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Glucosinolate Profile Variation of Growth Stages of Wild Radish (*Raphanus raphanistrum*)

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Wild radish (*Raphanus raphanistrum* L.) produces glucosinolates (GSL), which are important for its use as a biofumigation or allelopathic plant for weed management. Total GSL concentrations and individual GSLs were quantified in different plant parts at different developmental stages. Eight GSLs were found in various plant tissues but glucoerucin, glucoraphenin, and glucotropaeolin comprised >90% of the total GSLs. All three are degraded to isothiocyanates, which are associated with weed suppression. Maximum GSL concentration (1942.2 μmol/plant) occurred at 50% flowering stage prior to the time of maximum biomass production, when GSL concentration was 1246.65 μmol/plant. Roots contributed <15% of the total GSL. The highest concentration of GSLs was in flowers at flowering stage, but due to the low biomass they contributed only 11.83% to the total GSL. On the basis of these results, wild radish should be incorporated into soil at 50% flowering to provide the most GSLs for weed suppression.

KEYWORDS: Biofumigation; allelopathy; *Raphanus*; wild radish; glucosinolates; isothiocyanates

INTRODUCTION

Research has previously investigated the biofumigation potential of various Brassicaceae plants for weed management (*1*−*5*). Field tests incorporating Brassicaceae plants into soil as green manure have shown a reduction of weed emergence in the subsequent crop, but in many cases the green manure incorporation did not provide season-long weed management (*6*−*8*).

Wild radish (*Raphanus raphanistrum* L.), a Brassicaceae facultative winter annual broadleaf that emerges throughout the year in the southeastern United States (*9*), showed possible biofumigation potential when tested in controlled environments using aqueous extracts and soil-incorporated air-dried wild radish (*10*). Tested plants included crop species, cotton (*Gossypium hisutum* L.), corn (*Zea mays* L.), and wheat (*Triticum aestivum* L.); and weed species, pitted morningglory (*Ipomoea lacunosa* L.), sicklepod [*Senna obtusifolia* (L.) H. S. Irwin & Barneby], prickly sida (*Sida spinosa* L.), and yellow nutsedge (*Cyperus esculentus* L.). Cotton, sicklepod, and prickly sida were extremely sensitive to wild radish incorporation in soil, whereas yellow nutsedge rhizome production was decreased and morningglory emergence decreased linearly with increase in the percentage of wild radish amendment. In greenhouse studies of the inhibition of yellow nutsedge in wild radish-amended soil (1%), marginal necrosis of the leaf margins was observed in both bell pepper (*Capsicum annuum* L.) and tomato (*Lycopersicum esculentum* Mill.) after transplantation into the amended soil. Three weeks after transplanting, no necrosis was observed with tomato. Bell peppers continued to show a response to the amendments and did not accumulate as much biomass over the 9 week study when compared to those grown in nonamended soil (*11*). The mass of yellow nutsedge tubers was reduced from 0.32 to 0.05 g/tuber in the wild radish-amended soil, resulting in a competitive edge for bell pepper over yellow nutsedge, even with the decrease in biomass (*11*). Subsequent field studies incorporating flowering wild radish biomass into soil along with half the recommended rates of atrazine and *S*-metolachlor resulted in season-long weed control of Florida pusley (*Richardia scabra* L.) and large crabgrass [*Digitaria sanguinalis* (L.) Scop.] in sweet corn (*Z. mays* L.), indicating the potential for using wild radish as a biofumigant (*12*).

The biofumigation potential of Brassicaceae plants, including wild radish, has been linked to the ability of the plants to produce glucosinolates (GSL). Glucosinolates are sulfur-containing secondary metabolites commonly found in Brassicaceae plants. Glucosinolates are degraded by myrosinase (thioglucoside glucohydrolase) in the presence of water to produce glucose and unstable aglucones, which are further broken down into various compounds including isothiocyanates (ITC), organic thiocyanates, and nitriles, which are active in biofumigation (*13*, *14*) (**Figure 1**). In plants the action of myrosinase on GSLs has been suggested to be regulated by the separation of the enzyme from the presence of GSLs (*15*−*17*). This separation is disrupted upon tissue disruption (*18*) with the products produced

