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Biotic Elicitors Effectively Increase the Glucosinolates Content in *Brassicaceae* Sprouts

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Supporting Information

ABSTRACT: Several biotic elicitors have been used in *Brassicaceae* species to enhance their phytochemical quality. However, there is no comparison between elicitors under controlled growth conditions. In order to draw general conclusions about the use of elicitors to enrich ready-to-eat sprouts in health-promoting glucosinolates, the aim of this study was to unveil the effect of the phytohormones methyl jasmonate (25 μM), jasmonic acid (150 μM), and salicylic acid (100 μM), the oligosaccharides glucose (277 mM) and sucrose (146 mM), and the amino acid DL-methionine (5 mM) as elicitors over 8-day sprouting *Brassica oleraceae* (broccoli), *Brassica napus* (rutabaga cabbage), *Brassica rapa* (turnip), and *Raphanus sativus* (China rose radish and red radish), representative species high in glucosinolates previously studied. Results indicated that the phytohormones methyl jasmonate and jasmonic acid and the sugars acted as effective elicitors, increasing the total glucosinolate contents of the sprouts, particularly, glucoraphanin (from 183 to 294 mg·100 g^{-1} in MeJA-treated broccoli sprouts), glucoraphenin (from 33 to 124 mg·100 g^{-1} and from 167 to 227 mg·100 g^{-1} in MeJA-treated China rose radish and red radish, respectively), and glucobrassicin (from 23.4 to 91.0 mg·100 g^{-1} and from 29.6 to 186 mg·100 g^{-1} in MeJA-treated turnip and rutabaga sprouts, respectively).

KEYWORDS: *germinating seeds, Brassicaceae, elicitation, healthy edible sprouts, glucosinolates*

INTRODUCTION

Brassicaceae (cruciferous) sprouts are a good source of vitamin C, vitamin A, folic acid, dietary fiber, and minerals, which have higher levels of phytochemicals, glucosinolates (GLSs), and phenolic compounds compared to adult plants because of their physiological state.[1,2] As the phytochemical content of the sprouts decreases over the germination period due to a dilution effect of tissue expansion, 8-day-old sprouts were considered optimum for consumption, biomass, and size in order to deliver their health-promoting properties.[3]

Cruciferous vegetables have been widely investigated because of their economic importance and content of health-promoting phytochemicals with a positive effect against various pathologies and chronic diseases.[4] In particular, interest has been focused on GLSs, nitrogen- and sulfur-containing secondary metabolites mainly found in *Brassicaceae*, the precursors of bioactive isothiocyanates (ITCs), which are released by myrosinase (β-thioglucoside glucohydrolase; E.C. 3.2.1.147) hydrolysis upon chewing, cutting, or other mechanical disruption or by the intestinal microflora upon intake of vegetables tissues.[5] *Brassica oleraceae* is the mainly harvested species of this family, such as broccoli and cauliflower, and a variety of horticultural crops, such as *Brassica napus* (rutabaga), *Brassica rapa* (turnip and rapini), and *Raphanus sativus* (radishes). The differences in the phytochemical profiling among species are both qualitative and quantitative, finding characteristic GLSs in different species.[3,6] Broccoli sprouts have been intensively studied due to their high concentration of glucoraphanin and its hydrolysis product sulforaphane (4-methylsulfinylbutyl ITC). Also, the ITC iberin (3-methylsulfinylpropyl ITC) from its GLS glucoiberin has shown properties as inducer carcinogen detoxification (phase II enzymes).[7] Radish sprouts contain beneficial GLSs as well, such as dehydroerucin, also called glucoraphasatin, and glucoraphenin, which breakdown products, raphasatin (4-methylsulfanyl-3-butenyl ITC) and sulforaphane (4-methylsulfinyl-3-butenyl ITC), respectively, and show selective cytotoxic/apoptotic activity on three human colon carcinoma cell lines.[8] Indolic GLSs (glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin GLS) are present in *B. oleraceae, B. rapa, B. napus*, and *R. sativus* species, and their hydrolysis products, indoles, have also exhibited protective activities against many types of cancer.[9]

Elicitors are substances which induce physiological changes in the plant. Biotic elicitors have biological origin and are commonly applied to enhance the phytochemical composition of plants.[10,11] Depending on the type of compound, the plant activates different signaling pathways to synthesize an optimal mixture of defensive metabolites. The phytohormones salicylic acid (SA) and jasmonic acid (JA) play key roles in this signal interplay for defense gene expression, being accumulated following pathogenic or environmental stresses. Moreover, addition of exogenous JA and its methyl ester, methyl jasmonate (MeJA), or SA can also simulate pathogen-induced plant defense responses and lead to production of bioactive secondary metabolites though several mechanisms.[11,12] Sugars, such as glucose and sucrose, are also recognized as effective signaling molecules throughout plant life, modulating many developmental and metabolic processes including ROS-

