Biotic Elicitors Effectively Increase The Glucosinolates Content In *Brassicaceae* Sprouts

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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 unvail the effect of the phytohormones methyl jasmonate (25µM), jasmonic acid (150 µM), and salicylic acid (100 µM), the oligosaccharides glucose (277mM) and sucrose (146mM), and the amino acid DL-methionine (5mM), 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 and, particularly, health related compounds such as glucoraphanin, glucoraphenin, dehydroerucin and indoles, in all the *Brassicaceae* species studied.

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

INTRODUCTION

Ready-to-eat sprouts has recently caught the interest of scientists and consumers as providing fresh, safe, easy to consume, and environmental-friendly foods. Several studies have demonstrated that *Brassicaceae* (cruciferous) sprouts are a good source of vitamin C, vitamin A, folic acid, dietary fibre 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 their content of health-promoting phytochemicals with positive effect against various pathologies and chronic diseases.[4] In particular, the interest has been focused on GLSs, 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] GLSs are nitrogen- and sulphur-containing secondary metabolites mainly found in *Brassicaceae, a* family with a large number of crop species widespread and consumed worldwide. *Brassica oleraceae* is the mainly harvested species of this family, such as broccoli and cauliflower, and a variety of horticultural crops, such as *B. napus* (rutabaga), *B. 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 (GRA) and its hydrolysis product sulforaphane (SFN) (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, shown 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, such as phytohormones, oligosaccharides and amino acids, 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 signaling molecules 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, the addition of exogenous JA and its methyl ester, methyl jasmonate (MeJA), or SA can also simulate pathogen-induced plant defense responses and lead to the 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 scavenging functions, germination, development, photosynthesis, carbon and nitrogen metabolism, flowering, stress responses and senescence.[13]

Finally, previous experiments demonstrated that also the 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]

Pre-harvest variables, growth and environmental conditions (e.g. fertilizers, temperature, sunlight, stress), have been reported as factors influencing the GLSs content in plants and sprouts.[16,17] However, there is a lack of knowledge on the effects of elicitors on cruciferous species under controlled growth conditions.

The aim of this study was to investigate the effect of the most active elicitors found in literature, the JA,[18] methyl jasmonate,[19,20] salicylic acid,[20] glucose,[21] sucrose,[22,23] and DL-methionine,[20] using 5 days of treatment over 8-day sprouting period of *B. oleraceae* (broccoli), *B. napus* (rutabaga), *B. rapa* (turnip) and *R. sativus* (China rose and red radishes), rich in aliphatic and indole GLSs because their young physiological state.

MATERIAL AND METHODS

Standards and reagents

Jasmonic acid, sucrose and glucose were obtained from SIGMA-ALDRICH Co. (3050, Spruce Street, St. Louis, MO. 63103, USA); methyl jasmonate was purchased from SAFC (3050 Spruce Street, St. Louis, MO. 63103, USA) and 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 (64271 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 (3001, Leuven, Belgium). Sinigrin monohydrate was obtained from Phytoplan (Germany).

118

119 **Plant material and germination conditions**

120 Seeds provided by Intersemillas S.A (Valencia, Spain) were of commercial quality
121 for ready-for-sprouting lines. Five varieties from *Brassicaceae* family were used: broccoli
122 (*Brassica oleracea* L. var *italica*), rutabaga (*B. napus* L. var *napobrassica*), turnip (*B.*
123 *rapa* L. subsp. *rapa*), and China rose radish (*Raphanus sativus* L. cv. China rose) and red
124 radish (*Raphanus sativus* L. cv. Rambo). Seeds were rinsed in distilled water and
125 immersed in 5 gL^{-1} sodium hypochlorite under aeration during 24 h. After pouring off
126 the soaking water, the seeds were weighed (day 0) and spreaded evenly on trays (5 g per
127 tray) lined with cellulose growth pad (CN Seeds, UK) and irrigated everyday with Milli-
128 Q water. Aliquots of 5 g of seeds were frozen in liquid nitrogen and stored at −80ºC
129 pending phytochemical analysis.