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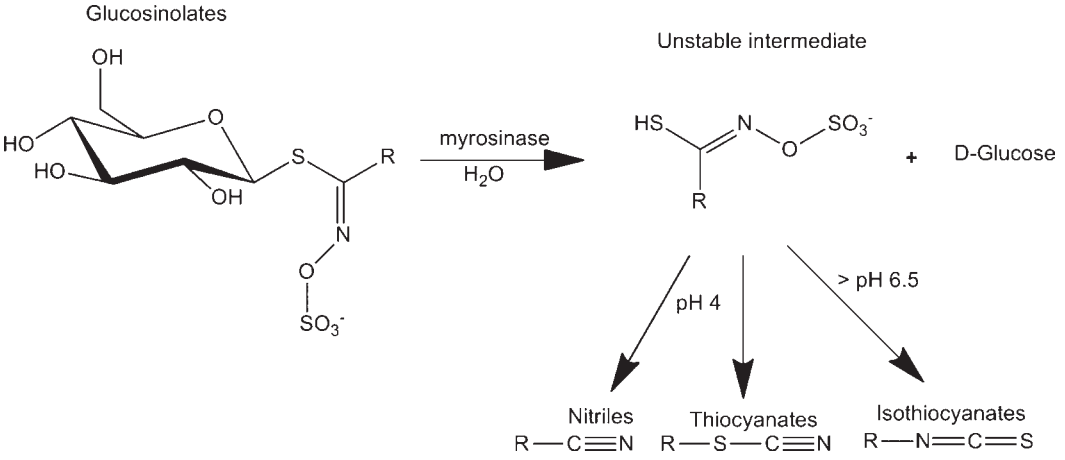

Figure 1. Toxic compounds normally produced during breakdown of glucosinolates by myroxinase.

depending on the original GSL structure and, more importantly, the R group. Variation of the identified R groups has resulted in approximately 120 different GSLs (*18*, *19*). The GSLs produced belong to several classes including aliphatic with a subclass of ω-methylthioalkyls, aromatic, and heterocyclic indoles containing either straight- or branched-chain carbons (*19*).

The ITCs formed following hydrolysis of GSLs are toxic to many organisms (*14*, *20*). They are volatile and highly unstable in soils, with maximum levels observed 30 h after tissue incorporation followed by levels dropping approximately 75% within 72 h (*21*). Levels after 72 h (3 nmol/g), however, remained relatively stable through 20 days. With rapeseed meal-amended soils ITCs were rapidly lost but ionic thiocyanates persisted longer (*22*). The release of isothiocyanates varies according to soil temperature, moisture, and amount of GSL released by plant tissue (*23*, *24*).

It has been suggested that isothiocyanates and other degradation products of GSLs interact with enzymes responsible for glycolysis and respiration, resulting in inhibition of seed germination (*10*, *25*−*28*). For example, benzyl ITC found in papaya (*Carica papaya* L.) inhibited velvetleaf (*Abutilon theophrasti* Medik.) germination (*29*). Metham, a synthetically produced fumigant, produces methyl ITC, which partially inhibited germination of large crabgrass seeds at 1.0 and 2.0 mM, whereas concentrations of ≥4.0 mM resulted in complete seed germination inhibition (*30*). Similarly, *n*-butyl and 2-phenethyl ITCs when incorporated into soil inhibited the germination of smooth pigweed (*Amaranthus hybridus* L.) (*28*).

The production of GSLs has been found to vary considerably among species (*1*, *31*), within species (*32*), with different environmental conditions (*1*, *33*−*36*), and with different growth stages and specific plant material of the same species (*35*−*43*). Because various factors affect GSL production and wild radish can be a problematic weed in the southeastern United States, it was important to determine GSL concentrations at different developmental stages to develop better weed control strategies for using wild radish as a cover crop and, thereby, reduce herbicide use. The overall goal is to maximize GSL content and especially ITC-producing GSL content in wild radish, which can be incorporated into soil. The objectives of this study were to quantify and compare GSL content and composition of wild radish in different organs at different developmental stages ranging from cotyledon to silique formation.

MATERIALS AND METHODS

Wild radish seeds were collected from Clemson, SC, during 2006. Seeds were stored at 4 °C prior to planting. In 2007 approximately 15 seeds were planted in 10 cm diameter pots containing commercial peat moss potting mix from Farfard Inc. (Anderson, SC) and placed in a growth chamber. The growth chamber was maintained with a 13 h day length at 23 °C followed by 8 °C during the dark phase (11 h). When wild radish reached the 2−3-leaf stage, plants were thinned to one plant per pot. The plants were watered three times each week with 0.4% w/v solution of Scotts Miracle Gro fertilizer (Marysville, OH) containing 24% N, 8% P, and 16% K.

