RESPONSE OF *BRASSICA CARINATA* TO BIOTIC AND ABIOTIC STRESS THROUGH GLUCOSINOLATE SYNTHESIS AND DISTRIBUTION

By

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To Silvia and Philip Stansly whose unyielding love, support, and guidance throughout my life have fostered an enlightened perspective on the importance of relationships, the wisdom of humility, and the power of perseverance

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BMP	Best management practices
BSM	*Brassica* seed meal
CP	Crude protein
CPC	Crude protein content
CEC	Cation exchange capacity
DAP	Days after planting
DM	Dry matter
EAC	Erucic acid content (C22:1)
GPS	Global positioning system
GSL	Glucosinolate
HSD	Tukey's honest significant difference
ITC	isothiocyanates
Met-GSL	Methionine derived glucosinolate
NIRS	Near-infrared spectroscopy
NFREC	North Florida Research and Education Center
ODT	Optimal defense theory
RCBD	Randomized complete block design
TGC	Total glucosinolate content
TOC	Total oil content
SRKN	Southern root-knot nematode
USDA	United States Department of Agriculture
UF/IFAS	University of Florida / Institute of Food and Agricultural Science
VLCFA	Very long-chain fatty acids

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Oilseed *Brassica carinata* produces seeds that are rich with erucic acid (C22:1), allowing for more efficient conversion into 'drop-in' fuel for the aviation industry. *B. carinata* has many desirable traits making it suitable for crop production in the southeastern United States during the winter season. However, questions remain on crop performance when grown in a variety of Florida soil types with increased disease pressure. In addition, a sulfur-rich secondary metabolite called glucosinolates (GSLs) are produced by *B. carinata* and may compete with oil production and reduce seed meal quality. GSLs are defensive compounds that aid in the protection against herbivores and soil-borne pathogens but with substantial energy costs, especially in response to environmental stress factors. It was determined that *B. carinata* follows a GSL distribution pattern that is consistent with optimal defense theory. GSLs initially accumulate in the roots and leaves during vegetative growth but are then redistributed to reproductive structures and ultimately to the seeds at maturity. *B. carinata* prioritizes the synthesis and transportation of GSLs to tissues of greatest fitness value which change with plant development or in response to environmental stress. Greenhouse trials on sulfur (S) fertility determined that seed GSLs had an inverse relationship between total oil content, but a positive impact on oil quality and protein

content in the seed. Similar results were found in field trials, except that S availability can differ depending on soil properties (i.e., soil texture). Biological productivity and seed composition were highly responsive to S fertility in deep sandy soils, but these effects were lost in heterogenous soils containing increased clay content illuviation. Lastly, several oilseed *Brassica* genotypes were evaluated for susceptibility to infection by *Meloidogyne incognita* or the southern root-knot nematode (SRKN) under optimal and sub-optimal S treatments. All *Brassica* genotypes had increased infection rates at sub-optimal S, but *B. carinata* had substantially reduced infection rates compared to low-GSL *B. napus.* There was an inverse relationship between GSLs in the seed and SRKN infection rates that continued when a susceptible host (cotton) was subsequently planted in post-harvest *Brassica* soil. These experiments demonstrate the importance of GSL interaction with seed composition and the potential benefits of integrating *B. carinata* into existing crop rotation systems under no-till or low-till soil management.

CHAPTER 1
INTRODUCTION

Plant-based Renewable Fuel

In the last century, there has been an exponential growth in the consumption of fossil fuel, including a dramatic shift from coal-based energy to natural gas and crude oil (Ritchie & Roser, 2020). According to the U.S. Energy Information Administration, the United States is the largest consumer of petroleum at 20.3%, of which about 70% is attributed to the transportation sector (EIA, 2019). Consequently, the transportation sector is also the largest contributor to greenhouse gas emissions in the US (EPA, 2019). Within the transportation sector, the aviation industry consumes over 18 billion gallons ($\approx 69.2 \times 10^9$ L) of fuel per year despite still recovering from the 2008 recession (Mazareanu, 2020). As the rate of consumption increases, so will the release of greenhouse gas emissions that will prompt a rise of global temperatures, extreme weather events (e.g. flooding, fire, and drought), and sea level rise. The volatility of Brent crude oil markets may be evidence that the United States is too reliant on readily available fossil fuel energy since disruptions can be easily influenced by actual or perceived interference on output productivity (EIA, 2020). This will contribute to an increase in conflict around the world and has prompted the US military to reevaluate existing infrastructure to meet the consequences of this increasing threat on our national security (DOD, 2014).

In an effort to increase energy independence and security, mitigate climate change by reducing the use of finite fossil-based resources and enhance existing agricultural systems, the US Department of Agriculture National Institute of Food and Agriculture (NIFA) Agriculture and Food Research Initiative (AFRI) committed over $183 mil to support integrated coordinated agricultural projects that would support the development of sustainable regional supply chains for advanced renewable fuel and bioproducts (USDA, 2017). This will also take advantage of

increasing the annual growth rate in the biofuel industry since North America is already holding a significant portion of the market share (Shah, 2019).

An important type of renewable fuel is called ‘drop-in’ biofuel that is produced from biomass through biological, thermal, or chemical processes. They are similar in specifications to fuel derived from petroleum and hence require no infrastructure modification in order to be used. Renewable gasoline, renewable jet, renewable diesel are all examples of renewable fuels that can be produced from various sources of biomass. Lipids (purpose-grown lipids, vegetable oils, used cooking oil, animal fats, algae, and others), cellulosic biomass (crop residues, woody biomass, other dedicated energy crops) are sources that can be converted to drop-in renewable fuels through various ASTM processes (Nadkarni, 2016).

Processes that are generally used for conversion of biomass to fuel include hydrotreating, gasification, catalytic conversion of sugars, pyrolysis, and others. Studies have found that the main drivers of cost for the conversion of plant-based oil into biojet fuel are fatty acid content and a lack of technology to increase the efficiency of refinement (Tao et al., 2017). The conversion efficiency of plant-derived oil to diesel and jet fuel is best when it contains high proportions of monounsaturated long-chain fatty acids (i.e., erucic acid; C22:1) and low amounts of polyunsaturated and saturated methyl esters (Ramos et al., 2009). More recently, Applied Research Associates Inc. (www.ara.com), a research and engineering company headquartered in Albuquerque, NM and Chevron Lummus Global developed the Biofuel ISOCOVERSION (BIC) process that can convert lipids into sustainable aviation fuel that is indistinguishable from petroleum (Sapp, 2020). These are goals that also align with the Commercial Aviation Alternative Fuels Initiative (CAAFI) and the U.S. Air Force that allow the drop-in fuel to be used in most of the airplane engines that exist today (Lew & Biddle, 2014).

A Brief Introduction on *B. carinata*

B. carinata A. Braun is a member of the mustard family (Brassicaceae, previously Cruciferae) which includes the model plant *Arabidopsis thaliana*. It is also known as Abyssinian or Ethiopian mustard, kale, or simply carinata. *B. carinata* is thought to have originated by a naturally occurring interspecific hybridization event 10,000 years ago between *B. oleracea* and *B. nigra* (black mustard) in the Ethiopian plateau and up to Kenya. (Dixon, 2006). They are traditionally eaten as a tender leaf vegetable known as 'goman' with the oil being used as lamp fuel (Vaughan & Hemingway, 1959; Westphal, 1975). The Highlands of Ethiopia tend to have a cool and wet climate with well-drained soils with textures ranging from sandy loams to heavier clays, including Ultisols and Alfisols (Deressa et al., 2018). Interestingly, this soil type is also common throughout Northern Florida, where winter-grown *B. carinata* produces high yields comparable to those from its native environment (Chala & Tesfaye, 2017; Seepaul et al., 2019).

The genetic history of *B. carinata* is intertwined with important *Brassica* crops making up the "Triangle of U" which share three genomes amongst each other as described by Nagaharu U[1], (1935). Through *in vitro* chromosome hybridization, he discovered that diploids *B. rapa* (AA, n=10), *B. nigra* (BB, n=8), and *B. oleracea* (CC, n=9) can hybridize into *B. juncea* (AABB, n=18), *B. napus* (AACC, 19), and *B. carinata* (BBCC, n=17). These amphidiploids have both pairs of chromosomes from each parent and, therefore, autogamous (self-pollinating) as opposed to the self-incompatible diploid parents (OECD, 2016). But, despite their close relationship among oilseed *Brassica,* important agronomic traits differ among species. For example, the genetic link to pod shattering resistance was much higher in *B. carinata* than in *B. napus* where

[1] Nagaharu U is actually Woo Jang-choon (1898-1959) who was a Korean-Japanese scientist that escaped Imperial Japan to pursue his passion for teaching and agricultural-based research in genetics and breeding.

there can be up to a 61% yield loss (Raman, 2017; Wang, Ripley, & Rakow, 2007). *B. carinata* also has a high heritability value with seed morphological characteristics and yield (Yohannes & Belete, 2013).

B. carinata possesses many desirable agronomic traits that include a robust root architecture for heat and drought resistance (Rana & Chaudhary, 2013; Rashidi, Majidi, & Pirboveiry, 2017), a refortified pod valve margin that reduces seed shattering and loss of yields at harvest (Zhang et al., 2016), and is mainly self-pollinated so it does not need to rely on pollinators for seed fertilization (Salisbury et al., 2017). *B. carinata* also has a high oil content (more than 40%) and a fatty acid composition that is high in erucic acid content (EAC) and other monounsaturated fatty acids and less than 6% in saturated fatty acids (Cardone et al., 2003). In addition, *B. carinata* also has a high protein content (35-40%), making it a good potential protein supplement source for beef, dairy cattle, poultry, and other animals similar to existing canola-based meal (Xin & Yu, 2014). One of the most important traits is the production of defensive compounds called glucosinolates (GSLs) that make *Brassica* naturally resistant to many pests and soil-borne pathogens (Navabi *et al.*, 2010; Subramanian, Bansal, & Kav, 2005; Uloth *et al.*, 2016).

B. carinata generally has seed with a high oil content (more than 40%) and a fatty acid composition that is rich in erucic acid and other monounsaturated fatty acids with less than 6% saturated fatty acids (Cardone et al., 2003). These traits along with previous studies indicating the possible integration into existing cropping systems for Winter production in Northern Florida, *B. carinata* was selected for further development as an industrial oilseed feedstock for renewable fuels and bioproducts in a collaboration effort called the Southeastern Partnership for Advanced Renewable from Carinata or SPARC (USDA-NIFA, 2017).

The Importance of Sulfur Fertility in *Brassica*

Sulfur (S) is an essential element in a plant's nutrient needs. It is required for the production of essential and non-essential amino acids such as methionine and cysteine that form disulfide bonds in protein and maintain their structure and function (Gilbert, 1951). Sulfur is especially important in *Brassica* because they have high contents of GSLs, an S-containing secondary metabolites. Furthermore, oil and protein quality are strongly linked with plant S status and S availability in the soil (Poisson, Trouverie, & Akmouche, 2019). High order plants have a natural ability to assimilate inorganic sulfur through the stomata in the form of sulfur dioxide (SO_2) and hydrogen sulfide (H_2S) from the atmosphere (Leustek & Saito, 1999). Due to increased regulations over the decades on industrial emissions, atmospheric S has decreased dramatically in Europe and the United States, increasing the risk of S deficiencies (Aas et al., 2019). Symptoms of S starvation in *B. carinata* include leaf mottling that progresses to intercostate chlorosis with a characteristic "spoon-shape" or cupping of leaves that can eventually lead to necrosis if left untreated (Schnug & Haneklaus, 2005).

At later stages of development, plants produce 'white blooms' with smaller flowers and petals that are noticeably lighter in coloration, followed by a reduction in pod development and ultimately yield loss. Sulfur deficiency in oilseed *Brassica* result in lower S contents in shoots, roots, and seeds especially affecting S-containing compounds like GSLs (Blake-kalff et al., 1998; Janzen & Bettany, 1984; Rathke et al., 2005). Previous studies on *Brassica* have shown that they have a positive response to S rates increasing seed total oil content (TOC), erucic acid EAC, and total GSL content (TGC) with increasing S. However, plants may display a dilution effect where there is a reduction in seed content of TOC, EAC, and GSL when overall yields increase (Grant, Mahli, & Karamanos, 2012; Malhi, Gan, & Raney, 2007). The range of S application rates for oilseed *Brassica* is wide (<15 to 60 kg S ha^{-1}) mainly attributed to the

environment rather than species or varietal S requirements (Grant, Mahli, & Karamanos, 2012). High-GSL oilseed *Brassica* is more S-use efficient than low-GSL genotypes (Malhi et al., 2007). Sulfur requirements remain similar despite differences in how much GSLs accumulate in the seed.

The southeastern US includes diverse soil types and land management practices that include intensive-tillage, strip-till, no-till, and pasture which can influence soil quality and microbial biodiversity. Pasture systems with deep-rooted perennial species such as bahiagrass (*Paspalum notatum* Flügge) have the propensity to accumulate organic matter (OM) over time (Matusinsky, Klem, Globe, & Urban, 2008). Most of the soils found in central and south Florida are classified as Spodosols; a poorly drained sandy soil with high OM and low pH. However, Ultisols are the major soil type on 6.9 million acres in northern Florida that often contains subsoils with high clay content, minerals with low reactivity, and base saturation that decreases with depth (Mylavarapu, Harris, & Hochmuth, 2016).

The availability of S for plant uptake can vary dramatically with the soil environment and weather patterns making soil tests for S highly unreliable in many instances (Franzen, 2018). When the soil texture contains high proportions of sand with little clay or OM, sulfate sulfur (SO_4-S) will readily leach after heavy rains. However, plants can overcome S scarcity if the roots can reach the clay layer (i.e., clay illuviation) that accumulate in Ultisol soils at 15 to 46 cm (≈ 6 to 18 in) below the soil surface (Roberson, 2012). It is also important to balance S inputs with nitrogen (N) to reduce the effects of S starvation (Anjum et al., 2012). About a 7:1 ratio between N and S application rates is optimal for plant growth and yields in *B. napus* (Janzen & Bettany, 1984) and 3:1 in *B. carinata* (Bhattarai, 2019; Seepaul et al., 2019). Without accurately measuring S in the soil there will inadvertently be an error for N applications that will either

increase S deficiency symptoms or the chances of leaching when under or over applied, respectively (Karamanos, Goh, & Flaten, 2007). The dynamics of S movement in certain soil environments require more research in a location-specific manner, especially for *Brassica*.

Glucosinolates in *Brassica*

Glucosinolates are S-rich secondary compounds that are constitutively present throughout the various tissues and organs of plants in the Brassicaceae family. The main function of these compounds is to aid in plant defense from herbivores and pathogens by becoming biologically active through hydrolysis by a GLS-specific enzyme called myrosinase into isothiocyanates (ITC) and other derivatives when cells are damaged (Redovnikovic et al., 2008). The quantity of GSLs present in plant tissue can vary with genetics and environment, but also fluctuate when experiencing biotic or abiotic stress (Halkier, 2016). *Brassica* contains higher quantities of GSLs in their shoots and roots early in their development that ultimately accumulate in the seeds at plant maturity (Bhandari, Jo, & Lee, 2015; De March, McGregor, & Seguin-Shwartz, 1989).

Glucosinolates in seeds are not made *in situ*, but instead, they are translocated by GSL-specific transporter proteins through phloem loading pathways (Nour-eldin et al., 2012). This behavior is consistent with the optimal defense theory (ODT), which posits that plants produce and distribute defensive compounds (i.e., GSLs) in anticipation or in response to threats to their fitness over time. This is an adaptation for a changing environment responding to biotic and abiotic stress, but with the tradeoff in energy allocation between defense and growth. The glucosinolate-myrosinase defensive mechanism is an example of ODT that is advantageous when plants are growing in hostile environments. Glucosinolates are initially prioritized to the leaves and roots during the vegetative stage of development but then accumulate in reproductive organs and ultimately in the seeds at maturity (Tsunoda, Krosse, & van Dam, 2017). The accumulation of GSLs in seeds at plant maturity will provide a layer of protection against pests

and pathogens, increasing the chances of survival to the next generation of plants. It is estimated that the metabolic cost of GSL production in *A. thaliana* requires an increase of photosynthate of at least 15% depending on the type being produced (Bekaert et al., 2012).

The two main ways to extract the oil out of *Brassica* seed are by pressing them or organic extraction, usually with hexane. In cold-pressing, the seeds are mechanically crushed, producing meal or cake that contains high GSL but similar crude protein content compared to those that are solvent extracted (Raikos et al., 2017). This method is economically practical when the seed contains low quantities of GSLs. However, high-GSL reduce meal palatability and can become toxic to animals by interfering with iodine uptake that could lead to goiters and other disorders (Alexander et al., 2008). Therefore, the international community has universally set requirements to < 30μmol g^{-1} of GSL in *Brassica* seed meal for use as animal feed (Alexander et al., 2008). Organic extraction has the advantage of removing an order of magnitude of intact GSLs from the seeds (Shahidi et al., 1988). Meal from *B. carinata* seeds extracted with hexane has shown to increase the bodyweight of heifers by a factor of 3 over 70 days (Schulmeister et al., 2019). Solvent extraction is a highly effective method in removing GSLs and often used repeatedly for GSL extraction methods, but the most desired way to reduce GSLs in seeds is through breeding low-GSL varieties (Nega & Woldes, 2018). However, breeding for low-GSL *B. carinata* (i.e., < 30μmols g^{-1}) has not yet been realized, and studies show that this task will be difficult due to its narrow genetic background (Alemayehu & Becker, 2002).

There are three main classes of GSLs derived from various amino acids. These include indole-GSL from tryptophan, aromatic-GSL from phenylalanine or tyrosine, and aliphatic-GSL from valine, isoleucine, leucine, or methionine (Met). Most of the GSLs that accumulate in the seeds of *Brassica* are in a class that includes an aliphatic functional group (Velasco et al., 2008)

with Met-GSL or sinigrin being particularly detrimental to the behavior of herbivores (Raybould & Moyes, 2001). Met-GSL (i.e., sinigrin) is one of the most common GSL in *Brassica* and has been identified in the roots and shoots of *B. carinata* (Gimsing & Kirkegaard, 2006).

There has been success in bringing down its production to undetectable levels through S scarcity (Falk, Tokuhisa, & Gershenzon, 2007). Repressor proteins reduce aliphatic GSL biosynthesis with increasing S scarcity (Aarabi et al., 2016). Therefore, it is possible to reduce the GSL content of oilseed *Brassica* through mild forms of S scarcity and without affecting TOC. Low GSL *B. napus* have been developed by incorporating a trait from a Polish variety (cv. 'Bronowski') discovered before WWII that disrupts the biosynthesis of Met GSLs (Finlayson, Krzymanski, & Downey, 1973; Knodel, Kandel, & Berglund, 2011). This increases the accumulation of GSL intermediates in plant tissues and disrupts translocation to the seed since only intact GSL can transport to the seeds (Bloem, Haneklaus, & Schnug, 2007). This may be why there are not any clear indications that seed TGC has a direct relationship to S requirements despite differences in seed TGC between species (Malhi, Gan, & Raney, 2007).

A different approach would be to maintain high levels of GSL in seeds if the end-use of the meal is used for soil amendments instead as an animal feed supplement. Several studies have reported *Brassica* seed meal's potential as an organic fertilizer (Balesh, Zapata, & Aune, 2005), in controlling weeds (Al-Khatib, Libbey, & Boydston, 1997; Boydston, Anderson, & Vaughn, 2008), and suppression of many types of soil-borne pathogens in a process called biofumigation (Gimsing & Kirkegaard, 2008). The volatility and toxicity of ITC have drawn interest as a soil fumigant and methyl bromide alternative in commercial production systems and not limited to organic farming systems. Vapam® is a soil fumigant used to control weeds, fungi, and nematodes and contains the active ingredient metam-sodium that is converted into methyl-ITC in

the soil (Matthiessen & Kirkegaard, 2007). They are synthetically produced to replace methyl bromide, a very effective broad-spectrum soil fumigant that is now phased out due to concerns on ozone depletion (Bruce, 2014). *B. carinata* seed meal high in GSLs could be a non-synthetic methyl-bromide alternative with similar action as its synthetic counterpart.

B. carinata and Plant Parasitic Nematodes

Root-knot nematodes (RKNs) are obligate plant endoparasitic roundworms of the genus *Meloidogyne* whose common name is derived from the gall or knot-like appearance of established feeding sites along the roots of plants. They are found in tropical and subtropical regions around the world with a vast range of plant hosts making them one of the most economically important plant pathogens in agriculture (Sasser, 1979). The most widely cited species is *M. incognita* (Chitwood 1949) or the southern root-knot nematode (SRKN) that often accounts for the majority of RKNs affecting crops (CABI, 2020). Despite limitations of mitotic parthenogenic reproduction, SRKN genome duplications and horizontal gene transfers that include cell wall degrading enzymes and repetitive elements have undoubtedly contributed to their adaptive success (Castagnone-Sereno, Danchin, Perfus-Barbeoch, & Abad, 2013).

The environmental conditions of the southeastern US are conducive to the survival and proliferation of SRKN. This includes soil texture with high sand content (Jaraba, Rothrock, & Kirkpatrick, 2014), moderate to high soil temperatures (Nardacci & Barker, 1978), and susceptible hosts that include vegetable and agronomic crops such as cotton (*Gossypium hirsutum* L.) or many species of native plants found throughout the region (Davis & Webster, 2005). Fields infested with SRKN reduce the efficiency of nutrient and water uptake resulting in plants with stunted growth and reduced yields (Grabau, 2017).

B. carinata has a low to moderate level of susceptibility to SRKN Race 1 and Race 3, respectively (E. C. Bernard & Allen, 1993; Liébanas & Castillo, 2004; Mcsorley & Frederick,

1995). *M. incognita* may bypass the GSL defense system through intracellular migration avoiding cell rupture as was observed in *Arabidopsis* (Wyss, Grundler, & Münch, 1992). The reduction of Met-derived GSLs, the uncertainty of soil tests, and the sandy soil environment in the region may exacerbate the effects of S deficiency on plants and influence *B. carinata*'s ability to respond effectively to SRKN infection. Conversely, high GSL *B. carinata* could play an important role in the suppression of plant-parasitic nematode suppression in crops following it in a rotation system.

