Increasing Accumulation of Glyceollins in Soybeans via Optimization of the Fungal Incubation Process

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INCREASING ACCUMULATION OF GLYCEOLLINS IN SOYBEANS VIA
OPTIMIZATION OF THE FUNGAL INCUBATION PROCESS

BY

STEPHANIE WOOTTON

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INCREASING ACCUMULATION OF GLYCEOLLINS IN SOYBEANS VIA
OPTIMIZATION OF THE FUNGAL INCUBATION PROCESS
STEPHANIE WOOTTON

This thesis is approved as a creditable and independent investigation by a
candidate for the Master of Biological Sciences degree and is acceptable for meeting the
thesis requirements of this degree. Acceptance of this does not imply that the conclusions
reached by the candidate are necessarily the conclusion of the major department.

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I dedicate this thesis to my family; Terrance and Anita for raising me to remember the importance of remaining down-to-earth while always looking through rose-colored glasses, and Kaylee and Austin for being the friends God wanted me to spend a lifetime with. This would not have been possible without their love and support.
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ABBREVIATIONS

DMSO- dimethyl sulfoxide
ER- endoplasmic reticulum
ESI- electrospray ionization
GES- Glyceollins enriched soybeans
GYE- glucose yeast extract
G4DT- glycinol 4-dimethylallyltransferase
HPLC- high performance liquid chromatography
IFS- isoflavone synthase
LC-MS liquid chromatography-mass spectrometry
MS- mass spectrometer
PAL- phenylalanine ammonia-lyase
PDA- photodiode array detector
PDA- potato dextrose agar
PPAR-γ- peroxisome proliferator-activated receptor-gamma
TFA- trifluoracetic acid
UV- ultra-violet
2-HIS- 2-hydroxyisoflavanone synthase
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Continual use of antibiotics in the feed of food animals was viewed a solution to the problem of disease outbreaks in livestock produced in confinement operations. This practice also improved animal performance, likely due to the reduction in sub-clinical infections. Unfortunately, this practice led to a new problem, the development of antibiotic resistant microbes. This increase in antibiotic resistance reduced the direct benefits of antibiotics in animal production. Moreover, as antibiotic resistance spread from animal to human pathogens, this practice created a major public health concern. This led the FDA to enact the Veterinary Feed Directive in 2017 that greatly restricts the use of antibiotics in feed or water for production purposes, limiting them instead to therapeutic uses.

The goal of this research thesis was to optimize a process for producing a natural antimicrobial (known as glyceollins) in soybeans, so that the glyceollins-enhanced soybeans (GES) could be used to replace antibiotics in the animal feed industry. Various processing factors for optimization were assessed including seed sterilization, pretreatment, fungal application, moisture maintenance, incubation and germination methods, and the use of filter paper. Possibly the most important variable related to production of glyceollins in soybeans is the variety of soybean seed used and how well it
responds to the elicitor or microbe introduced. Therefore, this study also compares several soybean varieties to determine which resulted in the most glyceollins production.

Controllable processing factors were individually tested by manipulating a standard production process developed previously by our team. This standard process included: 1) 10 min surface sterilization of soybeans in a 2% Micro-90 cleaning solution, 2) rinse with sterile water, 3) soak in 500 ml of sterile water overnight, 4) drain off water and aseptically dehull soybeans, 5) place 10 dehulled soybeans into Petri dish lined with sterile, moistened filter paper, 6) dropwise inoculate the 10 dehulled soybeans with 100 µl of previously grown inoculum, and 7) incubate plate at 30℃ in the dark for 120 hr. The typical fungal strain used to inoculate soybeans was *Trichoderma reesei* (NRRL-3653). *T. reesei* was prepared by inoculating a flask of glucose yeast extract broth (GYE) an incubating 48 hr at 30℃. Soybean samples were collected daily during incubation by harvesting all 10 soybeans on a plate, freezing and freeze-drying, and then grinding. Samples were extracted and glyceollins were measured by high performance liquid chromatography (HPLC).

Application of the surface sterilant showed no negative effect on germination percentage or rate. Comparison of glyceollins titer between sterile and non-sterile beans showed some increase in the former. Inoculum application method showed the best results for both fungal growth uniformity and production of glyceollins when using the standard dropwise method. Type of inoculant (mycelial or spore) had little to no effect on germination of the seeds. Comparison of incubation length showed the titer of glyceollins typically peaking within the 72 to 120 hr, with glyceollin levels dramatically falling after
144 hr. The use of filter paper had no effect on the inoculum's ability to stimulate production of glyceollins in the soybeans.

The variety of soybean used accounted for the largest difference in glyceollins levels. The MN01050628 soybean variety reached levels of glyceollins ~2 mg/g at the 96 hr, while the lowest producing variety Kouri peaked at ~0.03 mg/g at 48 hr. Investigation of total isoflavones of the soybean varieties and the precursor of glyceollins, daidzein, was also conducted and were shown to have no direct correlation to the glyceollins produced by the varieties examined. This suggests further study is warranted regarding the genetic makeup of the soybean variety and that variety’s response to a chosen fungal strain or physical elicitor.
CHAPTER I – LITERATURE REVIEW

1.1 Soybeans

1.1.1 Overview

Soybeans are an exceptionally important leguminous crop and are noted for enhancing energy and glucose homeostasis because of their isoflavonoid and soy protein components (Gudbrandsen, Wergedahl, & Berge, 2009). They are one of the most economically significant leguminous seed crops, supplying a large portion of world plant proteins, and more than a quarter of the world’s food and animal feed (Tian et al., 2010). The United States is the world’s largest producer and consumer of soybeans (Shiro et al., 2013). Soybeans are utilized for various food products, feedstuffs, and biofuel feedstocks (Shiro et al., 2013). Soybean meal is a huge source of protein in the swine and poultry industry and is also an important ingredient in the diets of pets and domesticated household animals. Additionally, it is a vital source for protein in aquaculture diets (Willis, 2003). Their status as the sole oil-bearing leguminous crop give them another boost in commercial utility (Leff et al., 2004). Soybean seed protein and oil composition are affected by the environment during seed development (Cherry et al., 1985). They are commonly grown as edible beans retaining several health-promoting properties (S. Kim et al., 2005).

1.1.2 Composition

Most legume species have a protein content around 20-30%, making soybeans the legume with the highest protein content (≥40%) on dry matter basis (Liu, 1997). Examples of proteins included in the soybean makeup are proteases inhibitors and
lectins (Ng et al., 2011). The fat content of soybeans (20% oil), make it the highest among food legumes (Salunkhe et al., 1982). Other components of soybeans include phospholipids, vitamins, and minerals (Liu, 1997), which make it a highly nutritious crop. Soybeans maintain a small amount of other minor compounds including biologically active ones, such as trypsin inhibitors, phytates, and oligosaccharides (Liu, 1997).

1.1.3 Phytochemical Components

Soybeans possess many beneficial phytochemicals, such as isoflavones like genistein and daidzein (Ng et al., 2011; Sirtori et al., 1997). The focus on the isoflavones of soybeans has mostly been directed to the health benefits of both the hormonal and non-hormonal properties and prevention of human cancer cells and diseases (M Messina et al., 1994; Mark Messina & Redmond, 2006). With soybean isoflavones, there is a much heavier expression in certain tissues, seeds and roots, than other tissues, hypocotyls and leaves. Further induction in these areas can be generated in response to elicitor treatment or pathogen confrontation (T. Graham & Graham, 1991).

Daidzein, genistein, and glycitein are examples of isoflavones produced (Yu et al., 2003), these three are products of the phenylpropanoid pathway (T. L. Graham, 1991). In the conjugate form of glucosyl- and malonyl-glucose, they can then be amassed in the vacuole (T. L. Graham, 1991). As described by Yu et al., (2003) in Figure 1.1, daidzein has a pathway that branches from the phenylpropanoid pathway. This branching is common for most plants in concurrent with the chalcone synthase catalyzed reaction through a legume specific enzyme, chalcone reductase (CHR). Glycitein (3) synthesis is not yet clearly defined but is likely derived from isoliguiritigenin. Genistein 2 synthesis
shares the naringenin intermediate with the flavonoid/anthocyanin branch of the phenylpropanoid pathway. With all of the isoflavones, the aryl migration reaction to create the isoflavone is mediated by isoflavone synthase (IFS), the encoding gene of which has been identified (Akashi et al., 1999; Jung et al., 2000; Steele et al., 1999; Yu et al., 2003).