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scavenging functions, germination, development, photosynthesis, carbon and nitrogen metabolism, flowering, stress responses, and senescence.[13] Finally, previous experiments demonstrated that also application of the amino acid methionine, as a biosynthetic precursor, led to enhanced GLSs contents in radish as well as in broccoli heads.[14,15]

The aim of this study was to investigate the effect of the most active elicitors found in the literature, the JA,[16] methyl jasmonate,[17,18] salicylic acid,[18] glucose,[19] sucrose,[20,21] and DL-methionine,[18] using 5 days of treatment from 3- to 8-day of sprouting under controlled growth conditions of *B. oleraceae* (broccoli), *B. napus* (rutabaga), *B. rapa* (turnip), and *R. sativus* (China rose and red radishes), rich in aliphatic and indolic GLSs because their young physiological state, in order to provide fresh, safe, and ready-to-eat sprouts, maximizing their health-promoting compounds.

MATERIAL AND METHODS

Chemicals. Jasmonic acid, sucrose, and glucose were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA); methyl jasmonate was purchased from SAFC (St. Louis, MO, USA); salycilic acid and ethanol absolute were obtained from Panreac S.A. (Barcelona, Spain). DL-Methionine was from Alfa Aesar GmbH & Co. (Karlsruhe, Germany). Formic acid (98−100%) for analysis was obtained from EMSURE, ACS, Reag. Ph Eur, Merck, KGaA (Darmstadt, Germany). Trifluoroacetic acid for optima LC/MS was purchased from Fisher Scientific Co. (New Jersey, USA). Methanol and acetonitrile were LC-MS grade from HiPerSolv Chromanorm, BDH Prolabo (Leuven, Belgium). Sinigrin monohydrate was obtained from Phytoplan (Germany).

Plant Material and Germination Conditions. Seeds provided by Intersemillas S.A. (Valencia, Spain) were of commercial quality for ready-for-sprouting lines. Five varieties from the *Brassicaceae* family were used: broccoli (*B. oleracea* L. var. *italica*), rutabaga (*B. napus* L. var. *napobrassica*), turnip (*B. rapa* L. subsp. *rapa*), China rose radish (*R. sativus* L. cv. China rose), and red radish (*R. sativus* L. cv. Rambo). Seeds were rinsed in distilled water and immersed in 5 $g \cdot L^{-1}$ sodium hypochlorite under aeration for 24 h. After pouring off the soaking water, the seeds were weighed (day 0) and spread evenly on trays (5 g per tray) lined with cellulose growth pad (CN Seeds, U.K.) and irrigated everyday with Milli-Q water with 5 $g \cdot L^{-1}$ sodium hypochlorite. Aliquots of 5 g of seeds were frozen in liquid nitrogen and stored at −80 °C pending phytochemical analysis.

The three replicates (trays) per sample were germinated for 2 days in a controlled dark chamber at 28 °C, for increasing the stem elongation of sprouts. Then, trays were transferred to a controlled environment chamber with a 16 h light/8 h dark cycle and air temperatures of 25 and 20 °C, respectively. The relative humidity (RH) was 60% (day) and 80% (night). Photosynthetically active radiation (PAR) of 400 $\mu mol\ m^{-2}\ s^{-1}$ was provided by a combination of fluorescent tubes (Philips TLD 36 W/83, Hamburg, Germany; Sylvania F36W/GRO, Danvers, MA, USA) and metal halide lamps (Osram HQI.T 400 W, Munich, Germany). Three replicates per treatment of *Brassicaceae* sprouts samples were rapidly and gently collected at day 8 after germination, in the middle of the light period, for analysis. All samples were weighed (fresh mass), collected separately, flash frozen in liquid nitrogen, and stored at −80 °C prior to analyses.

Treatments with Elicitors. The phytohormones jasmonic acid (JA) (150 μM), methyl jasmonate (MeJA) (25 μM), and salicylic acid (SA) (100 μM), the oligosaccharides glucose (277 mM) and sucrose (146 mM), and the amino acid DL-methionine (5 mM) were selected as elicitors according to a literature review. JA, MeJA, and SA were dissolved in 0.2% ethanol in Milli-Q water. Sucrose and glucose were also dissolved in Milli-Q water. DL-Methionine was dissolved in 0.04% ethanol in Milli-Q water. Elicitors were applied as exogenous spraying on the cotyledons (not as soaking or irrigation solution) with 30 mL of test solution per sample (10 mL per tray) from day 3 to day 7 of sprouting (5 days of treatment) using Milli-Q water as control.

Extraction and Determination of Glucosinolates. *Sample Extraction.* Freeze-dried samples (100 mg) were extracted with 1.5 mL of methanol 70% V/V in a US bath for 10 min, then heated at 70 °C for 30 min in a heating bath, with shaking every 5 min using a vortex stirrer, and centrifuged (17 500 × *g*, 15 min, 4 °C). Supernatants were collected, and methanol was completely removed using a rotary evaporator. The dry material obtained was redissolved in 1 mL of ultrapure water and filtered through a 0.45 μm Millex-HV13 filter (Millipore, Billerica, MA, USA).