130 The three replicates (trays) per sample were germinated during 2 days in a
131 controlled dark chamber at 28 ºC, for increasing the stem elongation of sprouts. Then,
132 trays were transferred to a controlled environment chamber with a 16 h light/8 h dark
133 cycle and air temperatures of 25 and 20 ºC, respectively. The relative humidity (RH) was
134 60% (day) and 80% (night). Photosynthetically active radiation (PAR) of 400 µmol m^{-2}
135 s^{-1} was provided by a combination of fluorescent tubes (Philips TLD 36 W/83, Hamburg,
136 Germany; Sylvania F36W/GRO, Danvers, Massachusetts, USA) and metal halide lamps
137 (Osram HQI.T 400 W, Munich, Germany). Three replicates per treatment of
138 *Brassicaceae* sprouts samples were rapidly and gently collected at day 8 after
139 germination, in the middle of the light period, for analysis. All samples were weighed
140 (fresh mass), collected separately, flash frozen in liquid nitrogen, and stored at −80 ºC
141 prior to analyses.

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Treatments with elicitors

The phytohormones jasmonic acid (JA) (150µM), methyl jasmonate (MeJA) (25 µM), salicylic acid (SA) (100 µM), the oligosaccharides glucose (277mM) and sucrose (146mM), and the amino acid DL-methionine (5mM) were selected as elicitors according to 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 ºC for 30 min in a heating bath, with shaking every 5 min using a vortex stirrer, and centrifuged (17 500 × *g*, 15 min, 4 ºC). The supernatants were collected and methanol was completely removed using a rotary evaporator. The dry material obtained was re-dissolved 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

Firstly, 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 x 1.0 mm, 3 µm particle size; Phenomenex, Macclesfield, UK). 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, 35% at 30, 50% at 35, which was maintained up to 45 min. The injection volume was 3 µL. Chromatograms were recorded at 227 nm. The 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). The 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 up to *m*/*z* 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 (20µL) were analysed and quantified in a Water HPLC-DAD system (Waters Cromatografia S.A., Barcelona, Spain) as described by Pérez-Balibrea et al.[24] The intact GLSs were identified following their UV spectra, and the order of elution previously described for similar acquisition conditions. Glucosinolates were quantified using sinigrin as standard, because of their similar structure to the others.[25]

Statistical methods

All assays were conducted by triplicate. The data were processed using the SPSS 15.0 software package (LEAD Technologies, Inc., Chicago, 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

Weight of seeds and sprouts were collected on day 0 (embebed seeds) and day 8. The ratio of fresh weight between sprouts and seeds as 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.[26] 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*.,[27] using a 146mM sucrose treatment. Stewart, *et al*.,[28] explained that sucrose (88mM) alters the growth rate and causes a dramatic increase in hypocotyl length. 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).[29] Stressful conditions such as starvation or hypoxia result in low energy status in the cell, Smeekens, *et al*.,[30] showed that sugars represses the bZIP growth regulatory system activity in a concentration-dependent, therefore, our employed dose (5g/100mL) resulted appropriate for biomass increase in sucrose treatment sprouts, but not in the case of glucose, according also with Mirnezhad. [31]

DL-Methionine also showed a positive effect increasing fresh weight of sprouts in almost all the varieties, 30% in broccoli, 4% in turnip, 57% in China rose radish and

75% in red radish, except for rutabaga, agreeing with previous reports.[32,33] Gigolashvili, *et al*.,[34] reported a relationship between the over-expression 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, even reduced the size as to 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*.,[35] 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,[35] supporting our result. In red radish sprouts, non-significant 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 necessary to find the required effective dose and time empirically.[36]

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 the identification of 17 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 sulphate group) by the disassociation of GLSs in the ion trap mass spectrometer, constituting a very useful preliminary screening method for determining the presence of GLSs in sprouts.[34] Sixteen GLSs, belonging to