The Overarching Project Goals

The overarching theme of this dissertation is the significance of GSL in *B. carinata* and crop rotations within the southeastern US context given the application of *B. carinata* as an industrial oilseed feedstock for renewable fuel and protein source in animal feed supplements and other byproducts (Figure 1-1). In keeping with the theme, this research aims to understand S nutrition in *B. carinata* as it impacts the production of GSLs. First, we set out to understand GSL production and allocation patterns in *B. carinata* tissues at different stages of development in various soil types. Then, we evaluate S requirements in *B. carinata* in various locations in northern and central Florida with similar climates, but different soil properties. Greenhouse S trials helped infer the relationship between seed contents (TOC, EAC, CPC, and TGC) and S fertility among *B. carinata* and *B. napus* genotypes that ranged in seed GLS content from low to high. Finally, the same genotypes were inoculated with the SRKN under optimal and sub-optimal S fertility to test susceptibility. Cotton was subsequently planted to test the ability of these genotypes to suppress SRKN over time.

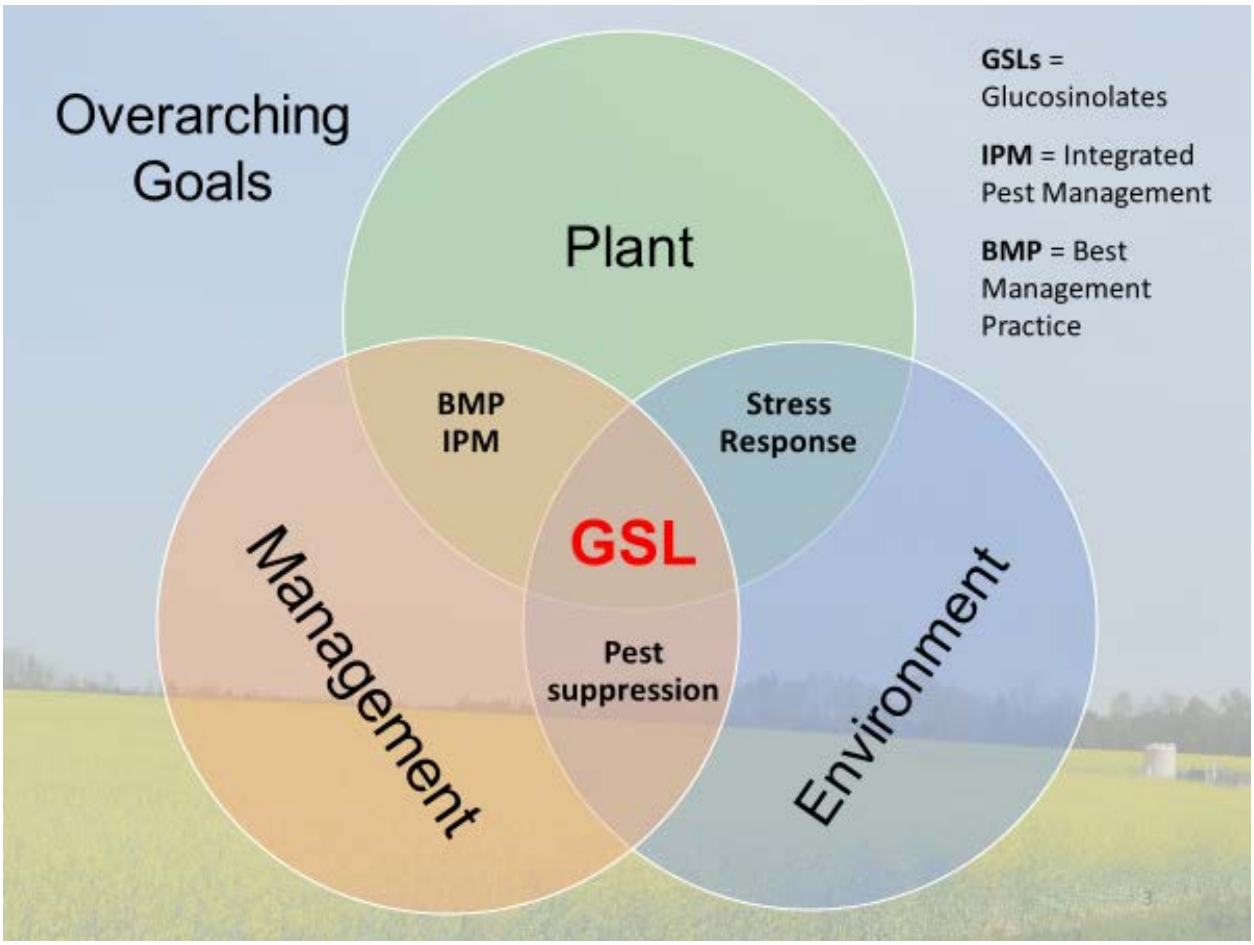

Figure 1-1. Comprehensive goals set for research on *B. carinata.* It incorporates impacts of glucosinolate (GSL) to aspects of plant physiology, responses to biotic and abiotic stress, and management of *B. carinata* in the southeastern US.

CHAPTER 2
GLUCOSINOLATE ALLOCATION PATTERNS IN *BRASSICA CARINATA*

Background

Oilseed *Brassica carinata* A. Braun has been introduced into the southeastern US for winter production as a feedstock for sustainable aviation fuel (Seepaul, Wright, & George, 2016). This region encompasses a variety 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 supports mitigating the negative effects of intensive farming by establishing programs like low-input sustainable agriculture (LISA), best management practices (BMP), and other conservation methods (Pimentel et al., 1989). *B. carinata* has desirable agronomic traits that allow for a seamless integration into existing crop rotation systems. They include a robust root architecture (Rana & Chaudhary, 2013; Rashidi et al., 2017), a refortified 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, Bansal, & Kav, 2005; Uloth et al., 2016).

Brassica plants initially contain higher quantities of GSLs in their leaves and roots during the vegetative stage of development that is then directed 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 areas suffering from attack by herbivores and plant pathogens (Keith & 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 deemed having 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 complex regulatory network between plant development, changes to its environment, and threats from pathogens.

It is thought that GSLs 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 varying OM will influence the production and allocation of GSLs. Our hypothesis states that *B. carinata* also follows the criteria of ODT and that the relative concentrations of GSLs will vary with plant tissues and stage of development. In addition, we hypothesize that there will be increased GSLs when *B. carinata* is grown in soils with increased OM (i.e., microbial diversity) than in sandier soils possibly altering oil quality that include 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 impacts to 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 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). They 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 (see Table 2-1), but all were fertigated through drip three times a day (0800, 1200, and 1600) with Hoagland nutrient solution (Hoagland & Arnon, 1938).

Tissue Sampling and Preparation

Five plants per soil type 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 4^{th} and 5th mainstem leaf during the rosette stage (30 DAP). The sampling site moved higher up the main stem with every subsequent sampling event in order to acquire leaves of similarcharacteristics. These characteristics include leaves that are young and fully expanded. Roots were sampled from the larger root mass after they were rinsed free of 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 (i.e., freeze-dried) 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 (i.e., vortex) before returning them 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 saturating it 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 run dry, 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) & Kumar et al. (2004). In separate vials, 300 μL 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. Seeds contents that include 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 (formally Agrisoma Biosciences) using a FOSS XDS^{TM} 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 5 replications. The two factors were soil types with 3 levels (BP, CF, and SP) and 4 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, $\alpha = 0.05$) 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.

Results

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 2-2.). 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 2-3). 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=0.9446), but no differences among the soil types. Soil type did not affect seed yields (12.8 g plant^{-1}) or harvest index (0.081). Although there were no interactions, Figure 2-1 has the average biomass of individual tissue and total at each stage of development.

Glucosinolates in Shoots and Roots

There were no interactions with soil type and stage of development on total glucosinolate content (TGC) in *B. carinata*. Main effects were found in stage of development or soil type, but the significance varied with each tissue (Table 2-2.). Leaves averaged 78.0 μmol g^{-1} from the rosette to the bolting stage and then decreased to 38.5 μmol g^{-1} at flowering (Figure 2-2.). TGC in the stem maintained a low concentration (≈ 11.2 μmol g^{-1}) from rosette to flowering but then

increased by a factor of 6.5 at maturity (72.7 μmol g^{-1}). The roots did not show significant change to TGC throughout stage of development and averaged 38.5 μmol g^{-1}. Soil type had no effect on TGC with the leaves, stems, and seeds in A110 averaging 64.2, 26.1, and 161.0 μmol g^{-1}, respectively. This included seed TGC that averaged 161.0 μmol 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 2-2). The main effects of soil type were found with roots only (Table 2-4). There was a doubling of root TGC in BP soil (50.9 μmol g^{-1}) compared to SP (25.2 μmol g^{-1}).

Glucosinolate, Oil, Erucic Acid, and Protein Contents in Seeds

Seed TGC, TOC, EAC, or CPC did not differ among soil types (Table 2-5.). Seeds contained a TGC average of 168.5 μmol g^{-1} with no difference from the colorimetrically determined TGC of 161 μmol g^{-1}. Seeds of A110 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).

Discussion

Changes in total GSL content (TGC) throughout plant development and with soil type 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, Walker, & Griffiths, 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, Burow, & Wittstock, 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, Sørensen, & Sørensen, 2004). They found that the highest concentration of TGC was in the reproductive structures in the shoots of *B. nigra* and *B. juncea* (120 μmol g^{-1}), while it was mainly concentrated in the roots *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 seed for redistribution to reproductive organs.

We found that variations with soil type did not influence shoot biomass or seed yield under excess 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 sand. The subtle responses of root TGC and soil type may be due to the induced production of GSLs in response to soil microbial microbiomes compared to sand (Van Dam, Tytgat, & Kirkegaard, 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 main taproot (Tsunoda et al., 2017). BP soils contained higher organic matter and potentially increased microbial diversity

compared to SP soil. Without any potential threats to the roots in SP soils, *B. carinata* may not need to allocate energy to increase its root TGC. To help evaluate this behavior, future studies should contain similar soils, but pasteurized so that the possible effects of soil microbial communities can be considered.

Finally, 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 between the different classes of GSLs. In this experiment, we used the wavelength of 405 nm to estimate TGC as was 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 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 non-sinigrin GSLs in the roots with variable functional groups were not accurately captured in this semi-quantitative approach.

Conclusion

The introduction of *Brassica carinata* into the southeastern US cropping system for Winter production has brought attention to the production of GSLs. These defensive compounds have provided plants with natural resilience against pests and diseases. Other plants in the family Brassicaceae have demonstrated a change in TGC with the stage of development and in response to soil-borne pathogens. However, this can change drastically with genotype and environment. Therefore, we investigated the accumulation of TGC in *B. carinata* cv. AAC A110 in various plant tissues, throughout plant development, and in soil types from various land management

practices. We found that *B. carinata* increases biomass accumulation and root TGC when grown in BP soil compared to soil derived from areas of intensive or conventional farming (CF) or sand from a local pit (SP) without affecting seed yield. The transition from vegetative to reproductive growth decreased leaf TGC indicating that it may be the primary source of GSLs being redistributed to the seeds by the time *B. carinata* reaches maturity. It is clear from this study that the GSL allocation patterns closely resemble those proposed by the optimal defense theory and could be an important trait for crops that may experience disease pressure.

Table 2-1. Analysis of the three soil types used to grow *B. carinata*. Soil from a bahiagrass pasture (BP), conventional farming (CF), and sand pit (SP).

					ppm†				mg kg^{-1}		
Soil	pH	OM (%)	EC (dS/m)	N (%)	P	K	Mg	Ca	Cu	Mn	Zn
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

Table 2-2. 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	-	-

Root to shoot ratio (R:S) †Derived using colorimetric methods

Table 2-3. 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 ($\alpha = 0.05$).

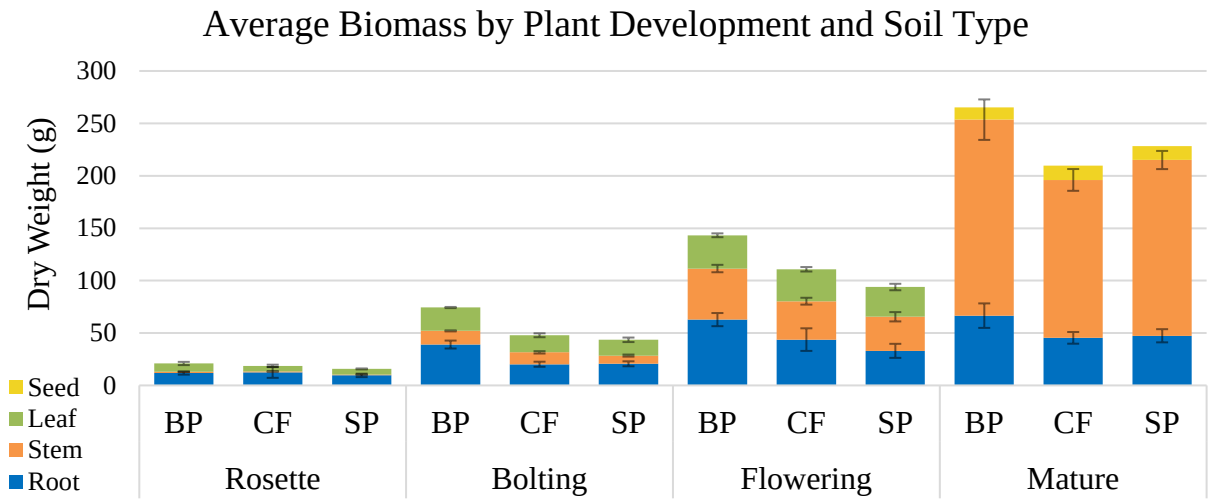

Figure 2-1. Average individual tissues and total biomass of *B. carinata* through plant development grown in soil collected from various land management practices. They include bahiagrass pasture (BP), conventional farming (CF), and sand pit (SP).

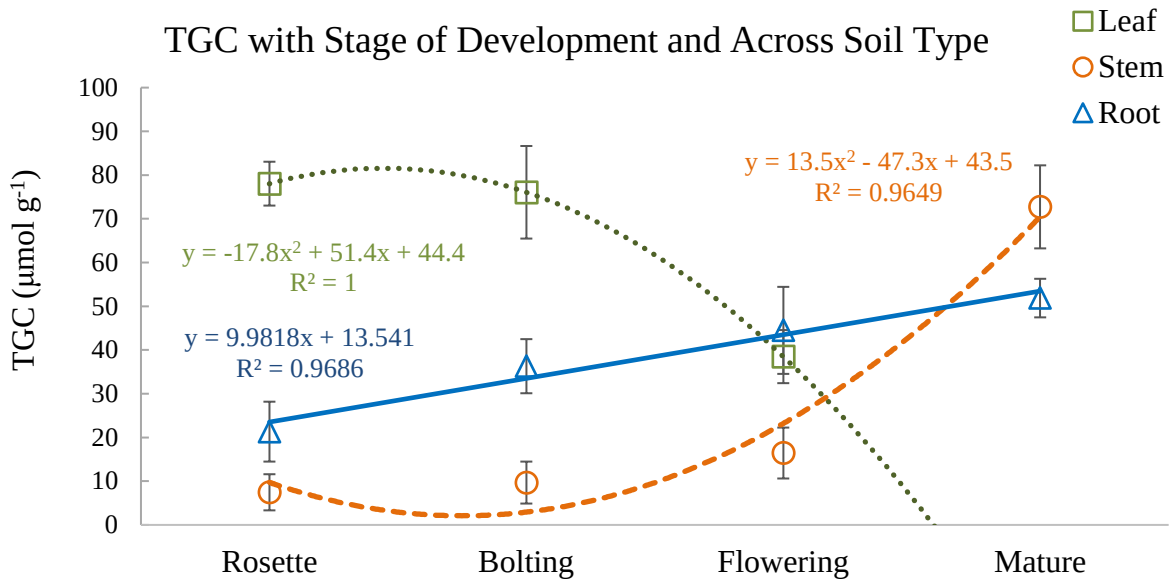

Figure 2-2. Total glucosinolate contents (TGC) of leaf, stem, and roots of *B. carinata* tissue with plant development. Each plant tissue has a corresponding line of best fit.

Table 2-4. Effect of soil type on the total glucosinolate contents (TGC) across the stage of development in *B. carinata*.

	TGC† (μmol 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 ($\alpha = 0.05$). Soil type includes those collected from bahiagrass pasture (BP), conventional farming (CF), and sand pit (SP).

Table 2-5. Effect of soil type on seed glucosinolate, oil, erucic acid, and crude protein content in *B. carinata*.

	Seed Content†							
	TGC (μmol 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 (C22:1ω9) 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).

CHAPTER 3
THE EFFECTS OF SULFUR AVAILABILITY ON GLUCOSINOLATE, OIL, AND PROTEIN CONTENT IN OILSEED *BRASSICA CARINATA*

Background

The United States Department of Agriculture (USDA) through the National Institute of Food and Agriculture (NIFA) supports research to develop sustainable biofuels and improve the bioeconomy including the recently funded Southeast Partnership for Advanced Renewables from Carinata (SPARC) (Seepaul et al., 2019; USDA, 2017). The conversion efficiency of plant-derived oil to diesel or jet fuel is best when there are high proportions of monounsaturated long-chain fatty acids (i.e., erucic acid; C22:1) and low amounts of polyunsaturated and saturated methyl esters (Ramos et al., 2009). *B. carinata* has a fatty acid composition that fits these requirements due to the high proportion of erucic acid content (EAC) in the seeds when compared to canola (*B. napus*) (Cardone et al., 2003). The overall goal is the commercialization of oilseed *Brassica carinata* as a biofuel feedstock into the southeastern cropping system through collaboration efforts by regional land-grant universities, USDA/ARS units along with private industry partners. SPARC objectives include crop improvement and management, development of the supply chain and outreach programs, co-products discovery, and byproducts research such as the meal for a dietary supplement for cattle (USDA-NIFA, 2017).

The inclusion of *Brassica* seed meal into the diets of livestock provides an excellent source of crude protein (CP) and sulfur-rich amino acids, but also increases the unsaturated fatty acids in milk fat and reduces fungal contamination of feed rations (Manoj K Tripathi & AS, 2017). Providing protein-rich *B. carinata* seed meal along with grass hay can significantly increase the weight of heifers in 70 days (Schulmeister et al., 2019). However, *Brassica sp.* produces defensive compounds called glucosinolates (GSLs) to defend against herbivory that can

reduce palatability of the seed meal or can become detrimental to the health of animals, so it is often regulated at ~<30 μmol g^{-1} (Nega & Woldes, 2018; M.K. Tripathi & Mishra, 2007).

Glucosinolates are S-rich secondary compounds that are constitutively present throughout the various tissues and organs of plants in the Brassicaceae family. The main function of these compounds is to aid in plant defense when cells are damaged from herbivores and soil-borne pathogens by becoming biologically active through hydrolysis using GLS-specific enzyme (i.e., myrosinase) into bioactive isothiocyanates (ITCs) and other derivatives (Redovnikovic et al., 2008). The quantity of GSLs present in plant tissue varies with genetics and environment, but also fluctuate when experiencing biotic or abiotic stress (Halkier, 2016). Initially, *Brassicas* accumulate GSLs in the leaves or roots during vegetative growth but then are redistributed to reproductive organs and to the seed at plant maturity (Bhandari et al., 2015). This type of defensive system is consistent with descriptions of the optimal defense allocation theory (ODT) where GSL are allocated to the most valuable and vulnerable organs throughout plant development and in response to threats that could risk biological fitness (Tsunoda et al., 2017).

For reasons not completely clear, this is not the case for *B. napus* (i.e., canola). A low GSL Polish variety called Bronowski was discovered in the 1970s, which allowed researchers to integrate this trait into existing high yielding varieties in which all commercial varieties are derived from today (Finlayson et al., 1973). Breeding for low GSL *B. carinata* has not yet been realized and studies show that this task will be difficult due to its narrow genetic background (Alemayehu & Becker, 2002). However, managing sulfur (S) availability for plant uptake may be an alternative method for reducing GSL in *B. carinata* seeds.

Most of the GSLs that accumulate in the seeds of *Brassica* are often in a class that includes an aliphatic functional group (Velasco et al., 2008). Aliphatic GSL derived from the

amino acid methionine (Met) has been particularly detrimental to herbivores (Raybould & Moyes, 2001). Met-GSL (i.e., sinigrin) is one of the most common GSL found, and S may be one way to manage its production and seed accumulation, as has been observed with many S deficiency studies in *Brassica* (Falk et al., 2007). In *B. carinata*, sinigrin was the most abundant GSL in the seeds and made up about half of the GSLs in the roots (Gimsing & Kirkegaard, 2006).

In this experiment, we hypothesize that limiting the amount of plant-available S will reduce the amount of total GSL in the seeds of *B. carinata* as was previously demonstrated in other species. We also included various *B. carinata* genotypes having a range of total GSL content (TGC) in seeds with low-GSL canola to evaluate the effect of S availability to seed TGC and S requirements for maximum yields. In addition, we analyzed interactions between seed TGC and other components such as oil and meal quality in response to S availability.

Materials and Methods

Plant Materials and Management

This 2-year greenhouse trial was conducted at the UF/IFAS North Florida Research and Education Center located in Quincy, FL (30° 32’ 44.7036’’ N, 84° 35’ 41.0820’’ W). The 1st year trial (Year 1) was planted on December 28th, 2016 and harvested June 7th, 2017 while the 2nd year trial (Year 2) on December 15th, 2017 and harvested on May 25th, 2018. Average relative humidity was 71.1% with evaporative cooling and propane heating provided when temperatures rose above 30°C or below 5°C, respectively. Three *B. carinata* genotypes included two that are commercially available (Avanza 641 and AAC A120) and one experimental line (AGR 137) representing a range of TGC in seeds (Table 3-1.) and acquired from NuSeed (formally Agrisoma Biosciences). These were compared to a low GSL (< 30 μmol g^{-1}) *B. napus* cv. Canterra 1918. They will be referred to as Canterra, Avanza, A120, and AGR137 hereafter.

Seeds were planted in 7.65 L tree pots (Stuewe and Sons, Tangent, OR 97389 USA) using fine sand as the growth medium with pH of 6.1 and containing negligible organic matter and nutrients, but supplemented with 15 ml of full-strength Hoagland nutrient solution (except S) 4 times a day and increasing at an average rate of 15ml every 3 weeks. This was set up as a randomized complete block design with 4 S rates that included 0, 33, 66, and 100% S with an estimated magnesium sulfate ($MgSO_4$) application using the formula of y = 200x, where y is grams of $MgSO_4$ and x is the percent S rate. Total S can then be estimated by multiplying the amount of $MgSO_4$ by 26.6%.