HPLC-DAD-ESI-MS^n Qualitative and Quantitative Analysis of Glucosinolates. First, the separate intact GLSs were identified from the extracted samples following their MS^2 $[M - H]^-$ fragmentations in HPLC-DAD-ESI-MS^n, carried out on a Luna C18 100A column (150 × 1.0 mm, 3 μm particle size; Phenomenex, Macclesfield, U.K.). Water:formic acid (99:1, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 20 μL/min. The linear gradient started with 1% of solvent B, reaching 17% solvent B at 15 min up to 17 min, 25% at 22 min, 35% at 30 min, 50% at 35 min, which was maintained up to 45 min. The injection volume was 3 μL. Chromatograms were recorded at 227 nm. HPLC-DAD-ESI/MS^n analyses were carried out in an Agilent HPLC 1200 (Agilent Technologies, Waldbronn, Germany) and coupled to a mass detector in series. The HPLC system consisted of a binary capillary pump (model G1376A), an autosampler (model G1377A), a degasser (model G1379B), a sample cooler (model G1330B), and a photodiode array detector (model G1315D) and controlled by ChemStation software (v.B.0103-SR2). The mass detector was a Bruker, model UltraHCT (Bremen, Germany), ion trap spectrometer equipped with an electrospray ionization interface (ESI) and controlled by Bruker Daltonic Esquire software (v.6.1). Ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range from m/z 50 to 600. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionization mode for glucosinolates. MS^n was carried out in the automatic mode on the more abundant fragment ion in $MS^{(n-1)}$. Then, the extracted samples (20 μL) were analyzed and quantified in a Water HPLC-DAD system (Waters Cromatografia S.A., Barcelona, Spain) as previously described by Pérez-Balibrea et al.[22] Intact GLSs were identified following their UV spectra and the order of elution previously described for the acquisition conditions. Glucosinolates were quantified using sinigrin as external standard, because of the similar structure to the glucosinolates in the sample.[22,23]

Statistical Methods. All assays were conducted by triplicate. Data were processed using the SPSS 15.0 software package (LEAD Technologies, Inc., Chicago, IL, USA). We carried out a multifactorial analysis of variance (ANOVA) and the Ducan's Multiple Range Test to determine significant differences at P values < 0.05.

RESULTS AND DISCUSSION

Biomass. The weight of seeds and sprouts was collected on day 0 (embebed seeds) and day 8. The ratio of fresh weight between sprouts and seeds as an indication of biomass production (Table 1) showed the expected increase in weight over sprouting and served as a quality index to select species with higher biomass production. Growing plants are exposed to a range of genetic, environmental, biotic, and abiotic factors which affect their growth and yield.[24] The biomass of the *Brassicaceae* sprouts treated with sucrose increased significantly over other treatments, ranging from about 15% in turnip and China rose radish to 80% in Red radish (Table 1), in agreement with results of Guo et al.[25] using a 146 mM sucrose treatment. Stewart et al.[26] explained that sucrose (88 mM) alters the growth rate and causes a dramatic increase in hypocotyl length.

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Table 1. Biomass of Sprouts:Seeds Ratio in Cruciferous Edible Sprouts on a Fresh Weight Basis

species	broccoli	rutabaga	turnip	china rose radish	red radish
control	2.62[a]c	4.34b	2.69c	3.29b	1.45b
methyl jasmonate	2.24 cd	5.59a	2.71 cd	3.78b	1.61b
jasmonic acid	1.33e	3.15c	1.37e	2.14c	2.28a
salicylic acid	1.50de	4.24b	1.54de	2.50c	1.59b
glucose	0.95e	3.94bc	1.03e	2.38c	1.92a
sucrose	4.21a	6.43a	3.17a	3.70b	2.65a
DL-methionine	3.47b	4.18b	2.81b	5.15a	2.54a
$LSD_{0.05}$ [B](ANOVA $P < 0.001$)	0.22[b]***	0.27**	0.47*	0.15***	0.21***

[a]Mean values ($n = 3$) comparing species for each elicitor treatment, followed by different lowercase letters are significantly different at $P < 0.05$. a–h, Different lowercase letters mean statistically significant differences among elicitor treatments ($p < 0.05$). [b]Least significant difference (LSD) for separating means in the respective column. The LSD was computed only after analysis of variance indicating a significant ($p < 0.05$) entry effect. Levels of significance for each sampling day between species. Nonsignificant at $P > 0.05$ (n.s.); significant at $P < 0.05$ (*); significant at $P < 0.01$ (**); significant at $p < 0.001$ (***).

