aims were (1) to assess whether using root exudates as a source of glucosinolates is possible and, if so, (2) to examine the effects of SA or MJ on glucosinolate exudation as well as to determine (3) differences in terms of glucosinolates concentration and composition and (4) optimal time for glucosinolate isolation during glucosinolate rhizosecretion.

MATERIALS AND METHODS

Plant Cultivation. Turnip (*Brassica rapa* ssp. *rapa*) was selected because of its high level of 2-phenylethyl glucosinolate associated with relatively high concentrations of indole glucosinolates compared to other *Brassica* vegetables.[3,19] Turnip seeds were germinated inside Grodan rockwool cubes (5 cm × 5 cm × 5 cm) placed in plastic trays (52 cm × 35 cm × 7 cm). Seeds were watered with half-strength

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Hoagland solution through an overhead misting system and were grown until the roots of the seedlings had emerged 2−4 cm out of the bottom of cubes (15−20 days). The cubes were then inserted at 10 cm × 11 cm intervals into holes in polystyrene plates installed in hydroponic systems (one system for each treatment: C, control; SA; MJ). The hydroponic systems were aerated by bubbling air through the nutrient solution at a flow rate of approximately 100 mL min^{-1} using 10 aeration tubes connected to a compressor (model 40A-10011; Hiblo, Berlin, Germany). Each system contained 25 L of nutrient solution. All plants were grown at 16 °C/12 °C (day/night) with a 12 h photoperiod (400 μmol m^{-2} s^{-1}) and 60% relative humidity. The nutrient solution used was 2× Hoagland with a 2-fold increase in sulfur concentration.[19]

Plant Elicitation. SA at 800 μM and MJ at 130 μM (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) were used according to the recommendations of Kneer et al.[9] SA or MJ was added to the nutrient solution and applied to the roots at the start of plant growth in the hydroponic system. Three different treatments were tested: control (no elicitor application), SA application, and MJ application.

Sampling of Exudates. A time course experiment was established for determining the kinetics of rhizosecreted indole, aromatic, and aliphatic glucosinolates, wherein root exudate samples were taken on days 10, 15, 20, 25, and 30 of postelicitation. Three replicates of 1 L nutrient solution containing root exudates were taken from the 10th day because at day 10, the plants had a root system (2.8 g fresh weight per plant) sufficient for gaining relatively high amounts of rhizosecreted glucosinolates. After sampling, the nutrient solution in the systems was changed. The samples were prefiltered with paper filters N 604 (Schleicher & Schuell, Dassel, Germany), then ultrafiltered, and finally concentrated using a ProScale system with Helicon-RO-4 cartridges containing Nanomax-95 membranes (Millipore GmbH, Eschborn, Germany). The samples were then transferred into 600 mL glass flasks (Christ, Osterode am Harz, Germany), frozen at −28 °C (Denley CFC-Free Deep Freeze, Life Science International Ltd., Denmark), and freeze-dried (Alpha 1-4, Christ).

Sampling of Secondary Roots. After 10, 15, 20, 25, and 30 days, 9 plants from each treatment (3 replicates, $n = 3$ for each replicate) were randomly harvested, and secondary roots were separated from the plants, then frozen at −28 °C (Denley CFC-Free Deep Freeze; Life Science International Ltd.), and freeze-dried (Alpha 1-4, Christ) to a constant weight for determining the root dry matter.