Sampling

During bolting, tissue samples from the 12^{th} and 13^{th} mainstem leaf were collected and immediately frozen in -80°C freezer then lyophilized (freeze-dried), ground, and sent for CNS analysis (Dubeux Lab at UF/IFAS NFREC, Marianna, FL). Seeds were collected at agronomic maturity, dried to <8% moisture, and weighed. Seed contents were quantified using near-infrared reflectance spectroscopy (NIRS) analyzed by Agrisoma Biosciences Inc. using a FOSS XDSTM Rapid Content Analyzer (FOSS Inc., Eden Prairie, MN) with the spectra evaluated using the ISIscan program (FOSS Analytical, Hilleroed, Denmark).

Experimental Design and Statistical Analysis

The study was set up as a randomized complete block design split by sulfur treatment with 6 replications per treatment. The treatments were factorial combination of S rates (4 levels), *Brassica* genotypes (4 levels), and year (2 levels). A 2-way ANOVA was generated using JMP Pro 15 (SAS Institute Inc., Cary, NC) with S rate and genotype as fixed effects and year as a random variable. The 0% S rate was excluded from seed component analysis between all *Brassica* genotypes due to the low seed productivity. However, Avanza and A120 will be subsequently analyzed independently (i.e., subset) since they produced enough seed for NIR

characterization at the 0% S rate. Q-Q plots and Anderson-Darling (AD) test was used to evaluate whether the residual was normally distributed. Multiple comparisons of means were analyzed using Tukey Honest Significant Difference (HSD) test (α=0.05). All correlations between yield and seed contents have been analyzed using the spearman's ρ nonparametric analysis.

Results

Seed Yields

There were no interactions between genotype and S rates on seed yields across trial-years, but main effects were observed with each factor (Table 3-2.). For *B. carinata,* Avanza had the greatest seed yield (37.1 g plant^{-1}) followed by A120, and AGR137 (Figure 3-1a.). Canterra produced yields similar to A120 averaging ≈34.4 g plant^{-1}. Seed yield increased quadratically ($y = -33.8x^2 + 71.0x + 4.8$; $R^2 = 0.9732$) with S rates across genotypes. The maximum yield was estimated at 42.1 g plant^{-1} at the 105% S rate (≈210 g $MgSO_4$ or 47.5 g of S) (Figure 3-1b.).

Seed Components

Total GSL contents (TGC)

There was an interaction between genotype and S rates to total GSL content (TGC) when excluding the 0% S rate (due to low seed productivity). AGR137 produced the greatest seed TGC among all *Brassica* genotypes averaging ≈162.1 μmol g^{-1} throughout all S rates (Figure 3-2a). Canterra produced the least seed TGC (≈24.4 μmol g^{-1}) compared to all *B. carinata* genotype, also without differences among S rates. Avanza and A120 had similar seed TGC at the 33% and 66% S rate (≈114.7 μmol g^{-1}) but diverged at the 100% S rate accumulating 118.1 and 138.4 μmol g^{-1}, respectively. Distinction between seed TGC occurred at the 100% S rate for all *Brassica* genotypes. When accounting for yields at the 100% S rate, Avanza produced the most

seed GSLs accumulating 7.1 mmol of GSL per plant followed by A120 (6.2 mmol), AGR137 (5.2 mmol), and Canterra (1.1 mmol) (Figure 3-2b).

An interaction was observed in the analysis of the subset that comprised *B. carinata* genotype's Avanza and A120 (Table 3-3). As previously noted, significant differences in TGC were found only at the 100% S rate but similar at the 0, 33, and 66% S rate averaging 34.1, 108.2, and 121.2 μmol g^{-1}, respectively (Figure 3-2c). Both genotypes had a quadratic relationship between S rates and seed TGC. For Avanza, it was estimated that the maximum seed TGC was 129.0 μmol g^{-1} at the 72.8% S rate (≈152.9 g $MgSO_4$ or 40.7 g of S) using the equation $y = -0.0172x^2 + 2.5045x + 37.845$ (R^2=0.9886). This was similar for A120 with max seed TGC estimated at 136.9 μmol g^{-1} at the 87.4% S rate (≈183.6 g $MgSO_4$ or 48.8 g of S) using the equation $y = -0.0132x^2 + 2.3084x + 35.945$ (R^2=0.9534). Although there were no interactions in total GSL yield (per plant) between Avanza and A120 and S rate, there were main effects. When accounting for seed production, average total GSLs is greater with Avanza (4.3 mmol) compared to A120 (3.7 mmol) and increases with S rate (Figure 3-2d.). The relationship between the accumulation of GSL and S rate is quadratic ($y = -0.0003x^2 + 0.0925x + 0.5302$, $R^2 = 0.9880$) but with an estimated maximum of 7.6 mmol (per plant) at the 154% S rate.

Total oil contents (TOC)

No significant interactions were observed with TOC in response to S rates and genotype. There was a main effect of genotype to TOC in seeds across S rate and trial years (*P*=<0.0001). Canterra produced the greatest seed TOC (40.1%) followed by Avanza and A120, and AGR137 (Figure 3-3a). No TOC interactions or main effects were observed between the subset of Avanza and A120 genotypes and all S rates across trial-years averaging 31.5% (±3.0 SE) TOC in seeds. However, interactions were observed when accounting for total oil yields per plant between Avanza and A120 and S rates (*P*=0.0031). Avanza and A120 increased the amount of oil with S

rate with Avanza accumulating 5.5 g at the 100% S rate compared to 13.5 g of oil per plant in A120.

Erucic acid contents (EAC)

There were no interactions, but the only main effects were observed with EAC and genotype and S rate. Avanza and AGR137 produced seed with the greatest EAC (≈40.0% EAC) followed by A120 and Canterra (Figure 3-3a.). Also, EAC increased from 37.4% at the 33% S rate to 38.8% at the 66% S rate but did not significantly increase thereafter. Interactions between genotype and S rate on total erucic acid yield showed a general increase with S rate. Avanza produced the greatest erucic acid yield (per plant) at each S rate with the highest quantity (7.8 g erucic acid) at the 100% S rate (Figure 3-3b). Despite the high seed EAC of AGR137, the yield was the lowest among the *Brassica* genotypes due to low seed production.

When a subset containing only Avanza and A120 as the genotypes but having all 4 S rates analyzed, an interaction was observed in both the EAC and total erucic acid yield. Distinctions between genotypes were observed at the 100% S rate with Avanza producing the greatest EAC (43%) and erucic acid yield (7.8g) compared to A120 (Figure 3-4c and Figure 3-4d). The relationship between EAC and S rates in Avanza was quadratic ($y = -0.002x^2 + 0.3141x + 31.257$, $R^2 = 0.9454$) with maximum EAC estimated at 43.6% at the 78.5% S rate. Erucic acid yield was closer to a linear relationship for Avanza ($y = 0.0597x + 1.7334$, $R^2 = 0.9057$) than it was for A120 ($y = -0.0003x^2 + 0.0668x + 1.579$, $R^2 = 0.9935$) whose maximum yield was estimated at 5.3 g of erucic acid at the 111% S rate.

Crude protein content (CPC)

There was not a significant interaction between genotype and S rate in response to seed crude protein content (CPC). *B. carinata* produced 24.6% more protein in seeds compared to *B. napus* (30.4%). AGR137 produced seed with the greatest CPC (41.5%) followed by A120,

Avanza, and Canterra (Figure 3-5a.). S treatment did not affect protein content, but there was a linear goodness of fit between protein and GSL (CPC = 0.09*TGC + 28.8; R^2 = 0.8861) across genotype and year. When accounting for yields, there was a significant interaction between genotype and S rate. Avanza was consistently a top producer from the 33% to the 100% S rate and had the greatest quantity of crude protein per plant than all other *Brassica* genotypes 23.6 g. This was followed by A120 (18.5 g) and both AGR137 and Canterra with the combined average of 12.7 g of crude protein.

Correlations

Yield was only minimally correlated (r=0.2227) with TOC. There was a strong inverse correlation between TOC and TGC (r = -0.9235) exhibiting a linear relationship (TOC = – 0.11*TGC + 43.5; r= 0.8637) across genotype and year. Total oil content also had an inverse correlation to CPC (r= -0.8811) and EAC (-0.4392). The total GSL content of seeds had a strong positive correlation to CPC (r = 0.8515). Moderate correlations were observed between EAC and TGC (r = 0.5552) and again with CPC (r = 0.5123).

Discussion

Plants expressed severe sulfur deficiency at the 0% S rate showing extreme leaf chlorosis and cupping in *B. carinata* and the reddish-purple leaf margins of *B. napus*, which lead to delayed maturity and reduced reproductive performance (Figure 3-6). Most oilseed *Brassicas* with moderate S deficiency sometimes resemble those experiencing N deficiency via leaf chlorosis (Gilbert, 1951), but also include leaf mottling and the appearance of reddish-purple tint around the margin due to the accumulation of anthocyanins in *B. napus* (Schnug & Haneklaus, 2005). Leaves can become highly chlorotic, forming a distinctive spoon-shaped leaf morphology (i.e., cupping) when under extreme S deficiency (Haneklaus & Schnug, 1992), especially with the middle and youngest leaves and when under low S but high N fertility conditions (Blake-

kalff et al., 1998). However, the development of S deficient plants was not evident in the early stages of growth (i.e., rosette stage) as in later stages; an observation that has been documented in other studies (Abdallah et al., 2010).

Sulfur deficiency was also apparent by the low seed productivity at the 0% S rate. Only Avanza and A120 were able to produce seeds at the 0% S rate with the low and high GSL genotypes (i.e., Canterra and AGR137) producing insignificant seed yield. Since no S was included at the 0% rate, this suggests that Avanza and A120 are better adapted to scavenge and assimilate S from the environment. *Brassica* can assimilate S from H_2S or SO_2 in the atmosphere to supplement its requirements for growth. A study on *B. juncea* and *B. rapa* found that seedlings can acquire sufficient S at the 0.25 μl L^{-1} rate without fertilization of sulfates (Aghajanzadeh, Hawkesford, & De Kok, 2016). They also observed a reduction of GSL with decreased sulfate availability, but the GSL composition (i.e., profile) was not affected when exposed to H_2S and SO_2. Avanza and A120 were able to produce seeds at the 0% S rate, which was roughly 1/3 of their seed TGC capacity with adequate S fertility. Future studies on *B. carinata* S use needs to account for this source to fully account for seed yield and GSL content.

Brassica species follow the optimal defense theory where defensive compounds (i.e., GSLs) are initially concentrated in the roots and shoots, but then accumulate in the seeds at maturity to increase the fitness for the next generation of plants (Tsunoda et al., 2017). Evidence suggests that GSLs are transported to the seeds via GSL-specific proteins rather than being produced in situ (Nour-eldin et al., 2012). However, it is thought that low GSL canola (i.e., Canterra 1918) have incorporated a trait that disrupts the biosynthesis of aliphatic GSL from a Polish variety (*B. napus* cv. ‘Bronowski’) discovered before WWII (Finlayson et al., 1973; Knodel et al., 2011). This increases the accumulation of GSL intermediates in plant tissues since

only intact GSL can be transported to the seeds (Bloem et al., 2007). This could be why we did not see strong indications that seed TGC has a direct relationship to S requirements despite the dramatic difference between species, similar to previous reports (Malhi et al., 2007).

Overall, TGC was high for all *Brassica* genotypes. The production of GSLs is dependent on genetics and responses to environmental stress factors which can drive the TGC in seeds up to a 50-fold increase (Falk et al., 2007), including under heat stress (Cocetta et al., 2018; Guo et al., 2019). Greenhouse conditions near the end of the season were warm with high humidity not allowing plants to use the properties of evaporative cooling efficiently that would ultimately help maintain GSL in the upper tier of their production potential. However, there was a strong correlation between TGC and EAC and CPC. This significantly improved oil quality by increasing the long-chain fatty acid composition needed for increased conversion potential to biojet fuel (Marillia et al., 2014). Avanza produced the greatest seed yield, oil, and almost 30% more erucic acid than Canterra 1918. These responses represent an improvement to the previous commercial variety, A120. This is despite the inverse correlation between TOC and TGC or CPC in seeds. This relationship is consistent with studies specifically looking at oil content in *B. napus* (Gu et al., 2017) and *B. carinata* (Mnzava & Olsson, 1990). Proteins such as myrosinase (Tahl, Erstin, & Undermann, 2009) or napin increase when S availability is limited (Poisson et al., 2019).

This study confirms that *B. carinata* TGC can be reduced through S management without affecting the TOC which may be the result of repressor proteins that have been documented to reduce aliphatic GSL biosynthesis with increasing S scarcity (Aarabi et al., 2016). Although, the TGC in *B. carinata* seed did not fall below 30 μmol g^{-1} as typically required for use as animal feed, studies using *B. carinata* seed meal indicate that oil extracted organically will allow some

flexibility as a protein supplement (Schulmeister et al., 2019). Several studies have reported *Brassica* seed meal's potential as an organic fertilizer (Balesh et al., 2005), weed control (Al-Khatib et al., 1997; Boydston et al., 2008), and suppression of many types of soil-borne pathogens (Brown et al., 1991; Curto, Dallavalle, Matteo, & Lazzeri, 2016; Mazzola, Granatstein, Elfving, & Mullinix, 2001; Oliveira et al., 2010). This could be an alternative use for the seed meal of high GSL seed meal, especially since methyl bromide alternatives using GSL derived products are being used under plastic mulch and in many organic farming practices as a biofumigation (Guerrero-Diaz et al., 2013).

Conclusion

The fatty acid profile of oilseed *Brassica carinata* has prompted interest as a potential biofuel feedstock for the aviation industry. In this study, we evaluated how sulfur (S) fertility affects TGC, total oil content (TOC), erucic acid (C22:1) content (EAC), and crude protein content (CPC) in three *B. carinata* genotypes and one low GSL canola (*B. napus*). A 2-year study found that decreasing S fertility did not affect TOC but did affect other seed traits. There were inverse correlations between TGC and TOC and direct relationships between TGC and EAC or CPC. Avanza 641 yielded the greatest quantity of seed, oil, erucic acid, and GSL of all genotypes despite having seeds with higher TGC and lower TOC compared to canola. We also observed that only Avanza 641 and AAC A120 produced seed at the 0% S rate. Although S scarcity did not reduce TGC below regulated standards (<30μmol g^{-1}) in *B. carinata*, organic extraction of the oil can still be used as a protein supplement or the high GSL seed meal could find a market as a fertilizer and suppressor of weeds and soil-borne pathogens in commercial and organic production systems.

Table 3-1. *Brassica* genotypes of 2016-2017 and 2017-2018 sulfur trials with expected range of seed GSL content.

Species	*Brassica* Genotypes	GSL† (µmol g^{-1})
B. napus	Canterra 1918	< 30
B. carinata	Avanza 641	40 - 60
B. carinata	AAC A120	50 - 70
B. carinata	AGR137	100 - 140

† Expected range per genotype based on previous data provided by NuSeed

Table 3-2. P-values of seed yield and components in responding to genotype (G) and S rates (S) with interactions (GxS) following a 2-way ANOVA. Total yield of individual seed components (per plant) is in parentheses.

		Seed Components† (x Yield)			
Factor	Yield	TGC	TOC	EAC	CPC
Genotype	<.0001	<.0001	<.0001	<.0001	<.0001
		(<.0001)	(<.0001)	(<.0001)	(<.0001)
S Rate‡	<.0001	<.0001	0.5279	<.0001	0.2600
		(<.0001)	(<.0001)	(<.0001)	(<.0001)
G x S	0.1244	0.0285	0.5805	0.5509	0.9134
		(0.0002)	(0.0529)	(0.0120)	(0.0176)

† Response includes total GSL content (TGC), total oil content (TOC), erucic acid content (EAC); crude protein content (CPC). ‡ The 0% S rate is excluded from the analysis of seed components due to low seed productivity.

Table 3-3. Subset P-values of seed yield and components responding to genotype (G) and S rates (S) with interactions (GxS) following a 2-way ANOVA. Total yield of individual seed components (per plant) is in parentheses.

		Seed Components† (x Yield)			
Factor	Yield	TGC	TOC	EAC	CPC
Genotype	0.0002	0.2036	0.2041	0.0011	0.0427
		(0.0093)	(0.0033)	(0.0044)	(0.0155)
S Rate	<.0001	<.0001	0.9161	<.0001	<.0001
		(<.0001)	(<.0001)	(<.0001)	(<.0001)
G x S	0.0950	0.0061	0.0807	0.0343	0.4625
		(0.3932)	(0.0031)	(0.0055)	(0.0358)

† Response includes total GSL content (TGC), total oil content (TOC), erucic acid content (EAC); crude protein content (CPC). Only *B. carinata* genotypes (Avanza and A120) were included with all S rates.

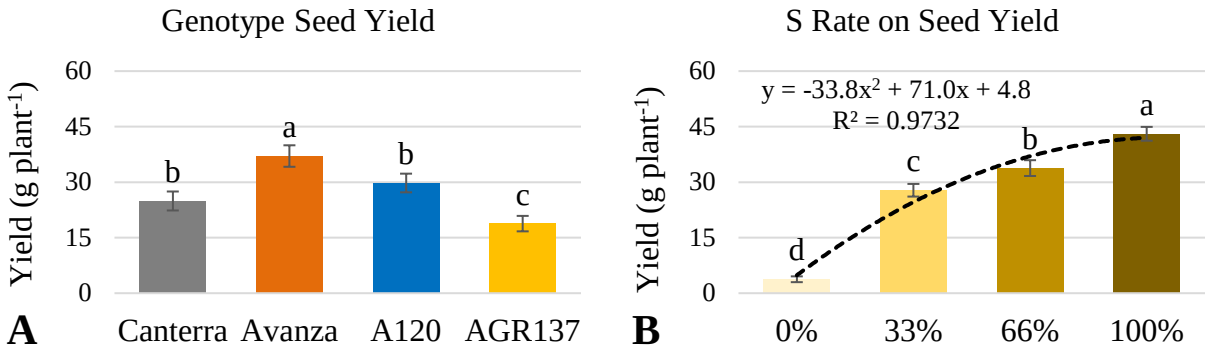

Figure 3-1. Seed yield response to S rates and *Brassica* genotype across trial-years. Seed yield varied by A) genotype and B) S rates with maximum yield (42.1 g plant^{-1}) estimated at the 105% S rate. Responses from a 2-way ANOVA with significant mean differences indicated with unique letters as determined by Tukey HSD test (α=0.05).

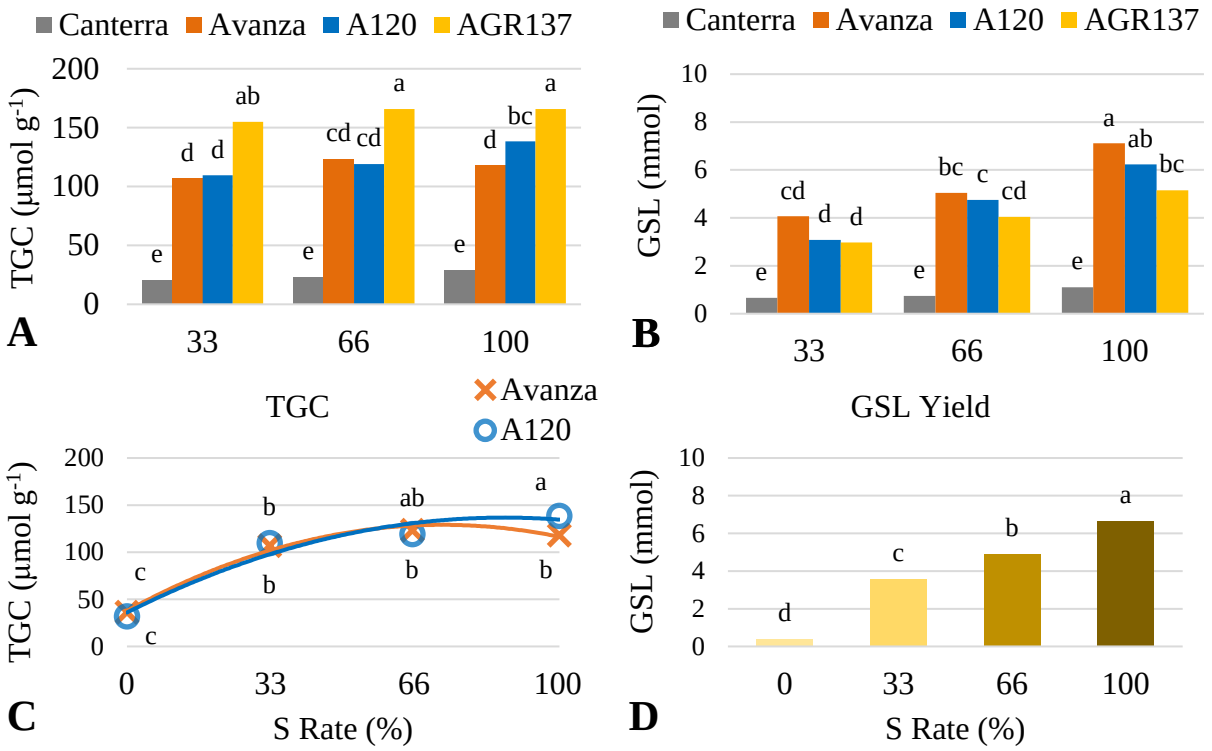

Figure 3-2. Total glucosinolates (GSLs) in seeds of *Brassica* genotypes across trial-years. The accumulation of seed A) total GSL content (TGC) and B) GSL yield per plant. Avanza and A120 were also analyzed individually for C) TGC and D) total GSL yield by S rate. Responses from a 2-way ANOVA with significant mean differences indicated with unique letters as determined by Tukey HSD test (α=0.05).

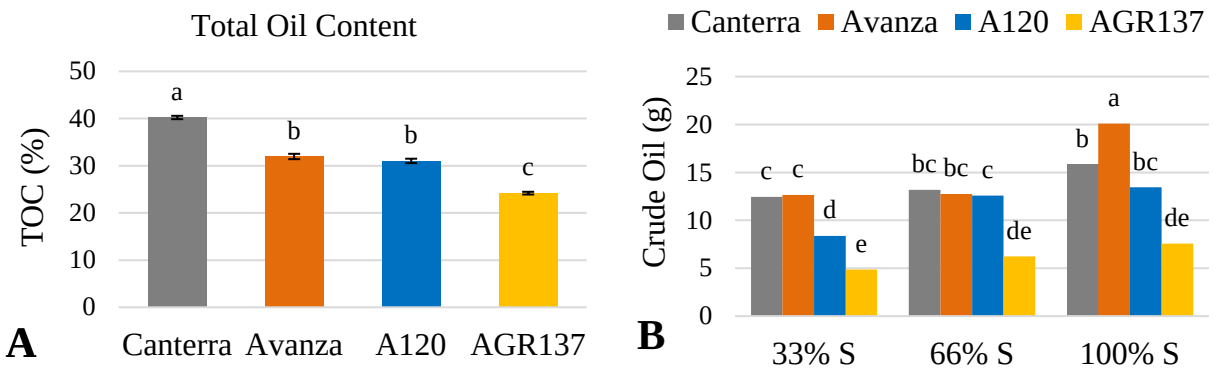

Figure 3-3. Total oil in seeds of *Brassica* genotypes across trial-years. The accumulation of seed A) total oil content (TOC) across S rates and B) total oil yield per plant by genotype and S rate. Responses from a 2-way ANOVA with significant mean differences indicated with unique letters as determined by Tukey HSD test (α=0.05).