Plants were harvested at five different growth stages: cotyledon (stage 1), 6−8-leaf (stage 2), bolting (stage 3), 50% flowering (stage 4), and 50% silique formation (stage 5). The plants were harvested at 50% flowering and 50% silique formation because wild radish is an indeterminate plant and flowering occurs from the base to apex of the flower stalk over several weeks. Plants were separated into roots, shoots (leaves), branches (primary and secondary), and flowers according to the developmental stage. At stages 1 and 2, only roots and shoots were harvested. For stage 1 sampling, which was at the time of thinning, five plants were combined from each replicate to obtain enough material for GSL analysis. At stage 3, roots, shoots, and primary branches were harvested. Roots, shoots, primary and secondary branches, and flowers were harvested at stage 4. Stage 5 samples included roots, shoots, and primary and secondary branches. Siliques could not be harvested at stage 5 because siliques were aborted soon after formation. The abortion of siliques was most likely due to the absence of insects and wind needed for cross pollination in the growth chamber and the minimal ability of wild radish to self-pollinate. After each sampling, samples were freeze-dried for 10−14 days. The biomass of each sample was determined prior to samples being ground to a fine powder that would pass through a 1 mm screen. Samples were stored at 4 °C in sealed plastic bags until analyzed for GSL content.

GSL Extraction and Analysis. GSL extraction from plant samples was conducted as described previously (*44*). Freeze-dried plant material (0.3 g) was combined with 10 mL of 70% methanol at 70 °C, vortexed, and placed in a water bath at 70 °C for 20 min. Glucotropaeolin and sinigrin (1.2 μM) were included as surrogates to a duplicate of each plant sample to correct for percent recovery of glucosinolates. After cooling, samples were centrifuged (3000*g*, 5 min). Supernatant (3 mL) was added to an A-25 DEAE Sephadex column (0.6 Ml) and allowed to pass through the column (see description below for column preparation). The column was washed twice with 1 mL of 0.02 M sodium acetate buffer, pH 4, and was end-capped to prevent drying. Sulfatase, previously purified (see description below), was added (100 μL) to the top of the column containing the supernatant, and one drop was allowed to elute through the column prior to the column being recapped. The sulfatase was allowed to interact with GSLs overnight. The desulfonated GSLs were eluted with 1.5 mL of Milli-Q water the following day. Samples were filtered through 0.2 μm nylon filters into autosampler vials.

Table 1. Common Names and R-Groups Associated with Glucosinolates Used as Standards during High-Performance Liquid Chromatography or Identified in Wild Radish Samples along with Degradation Products Produced following Myrosinase Activity[a]

Common Name	R-group	R group structure	Degradation Products
Aliphatic			
Glucoiberin	3-(Methylsulfinyl)propyl	—$CH_2CH_2CH_2SCH_3$ (=O)	ITC
Glucoraphanin	4-(Methylsulfinyl)butyl	—$CH_2(CH_2)_3SCH_3$ (=O)	ITC, nitriles
Glucoraphenin	4-Methylsulfinyl-3 butenyl	—$CH_2CH_2CH{=}CHSCH_3$ (=O)	ITC
Glucoerucin	4-(Methylthio)butyl	—$CH_2(CH_2)_2CH_2SCH_3$	ITC. nitriles
Sinigrin	2-Propenyl	—$CH_2CH{=}CH_2$	ITC, nitriles
Aromatic			
Gluconasturtiin	2-Phenylethyl	—CH_2CH_2–C$_6$H$_5$	ITC, nitriles
Glucosinalbin	4-Hydroxybenzyl	—CH_2–C$_6$H$_4$–OH	ITC, nitriles
Glucotropaeolin	Benzyl	—CH_2–C$_6$H$_5$	ITC, nitriles
Indole			
Glucobrassicin	Indol-3-ylmethyl		Indolyl-3-carbitol

[a] Compounds are grouped according to their major chemical class. Chemical classes and degradation products based on previous papers (*14*, *19*, *45*−*48*).

