Journal of Oilseed Brassica, 12 (1) : 14-20, January 2021

Glucosinolate allocation patterns in oilseed *Brassica carinata*

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(Received: 3 August 2020; Revised: 5 November 2020; Accepted: 8 December 2020)

Abstract

The introduction of oilseed *Brassica carinata* (carinata) into the southeastern US cropping system has fueled its prospects as a sustainable source of biojet fuel for the aviation industry. The presence of sulfur-rich defensive compounds called glucosinolates (GSLs) are effective against herbivores and soil-borne pathogens and understanding what biotic and abiotic factors trigger GSL synthesis and accumulation is important in the management of Total Glucosinolate Content (TGC) in various plant parts of carinata. In this study, we investigated the accumulation of TGC in *B. carinata* cv. AAC A110 in various plant tissues, throughout the plant life-cycle, and in soil types with differing land management practices. We found that *B. carinata* has greater biomass accumulation and root TGC when grown in soil with high organic matter compared to soil derived from areas of intensive or conventional farming (CF) or sand without affecting seed yield. Leaf TGC decreased during the transition from vegetative to reproductive growth indicating that it may be the primary source of GSLs, which are translocated to the developing seeds until physiological maturity. Our results demonstrate the GSL allocation patterns of carinata are like those proposed by the optimal defense theory and could be an important trait for crop defense and future production in the region.

Key words: *Allocation pattern, Brassica carinata, glucosinolate, oilseed Brassica*

Introduction

Oilseed *Brassica carinata* (A. Braun) or carinata has been introduced into the southeastern US for winter production as a feedstock for sustainable aviation fuel (Seepaul *et al*., 2016). The Southeast US encompasses a range of land management practices that can alter soil quality which is often measured by physical and biological characteristics of organic matter (OM), water and nutrient retention characteristics, and microbial and fungal diversity (Frac *et al*., 2020; Granada *et al*., 2019). The USDA continues to support mitigation of negative effects impacted by intensive farming practices by establishing programs that include low-input sustainable agriculture (LISA), best management practices (BMP), and other conservation methods or upgrading systems (Albertin, 2020; Pimentel *et al*., 1989). *B. carinata* has desirable agronomic traits that allow for a practical and sustainable integration into existing crop rotation systems. These include a robust root architecture (Rana and Chaudhary, 2013; Rashidi *et al.*, 2017), a fortified pod valve margin to reduce seed shattering (Zhang *et al.*, 2016), and the production of defensive compounds called glucosinolates (GSLs) that make it naturally resistant to many pests and soil-borne pathogens (Navabi *et al.,* 2010; Subramanian *et al.,* 2005; Uloth *et al.*, 2016).

Brassica plants contain higher quantities of GSLs in their leaves and roots during the vegetative stage of development, which are subsequently translocated to reproductive structures, including seeds at plant maturity (Bhandari *et al.*, 2015; De March *et al.*, 1989). However, the production of GSL can also be upregulated to tissues perturbed by herbivores and plant pathogens (Keith and Mitchell-Olds, 2017). This behavior is consistent with the optimal defense theory (ODT) which posits that plants will increase the relative concentration of defensive compounds in organs with a high fitness value or allocate them to sites that are under attack by disease-causing organisms (Tsunoda *et al.*, 2017). Kliebenstein *et al.* (2016), argues that the tradeoff between growth and resistance to disease is not linear, but instead is a dynamic response based on complex interactions among plant development, changes to its environment, and threats from pathogens.

Glucosinolates in seeds are not produced *in situ* but rather translocated by GSL-specific transporter proteins through phloem loading pathways (Nour-eldin *et al.*, 2012). It is not clear if *B. carinata* follows the GSL allocation patterns described in ODT or if soils with different levels of organic matter (OM) will influence the production and allocation of GSLs. Our hypothesis is that the pattern of *B. carinata* GSL production will be consistent with ODT and that the relative concentrations of GSLs will vary with plant tissues and stage of development. In addition, we

hypothesize that GSLs will increase when *B. carinata* is grown in soils with increased OM (i.e., microbial diversity) than in sandier soils possibly altering oil quality as measured by total oil content (TOC), erucic acid content (EAC), and crude protein content (CPC). Our objectives in this study were to 1) evaluate the dynamics of total GSL content (TGC) of the roots, stem, leaves, and seeds in *B. carinata* at different stages of development and 2) determine the possible impact on TGC in tissues and seed contents when grown in soils with varying OM.

