Zinc Availability in Hydroponic Culture Influences Glucosinolate Concentrations in *Brassica rapa*

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Abstract. Rapid cycling *Brassica rapa* L. were grown for 7 days in the presence of 11 levels of zinc (Zn) in hydroponic solution culture and evaluated for changes in Zn and glucosinolate (GS) content. Zinc levels were 0.05, 1, 5, 10, 25, 50, 75, 100, 125, 150, and 200 mg·L⁻¹ Zn. Plants grown in solutions with ≥50 mg·L⁻¹ Zn displayed severe Zn toxicity symptoms, grew little, or died and were not subsequently evaluated for GS content. Shoot Zn concentrations increased linearly with increasing Zn treatment levels. Gluconapin, which accounted for nearly 90% of the aliphatic GSs present, was the only aliphatic GS influenced by Zn, and decreased linearly with increasing Zn levels. Accumulation of glucobrassicin and 4-methoxyglucobrassicin, both indole GSs, responded with a linear increase and quadratically, respectively, to Zn fertility. An aromatic GS, gluconasturtiin, was also influenced by Zn levels in solution, and had a quadratic response to increasing Zn. This suggested that Zn fertility can influence changes in GS that may affect flavor (bitterness, etc.) or medicinal attributes associated with the GS and their breakdown products, as well as elevate the nutritional status of Zn in the leaves of *Brassica*.

Glucosinolates (GSs) are a broad class of sulfur-based secondary metabolites containing β-thioglucose-moiety found in 16 families of dicotyledonous plants (Mithen, 2001). At least 120 different GSs have been identified, many of these isolated from Brassicaceae and Capparaceae, which have species with economic importance (Fahey et al., 2001). When plant tissue is disrupted, myrosinases hydrolyze intact GSs, causing the formation of several biologically active compounds, including nitriles, thiocyanates, and isothiocyanates (Wittstock and Halkier, 2002).

Because many of the GS breakdown products have high biological activity, plant GS content has been of interest to researchers for some time. Early work conducted on *Brassica* was focused on the harmful effects of some GSs, which produced goitrogenic effects in livestock (Chesney et al., 1928; Fenwick et al., 1983; Stoewsand, 1995). Recently, however, GSs and their hydrolyzed products have been investigated for anticarcinogenic properties (Das et al., 2000; Fenwick et al., 1983; Kristal, 2002; McDanell et al., 1988; Mithen, 2001; Verhoeven et al., 1997), as well as the ability to act as soil fumigants and the suppression of pathogenic *Pythium* sp. and several types of nematode (Charron and Sams, 1999; Chitwood, 2002; Lazzeri and Manici, 2001;

Mizutani, 1999; Morra and Kirkegaard, 2002). Glucosinolates also influence the flavor of these plants. Researchers are now investigating ways to alter levels of GSs in some crops. Although genetics ultimately limits GS content in plants, the growing environment can dramatically influence GS levels as well. Water stress and high growing temperatures lead to increased GS content of several *Brassica* species (Mailer and Cornish, 1987; Mailer and Pratley, 1990; Rosa and Rodrigues, 1998). Sulfur and nitrogen fertility regimes have also influenced GS levels in plants, with S fertility generally having greater impact than N (Booth et al., 1991; Kim et al., 2002; Mailer, 1989; Milford and Evans, 1991; Zhao et al., 1994).

Zinc, an essential plant element and an important mineral for maintaining human health (Branca and Ferrari, 2002), was recently reported to influence GSs levels of *Thlaspi caerulescens*, which can hyperaccumulate Zn (Tolra et al., 2001). With increasing Zn levels supplied through hydroponic nutrient solutions, plant GS content generally decreased, especially sinalbin (p-hydroxybenzylglucosinolate). Earlier, Jiracek et al. (1974) reported that increasing Zn fertility levels increased glucobrassicin and neoglucobrassicin in etiolated seedlings of *B. napus*. The scope of that investigation was limited, however, as only the indole GSs were measured. Because Zn fertility influenced total S accumulation in *B. rapa* (Coolong and Randle, 2003), we felt it important to determine the impact of increasing Zn on GS content as well. Our purpose, therefore, was to evaluate the effects of a wide range of Zn fertility levels on detectable GSs in hydroponically grown *B. rapa*.