Sulfatase Preparation. Sulfatase Type H-1 from *Helix pomatia*, 10000 U/g (Sigma-Aldrich Co., St. Louis, MO) was combined with cold water (75 mg in 6 mL) and stirred to dissolve; then 4 mL of cold ethanol was added. After centrifugation (3000*g*, 6 min), the supernantant was transferred to another centrifuge tube and the precipitant was discarded. Cold ethanol (20 mL) was added to the supernatant, and the enzyme was obtained as a precipitant following centrifugation (3000*g*, 6 min). The remaining ethanol was allowed to evaporate for a few minutes before the precipitant was redissolved in 5 mL of cold water to obtain an approximate final concentration of 15 mg/mL (150 U/mL). Purified sulfatase could be stored at −20 °C for approximately 1 month.

DEAE A-25 Sephadex Column Preparation. DEAE Sephadex A-25 (Sigma-Aldrich Co.) was rehydrated in excess 2 M acetic acid. To prepare columns, 0.6 mL of column material was placed in a poly prep column, 0.8 × 4 cm (Bio-Rad Laboratories, Hercules, CA). After the material had been allowed to settle, the column was rinsed twice with 1 mL portions of distilled deionized water. Columns were end-capped until sample extracts were applied to prevent drying.

High-Performance Liquid Chromatography (HPLC) Analysis. GSL content and composition in samples were determined using a modification of a previous HPLC method (*44*). Desulfonated GSLs in samples (30 μL) were analyzed using a Hewlett-Packard 1090 HPLC equipped with diode array detector set at 229 nm and a reverse phase column (3.2 mm × 150 mm, 5 μm, LiChrosorb RP18, Alltech Associates, Inc., Deerfield, IL). The mobile phase was 100% water for 5 min followed by a linear elution gradient over the next 25 min to 65% water/35% acetonitrile, which was held for 3 min followed by 5 min at 100% acetonitrile and 5 min equilibration back to the starting condition of 100% water. The solvent flow rate was 0.5 mL/min. GSLs were determined by comparing samples to various standards and to previously published protocols (*1*, *44*). Glucoiberin, glucoraphanin, glucoraphenin, and glucoerucin standards were obtained from KVL (Copenhagen, Denmark). Glucotropaeolin and glucosinigrin were obtained from Sigma-Aldrich Co. R-groups, common names, and degradation products of the GSLs associated with this study are given in **Table 1**.

Total grams of each plant part was multiplied by the total micromoles of GSL per gram for that plant part, and then the various plant parts in each stage were added together to obtain the total micromoles per plant for that stage.

Statistical Analysis. Individual samples were extracted twice, and plants were replicated three times for each growth stage in each of two experiments. GSL concentrations and associated standard errors were calculated for each sampled part at each wild radish developmental stage. The total GSL and biomass data were subjected to ANOVA in SAS (Statistical Analysis Systems, version 9.1, SAS Institute Inc., Cary, NC). ANOVA was performed on rank transformed total GSL and biomass data, which is a form of Kruskal−Wallis nonparametric analysis. The means from ANOVA were separated using Fisher's protected LSD at 5% level of significance.

RESULTS AND DISCUSSION

Total micromoles of GSL production per plant varied at different wild radish developmental stages (**Table 2**). Total GSL production ranged from 5.70 μmol/plant at cotyledon stage to 1942.4 μmol/plant at the 50% flowering stage. Levels were significantly higher per plant at the 50% flowering stage when compared to the 50% silique stage. From the 50% flowering to

Table 2. Biomass and Total Glucosinolates among Different Growth Stages of Wild Radish

growth stage	biomass[a] (g/plant)	total glucosinolates[a] (μmol/plant)
1, cotyledon	0.05 d	5.79 e
2, 6−8-leaf	1.84 c	43.00 d
3, bolting	2.41 c	145.89 c
4, 50% flowering	16.16 b	1942.40 a
5, 50% silique	18.45 a	1246.65 b

[a] Means followed by the same letter are not significantly different using Fisher's protected LSD at $\alpha < 0.05$ based on the rank transformed data with three replicates.

the 50% silique stage there was an approximate 35% reduction in total GSLs per plant even though there was an approximate 14% increase in the total biomass associated with each plant. These results indicate that when wild radish is incorporated for the suppression of weeds, the wild radish should be incorporated at 50% flowering instead of later at the time of maximal biomass production to obtain maximal GSL incorporation into soils for weed suppression.