Materials and Methods

Plant Material and Management

This experiment was conducted at UF/IFAS North Florida Research and Education Center in Quincy, FL (30°32'44.7"N, 84°35'41.2"W) from February 21st to July 1st, 2015 under semi-controlled greenhouse conditions. *B. carinata* cv. AAC A110 (germplasm formerly developed by Agrisoma Biosciences) was planted in 7.65 L tree pots (Stuewe and Sons, Tangent, OR) filled with 3 different soil types where the top 15-20 cm of the soil surface was collected and sifted (1x1 cm) from a 30-yr bahiagrass pasture (BP; 30°32'59.6"N, 84°35'47.6"W), an intensive or conventional farming site (CF; 30°32'43.8"N, 84°35'59.9"W), and from a local sand pit (SP; 30°28'55.7"N, 84°39'35.6"W). All soils had similar pH (6.0-6.4) with BP soil containing 2.0% OM, CF 0.5% OM, and SP 0.0% OM. These soils were classified as Orangeburg loamy sand, Orangeburg-Norfolk complex, and Lakeland sand, respectively (Natural Resources Conservation Service, 2018). There were variations in soil nutrients (Table 1), but all were fertigated using a drip irrigation system three times a day (0800, 1200, and 1600) with Hoagland nutrient solution (Hoagland and Arnon, 1938).

Table 1: Analysis of the three soil types used to grow *B. carinata*. Soil from a bahiagrass pasture (BP), conventional farming (CF), and sand pit (SP)

Soil	pH	OM	EC	N	P	K	Mg	Ca	Cu	Mn	Zn
		%	dS/m	%	ppm†	mg kg^{-1}					
BP	6.4	2.00	0.12	0.10	72	125	192	753	0.58	23.6	6.2
CF	6.0	0.47	0.15	0.03	209	25	40	215	0.09	24.0	1.69
SP	6.1	0.00	0.15	0.01	42	0	11	82	0.02	1.90	3.11

† Derived using Mehlich 3 extraction

Tissue Sampling and Preparation

Five plants per treatment were destructively sampled at each of the 4 stages of development (rosette, bolting, flowering, and maturity) for TGC and biomass dry weights (DW). For all TGC tissue samples, at least 10 g (fresh weight, FW) was collected and immediately placed into prelabeled paper bags, stapled shut, snap-frozen using liquid nitrogen, and stored at -80°C. Leaves with corresponding stalk sections were collected starting with the 4th and 5th mainstem leaf during the rosette stage (30 DAP). The sampling site was adjusted higher up the main stem with every subsequent sampling event to acquire leaves of similar age. Roots were sampled from the larger root mass, followed by rinsing to remove sand and debris. Young fibrous roots were then collected from 3 random areas and stored at -80°C. The remainder of the shoot and root biomass was then separated by plant part, oven-dried at 48°C for 72 hours and weighed. Seeds were harvested at agronomic maturity and dried at 48°C to <8% moisture.

GSL Extraction and Purification

All tissues (except seeds) were lyophilized for 24h to 48h, ground while frozen in liquid nitrogen, and stored at -20°C until extraction. The GSL extractions followed a modified version of Gallaher *et al*. (2012). First, 200 mg of dried sample was transferred into labeled test tubes preheated to 80°C using a heating block (Barnstead Thermolyne, Dri-Bath 17600, Iowa, USA). Then, 4 ml of boiling 80% methanol was immediately added to each sample followed by 15s agitation before returning to the heating block. Heating prevented the hydrolysis of the GSLs by denaturing GSL-specific enzymes in the tissue. Boiling continued for a total of 15 minutes with a wooden toothpick added into each test tube to keep the methanol at a steady boil. Next, samples were centrifuged for 10 minutes at 6500 rpm (Eppendorf 5810R, Hamburg, Germany) and the supernatant transferred into a separate labeled glass test tube and repeated with supernatant of each sample pooled into its respective test tube.