Plant culture. On 10 Oct. 2001, seeds of a rapid-cycling *B. rapa* (Carolina Biological Supply, Burlington, N.C.) were sown into 16-cm³ rockwool cubes (Grodan, Hedehusene, Denmark). Seeds were germinated at 20 °C under a 24-h photoperiod in growth chambers that were tested for uniformity (Convicon, Pembina, N.Dak.). Average light intensity was 352 μmol·m⁻²·s⁻¹, measured at the base of the plant using a Basic Quantum Meter (Spectrum Tech., Plainfield, Ill.). Three-day-old seedlings were transferred to 5.4-L plastic containers (Rubbermaid Inc., Wooster, Ohio). Six plants were placed in holes in each container lid spaced ≈6 cm apart. The tubs were filled with 4 L of a modified Hoagland's #2 solution (Hoagland and Arnon, 1950). The solutions consisted of: 0.47 g·L⁻¹ Ca(NO₃)₂·4H₂O, 0.30 g·L⁻¹ KNO₃, 0.06 g·L⁻¹ NH₄H₂PO₄, 0.25 g·L⁻¹ MgSO₄·7H₂O, 0.1 g·L⁻¹ Iron Chelate (Sequestrene 330), 2.86 mg·L⁻¹ H₃BO₃, 1.81 mg·L⁻¹ MnCl₂·4H₂O, 0.22 mg·L⁻¹ ZnSO₄·7H₂O, 0.08 mg·L⁻¹ CuSO₄·5H₂O, and 0.02 mg·L⁻¹ Na₂MoO₄·2H₂O. Solutions were aerated via a 2.5-cm aquarium airstone. Plants were grown at 20 °C and a constant 24-h photoperiod for 6 d. Eleven Zn treatments were then imposed, with each treatment replicated three times with individual growth chambers acting as replications, in a completely randomized design. Zinc was supplied using ZnSO₄·7H₂O. The treatment levels were: 0.05, 1, 5, 10, 25, 50, 75, 100, 125, 150, and 200 mg·L⁻¹ Zn. The 0.05 mg·L⁻¹ Zn represented normal Zn levels in the standard nutrient solution. Sulfur levels were balanced with the addition of MgSO₄·7H₂O so that all treatments had 129 mg·L⁻¹ S. Magnesium levels were equalized by the addition of MgCl₂·6H₂O to the solutions. Each solution then contained 94 mg·L⁻¹ of Mg²⁺. Chloride levels, therefore, varied between solutions. The pH of the solutions ranged from 5.5 to 6.0 at the initiation of the experiment and did not change appreciably. Electrical conductivity (EC) (model 09-326-2; Fisher Scientific, Pittsburgh) measurements of the solutions were made at the beginning and end of the experiment. The average EC of solutions at the initiation of the experiment was 1148 μS·cm⁻². When Zn was added, the EC increased to 1487 μS·cm⁻² for the 0.05 mg·L⁻¹ Zn treatment and to 2200 μS·cm⁻² for the 200 mg·L⁻¹ Zn treatment. At harvest, EC ranged from 1045 to 2040 μS·cm⁻². Solution volumes were maintained daily with deionized water. After 7 d exposure to the Zn treatments, plants were harvested and weighed. The shoots were dried at 65 °C for 72 h in a forced-air oven (Linberg Blue, Asheville, N.C.). Dried tissue was weighed and then ground through a 0.5-mm screen attached to a Cyclotec Mill (model 1093; Tector, Hoganas, Sweden) and stored at 25 °C in 60-mL plastic vials.

Zinc determination

Zinc was extracted from 0.1 g tissue using a nitric acid digestion procedure. A national institute of standards and technol-

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ogy (NIST) standard 1573 (tomato leaves, NIST, Washington, D.C.) was also digested to establish Zn recovery and quantification. Following digestion at 125 °C in 5 mL of nitric acid, 5 mL of 30% H₂O₂ were added in 1-mL aliquots. The solutions were allowed to cool and brought to a final volume of 50 mL using HPLC grade water. Zn concentrations were determined using a Perkin-Elmer 5000 flame atomic absorption spectrometer and a Zn–Mn–Cu hollow cathode lamp (Intersitron Model 303-6105, Perkin-Elmer, Boston). The wavelength was set at 213.9 nm and slit width was 0.7 nm. Detection limits were 0.16 mg·kg⁻¹. Triplicate analysis of the NIST standard showed recovery between 93% and 106%.