The contributions of each plant part to the total GSL production in stages 2−5 are illustrated in **Figure 2**. Roots contribute between 5.3 and 12.92% of the total GSL concentration, which is similar to their contribution to the total biomass, 6.95−21.25%. A majority of the total GSL content is associated with the aboveground plant parts, and the percentage associated with the primary branches remains relatively consistent through stages 3−5. Flowers, even though they contain the highest concentration of GSL per gram (196.72 μmol/g), contribute only 11.83% of the total GSL due to the low total biomass per plant (1.13 g) for flowers. The contribution of the secondary branches increases into the final stage, as would be expected with the increasing percentage of the contribution of the secondary branches to the total biomass of the plant.

Total GSL concentrations (μmol) and GSL concentrations (μmol/g) from individual GSLs varied depending on the growth stage (**Figures 3** and **4**). To get enough biomass of cotyledon samples, five plants were combined and cotyledon samples were not separated into roots and shoots. Values at the cotyledon stage were approximately twice the values for the 6−8 leaf stage. Values then increased to the following stage. Values at silique stage were approximately half the values at flowering stage. Eight GSLs were identified in various wild radish samples in different organs and at various developmental stages. These GSLs included glucoerucin, glucotropaeolin, glucoraphenin, gluconasturtiin, glucobrassicin, glucosinalbin, glucoiberin, and glucoraphanin. The concentrations of individual GSLs in different organs of wild radish when present varied greatly, with a >800-fold difference from the lowest concentration of glucoraphanin (0.19 μmol/g) in roots at 50% silique formation to glucoerucin (157.30 μmol/g) in flowers at the flowering stage (**Figure 3**). Five GSLs were expressed at the cotyledon (stage 1), 6−8-leaf (stage 2), bolting (stage 3), and flowering stages (stage 4) from a low of 0.37 μmol/g (glucobrassicin) at bolting to a high of 157.30 μmol/g (glucoerucin) at the cotyledon stage. Three GSLs, glucotropaeolin, glucoerucin, and gluocoraphenin, were the predominant GSLs and contributed >90% of the GSL content at all developmental stages. At 50% silique formation (stage 5), three additional GSLs were expressed, glucoiberin, glucoraphanin, and glucosinalbin, which ranged in concentration from 0.19 to 5.58 μmol/g and contributed from 0.29 to 12.21% of the total GSL of the plant part. Glucoiberin was expressed in all plant organs at the silique stage, whereas glucoraphanin was expressed in roots and secondary branches and glucosinalbin was present in secondary branches.

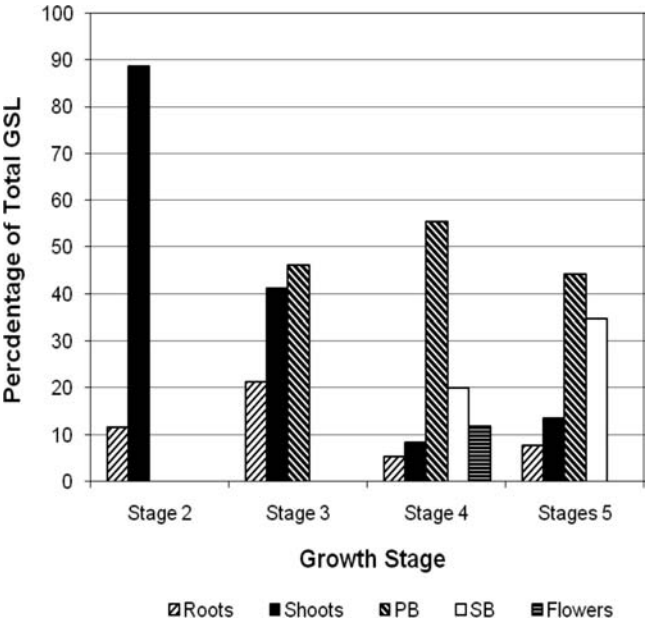

Figure 2. Percentage of total GSLs associated with roots, shoots, primary branches (PB), secondary branches (SB), and flowers at stage 2 (6−8 leaf), stage 3 (bolting), stage 4 (50% flowering), and stage 5 (50% silique).