A modified version of Widharna (2012) was followed for GSL purification. In brief, ion-exchange columns were prepared for each sample using 200 mg of DEAE Sephadex A-25 resin (GE Healthcare Biosciences, Pittsburgh, PA, USA) and saturated with double-distilled water (dd-H_2O). Then, two aliquots (x2), each consisting of 2 ml 0.5 M sodium hydroxide (NaOH) was added and allowed to pass through the column, followed by 4 ml of dd-H_2O to rinse

off any excess NaOH. Next, 2 ml of 0.5M pyridine acetate solution (x2) was added, followed by another series of rinses. The supernatant containing the GSL extract was then transferred into the prepared and primed ion-exchange column. The column was then rinsed with 2 ml of dd-H2O (x2), 2 ml of 30% formic acid (x2), and 2 mL of dd-H2O (x2), discarding the eluate. Finally, the purified GSL was eluted from columns using 2 ml of 0.3 M potassium sulfate into a new receptacle.

Estimation of Tissue Glucosinolates

The quantification of the GSL contents in plant tissue was estimated colorimetrically in duplicates following a modified version of Jezek *et al.* (1999) and Kumar *et al*. (2004). In separate vials, 300 ìL of 0.002 M sodium tetrachloropalladate (II) or TCPII was added to 1 ml sample aliquot and left standing for 30s to allow for complex formation. Then, each sample was vortexed for 3s, poured into a clean 1 cm cuvette, and the absorbance was measured using a UV-Vis spectrophotometer (Spectronic Unicam, Genesys 8, England) at 405 nm. To estimate total GSLs, a standard calibration curve was developed (TGC = (Abs_{405}/0.0026) – 60.54) through a series of dilutions using the allyl-GSL standard, sinigrin monohydrate (Sigma-Aldrich, St. Louis, MO, USA) and following the procedures as tissue extracted GSLs. Absorbance values, along with dilution factors and blanks were then used to estimate GSL concentrations. Seed contents that included TGC, total oil contents (TOC), erucic acid contents (EAC), and crude protein content (CPC) was evaluated using near-infrared reflectance spectroscopy (NIRS) analyzed by Nuseed (formerly Agrisoma Biosciences) using a FOSS XDS™ Rapid Content Analyzer (FOSS Analytical AB, Hogenas, Sweden) collecting the spectra at 0.5 nm increments from 400 to 2500 nm range. The spectra were analyzed using the ISIscan program (program version 4.10.0.15326, Database version 4.6.0.14416). Instruments were calibrated regularly using samples of known values.

Experimental Design and Statistical Analysis

This experiment was set up using a randomized complete block design with five replications. The two factors were soil types with three levels (BP, CF, and SP) and four stages of development (rosette, bolting, flowering, and agronomic maturity), totaling 60 experimental units. Vegetative growth included rosette to bolting and everything thereafter as reproductive growth stages. A two-way analysis of variance (ANOVA) was used to evaluate interaction and main effects for each tissue with block set as a random variable. Tukey's honest significant difference (HSD, á = 0.05) was then used to determine differences between average biomass, absorbance values, and estimates of individual seed constituents using JMP Pro 14.1.0 (SAS Institute Inc., Cary, NC). No biomass was collected for leaves at plant maturity due to senescence resulting in leaf drop.

Table 2: The response of height, biomass, and total glucosinolate content to soil type and stage of development using a 2-way ANOVA

	Soil	Stage	Soil*Stage
Height			
Shoot	0.0413	<0.0001	0.6003
Biomass			
Leaf	0.0204	<0.0001	0.6321
Stem	0.0319	<0.0001	0.1799
Root	0.0005	<0.0001	0.3369
R:S	0.8050	<0.0001	0.3934
Seed	0.7649	-	-
Total	0.0007	<0.0001	0.2930
Total GSL Content†			
Leaf	0.7705	0.0006	0.5421
Stem	0.8281	<0.0001	0.8051
Root	0.0257	0.0788	0.5707
Seed	0.2238	-	-