245 the aliphatic, indolic and aromatic classes based on their different side chain structure
246 were detected. Results showed significant differences of the characteristic GLSs profile
247 among cruciferous seeds and sprouts (**Tables 2-5**). The aliphatic GLSs were the major
248 group in *B. oleraceae*, *B. napus* and *R. sativus* sprouts, corresponding to 60% in *Brassica*,
249 and 90% in *Raphanus* varieties. In contrast, *B. rapa* sprouts showed higher amount of
250 indolic GLSs, corresponding to 65% of the total (**Table 4**). Seeds exhibited the largest
251 amount of GLSs being the nutrient reservoir organ, containing ranging concentrations
252 from 563.79 to 1731.32 mg·100g^{-1} F.W., in turnip and broccoli, respectively (**Table 2**),
253 of interest for the composition of the sprouts during germination. According to Pérez-
254 Balibrea, *et al*.,[24,25] the major source of glucoraphanin are broccoli seeds and sprouts
255 (987.02 and 182.46 mg·100g^{-1} F.W., respectively) (**Tables 2-3**), which has been
256 intensively studied because its derived product sulforaphane, a potential chemopreventive
257 beneficial compound against cancer, cardiovascular and neurological diseases.[4] Turnip
258 and rutabaga seeds and sprouts showed the antinutrient progoitrin as the major GLS, and
259 glucoraphanin and gluconasturtiin were absent in the sprouts, probably degraded or
260 diluted during germination.[24] In radish cultivars, specific GLSs in seeds were found as
261 well (traces of the aromatic glucoberteroin). The major characteristic GLS in this species
262 is glucoraphenin, containing 1051.88 and 32.78 mg·100g^{-1} F.W. in China rose radish and
263 887.20 and 166.93 mg·100g^{-1} F.W. in red radish in seeds and sprouts, respectively (**Table**
264 **5**). The bioactive sulforaphene, like sulphoraphane, is potential anti-cancer.[8] In *Brassica*
265 species, in addition to the parent indole GLS glucobrassicin, the 4-hydroxyglucobrassicin,
266 4-methoxyglucobrassicin, and neoglucobrassicin were also detected in the samples
267 (**Tables 2-5**). Only the indole 4-hydroxiglucobrassicin GLS was present in all species,
268 being also one of the major compounds in seeds (from 152.49 to 358.34 mg·100g^{-1} F.W.,

269 in China rose radish and broccoli, respectively). On the contrary, in *Raphanus* sprouts
270 only the 4-hydroxiglucobrassicin and the 4-methoxyglucobrassicin were detected.[6]
271
272 **Phytohormones as elicitors**
273 The jasmonates are signal compounds in the elicitation process leading to *de novo*
274 transcription, translation and, ultimately, the biosynthesis of secondary metabolites in
275 plant cell cultures. Methyl jasmonate (MeJA) is believed to be, at least, partially
276 hydrolyzed by endogenous esterases to free jasmonic acid (JA) within the plant tissue.[12]
277 MeJA elicitor (25 μM) was found highly effective for almost all the 8-day-old
278 *Brassicaceae* sprouts, increasing by 84, 50, 123, 25 and 23% the total GLSs amount in
279 broccoli, turnip, rutabaga, China rose radish and red radish, respectively, increasing more
280 the indoles than the aliphatic GLSs (**Tables 3-5**). After MeJA treatments, the broccoli
281 sprouts showed significantly much more glucoraphanin, glucobrassicin, 4-
282 methoxyglucobrassicin and neoglucobrassicin, by 60%, 241%, 48%, and 247%,
283 respectively, associated with potential health benefits due to the biological activity of
284 their products[4]. The enhancement of the aliphatic GLS glucoraphenin in China rose and
285 red radish sprouts after MeJA treatment was by 278 and 35 %, respectively. Indole GLSs
286 in turnip, rutabaga, red radish and China rose radish sprouts were also higher than the in
287 controls, and increased by 109, 223, 54, and 200%, respectively. The JA(150μM) also
288 produced an increase of total GLSs, especially in broccoli (by 50%), rutabaga (by 95%)
289 and turnip (by 24%), being higher the effect on the indoles than on the aliphatic GLSs
290 (**Tables 3 and 4**). In contrast, scarce differences were found in total GLSs in the treated
291 radish sprouts compared to control samples (**Table 5**). Salicylic acid (SA) caused an
292 increase of 20% in total GLSs in broccoli and radish sprouts, being aliphatic GLSs the
293 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**).