Glucosinolate Analysis. The HPLC method reported by Krumbein et al.[20] was used to determine the desulfoglucosinolate profiles. Duplicates of freeze-dried sample material (0.5 g from 1 L of freeze-dried nutrient solution) were heated to and incubated at 75 °C for 1 min, extracted with 4 mL of a methanol/water mixture (v/v = 7:3), and then, after the addition of 1 mL of 0.4 M barium acetate, centrifuged at 4000 rpm for 10 min. The residue was extracted twice more with 3 mL of the methanol/water mixture (v/v = 7:3, $T = 70$ °C). The supernatants were pooled and made up to 10 mL with the methanol/water mixture. From this, 5 mL of the extract was applied to a 250 μL DEAE-Sephadex A-25 ion exchanger (acetic acid-activated; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and rinsed with 10 mL of bidistilled water. Next, 250 μL of a purified solution of aryl sulfatase (Boehringer-Mannheim GmbH, Mannheim, Germany) was applied and left for 12 h before the desulfo compounds were flushed with 5 mL of bidistilled water. Desulfoglucosinolate analysis was conducted by HPLC using a Merck HPLC system (Merck-Hitachi, Darmstadt, Germany) with a Spherisorb ODS2 column (Bischoff, Leonberg Germany; 5 μm, 250 mm × 4 mm). A gradient of 0−20% acetonitrile in water was selected from 2 to 34 min, followed by 20% acetonitrile in water until 40 min and then 100% acetonitrile for 10 min. Determination was conducted at a flow of 1.3 mL min^{-1} and a wavelength of 229 nm. Glucosinolate concentration was calculated using 2-propenyl glucosinolate as internal standard (Sigma-Aldrich Chemie GmbH) and using the response factor of each compound relative to 2-propenyl glucosinolate (BCR-367R, Community Bureau of Reference, Brussels, Belgium). The well-known desulfoglucosinolates were identified according to previous work[21] from the protonated molecular ions $[M + H]^+$, and the fragment ions corresponded to $[M + H - \text{glucose}]^+$ by HPLC-ESI-MS^2 using Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) in the positive ionization mode. Determinations of desulfoglucosinolates were performed in duplicate. Because *B. rapa* ssp. *rapa* plants responded to SA and MJ applications with changes in dry matter content of the secondary roots, especially at day 10 after elicitor treatment and due to continuously increasing root dry matter content during plant development (Table 1), the rhizosecreted glucosinolate concentrations were expressed on the basis of dry matter of the secondary roots (mg g^{-1} dm).

Table 1. Dry Matter Content of Turnip Secondary Roots (Grams) per Plant

	days after treatment[a]				
treatment	10	15	20	25	30
control[a]	0.31c	0.83b	0.97a	1.27a	2.10a
salicylic acid	0.21b	1.25c	1.41b	1.72b	2.48b
methyl jasmonate	0.11a	0.63a	0.95a	1.64b	2.12a

[a] Each value represents the mean of three samples. Differences are compared between the treatments for each certain sampling date. Mean values, within a column (same sampling date), followed by the same letter are not significantly different.

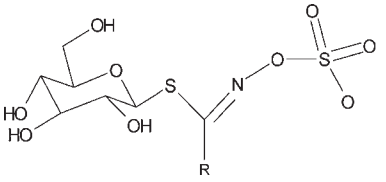
Statistical Analysis. Total and individual glucosinolate concentrations were analyzed as well as secondary root dry matter content using analysis of variance. Least significant differences were calculated with Tukey's Honest Significant Difference test (HSD; significance level $P \leq 0.05$). Analysis of variance was performed using Statistica for Windows (version 6.1, Statsoft Inc.).

RESULTS AND DISCUSSION

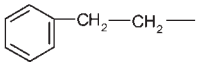
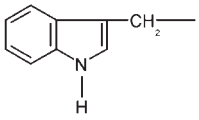
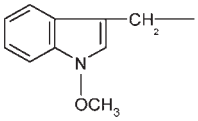
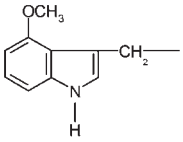
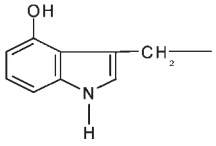
The total glucosinolate concentration as well as aromatic (2-phenylethyl), indole (3-indolylmethyl, 4-hydroxy-3-indolylmethyl, 4-methoxy-3-indolylmethyl, 1-methoxy-3-indolylmethyl), and aliphatic (3-butenyl, 2-hydroxy-3-butenyl, 4-pentenyl, 2-hydroxy-4-pentenyl) glucosinolates were quantitatively determined in the exudates of both elicited and nontreated turnip roots (Table 2). These individual glucosinolates were also determined in leaves (except 2-phenylethyl glucosinolate) and in primary and secondary roots of turnip.[19] One of the major rhizosecreted glucosinolates was 2-phenylethyl, and it was found at higher concentrations in the root exudate (Table 3) than in the turnip leaves and roots.[19]