**Figure 1.1:** Synthesis of Daidzein (1), Genistein (2), and Glycitein (3) (Yu et al., 2003).

### 1.1.4 Genetic and Environmental Influences on Soybean Composition

Final composition of a soybean seed is not only influenced by genotype but also influenced by environmental conditions during development (Brummer et al., 1997; Fehr et al., 2003; Nichols et al., 2006; Rotundo & Westgate, 2009; Vollmann et al., 2000;
Westgate et al., 1999; Wilson, 2004; Yaklich et al., 2002). There are numerous reports on regional soybean seed composition variations (Brumm & Hurburgh, 2006; Dardanelli et al., 2006; Hurburgh Jr, 1994; Hurburgh Jr et al., 1990; E. L. Piper & Boote, 1999; Rotundo & Westgate, 2009; Yaklich et al., 2002). A challenge faced by commercial soybean processors is the issue of maintaining a high-quality soybean while developing a somewhat universal model for possible environmental effects on soybean seed composition to foresee ideal regions and conditions (Rotundo & Westgate, 2009). A widely used approach to investigation of environmental factors on seed composition is manipulative experiment (Westgate et al., 1999). Buildup of phytic acid in soybean seeds is influenced by interaction between soil type and temperature (Kumar et al., 2006). Comparing growing locations and conditions, temperature is more influential to linolenic acid content than photoperiod (Howell & Collins, 1957; Kumar et al., 2006). For soybeans grown under warmer temperatures it is noted they will have elevated oleic acid and low polyunsaturated fatty acids (Kumar et al., 2006; Rennie & Tanner, 1989; Wolf et al., 1982). Low temperatures also have been shown to have an effect on protein and oil content, having a positive correlation with the former and a negative correlation with the latter (Kumar et al., 2006). In a study conducted by Kumar et al. (2006) it was noted that during seed development mean temperature had a significant correlation with protein in the positive direction and with oil and linolenic acid in the negative direction (P < 0.05).

### 1.1.5 Soybean Worldwide Production and Market Distribution

Production and consumption of soybeans is booming globally. This is evidenced by an increase of 200 million tons in the global consumption since the seventies (Boerema et al., 2016). Soybeans now occupy 5% of the world’s croplands (Leff et al., 2004). They,
like some other oilseeds such as canola and sunflower, prefer temperate conditions and are generally planted May through June and harvested October through November (Leff et al., 2004). The production of soybeans has grown considerably over the years, especially in the Americas, with a considerably positive growth trend (Leff et al., 2004). Its domestication is proposed to root from its annual wild relative, Glycine soja, in China approximately 5,000 years ago, which was later expanded to producing an assortment of soybean landraces in different climate environments (Tian et al., 2010). For the first three decades of the twentieth century, production of soybeans was largely restricted to the Orient regions such as China, Indonesia, Japan, and Korea (Burtis, 1950; Hymowitz, 1970; C. V. Piper & Morse, 1910, 1923). Somewhere in the late 1940's and early 1950's, the United States then overtook these Orient regions in production of soybeans (Hymowitz, 1970). By the late 1960's, roughly 28 million soybean hectares were sown in around 25 countries (Hymowitz, 1970). United States farmers grew nearly 76% of the total world production while China grew 17%. The areas of greatest soybean growth of the United States and China are located within the 35 to 45-degree north latitudes (Hymowitz, 1970). Other countries which continued or began to grow considerable numbers include Indonesia and South Korea, along with Brazil and U.S.S.R. (Hymowitz, 1970). Brazil has recently seen an incredible increase in soybean exportation; in fact, according to SoyStats.com, Brazil currently accounts for 47% (about 2,590 million bushels) of the international soybean exports in 2017 while the U.S. claims 37% (about 2,056 million bushels) of the exports. The American countries of Canada, Argentina, and Paraguay combine for 12% of exports and the remaining 4% is picked up by all other countries.
Over half of the soybean production in the U.S. comes from the eastern Corn Belt, which is through Wisconsin, Iowa, and Illinois, where it rivals corn for dominance as its single competitor (Hymowitz, 1970; Leff et al., 2004). The U.S. Soybean Belt into Missouri maintains roughly 1.1 million km$^2$ of crop space (Leff et al., 2004). Production in south central states accounts for an additional quarter of the country’s total soybeans produced with domination in lower Mississippi and areas of the Carolinas (Hymowitz, 1970; Leff et al., 2004).

The crop has high prevalence in eastern China and central India (Leff et al., 2004). Soybean production of China is most concentrated in Manchuria and Shantung. The provinces of Anhwei, Honan, Hopei, Kansu, Kiangsu, Shansi, Shensi, and Szechwan also maintain heavy soybean growth (Hymowitz, 1970).

In central South America soybeans form the second largest belt (southern Brazil, northern Argentina) for the continent. Here planting is done October through December and harvested March through May. In northern areas of the continent, soybeans once again rival corn for dominance, while in the rest of the continent maintaining a pretty steady second or third (Leff et al., 2004).

Today the value of soybeans is highly dependent on its protein concentration (Brumm & Hurburgh, 2006; Hurburgh Jr et al., 1990), but the overall market success of the crop comes from its multiple applications: use in food products (e.g. tofu, soybean sauce), as edible vegetable oil, as biofuel, and most notably as use for a protein source in livestock feeds (Boerema et al., 2016). The global food demand and global soybean trade will continue to rise because of growing populations and the growth in numbers of global meat consumption. This also will bring on the continued growth of soybean production.
expansion marginal value. According to Boereman et al., (2016), this can shift the optimum ‘conversion to soybean area’ to the right (Fig. 1.2B), whereas an influx in meat consumption would have negative health connotations and therefore added societal cost and thus a shift to the left (Fig. 1.2C). This model displays the multifaceted situation of the importance of tradeoff between increasing food production with environmental and societal costs.

**Figure 1.2:** A: Balance between soybean income (‘Soybean’) and environmental damage from deforestation and grassland conversion (‘Deforestation’). B: Consequences of increasing soybean income ex. due to a population increase (‘Soybean’ and ‘Net societal benefit’). C: Consequences of taking into account the health cost of increasing meat consumption (‘Health’ cost and ‘Net societal benefit’) (Boerema et al., 2016).

A big challenge for the processing industry has been to create an all-purpose model of environmental influence on seed composition to foresee regions and/or environmental conditions that yield reliably high quality soybeans (Rotundo & Westgate, 2009). Although the price of agricultural commodities fluctuates year-to-year, in the last two decades, the price of the soybean/metric ton has increased consistently (Fig. 1.3). Statistics show the United States is leader in soybean export (Fig. 1.4).
Figure 1.3: Annual prices for agricultural commodities (Nominal Dollars), 1960-2011. source: www.worldbank.org
1.1.6 Symbiotic Relationship with Microbes

Soybeans are able to form root nodules after introduction to soybean-nodulating rhizobia. These microbes are able to achieve a symbiotic relationship with the soybean plant for nitrogen fixation by taking in atmospheric nitrogen, in the form of ammonia, through root nodules (Shiro et al., 2013). As mentioned by Shiro et al. (2013), major soybean-nodulating rhizobia are *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, and *Sinorhizobium (Ensifer) fredii*. Others include, *Bradyrhizobium yuanmingense*, *Bradyrhizobium liaoningense*, *Sinorhizobium xinjiangense*, and *Mesorhizobium tianshanense* (Shiro et al., 2013).
1.1.7 Disease Resistance of Soybeans

There has been extensive work done to amass information concerning the disease resistance of soybeans. This work encompasses considerable investigation on the importance of the buildup and production of phytoalexins for various fungal tolerances among the soybean cultivars (Yoshikawa et al., 1978). It also includes work available on glucan elicitor recognition (Umemoto et al., 1997). Cultivated soybeans (Glycine max) have wild soybeans (Glycine soja) to thank for their gene source to resistance to insects and other pests, disease, and other stress factors (Dong et al., 2001). While we know glyceollins supply numerous health benefits to soybeans, consumer soy products available still maintain very small amounts of phytoalexins. The product gap created in the market by this is because of cultivated soybeans not regularly coming in contact with those stressors that induce glyceollin production. The trace levels of glyceollin found along with essentially no observation of glyceollins found in other plants, creates for a large break of knowledge that warrants further investigation (Eromosele et al., 2013).

1.2 Glyceollins

1.2.1 Glyceollins Composition

Glyceollins is the collective name for soybean-derived pterocarpan phytoalexins. They are one of the major phytoalexins and phytoestrogens of soybeans and are a significant prenylflavonoid in soybean (Ng et al., 2011). Glyceollins are referred to as phytoalexins because they are created by a defense mechanism in retort to pathogen invasion and stress (Quadri et al., 2013). In soybeans, five glyceollins have been identified from induction of soybeans by cupric chloride (Lyne & Mulheirn, 1978). The soybean glyceollins have three most notable isomers; i.e.: glyceollin I, II, and III, (Fig.
1.5). These three are produced by soybeans which have been introduced to fungal stressors, with glyceollin I being the most dominant of the three (Feng et al., 2007). Each glyceollin has a pterocarpanoid skeleton connected to a cyclic ether derived from a C$_5$ prenyl component (Akashi et al., 2009; Ebel & Grisebach, 1988; Ng et al., 2011). Gylceollin has been analyzed as a preventer of fungal infectors. The phytoalexin collects in the seeds as result to stimulation by several different elicitors (Ng et al., 2011; Nwachukwu et al., 2013).