The biosynthesis of glucosinolates can be drastically induced by wounding, hormone application, and pathogen or herbivore attack. Berger[33] 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.[33] 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 3-6**). Similar induction of GLSs by exogenous application of phytohormones as elicitors has been previously found by different authors, particularly, increased indole-GLSs.[18-20,32,35] Consistently with Brader, *et al*.,[35] 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, being the MeJA solution an interesting common elicitor to enrich in GLSs all the species studied.

Sugars as elicitors

318 Non-structural carbohydrates, both sucrose and glucose, used as elicitors,
319 enhanced the total GLSs amount in all the sprouts under study, in accordance with some
320 studies on broccoli, cabbage and radish sprouts.[21,22] Sucrose (146mM) showed higher
321 effects in *Brassica* species, increasing by 42, 31, and 159%, the total GLSs in broccoli,
322 turnip and rutabaga, respectively (**Tables 3 and 4**). By contrast, total GLSs in radish
323 sprouts were increased higher after glucose treatment (277mM), by 22 and 26 % in China
324 rose and red radish, respectively (**Table 5**). It must be emphasized the elicitation effect
325 observed in broccoli sprouts, where glucoraphanin was increased by 40% and 60% under
326 the sucrose and glucose treatments, respectively (**Table 3**). Glucoraphanin was enhanced
327 as well, by 50 and 30%, under both sucrose and glucose spray, in China rose and red
328 radish, respectively (**Table 6**). The other major aliphatic GLS from radish, dehydroerucin,
329 was increased by the glucose treatment, by 22 and a 33% in China rose and red radish,
330 respectively. In contrast to what was found by Wei, *et al*.,[21] who showed a decrease in
331 this compound. These results were consistent with those previously reported by Guo, *et*
332 *al*.,[26] indicating that the *Bo-Elong* gene involved in the aliphatic GLSs pathway was up-
333 regulated by sucrose. Gigolashvili, *et al*.,[31] described glucose as an important signaling
334 molecule that may induce transcriptional regulatory mechanisms, integrating
335 carbohydrate availability and hormone action, regulating this class of GLSs by the
336 HAG1/MYB28 gene, in response to carbohydrate availability, in *A. thaliana*.
337 As for the indole-GLSs, no effects were found on radish sprouts, while both sucrose and
338 glucose highly and significantly enhanced 4-hydroxiglucobrassicin, 4-
339 methoxyglucobrassicin, and neoglucobrassicin in *Brassica* species, being the
340 glucobrassicin mainly increased by sucrose (**Tables 3 and 4**). Sivanandhan, *et al*.,[36]
341 showed that the type and concentration of carbon source induces profound effects on
342 growth and quality of the metabolites produced. Sugars serve as carbon and energy source

and also affects the osmotic pressure of the medium, which stimulates mitochondrial activity and, hence, the energy production for metabolites synthesis.[37] 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 the gene expression involved in the biosynthesis of the target metabolites.[36,38]

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 (5mM) has been studied in order to increase the amount of GLSs in sprouts, mainly aliphatic ones.[15,20] 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.[31] Only in broccoli and rutabaga sprouts a significant effect after the application of this amino acid was found, where the total GLSs were increased by 19 and 85%, respectively (**Table 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**). Oppositely to our first hypothesis, aliphatic GLSs were not affected in higher degree than indole GLSs upon DL-methionine treatment, probably as resulting from expression of HAG1/MYB28 in young sprouts, reported by Gigolashvili *et al*.,.[31] 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