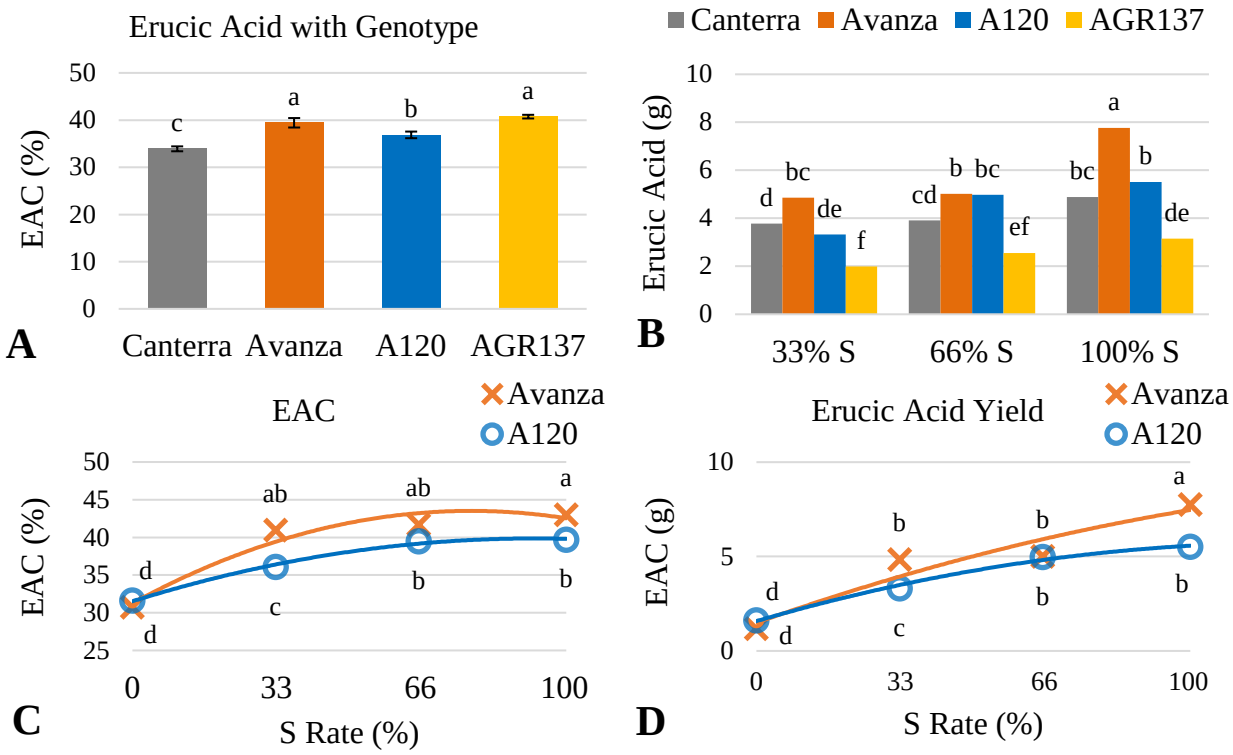

Figure 3-4. Erucic acid in seeds of *Brassica* genotypes across trial-years. The production of A) EAC (from TOC) across S rates and B) total erucic acid produced per plant by genotype and S rate interaction. Interactions were also observed from a data subset containing Avanza and A120 as genotypes and all 4 S rates on C) EAC and D) erucic acid yield. Responses from a 2-way ANOVA with significant mean differences indicated with unique letters as determined by Tukey HSD test (α=0.05).

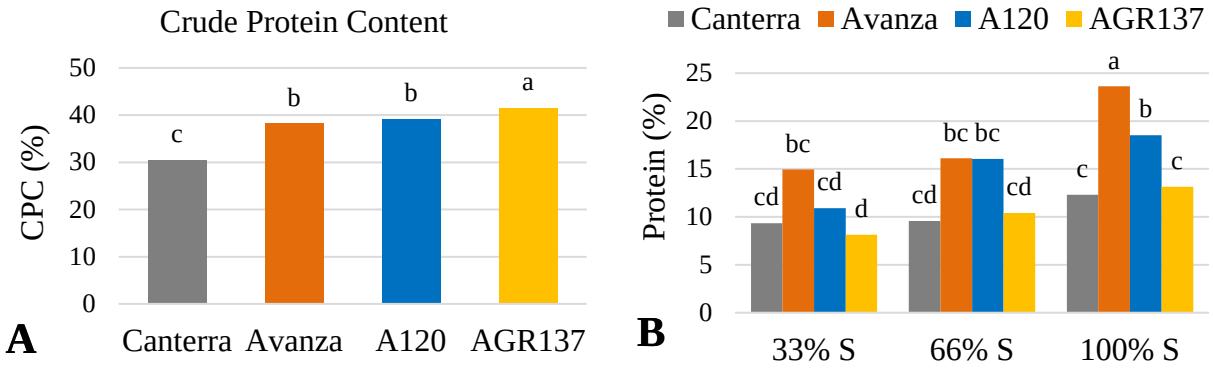

Figure 3-5. Crude protein in seeds of *Brassica* genotypes across trial-years. The accumulation of seed A) crude protein content (CPC) across S rates and B) crude protein yield per plant by genotype and S rate. Responses from a 2-way ANOVA with significant mean differences indicated with unique letters as determined by Tukey HSD test (α=0.05).

Table 3-4. Correlation between yield and seed contents of *Brassica* genotypes.

Variable	by Variable	Spearman ρ	Prob>|ρ|
TGC	Yield	-0.1773	0.0746
TOC	Yield	0.2227	0.0244
TGC	TOC	-0.9235	<.0001
EAC	Yield	0.1291	0.1960
EAC	TGC	0.5552	<.0001
EAC	TOC	-0.4392	<.0001
CPC	Yield	-0.1031	0.3024
CPC	TGC	0.8515	<.0001
CPC	TOC	-0.8811	<.0001
CPC	EAC	0.5123	<.0001

This table contains the Spearman's ρ correlation (nonparametric) between seed yields, total oil content (TOC), total glucosinolate content (TGC), erucic acid content (EAC) and crude protein content (CPC) with their respective p-value.

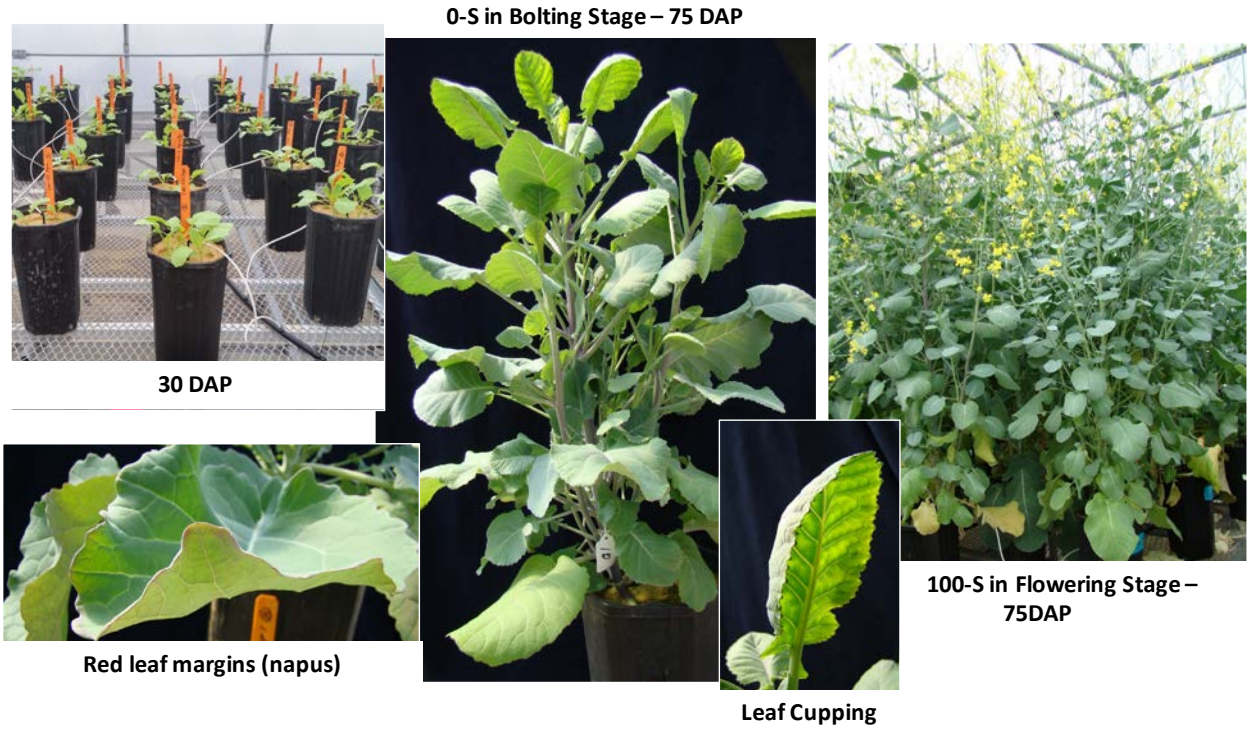

Figure 3-6. Photos of sulfur deficiency symptoms on *B. carinata* (AGR137) displays interveinal chlorosis with leaf cupping and reddish leaf margins in *B. napus*. In addition, there significant delays in development as seen in 0% S treatments compared with the complete Hoagland nutrient solution (100% S) at 75 days after planting (DAP).

CHAPTER 4
SULFUR FERTILITY MANAGEMENT OF OILSEED *BRASSICA CARINATA* IN FLORIDA

Background

The United States Department of Agriculture (USDA) is supporting research in sustainable biofuels for many types of renewable fuels in the US. In 2017, there was a solicitation for grant applications from the Agriculture and Food Research Initiative seeking proposals for research on sustainably produced bioenergy and development of bioproducts to enhance the bioeconomy (USDA, 2017). That following year the Southeastern Partnership for Advanced Renewable from Carinata (SPARC) was awarded a grant and launched a coordinated effort by the University of Florida and industry partners to commercialize *Brassica carinata* in the southeastern US (USDA-NIFA, 2017). *B. carinata* has many agronomic traits that make it a suitable winter oilseed crop for the region (Seepaul et al., 2019). They include resistance to pests and diseases (Gunasinghe et al., 2016; Navabi et al., 2010), drought tolerance (Cardone et al., 2003; Sharafi, 2015), low levels of seed shattering (Zhang et al., 2016), and the potential use of seed meal as a protein supplement for livestock feed (Xin, Falk, & Yu, 2013).

Plants in the Brassicaceae Family produce a defensive compound called glucosinolates (GSLs) that are hydrolyzed into its bioactive form commonly called isothiocyanate (ITC) when cells are ruptured by generalist herbivores or plant pathogens. These are secondary metabolites rich in sulfur (S) that aid in plant defense against a dynamic and hostile environment following the patterns of optimal defense theory (ODT) (Tsunoda et al., 2017). The basic premise states that plants under ODT produce defensive compounds (i.e., GSLs) that can be distributed to tissues in anticipation of or in response to biotic and abiotic stress. For this reason, much of the GSLs in the Brassicaceae family are concentrated in the leaves during vegetative growth and

then redistributed to reproductive organs like racemes and ultimately to the seed as the plant approaches maturity (Bloem et al., 2007).

Studies of *B. carinata* have found that most of the shoot and about half of the roots contain a methionine-derived GSL (Met-GSL) called sinigrin (2-phenylethyl GSL) (Kirkegaard & Sarwar, 1998). These GSLs often accumulate in the seeds and can be detrimental to the health of livestock when the meal contains high amounts, so it is regulated to concentrations under 30μm g^{-1} (Tripathi & Mishra, 2017). *Arabidopsis thaliana* and *Brassica* grown under reduced S availability have been found to negatively impact the accumulation of Met-derived GSL in seeds, but at some expense to seed yield (Falk et al., 2007). What is not clear is to what extent is *B. carinata* oil and seed meal quality affected by reduced S availability and the viability to reduce seed TGC through S management.

Plants readily assimilate sulfates (SO_4) from the soil through the roots and atmospheric sulfur dioxide (SO_2) and hydrogen sulfide (H_2S) through the stomata for the production of amino acids and other metabolites (Leustek & Saito, 1999). As a result of the US Clean Air Act and EU Clean Air Policy, atmospheric sulfur (S) emissions have decreased significantly (Aas et al., 2019). This reduction in atmospheric S has reduced the availability for plants to assimilation leading to increased occurrences of S deficiencies. To achieve optimum yields, it is recommended that S be included in the fertilization program for most crops. This is especially true for oilseed *Brassicas* due to the production of GSLs requiring between 15 to 60 kg S ha^{-1} usually in the form of SO_4 to maximize yields (Grant et al., 2012). Sulfur and nitrogen (N) are often used congruently to build molecules and proteins. Incidence of S deficiency are exacerbated when N inputs increase without accounting for S (Anjum et al., 2012). On the contrary, S can also be over-applied, leading to leaching events in sandier soil environments

(Karamanos et al., 2007). In *B. napus*, there is a 7:1 ratio between N and S applications for optimal plant growth and yields (Janzen & Bettany, 1984). While, *B. carinata* seems to have a reduced N:S ratio of about 3:1 (Bhattarai, 2019; Seepaul et al., 2019).

The nutrient requirements for maximum seed yields vary significantly with genotype and environmental conditions suggesting that specific management strategies must be developed for a given region and genotype (Pan et al., 2012). In Florida, it has been reported that crops planted in sandy soils have an increased propensity of nutrient deficiency, including S (Roberson, 2012). In addition, soil tests for S can be unreliable depending on soil texture and S movement (Franzen, 2018). This is especially true for SO_4 in sandy soils experiencing extensive rain events increasing the propensity of leaching and the loss of S. The relationship between S scarcity in oilseed *Brassica* grown in Florida soils and their interaction to GSLs and other seed components remain unclear.

In this experiment, we evaluated the effects of S in two contrasting soil environments to determine optimal S fertility. We hypothesize that S requirements of *B. carinata* may be location-specific with sandier soils requiring additional S inputs compared to areas with a higher clay content despite both soils being classified as S deficient. In addition, we believe that Met-GSLs may be affected disproportionally when S is limited leading to reduced total GSL content (TGC) in the seeds in *B. carinata* before there is a considerable impact on oil productivity or quality. Several *B. carinata* genotypes having a range of seed TGC and low-GSL *B. napus* will be evaluated to determine relationships between S requirements and seed yields and components. This study aids our understanding of S fertility and effects on oil productivity and chemical composition in *B. carinata* grown in variable soil environments.

Materials and Methods

Experimental Setup and Design

Sulfur deficient field sites included the North Florida Research and Education Center in Quincy, FL (30°32'19.6"N, 84°34'56.5"W) and the Plant Science Research and Education Unit in Citra, FL (29°24'28.4"N, 82°08'47.5"W) having similar climate (Figure 4-1), but with distinct soil texture and classification (Table 4-1). This trial was planted from Nov. 11-13, 2017 and harvested on May 14-16, 2018. It was set up as a randomized complete block design with four repetitions containing three factors that included four *Brassica* genotypes, four S rates, and two locations.

Three *B. carinata* varieties were selected including two commercial varieties, Avanza 641 and AAC A120, and an experimental line AGR 137 sourced from Nuseed (Sacramento, CA, USA) (formally Agrisoma Biosciences Inc) with seed TGC previously determined to contain the average range of 50-70, 60-90, and 90-140 μmol g-1, respectively. In addition, a low-GSL (<30 μmol g-1) canola (*Brassica napus* cv. Canterra '1918') was included for comparison. They represent *Brassica* genotypes with seeds that range in TGC from <30 to >140 μmol g^{-1} and will be referred to as Avanza, A120, AGR137, and Canterra for the remainder of this manuscript. The study was planted at the rate of 3.8 to 5.1 kg ha^{-1} (depending on germination rates) with 0.3 m row spacing in 1.5 x 6.1 m plots.

At both sites, fertilizer application rates were similar. Sulfate of potash or potassium sulfate (K_2SO_4) was the source of plant-available S at the rates of 0, 11.2, 22.4, or 33.6 kg ha^{-1} depending on treatment and supplemented with muriate of potash or potassium chloride (KCl) for additional K. All treatment also received 168.1 kg ha^{-1} N and 44.8 kg ha^{-1} P. The fertilizer was top-dressed and applied monthly (x4) with variable rates that included 10% at planting

followed by 20%, 40%, and 30% of total fertility thereafter to follow crop requirements. Plants were irrigated for the first few weeks until establishment and rainfed thereafter.

Site Selection and Management

These sites were prospected for S deficient soils from cores collected in various zones at each location using standard sampling procedures (15.2 cm depth) and analyzed by Waters Agricultural Laboratories Inc (Camilla, GA, USA). Soils with low S but with variable soil texture (i.e., clay content) were selected for possible trial sites. To confirm S deficient soils, the perimeter of each site was mapped, split into quadrants, and 40 soil core samples were collected systematically. Soil cores from each quadrant were thoroughly mixed and a subsample (4 subsamples per site) was sent for analysis. Each field site had similar S deficient soil estimated at ≈17.0 kg ha^{-1} (Table 4-2.).

The fields were tilled 2 weeks prior to planting, followed by the application of preemergent herbicide pendimethalin (Prowl® H2O, BASF, Florham Park, New Jersey, USA) to prevent germination of weed seeds. Buffer plots were included between each experimental unit and around the perimeter of the experimental site to reduce the variability of cross-plot contamination during major rain events and the possibility of border effects.

After the completion of the experiments, subsequent deep core (1.2 m) soil sampling revealed that plant-available S was not uniform with depth at the Quincy location. There was a dense clay layer below the soil surface where the concentration of S was initially 17.5 kg ha^{-1} from the surface to a depth of 30 cm, but increased to >138.3 kg ha^{-1} at 0.61 m and 207.7 kg ha^{-1} at a 1.2 m depth.

Data Collection and Seed Composition

Treatment plots were harvest using a plot combine (Wintersteiger Inc, Salt Lake City, UT) from an area of 9.15 m^2. Seeds were oven-dried at 50°C until moisture reached <8% as

measured by a Steinlite SI-95 moisture meter (Steinlite, Atchison, KS) to standardize moisture components to seed yields. Total glucosinolate content (TGC), total oil content (TOC), erucic acid content (EAC) and crude protein content (CPC) were estimated by NuSeed using near-infrared reflectance spectroscopy (NIRS) and analyzed by the FOSS XDS^{TM} Rapid Content Analyzer (FOSS Analytical AB, Hogenas, Sweden) that samples spectra at 0.5 nm increments (range of 400-2500 nm) using the ISIscan (Program 4.10.0.15326, Database 4.6.0.14416).

Statistical Analysis

This experiment was set up as a randomized complete block design with four blocks as replications. The 1st factor was *Brassica* genotypes (4 levels), the 2nd factor was S rates (4 levels), 3rd factor was location (2 levels). JMP Pro 13.2.0 was used for the analysis of variance (ANOVA). Tukey honest significant difference (HSD) test was used to make pairwise comparisons and mean separation ($\alpha = 0.05$). Wricke's ecovalence (W_i^2) was used to measure cultivar performance and stability between genotype and location (Wricke, 1962).

Results

Seed Yields

All interactions and main effect *P*-values from treatment responses are in Table 4-3. Average seed yields per plot in Quincy (1.61 kg) were more than double of what was observed in Citra (0.72 kg). This generated a S response curve of y (g)= $-0.457x^2+29.2x+869.5$ ($R^2 = 0.993$) with a maximum yield potential (1.34 kg of seeds per plot) at the 32.0 kg ha^{-1} S rate and an estimated 1.46 x 10^3 kg ha (64.4 bu ha^{-1}). The only positive response to S rate at the Quincy location occurred between 11.2 and 22.4 kg ha^{-1} which accounted for a 20.9% yield increase (Figure 4-3a.). Overall, Canterra was the highest yielding genotype across location and S rates with seed yield averaging 0.16 kg m^{-2} followed by A120 and Avanza, and AGR137 at 0.14, 0.12, and 0.08 kg m^{-2}, respectively. This pattern was mostly observed at Quincy, while in Citra, A120

produced the highest yield (0.10 kg m^{-2}) followed by Avanza, Canterra, and AGR137 (Figure 4-3b.). Compared to *B. carinata*, yields were greatly impacted by location in *B. napus* with a large reduction in productivity when grown at Citra. Wricke's ecovalence stability index found that Avanza was the most stable genotype, followed by AGR137, A120, and Canterra (Table 4-4.).

Seed Composition

Total oil content (TOC)

In both locations, seed TOC averaged >40% with seeds produced at Citra accumulating 46.2% while averaging 43.3% at Quincy. Oil increased with added S in Citra from 0 to 11.2 kg ha^{-1} with no increase thereafter (similar across locations) while no differences were observed in Quincy (Figure 3-3a). Overall, Avanza produced the highest TOC, but there was no difference between Avanza and Canterra among locations. There was a large decrease in TOC when AGR137 was grown at Citra and a smaller decline with A120, compared to Quincy (Figure 3-3b). However, when including seed yields and using the density of erucic acid (860 kg m^3) to estimate volume, the Quincy location would potentially account for two-thirds of the total oil production with an estimated 2174 L ha^{-1} compared to Citra (1015 L ha^{-1}). This extrapolation also produces a S fertility curve with the maximum oil productivity of 1,829.2 L ha^{-1} (≈195.6 gal $acre^{-1}$) with the addition of 33.0 kg ha^{-1} S.

Erucic acid content (EAC)

Avanza and AGR137 consistently produced the highest proportion of EAC as a percent of TOC, averaging 45.3% followed by A120 and Canterra at 40.0% and 26.7%, respectively (Table 4-5). There was a greater effect between treatments when accounting for yields. EAC at Quincy was estimated at 801.3 L ha^{-1} or nearly double than that of Citra (407.8 L ha^{-1}). Avanza and A120 were the highest producers of erucic acid overall, but these yields were similar to Canterra when accounting for location and S rates.