![Chemical structure of glyceollin molecules](Eromosele et al., 2013)

**Figure 1.5:** Chemical structure of glyceollin molecules (Eromosele et al., 2013).

### 1.2.2 Synthesis and Production

Glyceollins are phytoalexins produced from daidzein in soybeans with fungal infection (Paxton, 1991). The biosynthesis and accumulation of glyceollins in soybeans is closely associated with the inhibition of fungal growth (Welle et al., 1991), resistance to rot in the stem and phytophthora root (Bhattacharyya & Ward, 1987; T. Graham et al., 1990), along with reduced attack from insects (Fischer et al., 1990). In the biosynthetic process, first, phenylalanine is changed into trans-cinnamic acid by an enzyme called phenylalanine ammonia-lyase (PAL). Then the trans-cinnamic acid is converted to p-coumaric acid by a hydroxylation reaction catalysed by C4H (R. A. Dixon & Paiva,
1995). Work by Akashi et al. (2009) identified candidate genes of three soybean expressed sequence tag sequences homologous for gene encoding homogentisate phytanyltransferase as part of the tocopherol biosynthetic pathway. Additionally, the study recognized a cDNA-encoding dimethylallyl diphosphate: (6aS, 11aS)-3,9,6a-trihydroxypterocarpan [(-)-glycinol] 4-dimethylallyltransferase (G4DT) leading to the direct precursor (glyceollidin I) of glyceollin I. The final step of glyceollins biosynthesis is cyclization of glyceollidins to convert glyceollidin I to glyceollin I, and glyceollidin II to glyceollins II and III (Fig. 1.6) (Welle & Grisebach, 1988). A total syntheses of racemic glyceollin I and its enantiomers were described by Khupse and Erhardt (2008).

**Figure 1.6:** Biosynthetic pathway of glyceollins production from daidzein (Welle & Grisebach, 1988).
1.2.3 Elicitors of Glyceollins in Soybeans

Most of the secondary metabolites are isolated from plants due to their chemical synthesis being either problematic or infeasible. Biotechnological production in plant cell cultures is an alternative which could prove to be advantageous; however, currently this has only experienced curbed commercial success because of an absence of knowledge as to how the metabolites are created. Plants in vitro demonstrate physiological and morphological responses to microbial, physical, or chemical influencers known as ‘elicitors’ (Namdeo, 2007). ‘Elicitors’ are considered to be a substance or substances which can be presented in small concentrations to a living cell system in order to either start or enhance the biosynthesis of certain compounds (Namdeo, 2007; Stössel, 1982). Elicitation by a plant is a method of induced or improved synthesis of secondary metabolites to warrant the plant’s survival, perseverance, and competitiveness (Namdeo, 2007).

These elicitors include various extrinsic factors such as infection by fungus, ultraviolet (UV) irradiation, physical injury, and stress from chemicals or the environment (Alvarez et al., 2018; Feng et al., 2007; H. J. Kim, Suh, Lee, et al., 2010; Lyne & Mulheirn, 1978; Nwachukwu et al., 2013; I. S. Park et al., 2017). These stressing stimulants proved to be factors inducting alteration of the secondary metabolome of soybeans by means of initiation of glyceollins synthesis and accumulation (Nwachukwu et al., 2013). The best time and level of glyceollins accumulation in soybeans will differ between varieties and concentrations of the elicitor used (Stössel, 1982). Research has also shown that glyceollins is an efficient antifeedant for certain species of insect (Fischer et al., 1990).
1.2.3.1 Microbial Stimulation

Soybeans maintain a symbiotic relationship by infection with soybean-nodulating microbes and successive formation of root nodules. The soybean is then able to take in atmospheric nitrogen in the form of ammonia through said root nodules. Soybean-nodulating bacteria most likely differ among different places because various wild soybeans are widely distributed and cultivation is also widely distributed in both northern and southern regions (Saeki et al., 2006).

Many of these microbes are considered to be elicitors to glyceollins production. Glyceollins are produced by soybeans either in response to microbial infectors (Feng et al., 2007; M. R. Lee, Kim, et al., 2010; Ng et al., 2011) or some type of extrinsic factor such as environmental stress or physical injury (Lyne & Mulheirn, 1978; Nwachukwu et al., 2013). Production of glyceollins in soybeans through fungal processing is widely studied (S. Boué et al., 2008; S. M. Boué et al., 2000; Feng et al., 2007; Isaac et al., 2017). Differences among levels of collection will be unique for differing bean varieties and elicitors types and concentrations (I. S. Park et al., 2017; Stössel, 1982). Glyceollin production is going to be dependent on factors such as hull removal (M. R. Lee, Chun, et al., 2010), moisture levels and variations during germination, and compatibility of the fungus as an elicitor to the soybean variety (Feng et al., 2007; M. R. Lee, Chun, et al., 2010; I. S. Park et al., 2017; Stössel, 1982). Due to this a broad range of fungal strains have been studied. For example, Eromosele et al. (2013) and Kim et al. (2011) both looked at *Aspergillus* species, *Aspergillus oryzae* and *Aspergillus sojae* respectively, as their fungal infector. Wyss et al. (1990) used *Glomus mosseae* and *Rhizoctonia solani*, while another study by Feng et al. (2007) compared *Aspergillus niger*, *Aspergillus*
oryzae, Rhizopus oligosporus, and Aspergillus niger wry; where Park et al. (2017) compared Rhizopus oligosporus to Aspergillus sojae. Isaac et al. (2017), compared efficiency of glyceollin production using a series of fungal strains: Trichoderma reesei, Aureobasidium pullulans, Neurospora crassa, Fusarium venenatum, Rhizopus microsporus var. oligosporus, Paecilomyces variotii, Aspergillus niger, Mucor circinelloides and Pichia kudriavzevii. Experimental procedures for instigation of glyceollin production in soybeans by fungal stimulation have seen numerous alterations based on when and where the experimentation occurred. Examples of this can be seen in Feng et al. (2007), Boue et al. (2008), Lee et al. (2010), Park et al. (2017), and Isaac et al. (2017).

1.2.3.2 Physical and Other Elicitors

Other elicitors of glyceollins in soybeans have also been investigated. Although at lower concentrations than produced by fungal stimulation, some glyceollin accumulation has been successful with these methods. Exposure to UV light elicited some glyceollin production in soybeans and results show a range of capacities to produce by differing soybean varieties (I. S. Park et al., 2017; Reilly & Klarman, 1980). Production was also reported in a study by Graham et al. (1991) with glucan as the inducer. The fungal cell wall component of Phytophthora megasperma f. sp. glycinea elicited accumulation at levels of ~600 μg/g (T. Graham & Graham, 1991). In separate studies by Fliegmann et al. (2003) and Park et al. (2017), concentrations of jasmonate were investigated as an inducer of the glyceollins biosynthetic pathway and accumulation. Also investigated by Park et. al (2017) was incubation of soybeans at a designated concentration of an
aluminum solution for three days. Here Park et al. (2017) reported that glyceollins were accumulated, but at lower levels than treatments with UV, jasmonate, or fungi.

1.2.4 Biomedical Importance

Not much is known about the biological functions of glyceollins when related to other phytoalexins with much more investigative background (S. Park et al., 2012). Glyceollins frequently function as antifungal, antibacterial, or anticancer compounds (Park et al., 2010). Glyceollins show possible potential of prompting positive health effects with antiestrogenic and anticancer activity (H. J. Kim, Suh, Kim, et al., 2010), and helpful influence on metabolic ailments, like cardiovascular illness, obesity, type 2 diabetes, and inflammation (S. Park et al., 2010).

Antiestrogenic activity to estrogen receptor mediated activity has been noticed with glyceollins. Comparatively, glyceollins have higher antiestrogenic activity to hindering the success of gynecologic cancers than their production precursor daidzein (Salvo et al., 2006). Glyceollins show anticancer actions to cancer types, such as breast and ovarian, by inhibiting the development and progression of tumors (H. J. Kim, Suh, Kim, et al., 2010; Nwachukwu et al., 2013). There has also been reported inhibitory effects on human androgen-responsive prostate cancer cells (H. J. Kim, Suh, Kim, et al., 2010). Glyceollins also display these properties by prompting cell cycle arrest in cancer cells (Nwachukwu et al., 2013). Epidemiological (H Adlercreutz et al., 1986; Herman Adlercreutz et al., 1991; Herman Adlercreutz et al., 1992; Goldin et al., 1986; H. Lee et al., 1991), animal (Axelson et al., 1984; Baggott et al., 1990; Barnes et al., 1990; Hendrich et al., 1994; Sharma et al., 1992; Wei et al., 1993), and in vitro studies (Herman
Adlercreutz et al., 1992; Wei et al., 1993), all serve as evidence to the anticancer properties of isoflavones from soybeans.

A study by Park et al. (S. Park et al., 2010) displayed glyceollins enhanced insulin-stimulated glucose uptake in 3T3-L1 adipocytes without stimulating peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonists. It also showed, glyceollins somewhat enhanced glucose-stimulated insulin secretion and reduced β-cell apoptosis in Min6 cells. Glyceollins also potentiated insulinotropic activities by attenuating stress on the endoplasmic reticulum (ER) when β-cell dysfunction was stimulated by a 24-h treatment of 500 μM palmitate (S. Park et al., 2010). The results from this study indicate glyceollins might be useful to further develop glucose homeostasis and improve glucose application in diabetic animal models by potentiating β-cell function and mass (S. Park et al., 2012).