†Derived using colorimetric methods. Includes the root to shoot ratio (R:S)

Results and Discussion

Plant Height and Biomass

There were no interactions between soil type and stage of development on height and leaf, stem, root, or total biomass (Table 2.). However, all tissues had main effects differences with soil type or stage of development (except seed yield). There was an incremental increase in height and plant tissue biomass with plant development (Table 3). Soil type also contributed to differences in height and biomass accumulation. *B. carinata* grown in BP or CF soil had increased heights of 82.9 cm compared to SP (66.5 cm, *P*= 0.0413). BP and CF soil had increased leaf biomass averaging 19.0 g plant^{-1} compared to SP soil (16.3 g plant^{-1}). There was a 20.2% decrease in stem biomass when grown in CF soil (46.9 g plant^{-1}) than in BP soil (62.5 g plant^{-1}). This was similar for the roots where BP soil had greater biomass (45.0 g plant^{-1}) compared to both CF (30.4 g plant^{-1}) and SP (27.7 g plant^{-1}) soil. The total biomass was greater in BP soil (125.9 g plant^{-1}) than in CF and SP soil (≈ 96.1 g plant^{-1}). Root to shoot ratio decreased with the stage of development from 1.67 to 0.31 from the rosette stage to maturity at the rate of y= -0.44x + 1.98 (R^2=0.95), but no differences among the soil types. Soil type did not affect seed yields (12.8 g plant^{-1}) or harvest index (0.073).

Table 3: Average height, biomass, and root to shoot ratio of *B. carinata* with the stage of development across all soil types.

		Biomass (g plant^{-1})					
Stage	Height (cm)	Leaf	Stem	Root	Seed	Total	R:S
Rosette	11.9 c	6.2 c	1.0 c	11.3 c	-	18.4 d	1.67 a
Bolting	71.3 b	17.9 b	10.7 b	26.6 b	-	55.2 c	0.92 b
Flowering	149.1 a	30.2 a	39.3 a	46.5 a	-	116.0 b	0.66 c
Maturity	-	-	168.4 a	53.1 a	12.8	234.4 a	0.31 d

Values followed by the same letter within each parameter were not significantly different using Tukey's multiple comparison test (á = 0.05).

Glucosinolates in Shoots and Roots

There were no interactions with soil type and stage of development on total glucosinolate content (TGC) in *B. carinata*. Significant main effects were observed with stage of development and soil type, but the significance varied with each tissue (Table 2.). Leaves averaged 78.0 ì mol g^{-1} from the rosette to the bolting stage and then decreased to 38.5 ì mol g^{-1} at flowering (Figure 1). TGC in the stem maintained a low concentration (≈11.2 ì mol g^{-1}) from rosette to flowering but then increased by a factor of 6.5 at maturity (72.7 ì mol g^{-1}). The roots did not show significant change to TGC throughout stage of development and averaged 38.5 ì mol g^{-1}. Soil type had no effect on TGC with the leaves, stems, and seeds averaging 64.2, 26.1, and 161.0 ì mol g^{-1}, respectively. This included seed TGC that averaged 161.0 ì mol g^{-1} between soil type. Average TGC from the tissue indicated a quadratic relationship between TGC in the leaves and those in the stem with stage of development, but in opposite directions (Figure 1). Significant main effects of soil type were observed with roots only (Table 4). There was a doubling of root TGC with BP soil (50.9 ì mol g^{-1}) compared to SP (25.2 ì mol g^{-1}).

Table 4: Effect of soil type on the total glucosinolate contents (TGC) across the stage of development in *B. carinata*.

	TGC† (ì mol g^{-1})			
Soil Type	Leaf	Stem	Root	Seed
BP	60.0 a	27.2 a	45.3 a	159.8 a
CF	65.4 a	27.2 a	45.3 ab	160.9 a
SP	67.2 a	27.1 a	25.2 b	162.4 a

†Estimated colorimetrically at 405nm. Values followed by the same letter within each parameter were not significantly different using Tukey's multiple comparison test (á = 0.05). Soil type includes those collected from bahiagrass pasture (BP), conventional farming (CF), and sand pit (SP).