Sucrose could supply a balanced carbon source for cell growth by hydrolysis of invertase and sucrose synthase, with the resulting hexose directly participating in the glycolytic and pentose phosphate pathway (required for cells to synthesize nucleic acids and quickly replicate).[27] Stressful conditions such as starvation or hypoxia result in low energy status in the cell; Smeekens et al.[28] showed that sugars repressed the bZIP growth regulatory system activity in a concentration-dependent manner; therefore, our employed dose (5g/100 mL) was appropriate for biomass increase in sucrose treatment sprouts but not in the case of glucose, also in accord with Mirnezhad.[29]

DL-Methionine also showed a positive effect, increasing the fresh weight of sprouts in almost all varieties, 30% in broccoli, 4% in turnip, 57% in China rose radish, and 75% in red radish, except for rutabaga, agreeing with previous reports.[30,31] Gigolashvili et al.[32] reported a relationship between the overexpression of the HAG1/MYB28 gene, specific for methionine-derived GLSs (aliphatics), and strongest growth phenotype in *Arabidopsis thaliana*. On the other hand, glucose and the phytohormones (JA, MeJA, and SA) did not increase the fresh weight of sprouts and even reduced the size as for the control, as happened in broccoli, turnip, and China rose radish, founding a decrease around 60% in JA- and SA-treated sprouts, as also found by Kastell et al.[33] MeJA and SA regulate the overexpression of the OBP2 transcription factor involved in GLS biosynthesis, which altered the phenotype of *A. thaliana*, with smaller leaves,[34] supporting our result. In red radish sprouts, nonsignificant differences were found between the glucose- and phytohormones-treated sprouts and the controls. Higher values of biomass ratio not only means better growth (data not shown) but also higher fresh weight, making the sprouts more palatable. Concentration of elicitor and interval between treatment and harvest induce different responses characteristic of plant species, making it necessary to find the required effective dose and time empirically.[35]

Glucosinolate Profiles of *Brassicaceae* Sprouts. Identification and quantification of individual GLSs in seeds and 8-day-old sprouts of the five *Brassicaceae* cultivars are presented in Tables 2–5. The molecular ion $[M - H]^-$ (m/z) of GLSs, their fragment ion pattern, and retention times allowed identification of 16 different compounds.[3] The MS^2 fragmentation of aglycone side chain produces the most consistent ion at m/z 259, and the MS^3 fragmentation of this ion gives rise to fragments at m/z 97 (corresponding to the sulfate group) by disassociation of GLSs in the ion trap mass spectrometer, constituting a very useful preliminary screening method for determining the presence of GLSs in sprouts.[36] Sixteen GLSs, belonging to the aliphatic, indolic, and aromatic classes based on their different side chain structure, were detected. Results showed significant differences of the characteristic GLSs profile among cruciferous seeds and sprouts (Tables 2–5). The aliphatic GLSs were the major group in *B. oleraceae*, *B. napus*, and *R. sativus* sprouts, corresponding to 60% in *Brassica* and 90% in *Raphanus* varieties. In contrast, *B. rapa* sprouts showed higher amount of indolic GLSs, corresponding to 65% of the total (Table 4). Seeds exhibited the largest amount of GLSs being the nutrient reservoir organ, containing ranging concentrations from 563.79 to 1731.32 mg·100 g^{-1} F.W. in turnip and broccoli, respectively (Table 2), of interest for the composition of the sprouts during germination. According to Pérez-Balibrea et al.,[18,22] the major source of glucoraphanin are broccoli seeds and sprouts (987.02 and 182.46 mg·100 g^{-1} F.W., respectively) (Tables 2 and 3), which has been intensively studied because of its derived product sulforaphane, a potential chemopreventive beneficial compound against cancer, cardiovascular, and neurological diseases.[4] Turnip and rutabaga seeds and sprouts showed the antinutrient progoitrin as the major GLS, and glucoraphanin and gluconasturtiin were absent in the sprouts, probably degraded or diluted during germination.[22] In radish cultivars, specific GLSs in seeds were found as well (traces of the aromatic glucoberteroin). The major characteristic GLS in this species is glucoraphenin, containing 1051.88 and 32.78 mg·100 g^{-1} F.W. in China rose radish and 887.20 and 166.93 mg·100 g^{-1} F.W. in red radish in seeds and sprouts, respectively (Table 5). The bioactive sulforaphene, like sulphoraphane, is a potential anticancer agent.[8] In *Brassica* species, in addition to the parent indole GLS glucobrassicin, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin were also detected in the samples (Tables 2–5). Only the indole 4-hydroxiglucobrassicin GLS was present in all species, being also one of the major compounds in seeds (from 152.49 to 358.34 mg·100 g^{-1} F.W. in China rose radish and broccoli, respectively). On the contrary, in *Raphanus* sprouts only 4-hydroxiglucobrassicin and 4-methoxyglucobrassicin were detected.[6]