GSLs produced by wild radish were accumulated in roots, shoots, flowers, primary branches, and secondary branches. The highest concentration of GSLs was found in flowers at the flowering stage followed by primary branches at the flowering stage. Brown et al. (*38*) reported higher GSL concentrations in reproductive organs including seeds (63 μmol/g), inflorescences (25−30 μmol/g), and siliques (15−25 μmol/g) for *Arabidopsis thaliana*, which is similar to results reported here. Glucoerucin generally occurred at the highest concentration and glucobrassicin at the lowest level of all identified GSLs. Higher concentrations of glucoerucin, glucoraphenin, and glucotropaeolin were observed at the 50% flowering stage in roots, shoots, primary branches, and flowers and in primary branches at bolting. These GSLs hydrolyze to form ITCs with herbicidal properties. Overall, lower concentrations were observed at the 6−8-leaf and bolting stages in roots and shoots, ranging from 8.21 to 51.59 μmol/g.

Of the three major GSLs, which contributed approximately >90% of total GSLs, there were differences in levels across the developmental stages when similar plant organs were compared. Glucoerucin concentration decreased from the cotyledon to the 6−8-leaf stage and then increased to maximum levels at flowering followed by a decrease at silique formation, except in secondary branches. In contrast, glucoraphenin decreased from the cotyledon to the 6−8-leaf stage and then increased through silique formation. Overall, this study has shown that wild radish GSL content and composition vary among organs and developmental stages. The highest GSL concentrations occurred in flowers at flowering followed by primary branches. Among the GSLs detected, glucoiberin, glucoerucin, glucotropaeolin, glucoraphenin, glucoraphanin, gluconapin, and gluconasturtiin hydrolyze to form ITCs, which have herbicidal properties (*1*), and the three most prevalent GSLs detected in wild radish are among those that are precursors for ITCs. The increase in GSL production in wild radish at flowering is similar to that reported in other Brassicaceae plants (*38*, *42*). Flowers, leaves, and branches of wild radish were also shown to contain more GSLs than roots, similar to findings for other Brassicaceae species (*8*).

This research was conducted under controlled environmental conditions, and the GSL concentrations may change in different

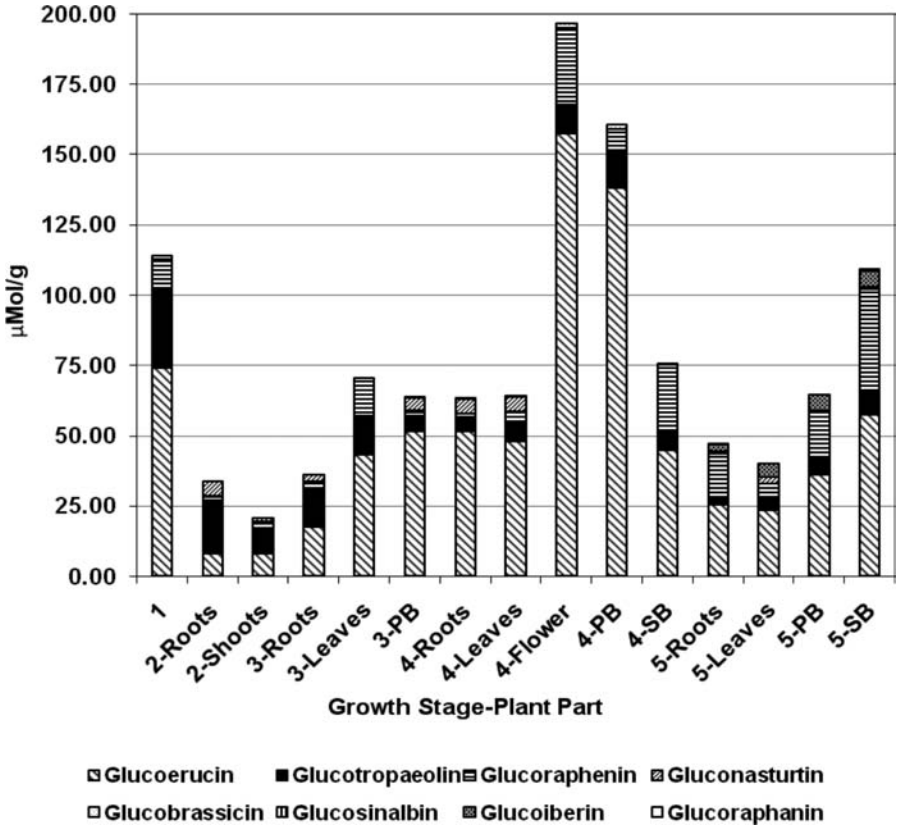

Figure 3. Individual GSL content in roots, shoots, primary branches (PB), secondary branches (SB), and flowers at each growth stage in wild radish: stage 1, cotyledon; stage 2, 6−8 leaf; stage 3, bolting; stage 4, 50% flowering; stage 5, 50% silique.