Effect of Elicitors on the Glucosinolate Profile in Root Exudates. Root elicitation with SA and MJ increased the concentrations of indole glucosinolates, especially 1-methoxy-3-indolylmethyl glucosinolate as well as 3-indolylmethyl and 4-methoxy-3-indolylmethyl glucosinolates, and 2-phenylethyl glucosinolate in the turnip exudate. MJ application caused a pronounced increase of these glucosinolates, resulting in a >2-fold increase of 2-phenylethyl glucosinolate and a 4-fold increase in total indole glucosinolates (Table 3). According to studies on *Arabidopsis thaliana*, this elicitation effect of indole glucosinolates is due to a jasmonate-induced expression of various glucosinolate biosynthetic genes of the core pathway of indole glucosinolates, for example,[22] as well as expression of genes involved in tryptophan biosynthesis.[23] In addition, SA signaling led to the induction of aromatic glucosinolates and, to a lesser extent, of indole glucosinolates in different Brassicales species, for example, *Brassica napus*,[18] *Tropaeolum majus* and *Carica papaya*,[24] and *Brassica*

Table 2. Structural Formulas of Glucosinolates Determined in This Study



Basic structure of glucosinolates

Glucosinolate (GS) group	trivial name	semisystematic name	structure of R group
Aliphatic GS			
Alkenyl GS	Gluconapin	3-Butenyl	CH_2=CH-CH_2-CH_2-
	Glucobrassicanapin	4-Pentenyl	CH_2=CH-CH_2-CH_2-CH_2
Hydroxy-Alkenyl GS	Progoitrin	2-Hydroxy-3-butenyl	CH_2=CH-CH(OH)-CH_2-
	Gluconapoleiferin	2-Hydroxy-4-pentenyl	CH_2=CH-CH_2-CH(OH)-CH_2-
Aromatic GS	Gluconasturtiin	2-Phenylethyl	
Indole GS	Glucobrassicin	3-Indolylmethyl	
	Neoglucobrassicin	1-Methoxy-3-indolylmethyl	
	4-Methoxy-glucobrassicin	4-Mythoxy-3-indolylmethyl	
	4-Hydroxy-glucobrassicin	4-Hydroxy-3-indolylmethyl	

oleracea and *Brassica nigra*.[25] Taken together, these findings suggest that SA and MJ applications lead to an accumulation of individual indole and aromatic glucosinolates not only in the plant organs of Brassicales species but also in Brassicales root exudates. This finding indicates an extended systemic response, which comprises the phyllosphere with all aboveground plant organs as well as the rhizosphere including the belowground root system and the root exudate.

This elicited increase of indole and aromatic glucosinolates in turnip root exudates might be due to de novo glucosinolate synthesis in turnip roots as already demonstrated for another rhizosecreted secondary plant metabolite, the isoflavonoid genistein.[9] In addition, we also found an increase in the levels of indole glucosinolates and 2-phenylethyl glucosinolate in the primary and secondary roots as well as in the leaves of turnip for both SA and MJ.[19] This finding supports the assumption that enhanced levels of rhizosecreted glucosinolates in turnip root exudate might be due to de novo glucosinolate synthesis in turnip roots. Moreover, de novo glucosinolate synthesis in turnip roots could be accompanied by an elicitor-induced change of the plasma membrane potential.[26] This change in potential could generate an electrochemical gradient, thereby enabling the energy-dependent glucosinolate transport process to proceed,[27] which subsequently leads to increased efflux of glucosinolates from the secondary roots to the exudates.

In contrast to the increase of indole glucosinolates and 2-phenylethyl glucosinolate after SA and MJ root treatment, both signaling molecules applied to the roots could also cause unchanged aliphatic glucosinolate concentrations or even some declines in aliphatic glucosinolates in *B. oleracea*, *B. nigra*, and *B. napus*.[25] Interestingly, we also found similar results in *B. rapa*

Table 3. Rhizosecreted Glucosinolates in Turnip Root Exudates (Milligrams per Gram, Dry Matter, Secondary Roots) Collected during 30 Days

	treatment[a]		
glucosinolate[b]	C	SA	MJ
total GS	21.66a	34.38b	45.46c
total aliphatic GS	10.72c	8.78b	7.19a
3-butenyl GS	2.68c	1.23a	1.75b
2-hydroxy-3-butenyl GS	4.14c	3.75b	2.93a
4-pentenyl GS	2.35b	2.15b	1.65a
2-hydroxy-4-pentenyl GS	1.55b	1.75b	0.85a
aromatic (2-phenylethyl) GS	4.25a	10.59b	10.13b
total indole GS	6.68a	15.02b	28.12c
3-indolylmethyl GS	2.16a	5.12b	8.93c
1-methoxy-3-indolylmethyl GS	3.07a	8.36b	13.59c
4-hydroxy-3-indolylmethyl GS	0.56a	2.45b	2.60b
4-methoxy-3-indolylmethyl GS	0.89a	3.09b	3.62b