Moreover, glyceollins were found to exhibit antimicrobial activity against pathogenic fungi, cellular antioxidant activities against endotoxin-induced oxidative stress, and regulatory effects on glucose metabolism and endotoxin-induced inflammatory response. The soybean-derived compounds warrant further studies to evaluate detailed molecular mechanisms during atypical cellular processes, safety and efficacy in animal disease models and human subjects, and potential use for food preservation. The bioactivity of glyceollins can result in the enrichment of soybean with these phytoalexins for improved health functions and other novel applications (Nwachukwu et al., 2013).
1.3 Isoflavones and Their Derivatives

Plants retain a large variety of ready-made and promotable defense mechanisms devised for resistance against prospective pathogens (Ebel & Grisebach, 1988). Isoflavones and their derivatives are used by plants as phytoalexin compounds to defend against infecting and pathogenic fungus and microbes. Isoflavonoids are a, often times biologically functioning, category of flavonoid phenolic compounds. Isoflavonoids are heterocyclic phenolic secondary metabolites biogenetically linked to flavonoids; however, they represent a notably different class with a C15 skeleton that has a different arrangement and can be considered as derivatives of 3-phenylchroman while flavonoids are 2-phenylchromans (Zheng et al., 1997). They are formed through an early branching pathway in flavonoid metabolism (Tahara & Ibrahim, 1995). Many compounds of isoflavonoid have biological effects by inhibiting activity of estrogen receptors. Isoflavonoid derivatives with some distinguishing species-specific alterations to their skeleton or linked components are what phytoalexins of the Fabaceae family commonly are (Dixon, 1999). By using the degree of oxidation in the skeleton and the intricacy of the skeleton as a categorizing tool, isoflavonoids can be subdivided into different sub-classes. Major sub-classes of isoflavonoids consist of isoflavones, isoflavanones, isoflavans, 6a-hydroxpterocarpans, coumestans, rotenoids, dehydro-rotenoids, isoflav—3-enes, 2-arylbenzofuran, isoflavanquinones, along with other comparatively rare classes.

1.3.1 Isoflavones Synthesis and Production

Production of isoflavones are largely limited to the family of beans, legumes, and peas (Steele et al., 1999; Yu et al., 2003), where they play an important role in facilitating various plant-microbial interactions (Yu et al., 2003). The most recognized source of
isoflavones is soybeans. Soybean isoflavone glycosides are comprised of genistin, daidzin, and glycitin, and the aglycones are genistein, daidzein, and glycine (Coward et al., 1993). In soybeans, isoflavones attract Rhizobial bacteria and also generate node gene expression to perpetuate the formation of nitrogen-fixing roots (Pueppke, 1996; van Rhijn & Vanderleyden, 1995; Yu et al., 2003). The exclusiveness of isoflavones among the Leguminosae family directs “isoflavone synthase” (IFS) activity. This jump starts the development of a flavanone, a ubiquitous in-between of the flavonoid production, into its isoflavone form. First in the IFS development is the construction of a 2-hydroxyisoflavanone from a cytochrome P450-mediated hydroxylation linked with an aryl ring migration (Kochs & Grisebach, 1986; Steele et al., 1999). 2-hydroxyisoflavanone is then spontaneously dehydrated to the isoflavone as shown in Figure 1.7 from Steele et al. (1999). 2-hydroxyisoflavanone synthase’s (2-HIS) action in soybean microsomes will act on both liquiritigenin and naringenin creating daidzein and genistein respectively (Hakamatsuka et al., 1990; Steele et al., 1999), also seen in Figure 1.7 from Steele et al. (1999).

![Figure 1.7: Isoflavone synthase reactions (Steele et al., 1999).](image)

Research with leguminous plants like Lotus japonicus, Medicago truncatula, alfalfa (Medicago sativa), and licorice (Glycyrrhiza echinate), along with soybeans (Glycine max), has led to the recognition and classification of several genes encoding
enzymes necessary to isoflavonid formation (R. Dixon, 1999; Shimada et al., 2007; Veitch, 2007). Most commonly, isoflavonoid skeletons of leguminous phytoalexins, such as in soybeans, are found to be pterocarpan (Tahara & Ibrahim, 1995). Over half of these phytoalexin pterocarpanoids have isoprenoid-derived substituents included in their makeup (Tahara & Ibrahim, 1995). The biosynthetic pathway of glyceollins utilized to investigate the characteristics of prenyltransferase, which catalyzes the prenylation reaction in a very small number of plant families (Ng et al., 2011).

1.3.2 Isoflavone Biomedical Importance

Among vegetables appropriate for human consumption, soy is the solitary one which has a high isoflavone content (Liu, 2004). Numerous health benefits associated with the isoflavone content of soybeans lead them to be regularly studied by researchers (Huber & Genazzani, 2016; Larkin et al., 2008; Tansaz & Boccaccini, 2016; Thorat, 2018). Research has shown various health benefits, such as preventing cardiovascular disease (Chan et al., 2007), alleviation from postmenopausal symptoms (Williamson-Hughes et al., 2006), protective effects to breast cancer (Wada et al., 2013), and effects protecting against bone loss in postmenopausal women (Atteritano et al., 2009).

1.4 Phytoalexins

Phytoalexins are low molecular mass secondary metabolites (Ahuja et al., 2012) produced by plants as an antimicrobial substance when they come in contact with a disease-causing infector or stressor (Ahuja et al., 2012; Feng et al., 2007; Nwachukwu et al., 2013). The type of phytoalexin produced is likely based on the existing preinfectional agents presents in a plant family (Harborne, 1999). Therefore, when considering the Leguminose family, pterocarpans and isoflavans are the readily produced phytoalexins
(Harborne, 1999). In legumes, phytoalexins often times belong to the isoflavonoid family, such as pisatin in *Pisum* species and medicarpin in *Medicago* species (Parniske et al., 1991). Undoubtedly, the most sizeable total of phenolic phytoalexins discussed thus far are the isoflavonoids of the Leguminosae family (Ingham, 1981).

### 1.4.1 Phytoalexin Synthesis and Production

The quantity of additional micromolecular and macromolecular barriers of infection already present might be useful for understanding how to record a plant phytoalexin response in a specific family (Harborne, 1999). As summed up in a review by Ahuja et al., (2012), “the biosynthesis of most phytoalexins, the regulatory networks involved in their induction by biotic and abiotic stress, and the molecular mechanisms behind their cytotoxicity are largely unknown”. When looking at structural activity, there does not seem to be any relationships for antimicrobial activity. An extensive array of phenolics, terpenoids and nitrogen-based molecules are maintained for affording fungitoxicity or inhibition of fungal growth (Harborne, 1999). It is known, however, they are a heterogeneous group of compounds (Huffaker et al., 2011; Shinbo et al., 2006) showing biological activity to a wide assortment of pathogens. Furthermore, they are also deemed to be molecular markers of disease resistance (Ahuja et al., 2012). Phytoalexins being produced are accumulated at the site of infection. There are several classes which are used to classify phytoalexins such as, alkaloids, terpenoids, and glucosteroids. Scientists tend to extend the phytochemical characterization to any phytochemical which is used by the plant for defense. Phytoalexins are inclusive inhibitors and vary chemically and characteristically between species of plants. They can be either constitutive or inducible (Dixon, 2001).
1.4.2 Biomedical and Nutritional Importance

The compounds are characteristically toxic to microbes and not to humans (Feng et al., 2007). As it happens, phytoalexins have the prospect of being valuable to human health because they are recognized to have antioxidant, anti-inflammatory, and anti-cancer activities (Kim et al., 2010). Specifically, the biological activities of (soybean) glyceollins as phytoalexins include antiproliferative and antitumor actions (Ahuja et al., 2012; Ng et al., 2011). Phytoalexins can also act as calorie restrictions (CR) potentially increasing the lifespan of lab animals (Baur & Sinclair, 2006; Fontana & Klein, 2007). Calorie restriction is referencing a decreased intake of calories by about 30-50% of what is considered to be normal intake levels without resulting in malnutrition.(Feng et al., 2007).

1.4.3 Phytoalexin Application

In order to expand and progress disease protection strategies, phytoalexin plant pathogen research has begun to gear towards understanding the biosynthesis pathways and regulation of the compound in varying crop plants by means of comparing and testing different cultivars, transgenic plants and mutants, and by applying -omics, molecular biology and biochemical methods (Ahuja et al., 2012). It is now well understood phytoalexins are often times vital to a plant’s resistance against pathogens, however, phytoalexins of a large Fage of species have not yet been distinguished and categorized (Ahuja et al., 2012; Harborne, 1999). Soybean phytoalexins and their biosynthetic mechanisms were investigated heavily through the 1970’s to 1990’s, a lot of this investigation being conducted by Grisebach et al. (Ebel and Grisebach, 1988), and
the pathway and biosynthetic enzymes engaged have been categorized extensively at the biochemical level (Ebel, 1986; Dixon, 1999).