Glucosinolate analysis

Extraction of glucosinolates. Glucosinolate analysis was performed using high performance liquid chromatography (HPLC) according to Raney and McGregor (1990), with several modifications. Two milliliters of methanol were added to 200 mg of dried tissue, then 1 mL of 1 mmol glucotropaeolin internal standard solution (Bioraf Denmark Foundation, Aakirkeby, Denmark) and 0.1 mL of barium lead-acetate were added in sequential order. The mixture was shaken for 1 h on a Vortex-Genie 2 shaker (model #G-560; Scientific Industries, Bohemia, N.Y.) at 60 rpm. This mixture was centrifuged at 2000 g_n for 10 min in a Centra-MP4R centrifuge [International Equipment Co. (IEC), Needham Heights, Mass.] using an IEC 224 swing bucket rotor (IEC). The supernatant was transferred to a diethylaminoethyl (DEAE) Sephadex A25 (Sigma-Aldrich, St. Louis) column, where it was subjected to ion exchange chromatography by washing with 1.8 mL of 2 methanol:1 water, 1.8 mL water, 0.9 mL 0.02 M pyridine-acetate, and 0.05 mL purified sulfatase in that order. After 24 h, samples were eluted from the column with 0.9 mL double deionized water for

HPLC analysis. DEAE columns were regenerated for reuse by rinsing with 1 mL 1 M sodium acetate, 1 mL 0.5 M sodium hydroxide, 1 mL 0.5 hydrochloric acid, and 3.6 mL nanopure water in sequential order.

Purification of sulfatase. Sulfatase was purchased, unpurified, from Sigma-Aldrich. Seventy milligrams of unpurified sulfatase was massed into a centrifuge tube. Three milliliters of nanopure water was added in order to dissolve the sulfatase, followed by 3 mL of absolute ethanol. The mixture was centrifuged for 20 min at 2000 g_n on the Centra-MP4R Centrifuge using an IEC 224 swing bucket rotor (IEC). The supernatant was decanted into a second culture tube, the precipitate discarded, and 9 mL of absolute ethanol added to the supernatant. The solution was centrifuged for 15 min at 2000 g on the Centra-MP4R Centrifuge using an IEC 224 swing bucket rotor (IEC). The supernatant liquid was discarded and the remaining precipitate dissolved in 2.5 mL of water. The purified sulfatase was then frozen until use.

HPLC analysis. The samples were separated on a Hewlett-Packard separations module coupled to a HP1050 Series Variable Wavelength Detector (Hewlett Packard, Palo Alto, Calif.), set at 227 nm. Fifty microliters of sample were injected into a 250 × 4.60 mm 5 μ C18 column (Phenomenex ODS Hypersil; Phenomenex, Torrance, Calif.). Mobile phase solvents were: 1) nanopure water and 2) acetonitrile (HPLC grade; Fisher Scientific, Atlanta). A gradient run with a constant flow-rate of 1 mL·min⁻¹ was programmed as follows: 100% ± 1 min, 75% ± 15 min, 75% ± 20 min, 100% ± 25 min. Identification of desulfonated GS peaks was determined by running desulfonated GS standards for 2(R)-2-hydroxy-3-butenyl GS (progoitrin), 4-methylsulfinyl-3-butenyl GS (glucoraphanin), 2-propenyl GS (sinigrin), 4-hydroxybenzyl GS (glucosinalbin), 3-butenyl GS (gluconapin), indolyl-3-methyl GS (glucobrassicin), 4-methoxyindolyl-3-methyl GS (4-methoxyglucobrassicin), 2-phenylethyl GS (gluconasturtiin), and 1-methoxyindolyl-3-methyl GS (neoglucobrassicin) using the above method and comparing retention times of standards to samples. Integration of peaks was performed on Chemstation Software Module, version 6.03 (Hewlett Packard).

Statistical analysis. All data was subjected to analysis of variance and linear or polynomial regression using SAS statistics software. (Version 8.2, SAS Institute, Cary, N.C.). Orthogonal contrasts among treatments were also performed. Data more than two standard

deviations from the mean of the particular treatment were treated as outlying data and removed from the analysis. As a result, one replication from three different treatments was removed from the analysis.