Phytohormones as Elicitors. The jasmonates are signal compounds in the elicitation process leading to de novo transcription, translation, and, ultimately, biosynthesis of secondary metabolites in plant cell cultures. Methyl jasmonate (MeJA) is believed to be, at least, partially hydrolyzed by endogenous esterases to free jasmonic acid (JA) within the plant tissue.[12] MeJA elicitor (25 μM) was found highly effective for almost all the 8-day-old *Brassicaceae* sprouts, increasing by 84%, 50%, 123%, 25%, and 23% the total GLSs amount in broccoli, turnip, rutabaga, China rose radish, and red radish, respectively, increasing the indoles more than the aliphatic GLSs (Tables 3–5). After MeJA treatments, the broccoli sprouts showed significantly much more glucoraphanin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin by 60%, 241%, 48%, and 247%, respectively, associated with

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Table 2. List of Individual Glucosinolates (mg 100 g^{-1} F.W.) Detected in the Seeds of *Brassicaceae* Varieties

				seeds					
peak	compound	GLS semisystematic name	class	broccoli	rutabaga	turnip	china rose radish	red radish	
1	glucoiberin	3-methylsulfinylpropyl-gls	aliphatic	26.0 ± 3.43[a]	n.d.	n.d.	n.d.	n.d.	
2	progoitrin	(*R*)-2-hydroxy-3-butenyl-gls	aliphatic	n.d.	1278 ± 65.5	177 ± 0.36	n.d.	n.d.	
3	glucoraphenin	4-methylsulfinyl-3-butenyl-gls	aliphatic	n.d.	n.d.	n.d.	1052 ± 16.8	887 ± 49.6	
4	glucoraphanin	4-methylsulfinylbutyl-gls	aliphatic	987 ± 51.4	40.6 ± 2.66	28.7 ± 1.42	n.d.	n.d.	
5	glucoalyssin	5-methylsulfinylpentyl-gls	aliphatic	10.1 ± 12.1	n.d.	2.30 ± 1.02	n.d.	n.d.	
6	gluconapoleiferin	(*R*)-2-hydroxy-4-pentenyl-gls	aliphatic	n.d.	11.9 ± 2.32	Tr	n.d.	n.d.	
7	gluconapin	3-butenyl-gls	aliphatic	n.d.	95.8 ± 4.62	44.9 ± 10.9	n.d.	n.d.	
8	4-hydroxiglucobrassicin	4-hydroxy-3-indolylmethyl-gls	indolic	358 ± 48.2	239 ± 23.5	290 ± 24.3	152.5 ± 19.4	223 ± 8.60	
9	glucobrassicanapin	4-pentenyl-gls	aliphatic	n.d.	Tr	Tr	n.d.	n.d.	
10	glucoerucin	4-methylthiobutyl-gls	aliphatic	324 ± 43.7	n.d.	n.d.	3.56 ± 0.36	n.d.	
11	dehydroerucin	4-methylthio-3-butenyl-gls	aliphatic	n.d.	n.d.	n.d.	85.1 ± 5.18	31.3 ± 2.23	
12	glucobrassicin	3-indolylmethyl-gls	aliphatic	14.9 ± 4.61	1.79 ± 0.05	14.3 ± 7.57	n.d.	n.d.	
13	gluconasturtin	2-phenylethyl-gls	aromatic	Tr	Tr	Tr	n.d.	n.d.	
14	4-methoxyglucobrassicin	4-methoxy-3-indolylmethyl-gls	indolic	6.23 ± 4.16	Tr	Tr	n.d.	n.d.	
15	*n*-hexyl	*n*-hexyl-gls	aliphatic	Tr	n.d.	n.d.	n.d.	n.d.	
16	neoglucobrassicin	*N*-methoxy-3-indolylmethyl-gls	indolic	7.36 ± 1.47	Tr	7.14 ± 3.56	n.d.	n.d.	
			total	1731a	1666a	564d	1293b	1142c	46.1^{be} ($LSD_{0.05}$)

[a]Mean values ($n = 3 \pm SD$). Tr, traces, not quantified. n.d., not detected. a–d, Different lowercase letters mean statistically significant differences in the total glucosinolates content between species. [b]Least significant difference (LSD) for separating means in the respective row. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value. [c](*) $p < 0.05$. [d](**) $p < 0.01$. [e](***)$p < 0.001$; n.s. $p > 0.05$.

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Table 3. List of Individual and Total Glucosinolates (mg 100 g^{-1} F.W.) in Broccoli (*B. oleraceae*) Sprouts under Elicitor Treatments

		broccoli							
peak	compound	control	MeJA	JA	SA	glucose	sucrose	DL-methionine	$LSD_{0.05}$ [b]
1	glucoiberin	10.8[a]a	7.68ab	9.33a	3.41b	6.35ab	11.9a	11.5a	1.86[c]
2	progoitrin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
3	glucoraphenin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
4	glucoraphanin	183c	294a	265ab	288ab	297a	253b	200c	13.2[e]
5	glucoalyssin	0.46b	Tr	0.70a	Tr	Tr	Tr	Tr	0.16[c]
6	gluconapoleiferin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
7	gluconapin	Tr	Tr	Tr	Tr	Tr	Tr .	Tr	
8	4-hydroxiglucobrassicin	39.9bc	40.1bc	32.4c	42.4b	54.7a	44.7b	55.6a	3.09[e]
9	glucobrassicanapin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
10	glucoerucin	39.1	31.7	36.5	39.1	36.8	41.0	37.8	4.35nd
11	dehydroerucin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
12	glucobrassicin	55.2 cd	188.5a	86.4b	43.0d	53.8 cd	92.3b	74.2bc	8.66[e]
13	gluconasturtin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
14	4-methoxyglucobrassicin	28.7c	42.7ab	49.2a	39.8b	43.4ab	45.4ab	40.2b	2.47[e]
15	*n*-hexyl	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
16	neoglucobrassicin	30.9c	107.0a	95.6a	25.5c	43.1bc	61.2b	43.1bc	6.69[e]
	total	388e	712a	575b	481 cd	536bc	549b	463d	21.1[e]