The study of these compounds could be beneficial to not only future plant health, but also future animal and human health. Future research directed towards phytoalexins could better advance innovations in areas such as anti-cancer, anti-inflammatory and infectious diseases. Aspects of phytoalexin mechanisms such as mode of action along with understanding of response of both the plant and the pathogen, could bring for potential to insight on how to control phytoalexin synthesis in a given tissue and stage of growth (Ahuja et al., 2012). New approaches, such as genome-wide analyses, could not only help us with the investigation of the role phytoalexins play in pathogen defense, but also provide new paths for research to better understand the regulatory networks governing the metabolism of phytoalexins (Ahuja et al., 2012).
CHAPTER II – INTRODUCTION

As world populations continue to increase, the ability to feed those populations becomes increasingly difficult. Increasing food demand brings about challenges we meet with several enhancing strategy applications tailored to the food production system. An illustration of this is the adding of antibiotics to the diets of food animals in order to improve gut health and growth performance. However, recurrent use of antibiotics prompts a foreseeable rise in antibiotic-resistant microorganisms (Landers et al., 2012; Witte, 2000). This then leads to a need of investigation into possible alternatives to antibiotics (feed additives). Many feed additives are found to be inconsistent concerning inclusive health and development of food animals (Landers et al., 2012; Levy, 2014). Hence, there is still necessity to develop effective, environmentally safe, and natural antibiotic alternatives.

Plants produce a class of antimicrobial secondary metabolite materials with low-molecular weights known as phytoalexins (Ahuja et al., 2012; Parniske et al., 1991). In a considerable amount of plant-pathogen interactions, induction of phytoalexins is thought to hold an indispensable role in the resistance of the host plant (Parniske et al., 1991). Moreover, phytoalexins are also considered as molecular markers to disease resistance (Ahuja et al., 2012). Scientists outspread the phytochemical characterization to any phytochemical used by the plant for defense. As inclusive inhibitors, phytoalexins vary chemically and characteristically between species of plants being either constitutive or inducible (R. A. Dixon, 2001). In legumes, phytoalexins often times belong to the isoflavonoid family, such as pisatin in Pisum species and medicarpin in Medicago species (Parniske et al., 1991).
Soybeans (*Glycine max*), one of most high-demand plants worldwide, not only for general feed purposes but also for oil and protein purposes (Al Loman & Ju, 2016), produces a phytoalexin known as glyceollin with three prominent isomers (glyceollin I, II, and III) (Isaac et al., 2017). Soybeans are leguminous crop well-known for boosting energy and glucose homeostasis because of their isoflavonoid and concentrated protein components (Gudbrandsen et al., 2009; Willis, 2003). Soybean meal is also lower in crude fiber than nearly all other oilseed meals (Willis, 2003).

Plant proteins in animal feeds are becoming significant due to consumer apprehensions relating to the health and safety of byproducts produced from animal proteins and a lessening in fish stocks (Willis, 2003). This concern for health from the consumer maintains demands upon soybean meal as a source of protein for livestock, even today as the animal feed market commands the most attention from the nonoil components of soybeans (Kerley & Allee, 2003). Production of soybeans is a booming international industry. The success and appeal of soybeans is explicated by an upsurge of 200 million tons in the global consumption since the seventies (Boerema et al., 2016). Soybeans maintain a protein content around 40% of their composition; comparatively, most other legume species maintain a protein content around 20-30% (Liu, 1997). Soy meal is a large source of protein in the swine and poultry industries and is furthermore an important component of animal food for pets and domesticated household animals. It is also an important source for protein in aquaculture diets (Willis, 2003). The important applications of soybean protein in feed creates a normalcy of soybean meal being used as a standard to which other protein sources are compared by feed manufacturers. In
addition, soybean meal has developed into the protein source that governs the price of proteins for feeding of all livestock (Willis, 2003).

Due to the widespread importance of soybeans in feedstock, it is a natural starting off point for investigation of naturally produced antimicrobials and applications for thereof. As a product of soybean interaction with various fungal stimulations and other elicitors, glyceollin is a promising candidate as a natural antimicrobial feed additive. Glyceollins is the collective name for soybean-derived pterocarpan phytoalexins. They are produced from an isoflavone precursor, daidzein. (Paxton, 1991). However, high or low concentrations of total isoflavone or daidzein is not associated with production of glyceollins in response to fungal or elicitor stimulation, as one might infer (I. S. Park et al., 2017). Rather, accumulation is largely affected by genetic variety of the soybean, elicitor type, and/or fungal strain used (I. S. Park et al., 2017). The three main isomers are one of the major phytoalexins and phytoestrogens of soybeans and are a substantial prenylflavonoid in soybean (Ng et al., 2011). Accumulation amount and rate of glyceollins in soybeans will be determined by variety and concentration of the elicitor used (Stössel, 1982). In the soybean plant, biosynthesis and accumulation of glyceollins in soybeans is linked with the prevention of fungal growth (Welle et al., 1991), with plant resistance to rot in the areas of the stem and phytophthora root (Bhattacharyya & Ward, 1987; T. Graham et al., 1990), and with reduced attack from insects (Fischer et al., 1990). Furthermore, they were found to show antimicrobial activity to pathogenic fungi, cellular antioxidant activities to endotoxin-induced oxidative stress, along with regulatory effects on glucose metabolism and endotoxin-induced inflammatory responses (Nwachukwu et al., 2013).
In a previous study, our research team at South Dakota State University recognized *Trichoderma reesei* NRRL 3653 as the best performing strain for stimulation of production of glyceollins in the soybean variety used. *T. reesei* prompted the greatest total concentration of glyceollins compared with several other tested fungal strains. A study conducted by Park et al. (2017), comparatively looked at soybean varieties and showed that levels of glyceollins were substantially influenced by soybean variety and were well increased by fungal infection. We hypothesized GES can be efficiently produced through a fungal incubation process and the resulting GES will be effective in replacing antibiotics in feed ingredients. Therefore, in this study our goal was to maximize production of glyceollins in soybeans by combining fungal metabolism with various processing parameters and to compare among several soybean varieties. The various processing parameters such as seed soaking time, inoculation method, incubation time, seed germination effect, seed varieties, etc. are assessed using *T. reesei* as a standard inoculum. These parameters are assessed for optimization and possibilities of upscale to efficiently increase total concentration. Chapter III outlines results and data found when looking at impact of seed sterilization before soaking, inoculum application, and incubation periods of inoculated soybeans. Results found in Chapter IV discuss comparison of concentration of glyceollins and isoflavones among several different soybean varieties.
CHAPTER III – EVALUATING THE PREPROCESSING CONDITIONS TO MAXIMIZE THE SYNTHESIS OF GLYCEOLLINS IN SOYBEANS DURING FUNGAL INFECTION

Abstract

Among other defense mechanisms, many plants have the ability produce compounds known as phytoalexins to increase chances of resisting infection of certain plant pathogens. In soybeans a commonly accumulated phytoalexin in response to infectors is glyceollin. Glyceollins have three distinguishing isomers (glyceollin I, II, and III). Levels of accumulated glyceollins are generally low and may vary at different stages of germination and fungal infection. However, these levels can be manipulated by the conditions of the fungal incubation process. Therefore, in this study our goal is to evaluate and compare the glyceollins producing abilities of soybeans by adjustments of processing parameters. In a preceding study, our research team at South Dakota State University recognized *Trichoderma reesei* NRRL 3653 as the best performing strain, prompting the highest total concentrations of glyceollins, after screening of eight different strains. For this reason, the study used *T. reesei* as the standard soybean inoculant throughout this work. Effect of processing conditions such as sterilization of soybeans prior to fungal inoculation, inoculum method and type, incubation period etc. were studied. The findings of this study highlighted the importance of preprocessing conditions in maximizing the glyceollins synthesis in soybeans. The data obtained clearly suggested that careful consideration must be given while selecting the preprocessing conditions to optimize the glyceollins synthesis.
3.1 Introduction

Investigation of the health benefits associated with the consumption of soybean-derived bioactive compounds such as isoflavones has been developing over the years (R. A. Dixon & Sumner, 2003; Eromosele et al., 2013; Júnior & Ida, 2015). Isoflavones, such as daidzein and genistein, tend to be the most seriously studied bioactive compounds (R. A. Dixon & Sumner, 2003) a group of phytoalexin compounds known as glyceollins have showed exploratory promise (Nwachukwu et al., 2013). Glyceollins are inducible phytochemicals synthesized by soybeans either in response to microbial infectors (Feng et al., 2007; M. R. Lee, Kim, et al., 2010; Ng et al., 2011) or some type of extrinsic factor such as environmental stress or physical injury (Lyne & Mulheirn, 1978; Nwachukwu et al., 2013). Further understanding of the antibiotic and antifungal (Huang et al., 2013; M. R. Lee, Kim, et al., 2010; Nwachukwu et al., 2013; S. Park et al., 2010; Weinstein & Albersheim, 1983) activities of gylceollins has been a central motive to a wide array of studies conducted via fungal processing (S. Boué et al., 2008; S. M. Boué et al., 2000; Feng et al., 2007; Isaac et al., 2017). Variances among levels of the accumulation of glyceollins exists between differing soybean varieties and elicitors types (I. S. Park et al., 2017; Stössel, 1982). Glyceollins synthesis is also dependent on a series of factors including hull removal (M. R. Lee, Chun, et al., 2010), maintaining moisture during germination and the compatibility of the fungus as a useful elicitor to the soybean variety (Feng et al., 2007; M. R. Lee, Chun, et al., 2010; Stössel, 1982), and types of soybean cultivar (Park et al., 2017). A broad range of fungal strains have been evaluated for their
abilities to induce glyceollins synthesis in soybeans. For example, Eromosele et al. (2013) and Kim et al. (2011) both looked at *Aspergillus* species, *Aspergillus oryzae* and *Aspergillus sojae* respectively, as their fungal infector. Wyss et al. (1990) used *Glomus mosseae* and *Rhizoctonia solani*, while another study by Feng et al. (2007) compared *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus oligosporus*, and *Aspergillus niger wry*. In a study by Isaac et al. (2017), authors compared glyceollins levels from a series of fungal strains (*Trichoderma reesei*, *Aureobasidium pullulans*, *Neurospora crassa*, *Fusarium venenatum*, *Rhizopus microsporus var. oligosporous*, *Paecilomyces variotii*, *Aspergillus niger*, *Mucor circinelloides* and *Pichia kudriavzevii*) and observed that some strains performed better than others under identical conditions. Likewise, a previous study by Park et al., (2017) on synthesis of glyceollins by sixty different soybean varieties using *Aspergillus sojae* and *Rhizopus oligosporous* reasserted that elicitors used had a high influence on the overall accumulation of glyceollins. Experimental procedures used for prompting glyceollins production in soybeans by fungal stimulation have seen several modifications based on when and where the experiments are being conducted. Examples of this can be seen in Feng et al. (2007), Boue et al. (2008), Lee et al. (2010), and Isaac et al. (2017). However, there are some key processing steps which are essential to soybean glyceollins stimulation. These key steps of processing warrant further examination to possibly optimize glyceollins concentration. Therefore, the aim of the study was to comparatively evaluate the effect of different processing conditions including sterilization methods, inoculation methods, and incubation period, types of fungal strain, importance of moisture during incubation period on the overall glyceollins synthesis was evaluated.
3.2 Materials and Methodology