Total glucosinolate content (TGC)

Plants grown at Quincy often produced higher seed TGC averaging 84.4μmol g^{-1} compared to those grown at Citra (36.0μmol g^{-1}). AGR137 consistently produced the most seed TGC (99.7 μmol g^{-1}) and Canterra the least (14.0 μmol g^{-1}) with Avanza and A120 having a similar GSL content of about 65.1 μmol g^{-1}. Avanza was the only variety to have a response to S in Quincy with its maximum occurred between 10 and 20 kg ha^{-1} (Figure 4-4). Instead, most of the effects of TGC with S rates were in plants that were grown in Citra which was often linear and positive to increasing S rates. There was a moderately negative correlation between GSL and TOC ($r = -0.6943$, $p < 0.0001$) and positive correlation to EAC ($r = 0.6306$, $p < 0.0001$).

Crude protein content (CPC)

Location and genotype had the greatest effect on crude protein content in seeds (Table 4-6). In Quincy, 28.7% of seed content was crude protein, whereas those grown in Citra had 23.1%. AGR137 produced seeds with the greatest quantity of CPC (across all S rates) containing an average of 35.9% CPC at Quincy and 24.6% CPC at Citra. The crude protein content of Avanza and A120 were similar at the Quincy location (27.4%). All genotypes grown in Citra had similar CPC as Canterra grown in Quincy (24.7%). There was a positive correlation between CPC and TGC ($r= 0.8608$, $p<0.0001$) and an inverse relationship with CPC and TOC ($r = -0.8901$, $p<0.0001$).

Discussion

The locations of these experiments represent some of the regional differences farmers may encounter when growing crops in the southeastern US. A soil texture consisting of mostly sand is conducive to the extensive SO_4 leaching, increasing the severity of nutrient deficiency (Camberato & Casteel, 2017; Gilbert, 1951; Scherer, 2009). This was evident by the quadratic response that produced the S fertility curve and the visual cues from plants grown in the deep

sands of Citra (Figure 4-6). Plants were observed to have many of the classic symptoms of plants suffering from S starvation ranging from leaf mottling to extreme intercostate chlorosis with a characteristic “spoon-shaped” or cupping of leaves (Schnug & Haneklaus, 2005).

Similar S deficiencies were reported from the test following standard soil sampling procedures at both locations. While S deficiencies continued at the Citra location, we observed *B. carinata* recover healthy plant characteristics in Quincy at the bolting stage. Ultisols comprise 6.9 million acres of the soils in northern Florida that often contains subsoils with high clay content, minerals with low reactivity, and base saturation that decreases with depth (Mylavarapu et al., 2016). This allows crops that are initially deficient in S to recuperate after roots reach a clay layer from 15 to 46cm (≈ 6 to 18 in) below the soil surface (Roberson, 2012). We also encountered increasing clay content with depth in Quincy where *B. carinata* has been previously observed of producing tap roots that reach a depth of 26.8cm (10.6in) below the soil surface (Seepaul et al., 2019). The accumulation of S in these clay-rich soils provided *B. carinata* enough S to fully recover after the roots reached the subsoil. Due to the heterogeneity of texture in Ultisols soils, estimations for S availability should include sampling soils at greater depths. Otherwise, standard soil testing will remain largely unreliable and inaccurate increasing input costs and the possibility of S leaching or runoff. There was a lack of treatment responses at the Quincy location suggesting that the timing of application may not have been as important and therefore fertilization should follow was has been recommended in the SPARC production manual (Seepaul et al., 2016).

B. napus yields were greater at Quincy than that at Citra, while *B. carinata* yields remained more stable across locations. This may be attributed to the extensive breeding history of *B. napus* being mostly focused on oil productivity rather stability across ecologically diverse

environments. Breeding for low erucic acid and low GSL (i.e., double-low or '00') varieties was not successful until the introduction of a Polish variety 'Bronowski' where breeding programs were finally able to reduce GSL below the acceptable limits despite the immense impact this had to yield potential (Jonsson, 1977). *B. carinata* is thought to have originated from the highlands of Ethiopia, where its soils are also comprised of Ultisols and Alfisols soils (Deressa et al., 2018). In addition, the harsh environment surrounding its point of origin may have driven *B. carinata* to become better adapted to stress and maintain stability across ecosystems.

A genetic analysis of *B. carinata* indicates that breeding for low GSL varieties will be difficult due to its narrow germplasm and a tight correlation to protein content (Alemayehu & Becker, 2002). However, we saw that *B. carinata* has an increased baseline for seed TGC that would not permit the use of its seed meal as feed unless they were removed using organic extraction methods. *B. carinata* seed extracted using hexane successfully increased the weight of cattle when the high-protein seed meal was supplemented with hay (Schulmeister et al., 2019). Additionally, high TGC seed meal does have its value as a soil amendment in organic farming or any fields found to be infested with soil-borne pathogens using the benefits of biofumigation (A. L. Gimsing & Kirkegaard, 2009b). Mazzola has found that using seed meal from *Brassica* has been effective for controlling soil-borne diseases with the purchasing price of $1,600 per ton at the rate of 3 tons ha^{-1} (Warner, 2014). Selling seed meal for soil amendment provides additional economic incentives and farmers can improve the soil quality of their fields, therefore, increasing the sustainability of renewable fuel production.

Conclusion

To better understand S fertility and interactions with GSL production in *Brassica carinata*, we evaluated the effects of S availability on growth, yield, and seed chemical composition. Maximum yields were obtained when applying S at the ≈ 32kg ha^{-1} rate with seed

yields doubling in Quincy compared to Citra. Soil texture may have been the greatest contributor to the disparity between yields between the two locations despite the initial similarities in S deficient soils using standard soil sampling procedures of the upper 15 cm. This study shows the importance of site-specific fertility management programs using deep soil samples to determine S availability. It is possible to reduce the seed TGC of *B. carinata* without affecting oil quality. However, the impact of seed yields is significantly affected when reducing TGC closer to acceptable levels (30μmol g^{-1}) for use as animal feed. TOC was above 40% for both locations, with *B. carinata* producing the greatest EAC compared to canola and Avanza 641, becoming the top EAC producer and the most stable variety across locations. *B. carinata* produces seeds with a favorable fatty acid composition, stable across variable soil types, and a suitable fit as a sustainable winter oilseed crop for the southeastern US cropping systems.

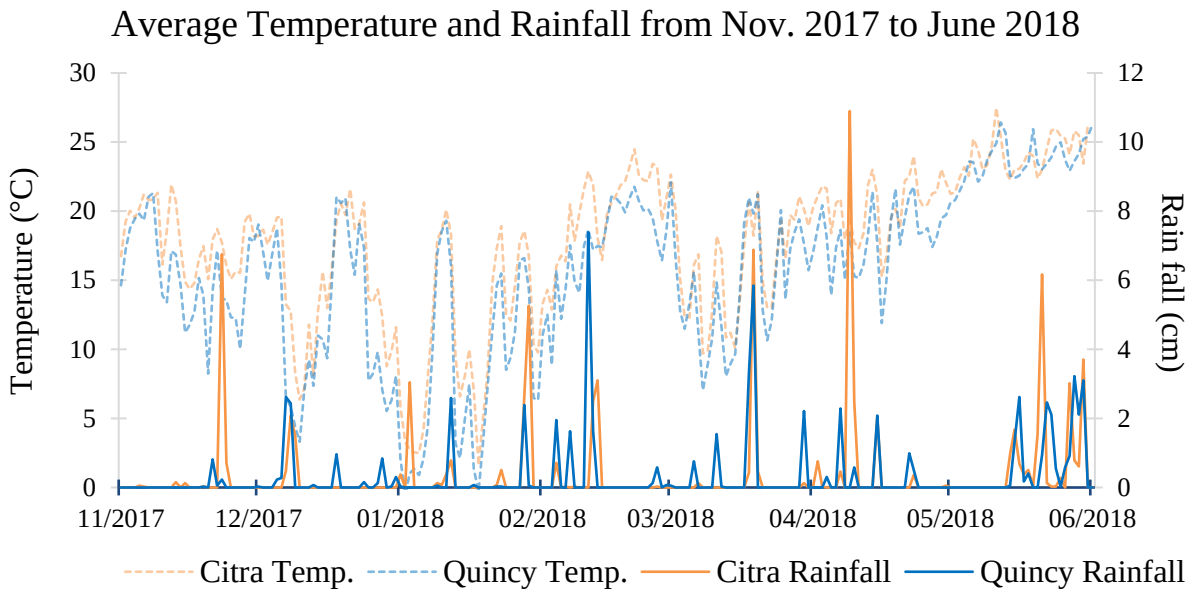

Figure 4-1. Average temperature and rainfall for Citra and Quincy during the trial period.

Table 4-1. The trial site and soil classification and texture.

				---------- % ----------				(dS/m)
Location	Soil ID†	Soil Type‡	Class	Sand	Silt	Clay	pH	CEC
Quincy	Orangeburg	Ultisols	loamy sand	82.1	12.3	5.6	6.43	3.6
Citra	Tavares	Entisols	sand (deep)	89.1	8.5	2.4	6.45	3.2

† USDA: Web Soil Survey (WSS). ‡ University of Florida (Soils and Water Dept)

Table 4-2. Soil nutrient composition from the 2017-2018 sulfur trials.

	Average Soil Nutrients† (kg ha^{-1})									
Location	P	K	Mg	Ca	S	B	Zn	Mn	Fe	Cu
Quincy	45	93.0	165	601	16.7	0	4.3	9.1	14	0
Citra	81	18.7	42	919	15.8	0	3.4	3.8	12	0

†Analysis by Waters Agricultural laboratories for the top 15 cm soil samples

Table 4-3. P-values of yield and seed content in response to location, S rates, and genotype.

Source	Yield	TGC	TOC	TOC*Y	EAC	EAC*Y	CPC
L	<.0001	<.0001	<.0001	<.0001	0.9297	<.0001	<.0001
S rate (S)	<.0001	<.0001	0.0002	<.0001	0.0002	<.0001	0.6837
L x S	<.0001	<.0001	<.0001	<.0001	0.0672	<.0001	0.003
G	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
L x G	<.0001	<.0001	<.0001	<.0001	0.1819	0.0016	<.0001
S x G	0.4988	0.0001	0.2216	0.4219	0.0003	0.5471	0.0231
L x S x G	0.0549	<.0001	0.8875	0.1030	0.0069	0.044	0.8528

P-value to the responses of a 3-way ANOVA for yield (Y) and seeds content that include total glucosinolate content (TGC), total oil content (TOC), erucic acid content (EAC) and crude protein content (CPC) with location (L), S rates (S), and genotype (G) with interactions.

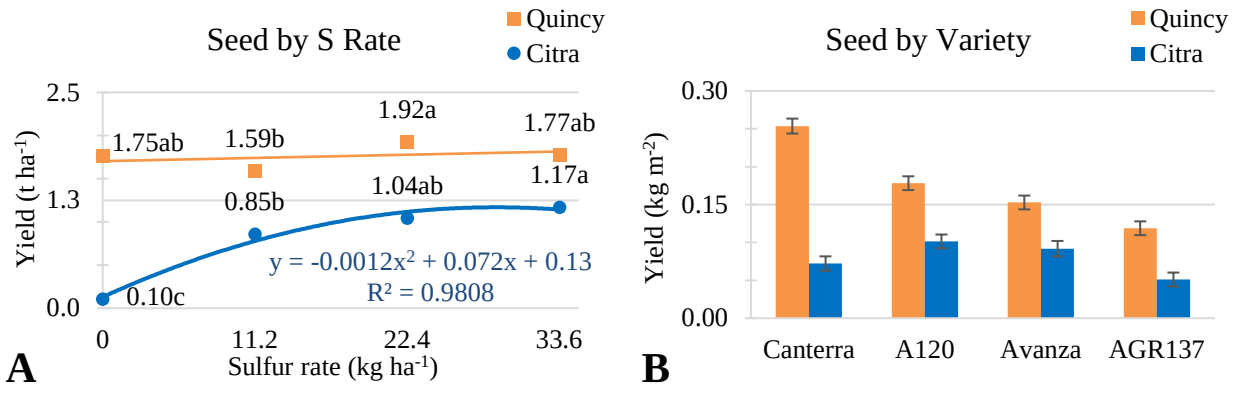

Figure 4-2. Average seed yields in Quincy and Citra experimental sites. They include yields by A) S rate and by B) *Brassica* genotypes. Tukey HSD test with significant mean differences indicated with unique letters (P<0.05).

Table 4-4. Overall stability of yields between *Brassica* genotype between experimental sites

		Yields (kg m^{-2})			Rank†
Genotype	S Rate	Quincy	Citra	Δ	W_i^2
Canterra	0	0.27	0.00	-0.27	4
	11	0.23	0.06	-0.17	
	22	0.28	0.12	-0.16	
	34	0.23	0.10	-0.13	
A120	0	0.18	0.02	-0.16	3
	11	0.18	0.10	-0.07	
	22	0.19	0.13	-0.05	
	34	0.17	0.15	-0.02	
Avanza	0	0.17	0.02	-0.15	1
	11	0.10	0.13	0.02	
	22	0.17	0.10	-0.07	
	34	0.17	0.13	-0.05	
AGR137	0	0.08	0.01	-0.08	2
	11	0.13	0.05	-0.08	
	22	0.13	0.06	-0.07	
	34	0.13	0.09	-0.04	

The changes (Δ) of average seed yields between location. † Wricke's ecovalence (W^2) was used to rank *Brassica* genotype stability across locations with low values indicated the highest stability.

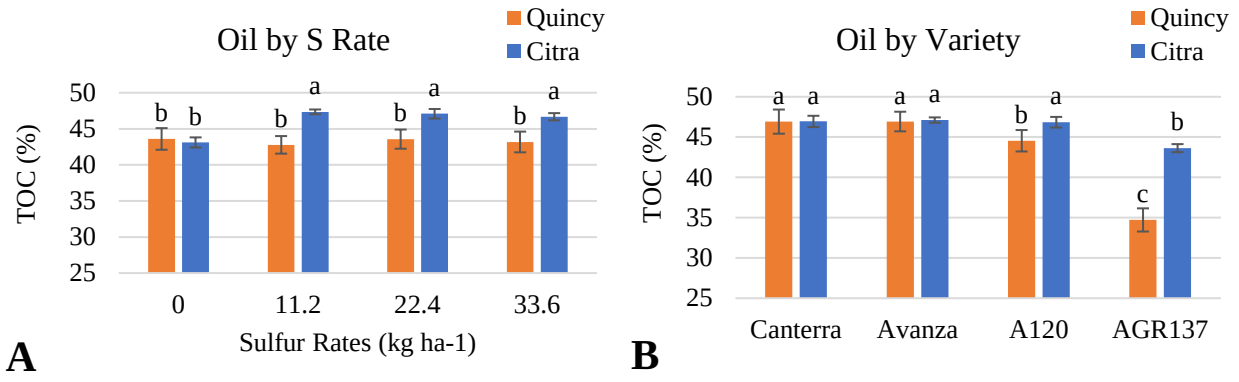

Figure 4-3. Estimated total oil content (TOC) between locations and by A) S rates or B) genotype. Tukey HSD test with significant mean differences indicated with unique letters (P<0.05).

Table 4-5. Estimated production volumes of EAC with location, S rates, and genotypes.

		EAC (L ha^{-1})							
		Quincy				Citra			
Genotype	S rate	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Canterra	0	26.8	0.6	962	50	29.0	0.4	15	0.2
	11	27.7	1.3	808	78	26.3	0.4	211	32
	22	26.4	0.8	1028	63	27.0	0.4	438	65
	34	26.9	0.8	911	151	26.5	0.5	355	100
Avanza	0	45.5	0.8	1006	34	41.6	0.5	86	43
	11	46.3	0.8	607	115	46.3	0.9	540	238
	22	46.1	1.4	1022	110	46.1	1.0	614	109
	34	45.6	0.8	1070	193	45.6	0.7	795	6
A120	0	39.3	0.3	890	145	40.0	1.2	80	26
	11	39.8	0.7	866	103	40.2	0.2	543	103
	22	39.7	0.6	918	79	40.6	0.5	732	62
	34	39.7	0.6	837	144	40.4	0.6	805	84
AGR137	0	44.3	0.8	355	67	40.0	0.3	27	8
	11	45.9	1.3	583	29	47.0	0.8	382	32
	22	46.3	0.8	602	71	47.0	0.7	336	90
	34	44.8	0.7	565	72	46.9	0.4	493	78

Means ± standard error (SE)

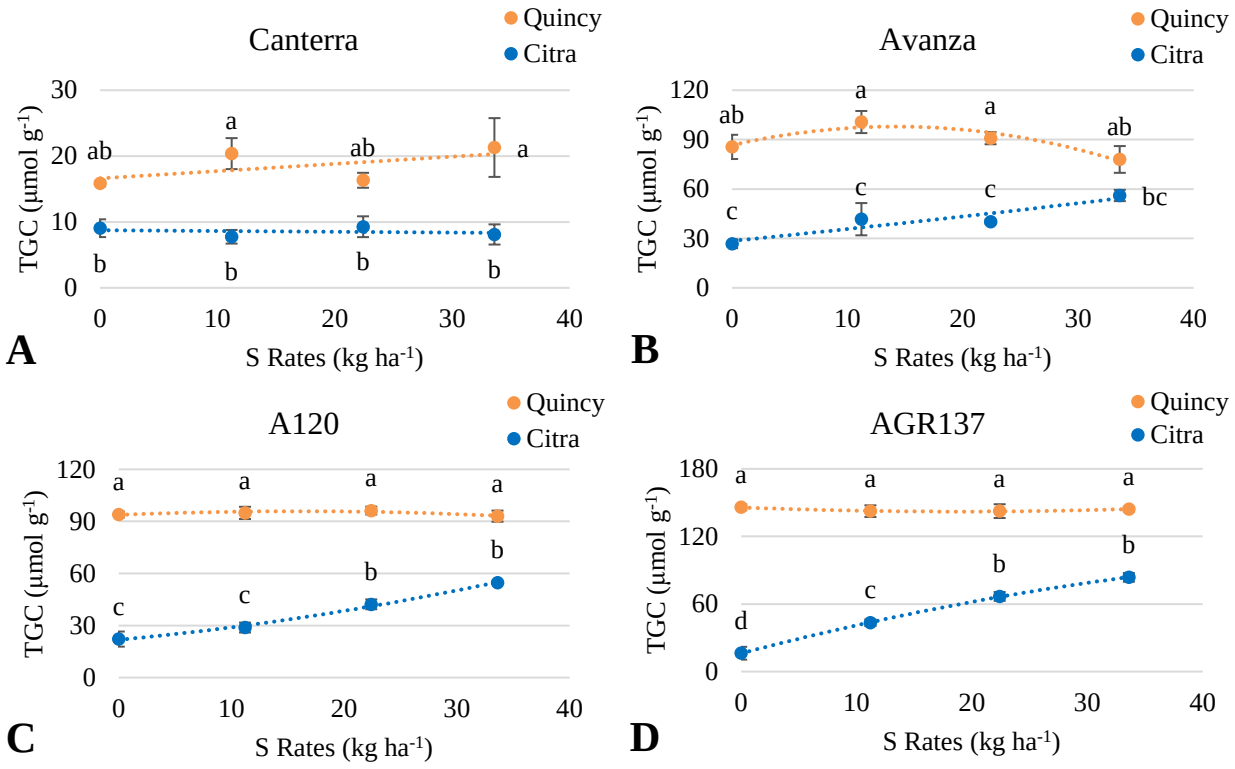

Figure 4-4. Total glucosinolate content (TGC) by location and genotype that includes A) Canterra, B) Avanza, C) A120, and D) AGR137. Tukey HSD test with significant mean differences indicated with unique letters (P<0.05).

Table 4-6. Average crude protein content (CPC) by location, S rates, and genotype.