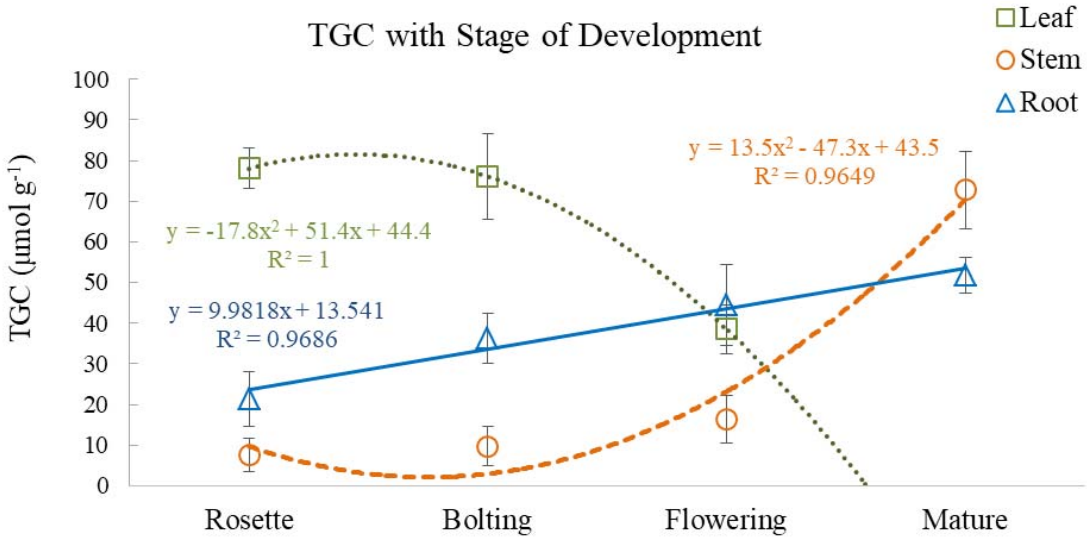

Figure 1: Total glucosinolate contents (TGC) of leaf, stem, and roots of *B. carinata* tissue with plant development. Each plant tissue has a corresponding line of best fit.

Table 5: Effect of soil type on seed glucosinolate, oil, erucic acid, and crude protein content in *B. carinata*.

	Seed Content†							
	TGC (ì mol g^{-1})		TOC (%)		EAC (%)		CPC (%)	
Soil Type	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE
BP	165.6	4.4	24.9	0.6	31.5	0.9	45.9	0.5
CF	167.4	7.9	24.4	1.4	30.4	2.1	45.2	0.5
SP	172.5	7.5	25.1	0.7	31.4	2.4	45.2	0.7
P-Value	0.7624	0.6295	0.8623	0.9047				

†Seed contents include total glucosinolate content (TGC), total oil content (TOC), erucic acid (C22:1) content (EAC), and crude protein content (CPC). The result of an ANOVA with p-value <0.05 indicates any significant differences among soil type, and they include bahiagrass pasture (BP), conventional farming (CF), and sand pit (SP). Means include standard error (SE).

Seed Contents

Seed TGC, TOC, EAC, or CPC did not differ among soil types (Table 5.). Near-infrared spectroscopy estimates an average TGC of 168.5 ì mol g^{-1} in seeds across all treatments and similar to colorimetrically determined TGC of 161 ì mol g^{-1}. Seeds also contained an average TOC of 28.8%, with 31.1% comprising of EAC and 45.4% CPC. There was a negative correlation between TGC and TOC (r= -0.6051, *P*= 0.0370).