3.2.1 Soybeans

Soybean varieties used were acquired through various sources such as farmers, processors, and seed distributors. Soybeans varieties used were also ensured to non-treated or enhanced in anyway. Brookings, Davison varieties were received from the South Dakota State University Seedhouse, and the MN01050628 variety was received by local farmer donation. All soybeans were kept in dry room temperature storage.

3.2.2 Inoculum Preparation

Fungal strains *Trichoderma reesei* NRRL 3653 (standard), *Aureobasidium pullulans* NRRL Y-2311-1 (for fungal comparison test only) and *Neurospora crassa* NRRL 2332 (for fungal comparison test only) were provided by the USDA National Center for Agricultural Utilization Research (Peoria, IL, USA). These strains were grown on potato dextrose agar (PDA) and kept at 4 °C for short term storage. The fungal spores were then suspended by adding ~1-5 ml of GYE media and were further transferred into a 250 ml flask containing 100 ml of GYE. The GYE flask was then incubated at 30°C at 150 rpms in a shaker incubator (New Brunswick Scientific Excella E25) for 24 hr. The fungal broth grown in GYE was further used for inoculating the soybeans to stimulate glyceollins production. Four different inoculation methods (mycelium, spores, filter paper spread, and inoculum submerge method) as described in the section below were tested using *T. reesei*. Inoculated plates were then incubated in an incubator at 30 °C for 5 days and samples were subjected for glyceollins determination.
3.2.3 Induction of Glyceollins Synthesis in Soybeans Using Filamentous Fungi

Fungal inoculation was conducted according to the method of Isaac et al. (2017) with slight modification. Briefly, soybeans were surface sterilized using Micro-90 solution (2%) and then rinsed with sterile water before being soaked in sterile water for 20 hr. After decanting the water, the beans were de-hulled and placed in sterile Petri dishes. The bottom of each Petri dish contained 2 pieces of filter paper (Whatman filter paper no.1) moistened with 1 ml of sterile water. Each Petri dish contained 8-10 soybeans and 100 µl of fungal inoculum was added to each Petri dish. Two more pieces of filter paper were added, also moistened with 1 ml of sterile water. Petri dishes were wrapped in parafilm and incubated at 30°C in dark conditions for 5 days. After incubation, soybeans were stored at 4°C. Frozen samples were then placed in a Labconco freeze drier and vacuum pump system (Kansas City, MO) to remove the moisture. Dried soybeans were ground and subjected to analysis for glyceollins measurement. To determine the ideal conditions for glyceollin stimulation in soybean, several microbial processing parameters were tested. The parameters studied include sterilization methods, various approaches to applying inoculum, length of soybean incubation period, effects of fungal strains, and effects of using filter papers during fungal incubation.

3.2.4 Fungal Inoculation with and without Sterilizing the Soybeans

As explained in the section above (3.2.3) sterilizing chemicals were applied to soybeans prior to being soaked and dehulled. The commonly used sterilizing chemicals, i.e., Micro-90 (International Products Corporation, Burlington, NJ, USA) was tested. This was tested for two soybeans varieties,
Davison and Brookings. To determine the effect of surface sterilization on glyceollins production, soybeans were soaked in a 2% Micro-90 cleaning solutions for 10 min. After 10 min soaking, soybeans were rinsed with sterile de-ionized water and soaked in sterile deionized water for 20 hr. After 20 hr soak, the soybeans were aseptically transferred onto sterile Petri dishes and incubated in the dark at 30°C for 120 hr. During the incubation periods, daily observations were made, and samples were withdrawn to determine the glyceollins production. The control samples were prepared by using the same protocol, but the soybeans were not sterilized. All the experiments were conducted in triplicate.

3.2.5 Evaluating the Different Approaches of Inoculum Application using *T. reesei* culture

3.2.5.1 Soybean inoculation using *T. reesei* mycelium

Soybeans were processed as described in the previous section and were inoculated with 100 µl of *T. reesei* mycelium suspended in GYE medium using pipette. Drops were added directly to the surface of the soybeans ensuring each bean came in contact with the fungal inoculum.

3.2.5.2 Soybean inoculation using *T. reesei* spores

Processed soybeans were inoculated with GYE suspended *T. reesei* spores taken directly from the PDA plates. Soybeans plated received 100µl of broth suspended spores per plate. Drops were added directly to the surface of the soybeans to ensure each bean was in contact with the fungal inoculum.
3.2.5.3 *Inoculating soybean by submerging into the T. reesei medium*

Prepared soybeans were immersed for 30 min in *T. reesei* GYE flask culture. Beans were removed from GYE using a sterile funnel and cheese cloth and were then transferred to sterile Petri dishes.

3.2.5.4 *Soybean inoculation using filter paper spread method*

For the spread plate method, *T. reesei* mycelium grown in the GYE medium was used. In this process, fungal inoculum was applied by pipetting directly onto the filter paper surface and then spread evenly across paper surfaces. Sterile filter paper was placed either in the bottom, top, or both side of the Petri plate during this test. Throughout these tests, inoculum was added to filter papers at varying rates. These rates included: 100 µl of inoculum on the bottom paper, 100 µl of inoculum on the top paper, 50 µl on both the top and bottom filter papers (for a plate total of 100 µl), and 100 ml on both the top and bottom filter papers (for a plate total of 200 µl). During each inoculation method, Petri dishes were sealed tightly using parafilm and incubated for 5 days.

3.2.6 *Effect of Incubation Period on Glyceollins Synthesis*

Soybeans plates inoculated with *T. reesei* (as described in section 3.2.3) were prepared in triplicate and incubated in a dark Napco Model 320 static incubator set to 30°C. Samples were removed every 24 hr until 336 hr and incubated plates were stored at 20°C until lyophilized.
3.2.7 Effect of Different Fungal Strains on Glyceollins Synthesis

Soybean variety MN01050628 was inoculated with three different fungal strains (\textit{A.~pullulans}, \textit{T.~reesei}, and \textit{N.~crassa}) individually according to the method described in section 3.2.3. All the experiments were conducted in triplicate.

3.2.8 Effect Cellulose Filter Papers During Fungal Incubation

The standard protocol used in this study (section 3.2.3) uses the moistened (1 ml sterile water) filter papers (2 pieces of Whatman filter paper no.1) on the bottom of Petri dish. To determine the effect of filter papers during the fungal fermentation, the soybean (variety MN01050628) was inoculated with \textit{T. reesei}, and incubated in a Petri dish with and without the filter papers. The plates without filter papers also contained 1 ml of water in the side of the plates. The incubated and lyophilized soybeans were then subjected to the glyceollins measurement. All the experiments were conducted in triplicate.

3.2.9 Analytical

Soybean flavonoids were analyzed based on standards and analysis previously defined by Berhow (2002) and Berhow et al. (2006). Soybean samples (~0.25 g) and 3 ml methanol or dimethyl sulfoxide (DMSO):methanol (1:1) solution were vialled for single-step extraction. Capping and sealing the vials, they were then sonicated for 15 min at 50°C. Once sonication was complete, they were placed in a static incubation at room temperature for 24 hr. Following this was HPLC. For HPLC analysis of phenolics and triterpenoids, an aliquot was procured from the vials to be filtered by a .45-µm nylon 66 filter.
A Shimadzu LC-20 HPLC system (LC-20AT quaternary pump DGU-20A5 degasser, SIL-20A HT auto-sampler, and SPD M20A photodiode array detector, running under Shimadzu LC Solutions v.1.22 chromatography software; Columbia, MD, USA) was used for HPLC analysis. The column used was Inertsil ODS-3 reverse phase C-18 column (5 µm, 250 x 4.6 mm, with a Metaguard column (GL Sciences, Torrance, CA, USA)). For phenolic analysis 20% methanol and 0.025% trifluoracetic acid (TFA) in water with a 1 ml/min flow rate was the original setting. The effluent was monitored at 280 nm on the variable wavelength detector of the UV–Vis detector. After injection of a ~25 µl sample, the column sustained the original setting for 2 min, then continued to 100% methanol and 0.025% TFA in a linear gradient over 50 min. Pure standards prepared and purchased commercially were used to produce standard curves by nmol injection.