		Quincy		Citra	
Genotype	S rate (kg ha^{-1})	Mean (%)	SE	Mean (%)	SE
Canterra	0	24.6	0.3	23.2	0.1
	11.2	25.2	0.3	21.3	0.3
	22.4	24.3	0.4	22.0	0.3
	33.6	24.8	0.7	22.1	0.1
Avanza	0	26.0	0.5	23.4	1.1
	11.2	30.3	1.1	22.6	0.8
	22.4	26.9	0.2	21.0	0.8
	33.6	26.1	1.6	21.5	0.4
A120	0	26.5	0.6	24.9	1.2
	11.2	27.9	1.3	22.7	0.8
	22.4	27.2	0.6	22.5	0.8
	33.6	28.1	1.4	24.1	0.4
AGR137	0	35.4	0.7	24.6	0.7
	11.2	35.3	0.4	23.5	0.3
	22.4	36.7	0.7	24.8	0.6
	33.6	36.2	0.7	25.2	0.6

Means ± standard error (SE)

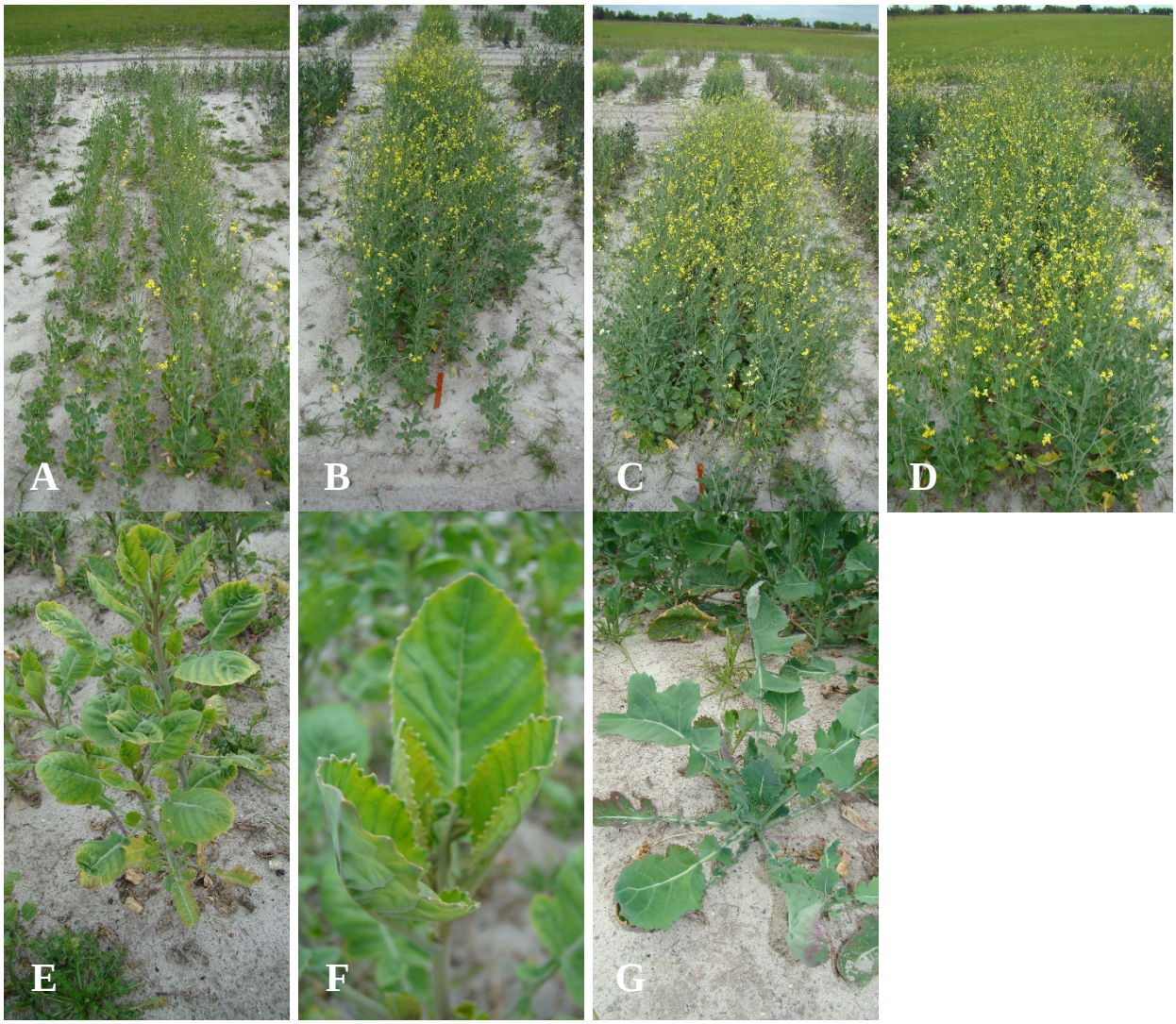

Figure 4-5. Examples of plots planted in Citra by S rates. They are all *B. carinata* cv. Avanza641 grown at the A) 0, B) 11.2, C) 22.4, and D) 33.6 kg ha^{-1} S rate. Symptoms of S deficiency include E) intercostate chlorosis throughout the entire plant and F) leaf cupping for *B. carinata* and G) red margins in leaves of *B. napus*.

CHAPTER 5
ROOT-KNOT NEMATODE INFECTION ON OILSEED *BRASSICA CARINATA*

Background

Root-knot nematodes (RKNs) are obligate plant endoparasitic roundworms of the genus *Meloidogyne*. Its common name is derived from the knots or gall-like appearance of RKN well established feeding sites along the roots of plants. *Meloidogyne* spp. are found in tropical and subtropical regions around the world with a vast range of plant hosts making them one of the most economically important plant pathogens in agriculture (G. C. Bernard, Egnin, & Bonsi, 2017; Sasser, 1979). The most widely cited species is *M. incognita* (Chitwood 1949) or the southern root-knot nematode (SRKN) that often accounts for the majority of RKNs affecting crops (CABI, 2020). Despite the limitations of mitotic parthenogenic reproduction, SRKN genome includes the production of plant hormones, cell wall degrading enzymes, and repetitive elements that helps adaptative success when experiencing various types of stress (Castagnone-Sereno et al., 2013).

Soils in the southeastern US provides ideal environmental conditions that allow SRKN to proliferate. This includes soil texture with high sand content (Jaraba et al., 2014), moderate to high soil temperatures (Nardacci & Barker, 1978), and susceptible hosts commonly used as vegetable and agronomic crops such as cotton but also many native plant species that act as alternative hosts and found throughout the region (Davis & Webster, 2005). Plants that are infested with SRKN reduce the efficiency of plant nutrient and water uptake resulting in stunted growth and reduced yield. To break the disease cycle of nematodes, crops such as cotton should be rotated with non-host cash or cover crops, managed with pesticides, and incorporate the use of resistant cultivars where available (Grabau, 2017).

Currently, oilseed *Brassica carinata* is being evaluated for winter production in the southeastern US as a potential source of renewable fuel for the aviation industry (Seepaul et al., 2019). The susceptibility of *Brassica* to RKN infection are highly variable and those on *B. carinata* are limited. Some research on *B. carinata* reported a low to moderate level of susceptibility to SRKN Race 1 and Race 3, respectively (E. C. Bernard & Allen, 1993; Liébanas & Castillo, 2004; Mcsorley & Frederick, 1995). Their low infection rates can be largely attributed to the production of a suite of sulfur-rich defensive compounds called glucosinolates (GSLs) that are hydrolyzed into phytotoxins called isothiocyanate (ITC) when cells are damaged. Also, methionine-derived GSL (Met-GSL) can be down regulated throughout the shoots, roots, and seeds of *Brassica* and Arabidopsis when the plant is experiencing S scarcity (Aarabi et al., 2016; Falk et al., 2007).

There are many types of GSLs that can vary considerably with genotype, stage of development, and environmental conditions. Sinigrin (2-propenyl-GSL) is derived from methionine and the most commonly found GSL in *Brassica* tissues that accumulates in the leaves and roots during vegetative growth but then in seeds at plant maturity (Bhandari et al., 2015). Glucosinolates that accumulate in the seeds are not produced *in situ*, rather they are being redistributed from other locations by specific GSL-transporters proteins (Nour-eldin et al., 2012). In *B. carinata*, sinigrin makes up the majority of total GSLs found in the shoots, roots, and seeds (Kirkegaard & Sarwar, 1998). They follow the concept of optimal defense theory (ODT) where a substantial portion of the plants energy is allocated for the production and transportation of defensive compounds (i.e., GSL) to plant tissues considered valuable to overall fitness heavily influenced by plant development and environmental stress (Keith & Mitchell-Olds, 2017; Tsunoda et al., 2017). However, it is possible that *M. incognita* are able to bypass the GSL

defense system without damaging root cells through intracellular migration as was observed in *Arabidopsis* (Wyss et al., 1992).

Most studies on the effects GSLs for weed and pest suppression have focused on the benefits of using *Brassica* seed meal (BSM) or green manure from high-GSL plant varieties as an effective soil amendment strategy in a process called biofumigation (A. L. Gimsing & Kirkegaard, 2009). This process has variable rates of effectiveness but has been used successfully to suppress soil-borne pathogens, including for the suppression of RKN (Boydston et al., 2008; Curto, Dallavalle, Matteo, & Lazzeri, 2016; Oliveira et al., 2011; Zasada & Ferris, 2004). The beneficial effects of *Brassica* amended soils have even been observed long after ITC has dissipated from the soil profile (Mazzola & Zhao, 2010). *Brassica* leaf tissue containing low concentration of sinigrin can significantly reduce RKN survival (Zasada & Ferris, 2004). While soil amendments strategies using *Brassica* biomass tissue can be effective against many soil-borne diseases, it can also incur additional economic costs that will reduce its adaptation into large-scale commercial crop production systems. Therefore, more research is needed to evaluate suppression potential of oilseed *Brassica* cash crops under conservation tillage. This will allow farmers to obtain the benefits of harvesting the crop for its oil production while leaving the roots intact for the possibility of gaining some level of protection to subsequent crops with a higher level of RKN susceptibility.

In this study, we are evaluating the susceptibility of oilseed *B. carinata* to SRKN infection and the relationship between seed total GSL content (TGC). This will include three *B. carinata* genotypes having the capacity to produce seeds with moderate to high TGC and comparing it to low-GSL *B. napus*. Our hypothesis is that increased GSLs in seeds is also associated with higher GSL throughout all of *B. carinata* plant tissues and therefore have a

higher level of protection against SRKN establishment in the roots. In addition, we will evaluate the effects between optimal and sub-optimal S fertility. We believe that the decrease of S availability will negatively impact GSL production and the ability of *Brassica* to defend against SRKN infection and establishment in the roots. Seed content of TGC, total oil content (TOC), erucic acid content (EAC), and crude protein content (CPC) of each genotype with and without SRKN inoculation will be determined. We hypothesize that TGC will be significantly higher when infected with SRKN and that this may interfere with TOC and yield but increase EAC or CPC as has been reported in other studies of *Brassica* oilseed crops (Shi et al., 2011). Lastly, we will plant a susceptible cotton variety directly in locations where *Brassica* had grown and later harvested to evaluate the lasting effects of root GSL decomposition in the soil. We believe that cotton planted in high GSL *Brassica* genotype with optimal S fertility will reduce the prevalence of SRKN in roots.

Materials and Methods

Plant Material and Management

On December 17th, 2018 oilseed *Brassica* was planted in a double-layer inflated greenhouse located at the University of Florida's North Florida Research and Education Center (NFREC) in Quincy, FL (30° 32' 44.7036" N, 84° 35' 41.0820" W). Three *B. carinata* varieties were used for this experiment that include Avanza 641, AAC A120, and AGR137 (Agrisoma Biosciences) with seed TGC ranging from 60 to 140 μmol g^{-1}. This was tested against a low GSL (<30 μmol g^{-1}) industry standard canola *B. napus* cv. 1918 (Canterra Seeds in Winnipeg, MB, Canada). These genotypes will be referred to as Avanza, A120, AGR137, and Canterra for the remainder of this manuscript (Figure 5-1.). Pots (31.8cm height × 19.7cm diameter, 7.65 L) were filled with a fine sand substrate with 0.5 kg of gravel and a 10cm x10cm section of landscape

clothe at the bottom to reduce the possibility of sand loss. The sand was not autoclaved but was obtained as 'washed' from a local sand pit (Roberts Sand Co., Quincy, FL).

Four seeds were planted per pot at 0 days after planted (DAP), but subsequently reduced to one during a 28-day period. During this time, plants received supplemental lighting under a 12-h light/12-h dark photoperiod using high-pressure sodium lamps (photon flux ≈1200 μmol m^{-2} s^{-1}) to increase rates of plant establishments, but then relied on natural sunlight throughout the remainder of the experiment. Initially, they were automatically irrigated with 10 ml of water (ESP-LX BASIC 12, Rain Bird Corp., Azusa, CA) four times a day (at 0600, 1000, 1400, and 1800) for 7 days. Thereafter, S treatments were introduced with an increasing volumes of 10 ml every 3 weeks, on average. Heating during the Winter and evaporative cooling during the Spring was used to maintain temperatures above 5°C and below 30°C, respectively. Insecticidal soap (Bonide® at half strength) and yellow sticky traps were set up throughout the greenhouse to control pests as needed.

Treatments

Due to constraints with fertigation applications and the possibility of SRKN cross contamination, this experiment was set up as a randomized block design split by S rate and SRKN inoculation. Treatments were separated into tables containing either S rate (+S and -S) or SRKN inoculation (+SRKN and -SRKN) with six replications of each *Brassica* genotype. An additional set of plants was included for destructive sampling during the bolting stage of development. Plants received either 80% (+S) for optimal or 20% (-S) for sub-optimal S treatments derived from a complete Hoagland nutrient solution using Epson salt ($MgSO_4$) standardized with additional Mg as needed. The SRKN treatments included plant inoculated with 5000 eggs (+SRKN) or they were not (-SRKN). These eggs *M. incognita* (Race 3) were acquired

from the University of Florida Department of Entomology and Nematology and extracted from susceptible tomato roots (cv. Rutgers) and tested for viability.

Extraction and Inoculation

Extraction followed a modified version of (Stetina, McGawley, & Russin, 1997). Infected roots are rinsed free of soil, cut into smaller pieces, and placed into a mixer grinder with a 2.5% bleach solution and mixed for 30s. Then the contents are poured into a nested 125, 75, and 25 μm pore sieve, rinsed, and counted. Inoculation of SRKN occurred 30 DAP by first making five holes around the base of each plant using a 1 ml pipette tip at a depth of 3 cm and at a distance of 3 cm. Then, 1 ml of SRKN eggs (standardized to ≈1000 eggs ml^{-1}) was injected into each of the five holes for +SRKN treatments or 1 ml of only water for -SRKN treatments followed by immediate covering by sand. Irrigation drip lines where then relocated from the middle of the pot and placed at the opposite ends from the plant's base, but 5 cm from pot wall. This will help to reduce the potential flooding the eggs away from area of inoculation. At this time, plant fertigation was withheld for 7 days to induce moderate drought stress and increase the probability of root localization by emerging SRKN infective juveniles (J_2)

Biomass and Soil Cores

An entire set of plants were destructively sampled when plants reached the threshold of ≈80% bolting stage (60 DAP). Plant height were measured from the soil surface to the uppermost node during the flowering stage of development. For biomass dry weight (DW) during the bolting stage, plants were clipped at the base and the leaves and stems were collected into separate paper bags. Newly expanded leaves (10th and 11th leaf from the base) was analyzed for nitrogen and sulfur content (Waters Agricultural Laboratories Inc., Camilla, GA, USA). Only stems were used collected at harvest due to leaf senescence during the seed-fill stage. After collecting shoot biomass for DW, 3 soil cores were collected using a soil auger (3 cm in

diameter) at a distance of 5 cm from the base of the plant and covering the length of the pots (Figure 5-2.). The cores were combined in a plastic bag (≈100cc total) and stored into a 4°C refrigerator. Once all cores have been collected, then each combined sample was placed on a 0.5 mm diameter sieve, rinsed free from sand and debris, and stored in its own labeled 50-ml Falcon tubes and stored at 4°C until staining. The remainder potted root mass is then rinsed, allowed to drip overnight to remove excess moisture, and placed in paper bags so that can be dried at 50°C for DW with leaves and stem tissue.

Soil cores for SRKN evaluation were collected from the second set of plants when 80% of the population was at the seed-fill stage of development (120 DAP). This was following the same procedures as in the bolting stage, except holes were filled with sand and plants left to continue until maturity. On May 20th, 2019 (154 DAP) plants clipped at the base, all above ground biomass collected into paper bags, and seeds dried to <8.0% moisture and stems until completion. The root mass was not used for dry mass but was left intact post-harvest analysis using cotton.

SRKN Quantification

The staining of SRKN followed procedures of a modified version of Byrd et al. (1983). First, prepare 500 ml of 10% bleach solution (5.25% NaOCl) and 100 ml of acid fuchsin stain (AFS) by combining 0.350g of acid fuchsin, 25 ml of glacial acetic acid, and 75ml of double-distilled water (ddH2O). When all materials are ready, add 30 ml bleach solution to a single 50-ml Falcon tube containing the rinsed roots, replace the lid, and gently agitate for a period of 45s. Then immediately pour the contents onto a fine mesh sieve and rinse continuously for 45s. With forceps, place the roots in a clean 50-ml beaker filled with dd-H2O for 15 min to remove any residual bleach. Thereafter, pour the beaker contents onto the sieve, remove excess water

surrounding the roots by allowing it to drip and blotting with paper towels, and place it into another clean 50-ml beaker.

Next, add 30 ml of dd-H2O with 1 ml of AFS to the beaker containing the roots and gently agitate to homogenize the solution. Microwave the beaker on high until if boils for 15 seconds then remove, reagitate, and boil for another 15s. Allow the contents to cool down to room temperature, pour contents onto a sieve, and gently rinse until it runs clear. Now, roots will be cleared using acidified glycerin. After removing excess water and placing the roots back into the beaker, add 30 ml of glycerin with a few drops of 5N HCl, and repeat microwave boiling procedures as before. The contents can then be placed back into its original labeled Falcon tube and stored in 4°C until SRKN quantification.

A stereomicroscope (Nikon SMZ1500) was used to quantify the SRKN within each root tissue sample and classified by 3 stages of development (Figure 5-3.) that include infective juveniles (J_2), advanced juveniles (J_+), and adults females ($A_♀$). Sample was poured into the lid of a large polystyrene tissue culture dish (Falcon, 150x25mm, item# 353025) and spread evenly using dissecting needles. Then the bottom portion was placed over the top keeping the roots pressed in between the two with grid to guide the quantification. This will maintain a consistent viewing plane and increase the amount of light able to penetrate through the root tissue. The entire root sample was evaluated for SRKN and counted according to stage of development. To be classified as SRKN, morphological characteristics of SRKN (e.g. size, shape, and location) and root tissues (e.g. increased cell size and number) were used. Any nematodes not falling under the set of criteria of a SRKN where counted separately.

Post-harvest Cotton

To evaluate if *Brassica* genotypes can help reduced infection to crops following its harvest from previously SRKN infested soils, we will plant a susceptible Cotton cv. DeltaPine

1646 directly into pots 1 week after harvest. This simulate some of the effects of a “no-till” soil management system that could be employed following a *B. carinata* cropping season. First seeds were soaked and rinsed multiple times over the period of 24 hours to remove any residual chemicals surrounding the seed coat that are commonly applied to commercial seeds. Two cotton seeds were planted in the same pots as oilseed *Brassica* 1 week after harvested and placed 3 cm from the middle of the pot and perpendicular to the direction of *Brassica* stem stubble remaining in the soil. They were fertigated similarly as the *Brassica* plants, except using 100% Hoagland nutrient solution. At 60 DAP, the entire root mass was quantified for SRKN eggs.

Experimental Design and Statistical Analysis

This experiment was set up as a randomized block design containing six replication of *Brassica* genotypes (4 levels) and split or blocked by S rate (2 levels), SRKN inoculation (2 levels), and plant stage of development (2 levels). Normal distribution was evaluated using a goodness of fit test (Andersen-Darling) and a Box-Cox transformation was used as needed. ANOVA of plant characteristics was analyzed using JMP Pro 15 with “block” as a random effect and nematodes analyzed using SAS 9.4 via ‘PROC GLIMMIX’ (SAS Institute Inc, Care, NC) under a binomial distribution. Although *B. napus* share many characteristics as *B. napus* for comparing infection rates, morphologically there can be large distinctions and therefore analyzed separately. In addition, *B. carinata* can be compared solely on its own premise for varietal/genotypic distinctions or comparisons.

Results

Plant Morphology

P-values of significant biomass responses (Table 5-1.) and node counts of all *Brassica* genotypes are included in (Table 5-2). The average height of *B. carinata* across all treatments at harvest was 42.7cm with an 18.8cm node count. For *B. napus*, its average height was 43.8%

shorter than *B. carinata* (18.7cm and 22.0% less nodes (14.7cm) ($p<0.0001$). Within *B. carinata*, there was a significant effect with height due to genotype and a trend with S treatment interaction. AGR137 produced the tallest plants measuring 48.3cm followed by Avanza and A120 both averaging about 40.1cm. The interaction between S and SRKN was only observed with A120 ($P=0.0373$). Instead of increasing height with S rate, the response reversed with the introduction to SRKN. For node counts, A120 produced the most per plant averaging 20.9, followed by both Avanza and AGR137 (≈17.7), and Canterra (15.0).

Biomass

The average DW of all plant tissues separated by *Brassica* genotype, S rates, and southern root-knot nematode inoculation are included in Table 5-3. They also contain the total biomass accumulated at the bolting stage and when plants were harvested.

Bolting

The biomass is given as dry weight (DW) and comprised of leaf, stem, and roots for the total DW during the bolting stage of development (Table 3). Analysis of total N and S contents in leaves between Avanza and Canterra found no significant differences with leaf N content (4.4% N), but there was with leaf S content ($P=<0.0001$) including an interaction with genotype ($P=0.0007$). In the 80% S rates, leaves contained 1.3% S which dropped to 0.37% S at the 20% S rate. The S content in the 20% S rate remained similar when comparing Avanza and Canterra but differed at the 80% S rate where Avanza had 1.48% S and Canterra had 1.2% S in leaves during the bolting stage. There were no differences between in leaf DW across *Brassica* genotypes except when inoculated with SRKN where there was a decrease of leaf biomass of 9.4% with increasing S rates (Figure 5-4a). However, within *B. carinata*, there were differences among varieties with AGR137 producing the highest leaf DW at the 20% S rate and Avanza the lowest at the 80% S rate (Figure 5-5). Stem biomass production slightly, but significantly

increased by 5.9% with S rates across *B. carinata* genotypes (*P*=0.0422). Overall, genotype was a significant factor on stem biomass production with AGR137 (15.7 g DW) producing the greatest quantities, followed by A120 and Avanza averaging 12.6g DW, and Canterra (9.2g DW ±0.4 SE).

AGR137 roots DW accumulated the least biomass at 14.8 g DW compared to an average of 19.5 g DW of A120 and Canterra with Avanza falling in between (28.9 g DW). When comparing root to shoot ratios (R:S), AGR137 was the lowest at 0.51 followed by Avanza (0.72g), A120 (0.78g), and Canterra (0.86g) who were all grouped together across S and SRKN treatments (*P*=0.0279). There were main effects with SRKN treatments (*P*=0.0377) and with its interaction with S treatments (*P*=0.0441) when R:S was analyzed by species largely centered with Canterra. Without SRKN treatments, no differences occurred between R:S with increasing S rates averaging 0.89. However, +SRKN treated plants increased their R:S by 41% with S rates from 0.61 at 20% S to 1.03 at 80% S.

Agronomic maturity

Leaves senescence and drop occurs by the time plants reach agronomic maturity and therefore not included in total biomass at harvest. AGR137 stem biomass (125 g DW) was 24.6% greater than all other *Brassica* genotypes that averaged 94.8 g DW. RKN increased stem biomass production by 8.6% from 98.0 g DW to 107.3 across genotype and S treatments. The increase in stem biomass production was largely centered at the 80% S rate by 13.0% with no differences at the 20% S rate and non-inoculated treatments (Figure 5-4b). There were main effects with seed yields to genotype (*P*=<0.0001) with Avanza and A120 producing the highest quantities that averaged 35.0 g of seed per plant compared to AGR137 and Canterra with an average of 20.1g.

Seed Contents

The responses to all seed contents to *Brassica* genotype, S rate, and root-knot nematode inoculations are located in Table 5-4.