[a] Each value represents the mean of three samples. Mean values of each glucosinolate group or each individual glucosinolate are compared between the three treatments (control, SA, MJ). Mean values, within a row, followed by the same letter are not significantly different. The data were calculated as sum of the exudate samples harvested at 10, 15, 20, 25, and 30 days. [b] Tentatively identified. GS, glucosinolate; C, control; SA, salicylic acid; MJ, methyl jasmonate.

ssp. *rapa* leaves and roots[19] as well as in root exudate (Table 3). This elicitor-mediated shift in the glucosinolate profile with pronounced increases of indole and aromatic glucosinolate levels might be caused by the reciprocal negative control of methionine- and tryptophan-derived glucosinolate pathways as demonstrated in *A. thaliana*.[28] In detail, overexpression of the positive regulator of indole glucosinolate genes (*IQD1*) leads to repression of the *CYP79F1* and *CYP79F2* genes involved in aliphatic glucosinolate biosynthesis. Conversely, positive regulators of aliphatic glucosinolate biosynthesis were shown to down-regulate the expression of regulators of indole glucosinolate (*ATR1/MYB34*, *HIG1/MYB51*, and *HIG2/MYB122*).[29]

Kinetics of Glucosinolates in Root Exudate. In the time course study, we monitored at 5 day intervals the concentration of rhizosecreted glucosinolates. The results demonstrate that SA and MJ increased the concentrations of indole glucosinolates and 2-phenylethyl glucosinolate immediately after root application (Figures 1 and 2). The maximal effect was observed at day 10 for MJ (total indole glucosinolates = 19.33 mg g^{-1} root dm; 2-phenylethyl glucosinolate = 6.15 mg g^{-1} root dm) and for SA to a lesser extent (total indole glucosinolates = 9.42 mg g^{-1} root dm; 2-phenylethyl glucosinolate = 5.36 mg g^{-1} root dm) compared to 2.77 mg g^{-1} root dm for total indole glucosinolates and 1.38 mg g^{-1} root dm for 2-phenylethyl glucosinolate for the nonelicited control (Figures 1 and 2). However, this distinct increase of total indole (3.4- and 7.0-fold increases for SA and MJ, respectively) and of 2-phenylethyl glucosinolate concentrations (3.9- and 4.5-fold increases for SA and MJ, respectively) compared to the control on day 10 thereafter steadily decreased until the end of the experiment (day 30), leveling down to the same concentration as for untreated control, suggesting repetitive elicitor applications for a continuous triggering of the glucosinolate synthesis. This short-term but pronounced elicitation effect on indole and aromatic glucosinolates indicates a temporal

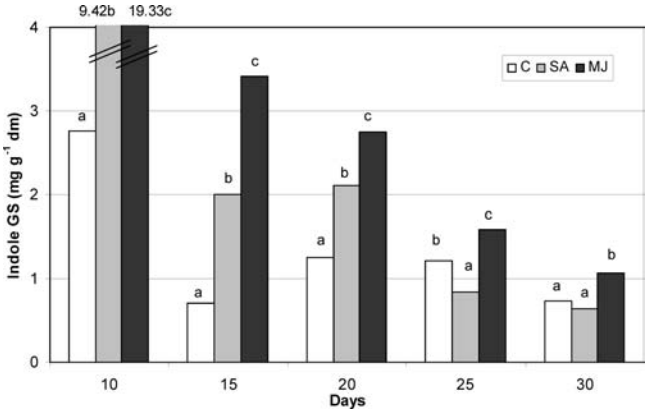


Figure 1. Rhizosecretion kinetics of indole glucosinolates (mg g^{-1} dm secondary roots) in turnip root exudates. Treatments are compared for each sampling date. Values designated by the same letter are not significantly different. Indole glucosinolates consist of 3-indolylmethyl, 4-hydroxy-3-indolylmethyl, 4-methoxy-3-indolylmethyl, and 1-methoxy-3-indolylmethyl glucosinolates. GS, glucosinolate; C, control; SA, salicylic acid; MJ, methyl jasmonate.