[a]Mean values ($n = 3$). Tr, traces, not quantified. n.d., not detected. a–d, Different lowercase letters mean statistically significant differences between treatments (for each variety). [b]Least significant difference (LSD) for separating means in the respective row. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value. [c](*) $p < 0.05$. [d](**) $p < 0.01$. [e](***) $p < 0.001$; n.s. $p > 0.05$.

potential health benefits due to the biological activity of their products.[4] The enhancement of the aliphatic GLS glucoraphenin in China rose and red radish sprouts after MeJA treatment was 278% and 35%, respectively. Indole GLSs in turnip, rutabaga, red radish, and China rose radish sprouts were also higher than the controls and increased by 109%, 223%, 54%, and 200%, respectively. The JA (150 μM) also produced an increase of total GLSs, especially in broccoli (by 50%), rutabaga (by 95%), and turnip (by 24%), having a higher effect on the indoles than on the aliphatic GLSs (Tables 3 and 4). In contrast, scarce differences were found in total GLSs in the treated radish sprouts compared to control samples (Table 5). Salicylic acid (SA) caused an increase of 20% in total GLSs in broccoli and radish sprouts, with aliphatic GLSs being the most affected (Tables 3 and 5), and no effects were found in turnip or rutabaga sprouts (Table 4). This phytohormone produced an increase in glucoraphanin in broccoli (by 58%) as well as in glucoraphenin (by 50% and 14%) and dehydroerucin (by 18% and 29%) in China rose and red radish sprouts, respectively (Tables 3 and 5).

Biosynthesis of glucosinolates can be drastically induced by wounding, hormone application, and pathogen or herbivore attack. Berger[34] demonstrated the induction of several pathway genes after phytohormones spraying application in *A. thaliana*, where IQD1 protein, OBP2 transcription factor, and ATR1/MYB34 and HIG1/MYB51 genes were overexpressed and regarded as a regulator with respect to increased concentrations of major indole GLSs. Nevertheless, the genes respond differently to biotic stress conditions in time and the site of metabolites accumulation in the plant.[34] These treatments increased the concentration of individual health-promoting glucosinolates (such as glucoraphanin, glucoraphenin, dehydroerucin, and indole GLSs) and also of great interest had not effect or even decreased the concentrations of the antinutrient progoitrin by JA and MeJA, present in rutabaga and turnip sprouts (Tables 3–5). Similar induction of GLSs by exogenous application of phytohormones as elicitors has been previously found by different authors, particularly, increased indole-GLSs.[16−18,33,34] Consistent with Brader et al.,[37] MeJA is able to trigger accumulation of the indole GLSs by inducting the tryptophan biosynthesis as demonstrated in *A. thaliana*, in contrast to SA, which seems to play a minor role in this response. The above-mentioned treatments, particularly, JA and its ester MeJA, were highly effective elicitors in *Brassica* sprouts. On the other hand, SA was more effective in radish sprouts than JA, with the MeJA solution being an interesting common elicitor to enrich in GLSs all species studied.

Sugars as Elicitors. Nonstructural carbohydrates, both sucrose and glucose, used as elicitors, enhanced the total GLSs amount in all sprouts under study, in accordance with some studies on broccoli, cabbage, and radish sprouts.[19,20] Sucrose (146 mM) showed higher effects in *Brassica* species, increasing by 42%, 31%, and 159% the total GLSs in broccoli, turnip, and rutabaga, respectively (Tables 3 and 4). By contrast, total GLSs in radish sprouts were increased higher after glucose treatment (277 mM) by 22% and 26% in China rose and red radish, respectively (Table 5). It must be emphasized the elicitation effect observed in broccoli sprouts, where glucoraphanin was increased by 40% and 60% under sucrose and glucose treatments, respectively (Table 3). Glucoraphanin was enhanced as well, by 50% and 30%, under both sucrose and glucose spray in China rose and red radish, respectively (Table 5). The other major aliphatic GLS from radish, dehydroerucin, was increased by the glucose treatment by 22% and 33% in China rose and red radish, respectively. In contrast to what was found by Wei et al.,[19] who showed a decrease in this compound. These results were consistent with those previously reported by Guo et al.,[25] indicating that the *Bo-Elong* gene involved in the aliphatic GLSs pathway was up-regulated by sucrose. Gigolashvili et al.[32] described glucose as an important signaling molecule that may induce transcriptional regulatory mechanisms, integrating carbohydrate availability and hormone action, regulating this class of GLSs by the HAG1/MYB28 gene, in response to carbohydrate availability, in *A. thaliana*.