Changes in total GSL content (TGC) throughout plant suggests that *B. carinata* does follow the pattern described for the optimal defense theory (ODT). For example, TGC in the leaves were greatly reduced as *B. carinata* transitioned from vegetative to reproductive growth. These fluctuations in TGC from various plant tissues have been documented in the Brassicaceae family (Booth *et al.*, 1991). In addition, there was a sharp increase in stem TGC at agronomic maturity. This is a strong indicator of the constant movement of GSLs from the leaves to reproductive organs that then abruptly halted when the mother plant is no longer providing nutrients and other metabolites to seeds at maturity. This is consistent with the discovery of GSL-specific transporter genes in *Arabidopsis* which transfers GSL from various organs (especially the leaves) to reproductive organs during plant development (Francisco *et al.*, 2016; Kuchernig *et al.*, 2012). Nour-Eldin *et al*. (2012) found that the disruption of GSL-transporter genes (i.e., GTR1 and GTR2) in *Arabidopsis* halted the accumulation of GSL in the seeds with a 10-fold increased accumulation in the shoots.

A similar study looking at GSL concentration and stages of development was conducted in Denmark using four *Brassica* species (Bellostas *et al.*, 2004) found that the highest concentration of TGC was in the reproductive structures in the shoots of *B. nigra* and *B. juncea* (120 ì mol g^{-1}), while it was mainly concentrated in the roots of *B. rapa*. In the current study, roots of *B. carinata* maintained a constant TGC throughout development with no indication that the stage of development influenced significant changes to TGC and instead relying on the GSLs from the leaves for redistribution to reproductive organs.

We found that variations with soil type did not influence shoot biomass or seed yield under conditions of adequate fertigation. Increasing organic matter in the soil can help with water and nutrient retention as well as increase soil biota and enzymatic functions important in nutrient cycling (Bowles *et al.*, 2014). By not inducing water or nutrient stress in these environments, the benefits or disadvantages of growing *B. carinata* could not be fully investigated. However, this may have contributed to differences in root TGC based only on the microbiota in the soil. We found that TGC in roots were being influenced by soil type with more accumulating in BP soil compared to SP. The subtle responses of root TGC to soil type may be due to the induced production of GSLs in response to soil micro-organisms in higher organic matter soil as compared to sand (Van Dam *et al*, 2009). Also, studies on the dynamics of TGC for optimal defense has found that distal or tertiary roots are not as important to plant fitness as the central taproot (Tsunoda *et al*., 2017). Bahiagrass soil contained higher organic matter probably translating into increased of microbial diversity compared soil derived from a sand pit. This is a flatwood habitat with the sand being mined from a marine terrace classified as Pits surrounded by Lakeland sand (National Resource Conservation Service). Entisol soil have a layer of topsoil followed by several layers of unaltered sand deprived of organic matter. When grown in soil with relatively little interaction to microbial populations or belowground threat to its roots, *B. carinata* would not need to allocate

extra energy for the production of GSLs and therefore increase its root TGC.

Colorimetry can be a relatively quick method for estimating TGC in plant tissue, but there are drawbacks. The most obvious is the inability to distinguish among the different classes of GSLs. In this experiment, we used the wavelength of 405 nm to estimate TGC, as developed by Thies (1982). Studies have shown that absorbance maxima are highly dependent on the type of GSL being analyzed due to the efficiency of functional groups forming a strong complex with TCPII (Mawlong *et al.*, 2017). *Brassica* species evaluated for GSLs have reported that *B. carinata* was one of the only species that consistently had a strong positive correlation between the root and shoot TGC and the roots contained about equal parts aliphatic GSL (i.e., sinigrin) to other classes of GSLs (Van Dam *et al.*, 2009). Although the estimated TGC of seeds derived colorimetrically did align closely with those from NIRS analysis, it is possible that GSLs in the roots with variable functional groups (i.e. non-sinigrin) were not accurately captured in this semi-quantitative approach. Nonetheless, these patterns still indicate that TGC in the seeds and other tissues do coincide with ODT. Future studies focused on individual GSLs responses to external biotic or abiotic environmental stress may help reveal the intricacies of *B. carinata* as a naturally resilient species, used as a feedstock for biojet fuel production, in areas of high pest and disease pressures such as the southeastern US.

Acknowledgements

USDA-NIFA Grant #2016-1123 for financial assistance and Nuseed for material and assistance with NIR analysis. We would like to thank the "Carinata crew" that includes C Dickson, M Douglas, GK Obrien, and the staff of Extension Agronomy at NFREC for their technical support throughout this project.

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