Total glucosinolate content (TGC)

Overall, *B. carinata* had more than a 4 factor increase of TGC (129.2 μmol g^{-1}) compared to *B. napus* (31.2 μmol g^{-1}). AGR137 had the greatest TGC in *B. carinata* seeds with 158 μmol g^{-1} followed by A120 and Avanza producing similar quantities of 113.7 μmol g^{-1}. TGC increased with S rates from 84.5 μmol g^{-1} at the 20% S rate to 125.7 μmol g^{-1} at the 80% S rate. Canterra had the lowest TGC of 31.2 μmol g^{-1} as expected, but with a trend indicating a decrease in TGC by 8.9% when infected with SRKN. When yield was factored into TGC, *B. carinata* produced 3.52 mmol compared to 0.67 mmol in *B. napus*. An interaction between S and SRKN treatments (P=0.0348) and a trend within genotypes (P=0.0775) was observed with *B. carinata*. Avanza at 80% S rate had the highest overall TGC yields and AGR137 at 20% S with the lowest averaging 5.01 mmol and 2.46 mmol, respectively.

Total oil content (TOC)

Seed TOC experienced main effects from *Brassica* genotype and S treatment with an SxSRKN interaction. Among *B. carinata* varieties, Avanza and A120 had similar TOC of 30.2% with AGR137 at 24.6%. Canterra produced seeds that contained 25% more TOC then all other *B. carinata* genotypes (37.0%), but not when including yields, so total oil production was slightly reduced by 5.6% (P=0.0451). Avanza and A120 both produced the most oil per plant (≈10.8 g) compared to AGR137 and Canterra (≈6.3 g). TOC decreased from 31.6% at 20% S to 27.8% at the 80% S rate, but this difference between S treatment was lost by the introduction of SRKN (P=0.0193). Although only a trend (P=0.0706), this amounted to the 80% S treatment producing the most oil (≈9.3% g) when inoculated with SRKN across genotypes.

Erucic acid content (EAC)

EAC in seeds are described as percent of TOC and responded significantly with S treatments and among *Brassica* genotypes (including yields). EAC increased slightly from 38.3% to 39.8% when plants were grown under the 20% S compared to the 80% S rates. All *B. carinata* varieties had higher EAC (P=0.0212) averaging ≈41.4% ±0.6 SE and total erucic acid yields (P=0.0343) compared to *B. napus*. Avanza and A120 produced more than double the amount of erucic acid per plant (≈4.4g) than what was produced by AGR137 and Canterra (2.3%).

Crude protein content (CPC)

B. carinata produces seeds with 27.1% higher CPC (42.6%) and 69.9% plants with more total crude protein (12.4g) than *B. napus*. The response of seed CPC was significant with S treatment and with its interaction to SRKN inoculation. Uninoculated plants produced seeds that averaged 39.4% CPC at the 20% S rate and increased slightly to 42.0% CPC at 40% S. However, there was no longer a significant difference between CPC and S rates when plants were grown in +SRKN treatments. Avanza and A120 produced more crude protein per plant (15.8g) than AGR137 and Canterra (7.1g) at the 20% S rate, but all with averages that lost significance from each other at the 80% S rate (7.7g).

SRKN in Roots

All of the responses between SRKN and *Brassica* genotypes, S treatments, plant stage of development are located in Table 5-5 and average counts of each SRKN constituent that include infective juveniles (J_2), advanced juveniles (J_+) and adult females ($A_♀$) are in Table 5-6. Only main effects were significant, and no interactions were observed.

Root samples were collected from each stages of plant development and found that the bolting stage had the highest SRKN counts averaging 9.8 SRKNs consisting mostly of J_2 and J_+

in about equal parts of 4.96 ct. and 4.61 ct., respectively. When plants were in the seed-fill stage, the population of SRKN decline 48.3% largely due to J_2 (62.2%) and J_+ (57.3%), but with an increase of $A_♀$ by a factor of 5 (Figure 5-6a). There was a consistent increase of SRKN infection rates when S treatment was reduced from optimal to sub-optimal rates. The total population increased 56.4% between 80% S and 20% S rates. $A_♀$ were impacted the greatest by 61.1%, followed by J_2 by 51.7%, and J_+ by 29.3%.

On average, Canterra always had the highest number of J_2, J_+, and $A_♀$ by 47.3%, 57.9%, and 65.8% compared to *B. carinata* genotypes, respectively (Figure 5-7). There was no difference with J_2 among *B. carinata* genotypes averaging 2.8 ct. across S treatments and plant stage. AGR137 had the lowest J_+ (2.0 ct.) and adult females counts (0.31 ct.). Although, Avanza and A120 had similar J_2 and J_+ counts (2.7 ct.), Avanza had about half (-45.6%) of the $A_♀$ then A120 (0.74 ct.). The highest total SRKN counts was with Canterra (12.6 ct.), followed by Avanza and A120 (6.2 ct.), and AGR137 (4.8 ct.).

SRKN eggs were extracted from cotton roots planted in the same pots as *Brassica* genotype after harvest. We observed that genotype had a significant effect on infection rates of SRKN in cotton (P=0.0014). It was estimated that the greatest number of SRKN eggs was found when cotton was planted with Canterra (4820 egg ct.) followed by both Avanza and A120 (2730 egg ct.) and AGR137 (280 egg ct.).

Correlations and Relationships

In Figure 5-8, the multivariate correlations between SRKN root counts, yield, and seed contents are listed with corresponding level of significance. Seed oil content has a strong negative correlation with TGC with a line of best fit of TOC = 40.3 - 0.097*TGC (R^2= 0.81; P=<0.0001) across *Brassica* genotypes and S rates. Seed GSL contents has a strong positive correlation with CPC and moderate with EAC. TGC in seeds had a mild to moderate negative

correlation with all SRKN stages that include J_2 (r= -0.2751; *P*= 0.0585), J_+ (r= -0.4226; *P*= 0.0340), $A_♀$ (r= -0.4592; *P*= 0.0221), and total SRKN (r= -0.4223; *P*= 0.0411) across all *Brassica* genotypes. Within *B. carinata*, a pairwise correlation found negative correlations between TGC and J_2 (r= -0.2667; *P*= 0.0332), J_+ (r= -0.2626; *P*= 0.0360), $A_♀$ (r= -0.2364; *P*= 0.0601), and total SRKN (r= -0.2364; *P*= 0.0263) while none was found with *B. napus*.

Discussion

The effects of S fertility on plant biomass and seed contents was characteristic with oilseed *Brassica*. We found that AGR137 had the greatest leaf DW at the 20% S rate and Avanza with the least leaf DW at the 80% S rate. The increase in leaf dry weights (DW) was considered the most sensitive measure in response to S deficiency in oilseed *Brassica* (Randall, Wang, Hocking, & Pinkerton, 1997). Total S content from leaf tissue decreased with S treatment identifying S deficient plants and the potential use of tissue analysis for diagnostic purposes or perhaps to differentiate between *Brassica* species (at optimal S only). The use of total S in leaves as a diagnostic tool could be employed, but only under certain conditions. Each *Brassica* crop would have to establish its own S fertility curve, not sampled early in the cropping season, and using leaves having similar physiological age (Blake-Kalff et al., 2000). The inclusion of other elements for this kind of diagnosis may increase the accuracy (Etienne et al., 2018), but it was not investigated in this study.

As expected, total glucosinolate content (TGC) was greater in the seeds of *B. carinata* compared to low-GSL *B. napus* and negatively linked total oil content (TOC). Despite this effect, Avanza and A120 produced high seed yields and were the top oil producers in the study. High-GSL oilseed *Brassica* have been reported to make better use of S than low-GSL genotypes which resulted in more seed yields in S-deficient soils even though they all required similar S fertility (Malhi et al., 2007a). The low-GSL trait in Canterra is thought to have been derived

from a Polish cultivar in the early 1970s containing a disruption in methionine-derived GSL (Met-GSL) like sinigrin (2-propenyl GSL) production pathways (Finlayson et al., 1973; Josefsson, 1971). This reduced the availability of GSL intermediates allowing only a small percentage of intact GSL making its way to the seed by GSL-specific transporter genes (Bloem et al., 2007).

This block of the Met-GSL pathway in low-GSL *B. napus* varieties explains why TGC was not affected by S treatments but had significantly affect on the TGC in *B. carinata*. Although the TGC in seeds may not be a good indicator for S requirement, it may be useful as a measure for resistance against the SRKN. For example, AGR137 had seeds with the greatest TGC and roots with the lowest SRKN infection rates. This is contrary to Canterra with the least TGC, but whose roots contained the greatest number of SRKN that include infective juveniles (J_2), advanced juveniles (J_+), and adult female ($A_♀$). Many studies report greater quantities of TGC in the shoots and roots of *Brassica* during the vegetative stage of plant development followed by the reallocation of GSLs as they are transported to the seeds at maturity (Bhandari et al., 2015; Falk et al., 2007; Madsen, Kunert, Reichelt, Gershenzon, & Halkier, 2015).

There is a close relationship between the GSL contents of the seed to that of shoots and roots in most *Brassica*. They follow the concept of optimal defense theory (ODT) where a substantial portion of the plants energy is allocated for the production and transportation of defensive compounds (i.e., GSL) to plant tissues considered of important to overall fitness heavily influenced by plant development and environmental stress (Keith & Mitchell-Olds, 2017; Tsunoda et al., 2017). Sub-optimal S fertility had a significant impact on the TGC in seeds, but it often exacerbated the effects of leaf, stem, and seed biomass and contents (TOC, EAC, & CPC).

TGC of the seeds is directly related to those that are in the roots of oilseed *B. carinata* allowing high-GSL varieties greater protection against SRKN. An analysis of many studies concerning GSL in *Brassica* plants report that roots often produce the most TGC with the highest diversity among 74 studies and 29 plant species (Van Dam et al., 2009). More than 40% of the TGC in roots were identified as 2-phenylethyl GSLs; an aromatic GSL that has been documented as an effective biocidal compound against many soil-borne pathogens. Six *B. carinata* varieties that were analyzed for GSLs found that the shoots are mainly comprised of 2-propenyl GSL and the roots up to 10.9% was aliphatic (2-propenyl GSL), 12.2% aromatic (2-phenylethyl GSL), and 1.2% indolyl with almost all successfully able to hydrolyze to ITC compared to only half in *B. napus* (Kirkegaard & Sarwar, 1998). The amino acid methionine contain sulfur as well as the core structure of GSLs, so the pervasiveness of Met-GSLs in the shoots and roots of *B. carinata* suggests why S scarcity would have a large impact on SRKN infection rates.

The inverse relationship between TGC in seeds and infection rates of SRKN continued with the planting of a susceptible cotton in the same soil as the oilseed *Brassica* simulating a no-till crop-rotation system. It is estimated that cotton roots contained more than 17 times the number of SRKN eggs when grown in Canterra soil compared to that of AGR137. Root infection rates at harvest give some information on SRKN populations but it is not completely clear how much of the cotton infection resulted from initial SRKN population in post-harvest *Brassica* soil or by the exposure to ITCs being released from the root residue through biofumigation. Usually, biofumigation is considered the process that takes advantage of high GSL plant residue from seed meal or green manure and incorporating it into the soil which then release ITCs to suppress soil-borne pathogens and improve soil quality as part of an integrated pest management program (Gimsing & Kirkegaard, 2009). However, to maximize the suppressive power of the ITCs

through constant exposure, measures must be taken to decrease plant residue and ITC decomposition since they are greatly affected by biotic and abiotic factors. More research is needed on the natural suppression of oilseed crops to SRKN and other soil-borne pathogens under a no-till or strip-till system. This will allow farmers to gain economic income while increasing the quality of their soils over time. Further investigations for the potential suppression of plant-parasitic nematodes using field-grown *B. carinata* are warranted. Especially, grown in various soil environments and under a no-till soil crop management system.

Conclusion

Oilseed *Brassica* produces a sulfur-rich defense compound called glucosinolates (GSL) that varies significantly with genetics and the environment. Under optimal S fertility, there is an increase in the accumulation of GSL in seeds that negatively impact oil production. However, sub-optimal S fertility will increase the infection rates of one of the most agriculturally important and most ubiquitous plant-parasitic nematodes in the world, *Meloidogyne incognita.* Compared to the commonly grown canola, *B. carinata* is significantly more capable of reducing infection rates and it increases with seed TGC. Despite the impacts of GSL to oil production, *B. carinata* cv Avanza 641 was one of the highest seed, oil, and erucic acid yielding varieties in this study, but more field-based research is needed to include the diverse soil environments located throughout the southeastern US.

Figure 5-1. Comparison of *Brassica* genotypes at the bolting stage of development.

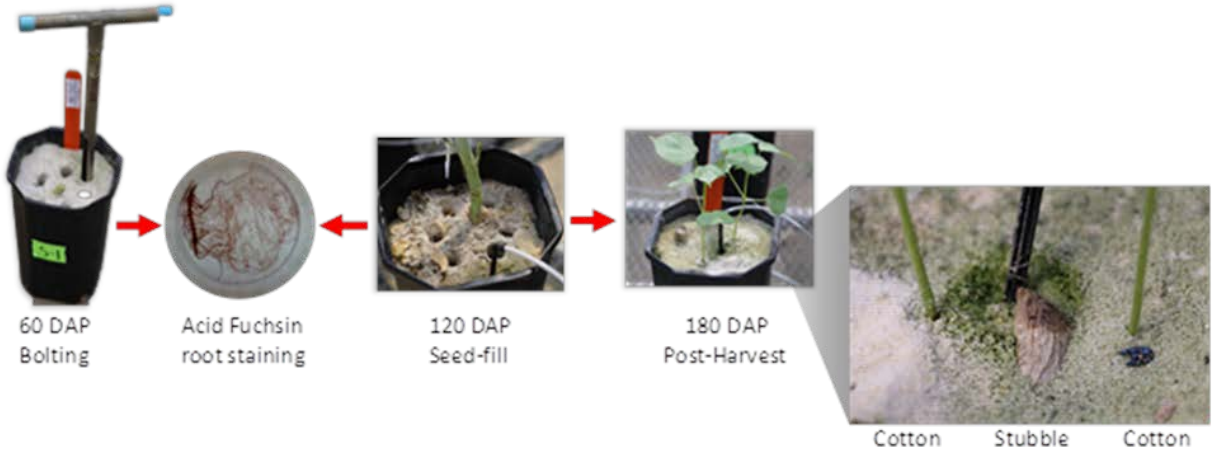

Figure 5-2. Soil core sampling at bolting and at seed-fill stage post-harvest cotton planted in the same pots as oilseed Brassica to test the infectivity of soil.

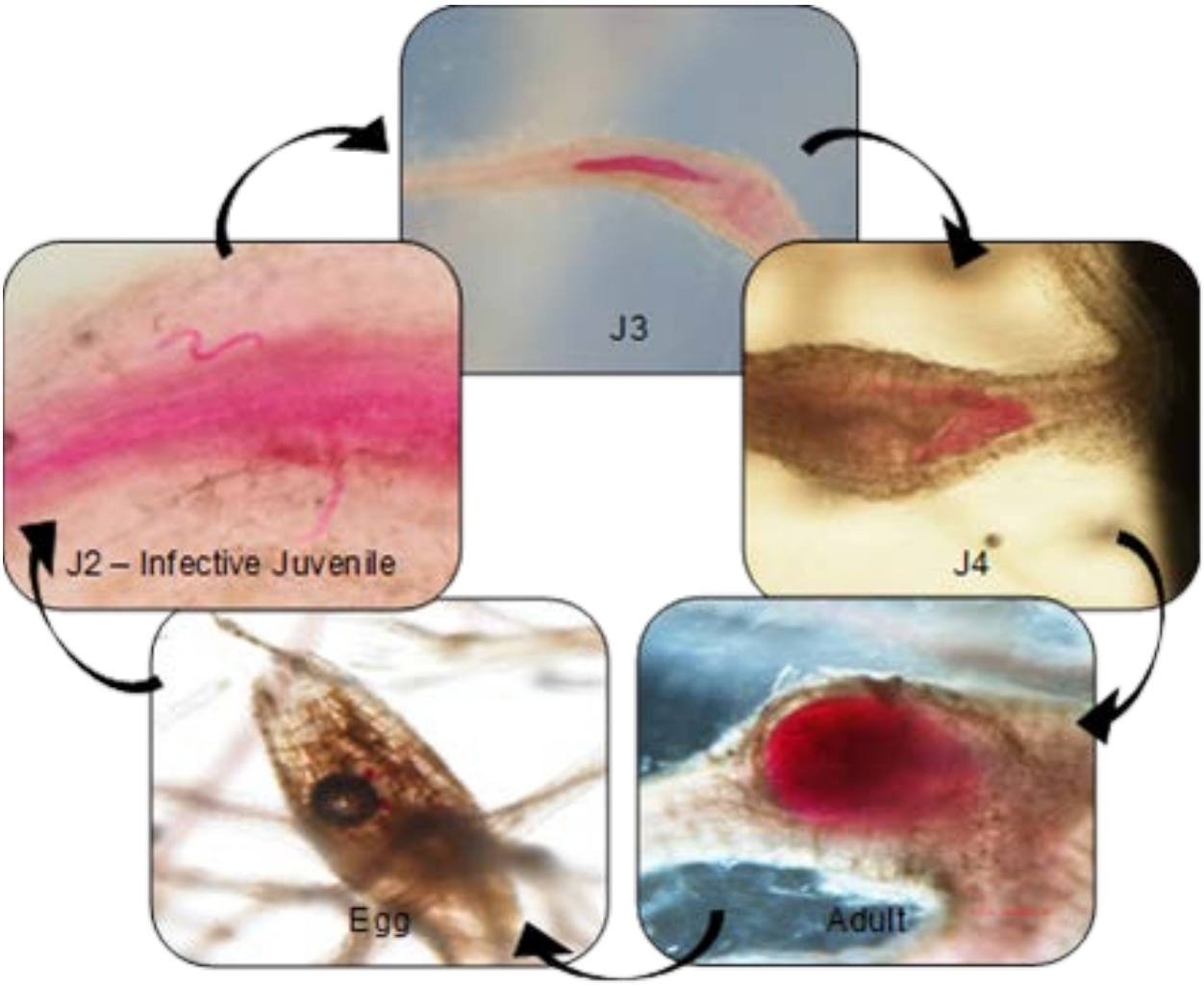

Figure 5-3. Southern root-knot nematodes stained in *Brassica* at each stage of development. The infective juvenile (J2) invades roots near the root meristem, they establish feeding sites and molt to stage J3 and J4, until becoming a mature adult female.

Table 5-1. The P-values of plant responses to genotype, southern root-knot nematode inoculation, and sulfur treatments.

Response	Data[†]	Genotype	SRKN	S Rate	NxS	GxS	GxN	GxNxS
Height $_H$	*carinata*	****	ns	ns	ns	.	ns	ns
Height $_H$	*Napus*$^{0.5}$	–	ns	ns	ns	–	–	–
Nodes $_H$	*Brassica*	****	ns	ns	ns	ns	ns	ns
Leaf $_B$	*Brassica*	.	ns	ns	**	.	ns	ns
Leaf $_B$	*carinata*	.	ns	.	**	*	.	ns
Stem $_B$	*Brassica*	****	ns	ns	ns	ns	ns	.
Stem $_B$	*carinata*	****	ns	*	ns	ns	ns	ns
Root $_B$	*Brassica* $^{0.4}$	**	ns	ns	ns	ns	ns	ns
Total $_B$	*Brassica*	ns	ns	ns	ns	ns	ns	ns
R:S $_B$	*Brassica* $^{0.2}$	*	ns	ns	ns	ns	ns	ns
R:S $_B$	*carinata* $^{0.2}$	ns	*	ns	*	ns	ns	ns
Stem $_H$	*Brassica*	****	***	ns	***	ns	ns	ns
Stem $_H$	*carinata*	****	***	ns	***	.	ns	ns
Yield $_H$	*Brassica*	****	ns	.	.	.	ns	ns
Yield $_H$	*carinata*	****	ns	*	.	ns	ns	ns

Total glucosinolate content (TGC), total oil content (TOC), erucic acid content (EAC), and crude protein content (CPC) in response to treatment factors genotype (G) sulfur treatments (S), and SRKN (N) with interactions. [†] Box-Cox transformation indicated with the value of λ in superscript and plant stage subscripts include bolting ($_B$), seed-fill ($_F$), and harvest ($_H$) stages of development. 3-way ANOVA and P-value: ****<0.0001, ***<0.001, **<0.01, *<0.05, .<0.1, and not significant (ns).

Table 5-2. Average plant height and number of nodes of *Brassica* genotypes with (+) and without (-) SRKN inoculations.

		− SRKN				+ SRKN			
	S Rate	Height (cm)		Node (ct.)		Height (cm)		Node (ct.)	
	(%)	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Canterra	20	18.9	3.3	14.6	0.6	20.9	3.8	15.5	0.6
	80	15.8	2.0	14.8	0.4	14.8	1.8	15.3	0.5
Avanza	20	37.4	1.6	18.5	0.3	42.7	3.7	18.3	0.7
	80	40.7	2.1	17.0	0.5	38.1	1.9	17.8	0.3
A120	20	38.4	1.3	21.5	0.5	43.6	2.3	21.1	0.7
	80	42.4	2.7	20.3	0.5	37.5	1.2	20.8	0.9
AGR137	20	46.1	1.4	18.4	0.4	46.1	1.7	17.2	0.3
	80	52.3	1.5	17.1	0.5	48.5	1.3	17.3	0.3

S treatments derived from the percent (%) of S of a complete Hoagland nutrient solution.

Table 5-3. Average biomass of plant tissue between *Brassica* genotypes, S rates, and southern root-knot nematode inoculations.