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Table 4. List of Individual and Total Glucosinolates (mg 100 g^{-1} F.W.) in Turnip (*B. rapa*) and Rutabaga (*B. napus*) Sprouts under Elicitor Treatments

		turnip								rutabaga							
peak	compound	control	MeJA	JA	SA	glucose	sucrose	DL-metionine	$LSD_{0.05}$ [b]	control	MeJA	JA	SA	glucose	sucrose	DL-metionine	$LSD_{0.05}$ [b]
1	glucoiberin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
2	progoitrin	41.9[a]bc	27.5c	5.69d	40.7bc	50.5ab	63.8a	43.5b	4.92[e]	185e	292c	240d	198e	253d	444a	343b	8.64[e]
3	glucoraphenin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
4	glucoraphanin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
5	glucoalyssin	1.09	0.96	Tr	Tr	Tr	0.99	1.27	0.23nd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
6	gluconapoleiferin	Tr	Tr	Tr	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	Tr	Tr	Tr	
7	gluconapin	7.81	0.38	1.50	5.19	1.06	0.92	1.41	2.34 [nd]	15.8b	17.8ab	5.36 cd	1.22d	Tr	11.8bc	24.0a	2.51[e]
8	4-hydroxiglucobrassicin	23.6bc	25.5ab	31.9a	17.6c	32.0a	30.5ab	23.8bc	2.28[d]	17.1c	21.5b	22.7b	17.0c	23.8b	28.7a	14.8c	1.24[e]
9	glucobrassicanapin	Tr	Tr	Tr	Tr	Tr	Tr	Tr		Tr	Tr	Tr	Tr	Tr	Tr	Tr	
10	glucoerucin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
11	dehydroerucin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
12	glucobrassicin	23.4c	91.0a	41.1b	18.6c	26.1c	37.7b	25.0c	3.26[e]	29.6e	186a	158b	24.2e	46.4e	132c	94.6d	8.22[e]
13	gluconasturtin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
14	4-methoxyglucobrassicin	22.4b	22.3b	42.8a	23.0b	25.0b	23.9b	18.5b	2.08[e]	37.8de	61.9bc	33.6de	25.2e	48.3 cd	93.6a	73.5b	5.03[e]
15	*n*-hexyl	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
16	neoglucobrassicin	24.2 cd	48.8a	56.7a	28.0bcd	41.4ab	31.4bc	12.7d	5.22[e]	33.9d	131b	162a	47.9d	73.8c	116b	38.4d	5.53[e]
	total	150 cd	216a	180b	133d	176bc	189b	126d	9.13[e]	319e	710b	621c	314e	445d	826a	588c	12.3[e]

[a]Mean values (n = 3). Tr, traces, not quantified. n.d., not detected. a–d, Different lowercase letters mean statistically significant differences between treatments (for each variety). [b]Least significant difference (LSD) for separating means in the respective row. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value. [c](*) $p < 0.05$. [d](**) $p < 0.01$. [e](***) $p < 0.001$; n.s. $p > 0.05$.

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Table 5. List of Individual and Total Glucosinolates (mg 100 g^{-1} F.W.) in China Rose Radish and Red Radish (*R. sativus*) Sprouts under Elicitor Treatments

		China rose radish								red radish							
peak	compound	control	MeJA	JA	SA	glucose	sucrose	DL-methionine	$LSD_{0.05}$ [b]	control	MeJA	JA	SA	glucose	sucrose	DL-methionine	$LSD_{0.05}$ [b]
1	glucoiberin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
2	progoitrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
3	glucoraphenin	32.8[A]d	124a	88.7b	50.5c	51.4c	50.6c	31.6d	3.18[e]	167 cd	227a	185bc	191ab	210ab	226a	146d	12.8[d]
4	glucoraphanin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
5	glucoalyssin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
6	gluconapoleiferin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
7	gluconapin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
8	4-hydroxiglucobrassicin	15.2b	22.6a	22.5a	16.1b	16.1b	12.7b	12.8b	1.31[e]	27.3b	44.2a	31.2b	21.1c	22.2c	41.7a	27.0b	1.42[e]
9	glucobrassicanapin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
10	glucoerucin	1.95b	10.9a	0.59b	1.61b	0.98b	1.73b	1.95b	0.67[e]	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
11	dehydroerucin	411bc	434b	370c	489b	502a	439b	403c	9.33[e]	172c	182bc	180bc	222ab	229a	183bc	150c	14.4[c]
12	glucobrassicin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
13	gluconasturtin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
14	4-methoxyglucobrassicin	27.2ab	19.8c	15.5d	30.8a	25.9b	17.8 cd	29.0ab	1.31[e]	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
15	*n*-hexyl	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
16	neoglucobrassicin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
	total	488bc	611a	498bc	588a	596a	520b	478.c	12.1[e]	366d	452ab	396c	431b	461a	451ab	323e	8.04[e]