Results and Discussion

Shoot dry weight, zinc uptake, and plant condition

Shoot dry weight (DW) was influenced ($P < 0.01$) by Zn treatments and ranged from 0.9 to 2.6 g/plant (Table 1). The trend was quadratic [$y = 2.42 - 0.22(\text{Zn}) + 0.0062(\text{Zn})^2$], $r^2 = 0.78$), increasing from 0.05 to 1.0 mg·L⁻¹ Zn and then decreasing as the Zn solution concentrations increased to 25 mg·L⁻¹ Zn. Plants grown with 5 and 10 mg·L⁻¹ Zn exhibited toxicity symptoms (marginal chlorosis, decreased dry matter accumulation, and some root death). Above 25 mg·L⁻¹ Zn, plants grew little and exhibited strong symptoms of Zn toxicity. Nearly all plants grown in solutions ≥50 mg·L⁻¹ Zn were severely stunted, dead, or were dying following 7 d of exposure. As such, plants from ≥50 mg·L⁻¹ Zn treatments were not analyzed for GS content due to insufficient tissue.

Shoot Zn concentrations were affected by solution Zn levels ($P < 0.01$) and ranged from 65 to 4238 mg·kg⁻¹ DW. The response was linear to increasing Zn treatment [$y = 429.78 + 167.01(\text{Zn})$, $R^2 = 0.92$] (Table 1). Despite the observation that the plants grew very little, especially following exposure to the higher Zn concentrations, the plants continued to accumulate Zn in shoots. Subsequent preliminary studies indicate that Zn accumulates in the shoots of *Allium cepa* seedlings rather quickly, perhaps accumulating in large amounts prior to the exhibition of toxicity symptoms and the cessation of growth (Coolong and Randle, unpublished data).

Shoot glucosinolate content

Aliphatic glucosinolates. Of the five aliphatic GSs measured, gluconapin was found in highest concentration and was the only one that responded to increasing Zn levels ($P < 0.02$). Progoitrin, glucoraphanin, sinigrin, and glucosinalbin were not significantly influenced by Zn fertility (Table 2). Gluconapin concentrations decreased linearly to increasing Zn [$y = 9.98 - 0.23(\text{Zn})$, $R^2 = 0.66$]. Correspondingly, small decreases in root gluconapin concentrations were reported when the Zn hyperaccumulator *T. caerulescens* was grown at high Zn

Table 1. Mean (±SE) dry weight (DW) and zinc (Zn) uptake for *Brassica rapa* grown with different levels of Zn in solution culture.

Solution Zn (mg·L ⁻¹)	Shoot DW (g/plant)	Shoot Zn (mg·kg ⁻¹ DW)
0.05	2.1 ± 0.1	65 ± 6
1	2.6 ± 0.2	304 ± 1
5	1.2 ± 0.1	1764 ± 161
10	1.0 ± 0.2	2433 ± 335
25	0.9 ± 0.1	4238 ± 101
Regression ^a	Q	L

^aSignificant at $P < 0.01$; linear (L), quadratic (Q) regression.

Table 2. Mean (±SE) shoot glucosinolate concentrations for *Brassica rapa* grown at different levels of zinc (Zn) in solution culture (nd indicates not detectable).

Solution Zn (mg·L ⁻¹)	Progoitrin	Glucoraphanin	Sinigrin	Gluconapin	Glucosinalbin	Glucobrassicin	4-Methoxy-glucobrassicin	Glucobrassicin	Neoglucobrassicin
	(μmol·g ⁻¹ DW)								
0.05	0.04 ± 0.01	0.48 ± 0.04	0.07 ± 0.01	10.42 ± 0.44	0.15 ± 0.01	1.04 ± 0.12	0.16 ± 0.02	0.76 ± 0.05	0.34 ± 0.09
1	nd	0.47 ± 0.14	0.06 ± 0.03	10.48 ± 1.43	0.17 ± 0.04	0.89 ± 0.12	0.14 ± 0.003	0.64 ± 0.11	0.28 ± 0.06
5	0.07 ± 0.01	0.69 ± 0.10	0.06 ± 0.01	8.74 ± 0.16	0.26 ± 0.04	1.81 ± 0.04	0.32 ± 0.06	0.93 ± 0.03	0.52 ± 0.06
10	0.05 ± 0.01	0.78 ± 0.11	0.09 ± 0.03	6.58 ± 1.58	0.18 ± 0.03	2.17 ± 0.38	0.39 ± 0.08	0.98 ± 0.17	0.44 ± 0.06
25	0.05 ± 0.003	0.91 ± 0.32	0.09 ± 0.02	4.79 ± 0.74	0.16 ± 0.03	3.09 ± 0.63	0.35 ± 0.02	0.51 ± 0.03	0.29 ± 0.03
Regression ^a	NS	NS	NS	L	NS	L	Q	Q	NS