Using a LC-ESI high-resolution mass spectrometer (MS), isoflavones and glyceollin were confirmed. Samples were run on a Thermo Electron LTQ Orbitrap Discovery MS (a linear ion trap linked to a high precision electrostatic ion trap MS) (LTQ XL; Thermo Scientific Waltham, MA, USA), using an Ion Max electrospray ionization (ESI) source (Thermo Scientific), under Thermo Scientific Xcalibur 2.1.0.1140 liquid chromatography-mass spectrometry (LC-MS) software. Thermo Scientific recommended a standard calibration mixture used for weekly calibration as needed. Signal detection was adjusted for best performance by running necessary auto-tune software features. Source inlet temperature was 350°C and the ESI probe in the negative mode. The setting for sheath gas rate was 35 arbitrary units, the setting for the auxiliary gas rate was 2 arbitrary units, and the setting for sweep gas rate was 2 arbitrary units.
Maximal mass resolution was 30,000, and spray voltage was 3.0kV. Tube lens was – 100V and the column used was Inertsil ODS-3 reverse phase C-18 column (3 µm, 150 × 3 mm) (GL Sciences). Effluent was attended to using a photodiode array detector (PDA) at 280 nm. The column was kept at the initial conditions for 2 min; initial condition being 90% water, 10% methanol, and 0.1% formic acid with the flow rate 0.25 ml/min. The column was then advanced with a binary gradient up to 100% methanol and 0.1% formic acid over 50 min.

### 3.3 Results and Discussion

#### 3.3.1 Effect of Sterilization on Glyceollins Synthesis by Soybeans

Although several studies have emphasized the importance of microbial elicitors in stimulating the glyceollins synthesis (Park et al., 2017), there is lack of knowledge on how preprocessing conditions might influence the overall glyceollins synthesis. Previous reports on glyceollins synthesis oftentimes do not mention details on whether soybeans seeds were sterilized prior to subjecting to the microbial inoculation. Therefore, with the aim of determining the important preprocessing conditions, impact of sterilizing agent on the seed germination and glyceollins synthesis was evaluated. Soybean seeds with and without surface sterilization were observed daily for their growth. Application of the surface sterilant had no apparent negative effect on germination or rate of soybean growth. Daily observation of shoot and root length, displayed by the soybeans soaked for 10 min in 2% Micro-90, showed no difference to those which were unsterilized (data not shown).
Comparing concentration of glyceollins of the surface sterilized soybeans to the unsterilized soybeans showed a positive correlation in concentration for all sterilized daily sampling time points and most notably for the 96 and 120 hr (Fig. 3.1). This difference between levels of glyceollins with and without surface sterilization could be caused by possible factors such as the application of the surface sterilant adding an extra mode of stress to the soybean, or the surface sterilant removing microbes which would be competitors or inhibitors of the applied fungal infector. To the best of our knowledge, no data is reported on use of Micro-90 as sterilizing agent for soybeans prior to fungal inoculation for glyceollins synthesis. Park et al., (2017) mentioned the use of 70% (v/v) ethanol for 3 mins. In the future, it will probably be worthwhile to compare the impact of different chemical sterilant to optimize the glyceollins synthesis in soybeans. Data also suggested that there is notable difference in the glyceollins concentration of two different soybean varieties under the identical conditions (Davison vs Brookings soybean variety). Similar phenomenon has also been previously reported (I. S. Park et al., 2017; Stössel, 1982).
3.3.2. Effect of Inoculum Application Approach on Glyceollins Concentration

The Figures 3.2A and 3.2B represent *T. reesei* growth on Petri dishes inoculated with 200 µl and 100 µl of *T. reesei* mycelium in dropwise manner, respectively. Whereas, Figure 3.2C shows the fungal growth on the plates that were completely submerged into the GYE media containing the *T. reesei* mycelium. Figure 3.2D is showing the *T. reesei* growth after spore inoculation. Soybean plates inoculated either with mycelial growth or spore suspension showed fair amount of fungal growth throughout the plate at 120 hr of incubation. Also, the type of inoculant (mycelium vs. spores) did not have any impact on the soybean germination. However, the rate of fungal growth was slower in the plates that were inoculated with the spores. It took about 48 to 72 hr more for the fungi to
appear in the plates when compared to the mycelium inoculated plates. This delayed fungal growth is expected with the spore inoculation due to the longer lag phase of the spores as compared to the mycelium growth. Contrary to the mycelium and spore inoculation, the soybeans submerged for 48 hr in culture of T. reesei (GYE media) did not germinate (Fig. 3.2C). Though plates were full of T. reesei growth, soybeans became mushy and fragmented and eventually decayed. This could probably be due to the longer soaking period in broth (30 min) or too heavy T. reesei growth on the plates. This could probably be improved by using a shorter submerged period and use of a less concentrated T. reesei broth. When fungal culture was spread on the filter papers instead of directly applying to the soybeans (Fig. 3.2E), soybeans germinated normally but fungal growth mostly occurred on the surface of the cellulose filter papers.

![Figure 3.2: Effect of T. reesei inoculation method on soybean; 3.2A- inoculated with 200 µl of mycelium. 3.2B- inoculated with 100 µl of mycelium; 3.2C- Soybeans submerged in T. reesei mycelium and GYE culture for 30 min with mild agitation, aseptically removed, and plated; 3.2D- inoculated with spores; 3.2E- inoculum was spread on the filter papers (soybeans at 120 hr of incubation).](image-url)
The mycelium inoculation resulted in the higher concentration of glyceollins as compared to that of spore inoculation (Fig. 3.3). There was no glyceollin in the beginning of incubation (0 day), and it reached up to 0.812 mg/g and 0.452 mg/g after 120 hr of incubation for mycelium and spore inoculated soybeans, respectively. For the inoculum spread method, the standard mycelium in GYE broth was applied to the filter paper and spread using a sterile spreader on either the top, bottom or both filter papers. Levels of growth were observed to be similar amongst all variations with those filter papers with inoculum applied to both top and bottom being the most consistent. Those with top and bottom spreading also had more consistency when referring to glyceollins concentration (Fig. 3.4). There was no difference in the glyceollins concentration of the soybeans that were inoculated with fungal mycelium dropwise or spread method (Fig. 3.4).

**Figure 3.3:** Glyceollins concentration of dropwise application compared to spread methods (applied on top and bottom of the filter paper).
These results clearly indicated inoculum application method influences the degree of glyceollins synthesis in soybeans. Based on these findings, inoculating the soybeans with fungal mycelium in dropwise manner was selected to be the most effective approach.

3.3.3 Effect of Incubation Period on the Synthesis of Glyceollins

With increased incubation time a correlating increase in concentration of glyceollins is observed through the daily sampling (0 to 120 hr). Extending incubation time past the 120 hr used in the standard procedure did see an increase in concentration of glyceollins until 144 hr, beyond 144 hr the levels are reduced (Fig. 3.5). This reduced level of glyceollins after certain period of incubation (beyond 96 to 144 hr) could possibly be due to the decaying of soybeans by factors of either dryness of the soybean,
increased crowding on the plate, fungal infection, or breakdown of the metabolites to be used for other plant processes as the plant matures.

**Figure 3.5:** Glyceollins concentration during the extended incubation period.

![Glyceollin concentration during the extended incubation period](image)

### 3.3.4 Effect of Different Fungal Strains on Glyceollins Synthesis

In a study conducted by Isaac et al. (2017), three strains (*T. reesei, A. pullulans, N. crassa*) were found to be the best performing strains in inducing the glyceollins synthesis in soybeans. Hence, these three strains were chosen to determine the efficiency of the fungal strains in stimulating the glyceollins synthesis in soybeans. Among three different strains that were evaluated, *T. reesei* prompted the highest level of glyceollins followed by *A. pullulans* and *N. crassa*, respectively (Fig. 3.6). These results further validated the findings of Isaac et al., (2017) and Park et al., (2017), where authors have noted the difference in the glyceollins level depending on the fungal strains used. Data also
suggested fungal incubation period of 96 to 120 hr is needed to achieve the highest level of glyceollins (Fig. 3.6).

![Graph showing glyceollins concentration of soybean inoculated with three different fungal strains]

**Figure 3.6:** Comparing the glyceollins concentration of soybean inoculated with three different fungal strains.

### 3.3.5 Glyceollins synthesis in Soybean with and without the Cellulose Filter Papers

Maximum levels of glyceollins synthesis is reported to have occurred during the soybean sprouting (germination) process. Therefore, the standard protocol of glyceollins induction includes the water soaking for appropriate period of time as one of key preprocessing steps. After fungal inoculation when soybeans are incubated for several days it is important to maintain the certain levels of moisture, hence moistened cellulose filter papers are used. But for the scaling up of the process, this step may become somewhat unpractical; hence we conducted experiments with and without filter papers. There was no difference in the glyceollins titer of soybeans with and without the use of filter papers (Fig. 3.7). This indicated that keeping the moisture level is important but use
of filter papers could be avoided without having any adverse impact on the level of glyceollins production.