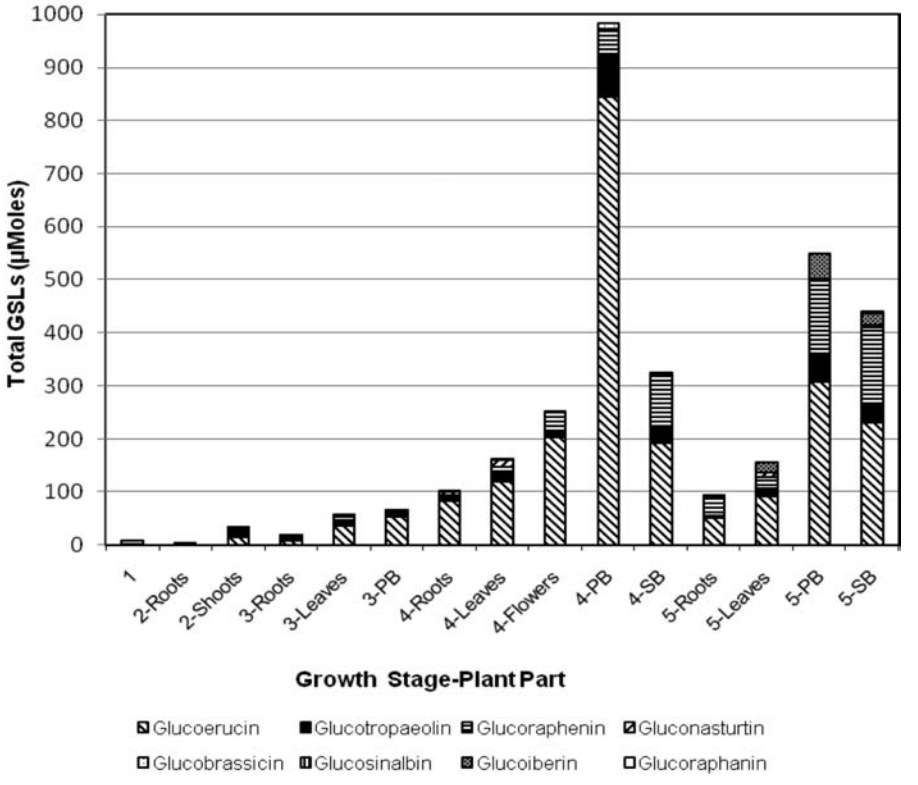

Figure 4. Total GSL content of each GSL in roots, shoots, primary branches (PB), secondary branches (SB), and flowers at each growth stage in wild radish: stage 1, cotyledon; stage 2, 6−8 leaf; stage 3, bolting; stage 4, 50% flowering; stage 5, 50% silique.

environments. GSL concentrations are known to be dependent on changes in biotic and abiotic conditions (*33*). Knowledge of GSL accumulation in different organs at different wild radish developmental stages, however, is important for determining at

which stage GSL concentration is maximized along with its allelopathic potential. On the basis of this research, if wild radish is used as a cover crop, it should be incorporated into the soil at the flowering stage for maximum GSL production, which in turn can result in higher ITC levels and greater weed management. It is important to ensure that maximum disruption of wild radish tissue occurs prior to its incorporation into soil. More hydrolysis of plant tissue takes place following increased tissue maceration, resulting in more ITCs being produced and maximum ITC production during the first 24 h following incorporation of cover crops and, therefore, better pest suppression (*45*). Incorporating wild radish biomass into soil at flowering can serve as one means of using this natural cover crop to augment pest suppression in vegetable crops in the southeastern United States. Incorporating wild radish prior to silique formation also reduces the possibility of wild radish becoming a problem weed following its usage as a cover crop. It should not be allowed to produce mature siliques prior to its incorporation. Ultimately, the use of wild radish cover crops can result in more environmentally friendly approaches to weed and disease management (*45*) by reducing the need for herbicides as was shown in a field study with corn in which herbicide usage could be decreased 50% (*12*). Further studies are being conducted to determine if there are wild radish accessions that have higher levels of glucosinolates.

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