[a]Mean values (n = 3). Tr, traces, not quantified. n.d., not detected. a–d, Different lowercase letters mean statistically significant differences between treatments (for each variety). [b]Least significant difference (LSD) for separating means in the respective row. The LSD was computed only after analysis of variance indicated a significant ($p < 0.05$) entry effect. ANOVA p value. [c](*) $p < 0.05$. [d](**) $p < 0.01$. [e](***) $p < 0.001$; n.s. $p > 0.05$.

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As for the indole-GLSs, no effects were found on radish sprouts, while both sucrose and glucose highly and significantly enhanced 4-hydroxiglucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin in *Brassica* species, with the glucobrassicin being mainly increased by sucrose (Tables 3 and 4). Sivanandhan et al.[38] showed that the type and concentration of carbon source induces profound effects on growth and quality of the metabolites produced. Sugars serve as the carbon and energy source and also affect the osmotic pressure of the medium, which stimulates mitochondrial activity and, hence, energy production for metabolites synthesis.[39] The secondary product formation after sugar application could be attributed to a certain level of osmotic stress, which initiated the signal perception through a receptor in the cell membrane to activate the signal transduction network. This activates the transcription factors, which regulates gene expression involved in biosynthesis of the target metabolites.[38,40]

DL-Methionine as Elicitor. Aliphatic GLSs, such as glucoraphanin and glucoiberin, are secondary metabolites derived from amino acids, mainly methionine.[31] The effect of the exogenous spray application of this amino acid (5 mM) has been studied in order to increase the amount of GLSs in sprouts, mainly aliphatic ones.[17,18] In the biosynthesis of glucosinolates, first, methionine is transaminated to the corresponding α-keto-acids, and subsequently, the side-chain elongation of the amino acid is produced, followed by formation of the GLS core structure mediated by cytochrome P450 mono-oxygenase.[32] Only in broccoli and rutabaga sprouts a significant effect after application of this amino acid was found, where the total GLSs were increased by 19% and 85%, respectively (Tables 3 and 4). China rose radish sprouts remained without changes in GLSs contents, while turnip and red radish sprouts showed a small decrease in total GLSs after the DL-methionine applications (Tables 4 and 5). Opposite to our first hypothesis, aliphatic GLSs were not affected to a higher degree than indole GLSs upon DL-methionine treatment, probably resulting from expression of HAG1/MYB28 in young sprouts, reported by Gigolashvili et al.[32] Broccoli-treated sprouts showed a weak increase of 7% and 28% in aliphatic (glucoiberin and glucoraphanin) and indole GLSs (4-hydroxiglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin), respectively. Rutabaga sprouts registered a significant increase (by 85%) in both aliphatic (progoitrin and gluconapin) and indole GLSs (glucobrassicin and 4-methoxyglucobrassicin). Some authors reported that application of methionine to growing broccoli plants increased not only their aliphatic GLSs content but also the indolic GLSs.[41] Few reports on the effect of methionine elicitor have been found, and based on our results we may conclude that low concentrations of methionine, such as 5 and 10 mM applied by Pérez-Balibrea et al.,[18] allowed a certain increase of total GLSs (23% and 21% respectively) than higher concentrations, such as 200 mM applied by Scheuner et al.,[41] where a similar increase by 28% was found in broccoli at the time of head formation, while no significant impact on total GLSs was found in broccoli heads or radish hypocotyls.

All elicitors promoted the accumulation of GLSs in *Brassicaceae* sprouts. Detected differences in the quantified total and individual GLSs between controls and treated sprouts were not only due to cultivar differences but also due to the specific elicitor nature used. Indole GLSs in all species were found to either increase or remain stable after elicitor treatments. The total GLSs performed in similar way. Major desirable aliphatic GLSs, such as glucoraphanin, glucoraphenin, and dehydroerucin, were increased by elicitors, except with DL-methionine. Only undesirable aliphatic progoitrin and the glucoiberin decreased after the treatments, and minor GLSs, such as glucoerucin or gluconapin, were not affected. Elicitation practices, particularly using MeJA, could be established as an effective treatment to enrich in health-promoting GLSs cruciferous sprouts, for natural functional foods, a source of bioactive ingredients. The increase in the production of desirable healthy GLSs (glucoraphanin, glucoraphenin, dehydroerucin, and indole-GLSs) is important in order to enhance the intake of beneficial phytochemicals on a daily basis. Understanding the changes in the metabolism of sprouts is crucial to design strategies that would enhance the biosynthesis of secondary metabolites as novel cost-effective tools for nutrition and health applications that guarantee further research.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Intact glucosinolates identified by HPLC-DAD-ESI-MSn in the Brassicaceae sprouts under study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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