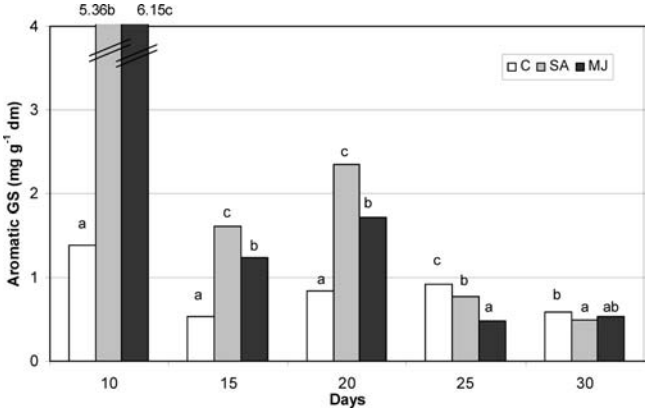


Figure 2. Rhizosecretion kinetics of aromatic (2-phenylethyl) glucosinolate (mg g^{-1} dm secondary roots) in turnip root exudates. Treatments are compared for each sampling date. Values designated by the same letter are not significantly different. GS, glucosinolate; C, control; SA, salicylic acid; MJ, methyl jasmonate.

limited plant defense response due to MJ and SA mimicking a pathogen or herbivorous attack.[22]

Both elicitors decreased aliphatic glucosinolate concentrations (Figure 3). This observed decrease compared to untreated control may be due to the already mentioned reciprocal negative control of methionine- and tryptophan-derived glucosinolate biosynthesis performing a metabolic homeostasis.[28]

We propose that the efficiency of glucosinolate isolation from exudates depends on the glucosinolate subgroup as well as the type of elicitor application. In general, the isolation of potentially anticarcinogenic indole and aromatic glucosinolates from the turnip root exudates should be performed within 10 days of single-treatment application with either 800 μmol of SA or 100 μmol of MJ. However, multiple elicitor applications, for example, in a 10 day rhythm, could possibly lead to permanent glucosinolate accumulation in root exudates, thereby enabling continuous glucosinolate isolation from exudates over the entire cultivation time of the turnip plants. Both elicitors raised essentially the concentration of indole glucosinolates and 2-phenylethyl glucosinolate. However, MJ

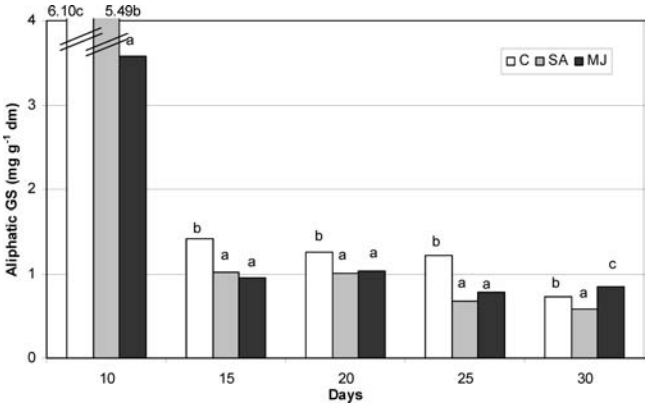


Figure 3. Rhizosecretion kinetics of aliphatic glucosinolates (mg g^{-1} dm secondary roots) in turnip root exudates. Treatments are compared for each sampling date. Values designated by the same letter are not significantly different. Aliphatic glucosinolates consist of 3-butenyl, 2-hydroxy-3-butenyl, 4-pentenyl, and 2-hydroxy-4-pentenyl glucosinolates. GS, glucosinolate; C, control; SA, salicylic acid; MJ, methyl jasmonate.

elicited a stronger increase in indole glucosinolate concentration compared to SA, whereas 2-phenylethyl glucosinolate was enhanced by both SA and MJ treatment, thereby suggesting that depending on the targeted glucosinolate or glucosinolate subgroup, the most inducible elicitor has to be identified as elicitors differ in their induction of individual glucosinolate accumulation. Different elicitation effects of MJ and SA on individual glucosinolates might be explained by the differentiated defense strategy of brassicaceous plants as both signaling pathways can be triggered depending on the type of pathogen or herbivorous attack (see, e.g., refs 22 and 30).

Finally, we established a plant-based system to produce a high level of health-promoting glucosinolates in root exudates with the aim that these secondary plant metabolites could be used as bioactive ingredients in functional foods and nutraceuticals. Also, to the best of our knowledge, this study is the first to report that the occurrence of potentially anticarcinogenic glucosinolates is not restricted to the plant organs and that substantial amounts of indole glucosinolates and 2-phenylethyl glucosinolate can also be isolated from root exudates in turnip. Moreover, this is the first study to provide a detailed profile of glucosinolate composition as well as the kinetics of glucosinolate secretion in root exudates after elicitor treatment.

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