			Bolting (g DW)								Maturity (g DW)					
			Leaf		Stem		Root		Total		Stem		Seed		Total	
		S Rate	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Canterra	− SRKN	20	13.1	0.4	8.4	0.4	19.7	2.2	41.2	2.4	84.4	5.3	15.1	4.7	99.5	9.7
		80	13.7	0.5	9.4	0.9	20.4	3.1	43.5	3.9	81.6	5.3	21.7	3.6	107.5	5.8
	+ SRKN	20	13.0	0.8	10.5	0.9	14.2	0.7	37.7	1.2	86.0	5.7	21.9	4.4	107.9	9.8
		80	12.5	0.5	8.3	0.9	21.4	2.5	42.3	3.1	96.0	3.9	28.5	3.0	124.5	6.5
Avanza	− SRKN	20	12.6	0.3	12.5	0.6	21.6	3.5	46.7	3.0	98.4	3.7	41.4	1.9	139.8	5.0
		80	11.8	0.8	12.1	1.1	16.9	2.4	40.8	3.9	98.7	5.9	31.0	4.3	129.7	4.8
	+ SRKN	20	13.5	0.6	10.0	1.8	16.4	1.2	39.9	0.4	101.1	3.3	36.4	2.0	137.5	3.5
		80	12.0	0.4	13.8	0.9	17.9	3.1	43.7	3.5	118.0	5.3	41.2	3.5	159.2	7.0
A120	− SRKN	20	11.8	0.5	12.5	0.2	20.3	2.9	44.6	3.2	98.7	3.5	39.2	1.9	137.9	3.6
		80	13.7	0.3	13.8	1.1	22.3	1.6	49.8	1.8	81.6	5.0	24.6	4.9	110.7	7.5
	+ SRKN	20	12.6	0.6	12.8	0.3	19.0	1.0	44.3	1.7	95.1	8.0	36.3	5.1	131.4	4.0
		80	11.7	0.5	14.1	0.8	18.6	2.7	44.3	3.3	99.5	7.4	29.4	5.2	128.8	6.2
AGR137	− SRKN	20	13.5	0.4	15.1	0.6	14.6	0.8	43.7	1.1	123.5	1.8	16.0	3.4	139.5	4.1
		80	13.4	0.5	16.5	1.1	13.3	1.4	43.1	2.8	117.4	3.2	13.3	3.6	130.7	5.8
	+ SRKN	20	13.6	0.4	15.8	0.4	16.6	2.2	46.0	2.7	120.6	5.1	22.0	3.8	142.6	6.4
		80	12.0	0.3	15.6	0.5	14.6	2.0	42.2	2.5	141.7	6.4	22.5	4.3	164.2	10.2

S rates are in percent (%S) of a full-strength Hoagland nutrient solution and average DW biomass is included with standard error (SE)

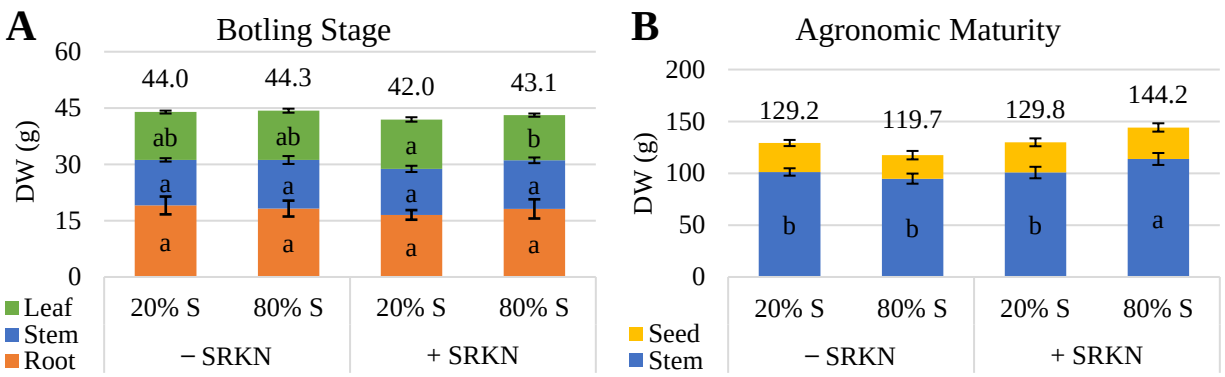

Figure 5-4. Total and individual tissue biomass by S rate and SRKN of *Brassica* genotypes. They include significant differences of tissue biomass from the A) bolting stage and B) at harvest with unique letters indicate the separation of means using Tukey HSD test (P<0.05).

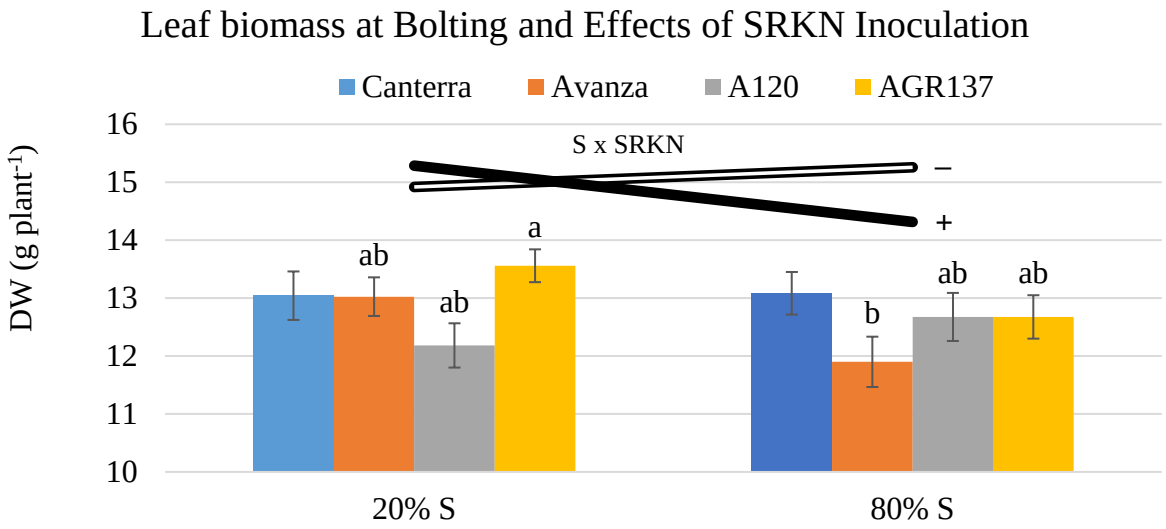

Figure 5-5. Leaf biomass by S rate at bolting and the effect of SRKN inoculation. Only *B. carinata* was included for the Tukey HSD test (P<0.05) with separation of means indicated by unique letters following an ANOVA with significant differences between varieties and a trend (*P*=0.0505) with the interaction of S rates with (+) and without (-) SRKN inoculations.

Table 5-4. The response of seed components and yields to *Brassica* genotypes, S rates, and root-knot nematode inoculations.

Response	Data†	Genotype	SRKN	S Rate	NxS	GxS	GxN	GxNxS
TGC_C	*carinata*	****	ns	****	ns	ns	ns	ns
TGC_C	*napus*	–	.	ns	ns	–	–	–
TGC_Y	*carinata*	.	ns	ns	*	ns	ns	ns
TGC_Y	*napus*	–	ns	ns	ns	–	–	–
TOC_C	*Brassica*	****	ns	****	*	ns	ns	ns
TOC_Y	*Brassica*	****	ns	*	.	*	ns	ns
EAC_C	*Brassica*	*	ns	*	ns	ns	ns	ns
EAC_Y	*Brassica*	****	.	*	ns	.	ns	ns
CPC_C	*carinata*	ns	ns	****	***	ns	ns	ns
CPC_C	*napus*	ns	ns	ns	ns	ns	ns	ns
CPC_Y	*Brassica*	****	ns	ns	ns	.	ns	ns

Seed contents include total glucosinolate content (TGC), total oil content (TOC), erucic acid content (EAC), and crude protein content (CPC) in response to treatment factors. Treatment factors include genotype (G) sulfur treatments (S), and SRKN (N) with interactions following a 3-way ANOVA. Datasets included all 4 genotype (*Brassica*) or split by species (carinata or napus). Subscripts: C = content; Y = yields. P-value: ****<0.0001, ***<0.001, **<0.01, *<0.05, .<0.1, ns= not significant.

Table 5-5. The response of root-knot nematode infection rates to *Brassica* genotypes, S rate, and by stage of plant development.

Response	SRKN†	Genotype	S Rate	Stage	DxS	GxS	GxD	GxDxS
SRKN	J_2	*	****	****	ns	ns	ns	ns
SRKN	J_+	****	*	****	ns	ns	ns	ns
SRKN	$A_♀$	**	*	***	ns	ns	ns	ns

Datasets include all 4 *Brassica* genotypes and only +SRKN treatments for the analysis since none was observed during sample staining quantification. † SRKN infective juveniles (J_2), advanced juvenile (J_+) and adult females ($A_♀$). P-value: ****<0.0001, ***<0.001, **<0.01, *<0.05, .<0.1, ns= not significant.

Table 5-6. The average number of root-knot nematodes in the roots of *Brassica* genotypes with plant development.

		J_2				J_+				$A_♀$			
		Bolting		Seed-fill		Bolting		Seed-fill		Bolting		Seed-fill	
	S Rate	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Canterra	20%	12.5	4.2	3.2	1.4	11.2	3.5	3.7	1.5	0.5	0.5	3.0	1.2
	80%	4.5	1.5	1.1	0.4	5.6	2.5	2.9	1.6	0.3	0.2	1.9	1.0
Avanza	20%	5.8	2.0	2.2	1.1	4.3	1.6	1.2	0.5	0.5	0.3	0.7	0.3
	80%	3.1	1.3	0.7	0.4	4.6	2.0	0.8	0.4	0.1	0.1	0.3	0.2
A120	20%	3.9	1.5	3.5	1.7	2.9	1.1	3.5	1.7	0.3	0.2	2.4	1.4
	80%	3.1	1.4	1.3	0.7	3.6	1.7	0.7	0.3	0.1	0.1	0.3	0.2
AGR137	20%	3.9	1.4	2.0	0.9	2.7	1.1	1.5	0.7	0.0	0.0	0.8	0.3
	80%	2.9	1.2	1.0	0.6	2.3	0.8	1.3	0.5	0.1	0.1	0.3	0.2

The counts of southern root-knot nematodes (SRKN) are split by their sampling period in terms of plant development (bolting and seed-fill) and SRKN stage that include infective juveniles (J_2), advanced juveniles (J_+), and adult females ($A_♀$) derived from 100cc soil cores and can be multiplied by 75 for whole-pot estimates.

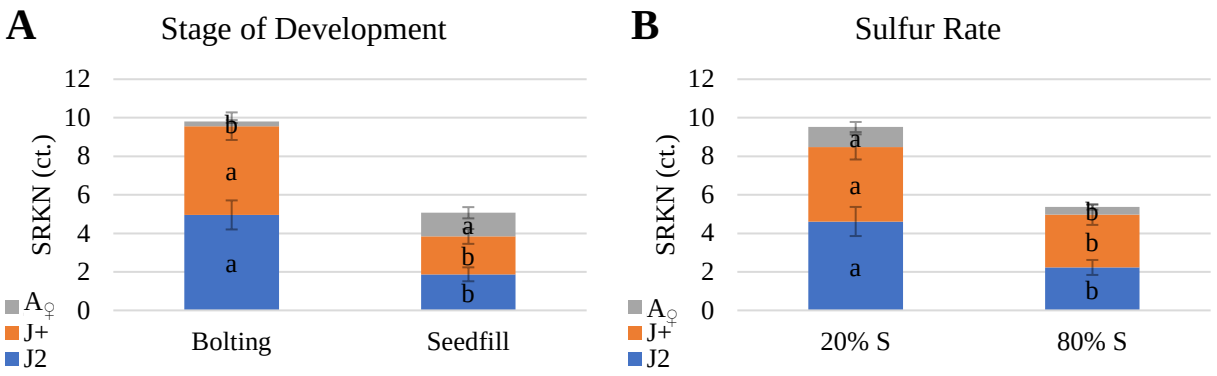

Figure 5-6. Total and individual number of root-knot nematodes in root samples across *Brassica* genotypes. They include infective juveniles (J_2), advanced juveniles (J_+), and adult females ($A_♀$) by A) plant stage of development and by B) S rate. Separation of means (between factors) has unique letters using Tukey HSD test ($P<0.05$).

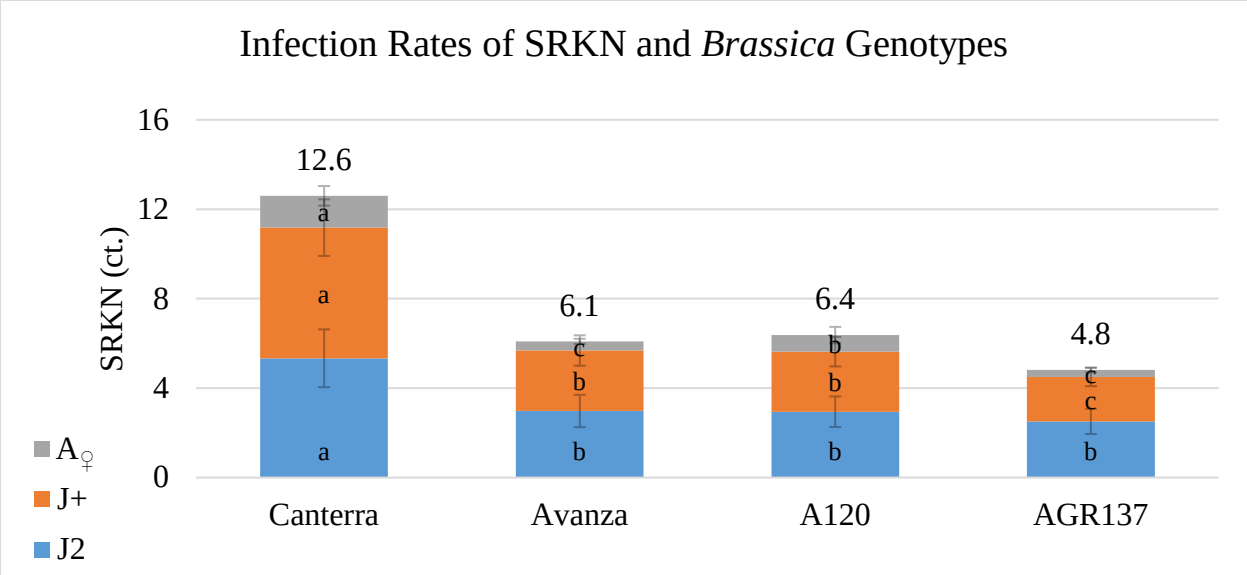

Figure 5-7. Total and individual root-knot nematode across plant stages and S treatment. The southern root-knot nematode includes infective juveniles (J2), advanced juveniles (J+), and adult females (A♀). Separation of means has unique letters using Tukey HSD test (P<0.05).

Figure 5-8. Correlations and P-values between root-knot nematode infection rates and seeds contents.

	J_2	J_+	$A_♀$	Total	Yield	TGC	TOC	EAC	CPC
J_2		0.7348	0.6102	0.8710	0.0288	-0.2751	0.1748	0.0680	-0.0961
J_+	*** *		0.8392	0.9514	-0.0569	-0.4226	0.3066	-0.0763	-0.3265
$A_♀$	*** *	****		0.8911	-0.0756	-0.4592	0.3297	-0.1781	-0.3783
SRKN T	*** *	****	****		-0.0364	-0.4223	0.2959	-0.0623	-0.2898
Yield	ns	ns	ns	ns		-0.0297	0.3358	0.3343	0.0579
TGC	.	**	**	**	ns		-0.8886	0.4988	0.8490
TOC	ns	*	*	*	*	****		-0.3239	-0.8288
EAC	ns	ns	ns	ns	*	***	*		0.6230
CPC	ns	*	**	*	ns	****	****	****	

Includes infective juveniles (J2), advanced juveniles (J_+), adult females ($A_♀$) and total SRKN with seed yields, total glucosinolate content (TGC), total oil content (TOC), erucic acid content (EAC), and crude protein content (CPC). The shade and color indicate relative characteristics about their correlations that include decreasing in strength (darker to lighter) and direction (positive/direct as orange and negative/inverse as blue). P-value: ****<0.0001, ***<0.001, **<0.01, *<0.05, .<0.1, ns= not significant.

CHAPTER 6
SUMMARY

The overarching objectives presented in these studies was to evaluate the dynamics of GSL production in *B. carinata* under biotic and abiotic stress to help improve oil quality and integration into the southeastern US cropping systems. We first followed the accumulation of GSLs in various plant tissues through development when grown in soil derived from areas with various land management practices and soil types where *B. carinata* may be potentially grown. Changes in TGC throughout plant development suggests that *B. carinata* does follow the pattern described for the optimal defense theory. Leaves contained the greatest TGC during the rosette and bolting stage producing an average of 78.0 μmol g^{-1} but decreased by 50% by the time it had reached the flowering stage. The stem maintained an average TGC of 11.2 μmol g^{-1} from rosette through flowering, followed by a sharp increase of a factor of 5.5 to a TGC of 72.7 μmol g^{-1} at maturity. This is a strong indicator that the flow of GSLs from the leaves and throughout the plant structure was abruptly halted and no longer being transported to the seeds, but instead accumulating in the stem. There was a doubling of root TGC in bahiagrass pasture soil compared to sand. This change in GSLs may be indicative of increased interactions by an increase microbial and fungal presences between soil types but is did not affect seed yield or oil quality.

Sulfur fertility studies were preformed in a semi-controlled environment evaluating 4 *Brassica* genotypes to effects of S availability. Two commercial *B. carinata* cultivars (AAC-A120 and Avanza 641), a high GSL experimental genotype (AGR 137), and a low GSL canola (*B. napus* cv. Canterra 1918). Seed yield increased quadratically ($y = -33.8x^2 + 71.0x + 4.8$; $R^2 = 0.9732$) with S rates across genotypes. Overall, the maximum seed yield was estimated at 42.1 g $plant^{-1}$ at the 105% S rate (≈210 g $MgSO_4$ or 47.5 g of S). Avanza produced this greatest seed yield and total GSLs, erucic acid, and crude protein among all *Brassica* genotypes. This was

despite AGR 137 having the greatest content of each seed component (per seed) but also producing the lowest seed yield. Yield was only minimally correlated with TOC, but there was a strong inverse correlation between TOC and TGC exhibiting a linear relationship across genotype and year. Total oil content also had an inverse correlation to CPC and EAC. The total GSL content of seeds had a strong positive correlation to CPC and moderate correlations were observed between EAC and TGC or CPC.

We evaluated these same *Brassica* genotypes for S availability grown under field conditions at two locations having similar climates but variable soil texture. *B. carinata* doubled its seed, EAC, and nearly tripled its TGC productivity in Quincy, where soils had increased clay content and heterogeneity compared to the deep sands of Citra. Quincy soil is classified as Ultisols and resembles those from where *B. carinata* had originated (i.e., the Ethiopian Highlands). In Citra, *B. carinata* had reduced yield, but to a much lesser extent than canola. Amongst all *Brassica* genotypes, *B. carinata* cv. Avanza 641 yielded the greatest EAC and ranked the most stable across the two difference environments. There was an S fertility curve of $Yield(g)=-0.457x^2 + 29.2x + 869.5$ ($R^2 = 0.993$) with a maximum yield potential at the 32.0 kg ha^{-1} S rate and an estimated 1.34 kg of seeds per plot or 0.146 kg m^{-2} (≈ 1.46 t ha^{-1} or 64.4 bu ha^{-1}). Correlations between seed yield and components remained similar between greenhouse and under field conditions. It was visually evident that plants from these experiments suffered from severe S deficiency at the 0% S rate or 0 S kg ha^{-1} by the shortage of seed production, delayed maturity, extreme leaf chlorosis, and cupping in *B. carinata* or the reddish-purple leaf margins of *B. napus*. Although similarities in the greenhouse and field trials were initially observed, they were lost in the Quincy location during the bolting stage of development, indicating that roots may have reached the clay layer residing underneath the soil surface.

One of the most asked questions by farmers inquire about the ability of root-knot nematodes (*Meloidogyne spp.*) to infect *B. carinata*. We tested the infection rate of the same *Brassica* genotypes having variable seed TGC and under optimal and sub-optimal S fertility treatments. Although relatively low compared to susceptible crops, we found that *B. carinata* can get infected by the SRKN and rates increased by over 50% when they are under suboptimal S fertility treatments. The biggest impact with increasing S rates was the number of adult females who suffered a 61.6% decline. Canola always had the highest infection rates compared to *B. carinata* with an increase of 47.3% to 65.8% between infective juveniles and adult females, respectively. There was also a negative correlation between TGC and infection rates, but not with yields indicating a tolerance to SRKN in *B. carinata.* Female adult SRKN declined sharply by 50% in Avanza 641 indicating that mortality of SRKN increase over time. Similar response between TGC and EAC, TOC were observed as previous S fertility studies. The potential of SRKN infection to subsequent crops was tested by planting a susceptible cotton following *Brassica.* The same pattern emerged where SRKN infection rates declined with increasing seed TGC. All cotton planted after *B. carinata* had significantly less SRKN infection then after canola. It would be interesting to see if this translated into the possibility of using *B. carinata* for suppressing SRKN in infested soils during the Winter season before the planting of cotton in the Spring. It would allow farmers to make additional economic gains by harvesting the seeds of *B. carinata* or incorporate all of the biomass into the soil to maximize the suppressive power of ITC as it commonly done with high-GSL mustards for biofumigation following soil amendment strategies under various types of sustainable farming practices.

These experiments were able to answer a significant amount of questions concerning the dynamics of *B. carinata* and the effects of GSL on yield and seed components with S fertility,

soil environment, and when under attack from soil-borne pathogens. Avanza was the best preforming genotype among all *Brassica* tested. Having increased EAC is a sought-after trait in oilseed crops to achieve greater conversion efficiency into biodiesel and biojet fuel. These studies will not only help to increase economic gains due to oil production but also add value to *B. carinata* on an agroecological level by improving soil health. Florida's agricultural community can take advantage of growing a winter cash crop but also reap some of the benefits that are usually provided by cover crops in a variety of soil environments throughout the region.

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BIOGRAPHICAL SKETCH

Theodor L. Stansly (Ted) spent most of his childhood years in Labelle, FL where he earned his high school diploma and was involved in extracurricular activities such as baseball, track, cross-country, and wrestling. After graduation, Ted and his family spent a year living in Spain and traveling throughout Europe during the time when his father helped to effectively eliminate the use of pesticides for vegetables grown in enclosed environments by exploiting introduced and native beneficial insects.

Once back in the US, Ted began working at the UF/IFAS SFREC in Immokalee for the Department of Plant Pathology maintaining field and laboratory research trials. Thereafter, he worked for private industry as a quality control specialist for an electronics company. He was also responsible for the procurement of obsolete military parts and negotiations to acquire computer components from locations around the world. Ted then spent some time working for a waterway management company that helped control invasive exotic plant species including the removal of Melaleuca trees that are invading protected natural habitats.

It was about this time that Ted decided to get back into academia and follow his passion for science. In 2010, he acquired his AA from Edison College and enrolled at the Florida Gulf Coast University (FGCU). Ted attended the Summer Institute for Biostatistics (SIBS) at the University of South Florida exploring aspects of epidemiology and advances in cancer research. After a year, Ted transferred to the University of Florida pursuing a BS degree in molecular plant biology. During this time, he worked for the USDA studying the behavior of root-knot nematodes and identifying repellents made naturally by plants. Ted then continued his interest in plant research and was introduced to *Brassica* carinata during his MS (horticulture) followed by his PhD (agronomy) at the University of Florida.