368 significant increase (by 85%) in both aliphatic (progoitrin and gluconapin) and indole
369 GLSs (glucobrassicin and 4-methoxyglucobrassicin). Some authors have reported that the
370 application of methionine to growing broccoli plants increase not only their aliphatic
371 GLSs content, but also the indolic GLSs.[39] Few reports on the effect of methionine elicitor
372 have been found, and based on our results we may conclude that low concentrations of
373 methionine, such as 5 and 10 mM applied by Pérez-Balibrea *et al*.,[24] allowed certain
374 increase of total GLSs (23 and 21% respectively), than higher concentrations, such as 200
375 mM applied by Scheuner *et al*.,[39] where a similar increase by 28% was found in broccoli
376 at the time of head formation, while not significant impact on total GLSs was found in
377 broccoli heads or radish hypocotyls.

378 All elicitors promoted the accumulation of GLSs in *Brassicaceae* sprouts.
379 Detected differences in the quantified total and individual GLSs between controls and
380 treated sprouts were not only due to cultivar differences, but also to the specific elicitor
381 nature used. Indole GLSs in all species were found either increased or remained stable
382 after elicitor treatments. The total GLSs performed in similar way. Major desirable
383 aliphatic GLSs, such as glucoraphanin, glucoraphenin and dehydroerucin, were increased
384 by elicitors, except with DL-methionine. Only undesirable aliphatic progoitrin and the
385 glucoiberin decreased after the treatments and minor GLSs, such as glucoerucin or
386 gluconapin were not affected. Elicitation practices, particularly using MeJA, could be
387 established as effective treatment to enrich in health-promoting GLSs cruciferous sprouts,
388 for natural functional foods, source of bioactive ingredients. The increase in the
389 production of desirable healthy GLSs (glucoraphanin, glucoraphenin, dehydroerucin and
390 indole-GLSs) is important in order to enhance the intake of beneficial phytochemicals on
391 daily basis. Understanding the changes in the metabolism of sprouts is crucial to design

392 strategies that would enhance the biosynthesis of secondary metabolites as novel cost-
393 effective tools for nutrition and health applications that guarantee further research.
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TABLES

Table 1.

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[†]c	4.34b	2.69c	3.29b	1.45b
Methyl Jasmonate	2.24cd	5.59a	2.71cd	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 [‡***]	0.27[**]	0.47[*]	0.15[***]	0.21[***]

[†]Mean values (n=3) comparing species for each elicitor treatment, followed by different lowercase-letters are significantly different at P<0.05.

[‡]Least Significant Difference (LSD) for separating means in the respective column. The LSD was computed only after analysis of variance indicated a significant (p<0.05) entry effect. Levels of significance for each sampling day between species. Non-significant at P>0.05 (n.s.); significant at P<0.05 (*); significant at P<0.01 (**); significant at p<0.001 (***).

a-h, Different lowercase-letters mean statistically significant differences among elicitor treatments (p<0.05).

Table 2.

Table 2. List of individual glucosinolates (mg $100g^{-1}$ F.W.) detected in the seeds of *Brassicaceae* varieties.