^aNon-significant at $P < 0.05$; linear (L), quadratic (Q).

concentrations (Tolra et al., 2001). Gluconapin was also reported to constitute a large portion of the aliphatic GS content in *B. rapa* (Kim et al., 2002). In our study, gluconapin was responsible for between 81% to 94% of the total aliphatic GSs measured, and ranged between 10.5 and 4.8 $\mu\text{mol}\cdot\text{g}^{-1}$ DW. Our data suggests that higher Zn availability may be a way to suppress gluconapin accumulation in the plant. Gluconapin content was highly correlated with bitterness and using Zn fertility to decrease gluconapin concentrations may be useful for improving tissue palatability (Fenwick et al., 1990; Mithen, 2001). However, decreased gluconapin content was highly correlated to decreased shoot DW ($r = 0.84$) and suggests that gluconapin suppression was linked to Zn toxicity. Further work with Zn on edible *Brassica* sp. may prove useful as a way of increasing Zn content in the diet and reducing potentially unpalatable glucosinolates. Edible *Brassica* are low in oxalates, which bind elements like Zn, and could be an excellent vehicle for delivering micronutrients to our diet.

Indole and aromatic glucosinolates. Of the indole GSs measured, glucobrassicin ($P < 0.006$) and 4-methoxyglucobrassicin ($P < 0.008$) were affected by Zn in solution, whereas neoglucobrassicin was not. Glucobrassicin responded linearly, increasing as Zn levels increased [$y = 1.1 + 0.085(\text{Zn})$, $R^2 = 0.68$] (Table 2). Shoot glucobrassicin concentrations ranged from 0.89 to 3.09 $\mu\text{mol}\cdot\text{g}^{-1}$ DW, and were within the range to those previously reported for *B. rapa*. (Kim et al., 2002). Similarly, Jiracek et al. (1974) reported a slight increase in glucobrassicin content in *B. napus* when etiolated seedlings were exposed to increasing levels of Zn. 4-Methoxyglucobrassicin responded quadratically to increasing Zn [$y = 0.13 + 0.039(\text{Zn}) - 0.0012(\text{Zn}^2)$, $r^2 = 0.69$]. Shoot 4-methoxyglucobrassicin concentrations ranged from 0.14 to 0.39 $\mu\text{mol}\cdot\text{g}^{-1}$ DW (Table 2). Therefore, unlike the responsive aliphatic GS that decreased at higher Zn fertility levels, the response of the indoles generally increased.

Gluconasturtiin, the only aromatic GS measured, was influenced by Zn ($P < 0.03$) with a quadratic response to increasing Zn in the solutions ($y = 0.68 + 0.056(\text{Zn}) - 0.0025(\text{Zn}^2)$, $r^2 = 0.58$). Gluconasturtiin shoot concentrations ranged from 0.51 to 0.93 $\mu\text{mol}\cdot\text{g}^{-1}$ DW (Table 2), which were lower than values obtained by Kim et al., (2002) for *B. rapa*. A breakdown product of gluconasturtiin, phenylethyl isothiocyanate, has been reported to have watercress-like flavor attributes with very low odor threshold when compared to the breakdown products of other GSs (Fenwick et al., 1983).

Conclusions

Zinc fertility changes were associated with changes in gluconapin, glucobrassicin, 4-methoxyglucobrassicin, and gluconasturtiin levels with the plant. This confirms earlier reports that

Zn levels can influence concentrations of the indole GSs, but also indicates that the aliphatic and aromatic GSs in *Brassica* are influenced by Zn fertility. Although the full range of available Zn used in this experiment would be difficult to duplicate in a terrestrial grown crop, orthogonal contrasts among treatments indicate that statistically significant changes in GS content occurred among relatively small changes in Zn levels (1–5 $\text{mg}\cdot\text{L}^{-1}$ Zn). Our data also supports the findings that the changes in GS resulting from Zn fertility differences may affect the flavor (bitterness, etc.) or medicinal attributes associated with the GS and their breakdown products. Furthermore, the combination of enhanced Zn accumulation along with an overall depression in GS accumulation following an elevated Zn fertility program may be worthy of future study within *Brassica* as a way of addressing micronutrient malnutrition through improved food palatability.

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