Peak	Compound	GLS Semisystematic Name	Class	Seeds					
				Broccoli	Rutabaga	Turnip	China rose radish	Red radish	
1	Glucoiberin	3-methylsulfinylpropyl-gls	Aliphatic	26.02±3.43†	n.d.	n.d.	n.d.	n.d.	
2	Progoitrin	(R)-2-hydroxy-3-butenyl-gls	Aliphatic	n.d.	1277.72±65.48	176.90±0.36	n.d.	n.d.	
3	Glucoraphenin	4-methylsulfinyl-3-butenyl-gls	Aliphatic	n.d.	n.d.	n.d.	1051.88±16.78	887.20±49.60	
4	Glucoraphanin	4-methylsulfinylbutyl-gls	Aliphatic	987.02±51.39	40.57±2.66	28.73±1.42	n.d.	n.d.	
5	Glucoalyssin	5-methylsulfinylpentyl-gls	Aliphatic	10.05±12.14	n.d.	2.30±1.02	n.d.	n.d.	
6	Gluconapoleiferin	(R)-2-hydroxy-4-pentenyl-gls	Aliphatic	n.d.	11.87±2.32	Tr	n.d.	n.d.	
7	Gluconapin	3-butenyl-gls	Aliphatic	n.d.	95.77±4.62	44.89±10.87	n.d.	n.d.	
8	4-Hydroxiglucobrassicin	4-hydroxy-3-indolylmethyl-gls	Indolic	358.34±48.20	238.65±23.48	289.50±24.26	152.49±19.38	222.99±8.60	
9	Glucobrassicanapin	4-pentenyl-gls	Aliphatic	n.d.	Tr	Tr	n.d.	n.d.	
10	Glucoerucin	4-methylthiobutyl-gls	Aliphatic	324.85±43.72	n.d.	n.d.	3.56±0.36	n.d.	
11	Dehydroerucin	4-methylthio-3-butenyl-gls	Aliphatic	n.d.	n.d.	n.d.	85.10±5.18	31.31±2.23	
12	Glucobrassicin	3-indolylmethyl-gls	Aliphatic	14.93±4.61	1.79±0.05	14.32±7.57	n.d.	n.d.	
13	Glucoberteroin	5-methylthiopentyl-gls	Aromatic	n.d.	n.d.	n.d.	Tr	Tr	
14	Gluconasturtin	2-phenylethyl-gls	Aromatic	Tr	Tr	Tr	n.d.	n.d.	
15	4-Methoxyglucobrassicin	4-methoxy-3-indolylmethyl-gls	Indolic	6.23±4.16	Tr	Tr	n.d.	n.d.	
16	n-hexyl	n-hexyl-gls	Aliphatic	Tr	n.d.	n.d.	n.d.	n.d.	
17	Neoglucobrassicin	N-methoxy-3-indolylmethyl-gls	Indolic	7.36±1.47	Tr	7.14±3.56	n.d.	n.d.	
			Total	1731.32a	1666.36a	563.79d	1293.03b	1141.50c	46.05‡*** ($LSD_{0.05}$)

†Mean values (n=3 ± SD) Tr, traces, not quantified. n.d., not detected.
a-d, Different lowercase-letters means statistically significant differences in the total glucosinolates content between species.
‡, Least Dignificant 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, * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. $p>0.05$

Table 3.

Table 3. List of individual and total glucosinolates (mg $100g^{-1}$ F.W.) in broccoli (*Brassica oleraceae*) sprouts under elicitor treatments.

Peak	Compound	Broccoli							
		Control	MeJA	JA	SA	Glucose	Sucrose	DL-Methionine	$LSD_{0.05}$ ‡
1	Glucoiberin	10.82†a	7.68ab	9.33a	3.41b	6.35ab	11.94a	11.50a	1.86*
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	182.46c	294.30a	265.03ab	287.55ab	297.37a	252.61b	200.32c	13.18***
5	Glucoalyssin	0.46b	Tr	0.70a	Tr	Tr	Tr	Tr	0.16*
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	39.93bc	40.12bc	32.37c	42.35b	54.68a	44.68b	55.58a	3.09***
9	Glucobrassicanapin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
10	Glucoerucin	39.10	31.70	36.52	39.09	36.78	40.99	37.76	4.35nd
11	Dehydroerucin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
12	Glucobrassicin	55.20cd	188.52a	86.40b	43.04d	53.82cd	92.33b	74.16bc	8.66***
13	Gluconasturtin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
14	4-Methoxyglucobrassicin	28.70c	42.69ab	49.22a	39.84b	43.41ab	45.37ab	40.17b	2.47***
15	n-hexyl	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
16	Neoglucobrassicin	30.89c	107.04a	95.64a	25.47c	43.06bc	61.15b	43.09bc	6.69***
	Total	387.56e	712.05a	575.21b	480.75cd	535.47bc	549.07b	462.58d	21.06* **

†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).
‡, Least Dignificant 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, * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. $p>0.05$

Table 4.

Table 4. List of individual and total glucosinolates (mg $100g^{-1}$ F.W.) in turnip (*Brassica rapa*) and rutabaga (*Brassica napus*) sprouts under elicitor treatments.

Peak	Compound	Turnip								Rutabaga							
		Control	MeJA	JA	SA	Glucose	Sucrose	DL-Metionine	$LSD_{0.05}$‡	Control	MeJA	JA	SA	Glucose	Sucrose	DL-Metionine	$LSD_{0.05}$‡
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	41.90†bc	27.46c	5.69d	40.72bc	50.49ab	63.84a	43.49b	4.92^{***}	184.46e	292.18c	239.82d	198.14e	252.68d	443.74a	342.79b	8.64^{***}
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	0.23^{nd}	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}	15.77b	17.78ab	5.36cd	1.22d	Tr	11.83bc	24.02a	2.51^{***}
8	4-Hydroxiglucobrassicin	23.63bc	25.47ab	31.87a	17.62c	31.97a	30.46ab	23.79bc	2.28^{**}	17.06c	21.45b	22.66b	17.04c	23.75b	28.71a	14.80c	1.24^{***}
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	23.37c	91.01a	41.06b	18.61c	26.12c	37.68b	24.98c	3.26^{***}	29.55e	185.56a	157.63b	24.16e	46.43e	132.06c	94.58d	8.22^{***}
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	22.37b	22.31b	42.83a	23.00b	24.98b	23.91b	18.54b	2.08^{***}	37.77de	61.88bc	33.56de	25.23e	48.34cd	93.60a	73.50b	5.03^{***}
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	24.23cd	48.75a	56.70a	27.99bcd	41.36ab	31.35bc	12.66d	5.22^{***}	33.93d	131.21b	162.24a	47.86d	73.76c	115.83b	38.42d	5.53^{***}
	Total	149.99cd	216.11a	179.77b	132.80d	175.66bc	189.14b	126.14d	9.13^{***}	318.54e	710.06b	621.27c	313.65e	444.96d	825.77a	588.11c	12.30^{***}

†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).

‡, Least Dignificant 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, * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. $p>0.05$

Table 5.

Table 5. List of individual and total glucosinolates (mg $100g^{-1}$ F.W.) in China rose radish and red radish (*Raphanus sativus*) sprouts under elicitor treatments.

Peak	Compound	China rose radish								Red radish							
		Control	MeJA	JA	SA	Glucose	Sucrose	DL-Methionine	$LSD_{0.05}$ ‡	Control	MeJA	JA	SA	Glucose	Sucrose	DL-Methionine	$LSD_{0.05}$‡
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	32.78^Ad	124.12a	88.73b	50.54c	51.40c	50.57c	31.57d	3.18***	166.93cd	226.98a	184.83bc	191.01ab	209.98ab	225.76a	145.69d	12.83**
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	15.19b	22.64a	22.51a	16.05b	16.06b	12.72b	12.81b	1.31***	27.30b	44.19a	31.16b	21.10c	22.19c	41.72a	26.99b	1.42***
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	10.89a	0.59b	1.61b	0.98b	1.73b	1.95b	0.67***	Tr	Tr	Tr	Tr	Tr	Tr	Tr	
11	Dehydroerucin	411.28bc	433.79b	370.42c	488.72b	502.33a	438.68b	402.78c	9.33***	171.88c	180.65bc	179.71bc	222.32ab	228.76a	183.14bc	149.96c	14.39*
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	27.20ab	19.80c	15.50d	30.76a	25.90b	17.75cd	28.99ab	1.31***	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	488.40bc	611.24a	497.75bc	587.68a	595.69a	519.72b	478.10c	12.12***	366.01d	451.83ab	395.70c	431.12b	460.94a	450.63ab	322.65e	8.04***

†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).

‡, Least Dignificant 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, * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. $p>0.05$