![Glyceollins concentration during fungal inoculation with and without cellulose filter papers.](image)

**Figure 3.7:** Glyceollins concentration during fungal inoculation with and without cellulose filter papers.

### 3.4 Conclusion

Several studies have previously demonstrated that glyceollins synthesis in soybeans could be impacted by several factors. The findings of this study indicated various factors need to be considered carefully while maximizing the glyceollins synthesis during the fungal infection. Preprocessing conditions such as surface sterilization, fungal inoculum type, method of inoculation, fungal incubation period, had direct impact on the level of glyceollins accumulated. Surface sterilization had a positive impact on the glyceollins concentration levels giving a high level of glyceollins compared to unsterilized soybeans. Inoculation using either the dropwise or spread method resulted in higher concentrations compared to spore or submerged inoculation methods. In addition, incubation of
96 to 120 hr at 30°C in dark conditions produced the highest concentrations when comparing sampling hours. Fungal type used has a great impact on the production of glyceollins highlighting the important role of elicitors used in glyceollins induction. Further research needs to be conducted focusing on optimizing the preprocessing conditions to maximize the glyceollins synthesis in soybeans.
CHAPTER IV – COMPARISON OF SOYBEAN VARIETIES FOR THEIR ABILITIES TO STIMULATE GLYCEOLLINS PRODUCTION BY FUNGAL INCUBATION

Abstract

Plants produce a class of antimicrobial materials known as phytoalexins. Glyceollins is the name for soybean-derived phytoalexins with three prominent isomers (glyceollin I, II, and III). Glyceollins naturally collect in soybean seeds in response to microbial (typically fungal) infection, but, glyceollin titers are at low levels and vary among soybean varieties. Therefore, in this study our goal was to assess and compare the glyceollins producing capabilities and potentials of different soybean varieties over the period of fungal incubation. In a preceding study, our research team at South Dakota State University identified Trichoderma reesei NRRL 3653 as the best performing strain, stimulating the highest total glyceollins concentration among many other tested strains. Hence, in this study, eleven different soybean varieties were inoculated with T. reesei for five days. The level of glyceollins produced varied among the soybean varieties. The MN01050628 reached glyceollin concentration levels ~2 mg/g after 96 hr of incubation, while the lowest producing variety Kouri peaked at ~.03 mg/g. Our data suggested that total isoflavone content and the glyceollin precursor daidzein did not have any direct correlation to the glyceollin produced by the soybean varieties. This validated the previous findings that glyceollins production is largely affected by the genetic makeup of the soybean variety and the elicitors used. Our results indicated that wide range of soybean varieties further needs to be evaluated for their abilities to stimulate the glyceollins synthesis in response to the strong elicitors.
4.1 Introduction

In recent years, several published studies have expounded on the health benefits associated with the consumption of soybean-derived bioactive compounds such as isoflavones (R. A. Dixon & Sumner, 2003; Eromosele et al., 2013; Júnior & Ida, 2015). Although isoflavones, like daidzein and genistein, are the most heavily studied bioactive compounds (R. A. Dixon & Sumner, 2003) another group of compounds known as glyceollins have showed exploratory promise. Glyceollins are inducible phytochemicals and are well known to have both antibiotic and antifungal activities (Huang et al., 2013; M. R. Lee, Chun, et al., 2010; Nwachukwu et al., 2013; S. Park et al., 2010; Weinstein & Albersheim, 1983). Glyceollin production in soybeans either in response to microbial infectors (Feng et al., 2007; M. R. Lee, Kim, et al., 2010; Ng et al., 2011) or some type of extrinsic factor such as environmental stress or physical injury (Lyne & Mulheirn, 1978; Nwachukwu et al., 2013). Glyceollins production in soybeans via fungal processing is widely studied (S. Boué et al., 2008; S. M. Boué et al., 2000; Feng et al., 2007; Isaac et al., 2017). Differences among levels of accumulation is unique for differing bean varieties and elicitors types used (I. S. Park et al., 2017; Stössel, 1982). Glyceollin in soybeans is synthesized de novo from daidzein through a series of enzymatic steps and the concentration of daidzein, and other isoflavones, widely varies among soybean varieties (I. S. Park et al., 2017). In a study by Park et al. (2017), it was found that concentration of total isoflavone or daidzein was not associated with glyceollin accumulation in response to fungal stimulation. Instead it was shown that glyceollin levels were considerably affected by soybean variety and could be effectively amplified by fungal infection (I. S. Park et al., 2017).
The differences among the varieties that is being reported previously, call for further investigation to optimizing glyceollin concentration among soybeans. Hence, we were interested in exploring the glyceollins producing abilities of different soybean varieties that are primarily grown in the US mid-western region. The specific goal of this study was to comparatively evaluate differences in glyceollins produced among a series of soybean varieties. Varieties are differing both phenotypically, as recorded by vendors, as well as geographically by location of plant grown to produce seeds.

4.2 Materials and Methodology

4.2.1 Soybeans

The eleven soybean varieties used were acquired through various sources such as farmers, processors, and seed distributors. Soybeans varieties used were also ensured to non-treated or enhanced in anyway. Five varieties were received from the South Dakota State University Seedhouse, Brookings, Codington, Davison, MN1806CN, and NE1318382. Two varieties, Tankuro and Kouri, were purchased from the Kitazawa Seed Company in Oakland California. The remaining varieties, Deuel, Dixon, 27130, and MN01050628 were received by donation of farmers in the South Dakota and Nebraska area. All soybeans were kept in dry room temperature storage.

4.2.2 Inoculum Preparation

Fungal strain Trichoderma reesei NRRL 3653 was provided by the USDA National Center for Agricultural Utilization Research (Peoria, IL, USA). The Trichoderma reesei strain was grown on PDA and kept at 4°C for short term storage. The fungal spores were then suspended by adding ~1-5 ml of GYE media and were further
transferred into a 250 ml flask containing 100 ml of GYE. The GYE flask was then incubated at 30°C at 150 rpms in a shaker incubator (New Brunswick Scientific Excella E25) for 24 hr. The fungal broth grown in GYE was further used for inoculating the soybeans to stimulate glyceollin production. Dropwise inoculation as described in the section below was used. Inoculated plates were then incubated in an incubator at 30 °C for 5 days and samples were subjected for glyceollin determination.

4.2.3 Stimulation of Glyceollin Production in Soybeans Using Filamentous Fungi

Fungal inoculation was conducted according to the method of Isaac et al. (2017) with slight modification. Briefly, soybeans were surface sterilized using Micro-90 (2%) and then rinsed with sterile water before being soaked in sterile water for 20 hr. After decanting the water, the beans were de-hulled and placed in sterile Petri dishes. The bottom of each Petri dish contained 2 pieces of filter paper (Whatman filter paper no. 1) moistened with 1 ml of sterile water. Each Petri dish contained 8-10 soybeans and 100 µl of *Trichoderma reesei* inoculum was added to each Petri dish. Two more pieces of filter paper were added, also moistened with 1 ml of sterile water. Petri dishes were wrapped in parafilm and incubated at 30°C in dark conditions for 5 days. After incubation, soybeans were stored at 4°C. Frozen samples were then placed in a Labconco freeze drier and vacuum pump system, serial number 040317525M, Kansas City, MO, to remove the moisture. Dried soybeans were ground and subjected to analysis for glyceollins measurement. To determine the ideal conditions for glyceollin stimulation in soybean, several soybean varieties were tested. The varieties studied include Brookings, Codington, Davison, MN1806CN, NE1318382, Tankuro, Kouri, Deuel, Dixon, 27130, and MN01050628.
4.2.4 Inoculum Application

All soybeans of each variety were processed as described in the previous section and were inoculated with 100 µl of *T. reesei* mycelium suspended in GYE medium using pipette. Drops were added directly to the surface of the soybeans ensuring each bean came in contact with the fungal inoculum.

4.2.5 Effect of Incubation Period on Glyceollins Synthesis

All soybeans plates of each variety inoculated with *T. reesei* (as described in section 4.2.4) were prepared in triplicate and incubated in a dark Napco Model 320 static incubator set to 30°C. Samples were removed every 24 hr until 120 hr and incubated plates were stored at 20°C until being lyophilized.

4.2.6 Analytical

Soybean flavonoids were analyzed based on standards and analysis previously defined by Berhow (2002) and Berhow et al. (2006). Soybean samples (~0.25 g) and 3 ml methanol or DMSO:methanol (1:1) solution were put in a vial for single-step extraction. After being capped and sealed, the vials were sonicated for 15 min at 50°C. Next, they were put in static incubation at room temperature for 24 hr. For HPLC analysis of phenolics and triterpenoids, an aliquot was taken from the vials to be filtered by a .45-µm nylon 66 filter.

A Shimadzu LC-20 HPLC system (LC-20AT quaternary pump DGU-20A5 degasser, SIL-20A HT auto-sampler, and a SPD M20A photodiode array detector, running under Shimadzu LCSoultions v.1.22 chromatography software; Columbia, MD, USA) was used for HPLC analysis. The column was an Inertsil ODS-3 reverse phase C-18 column (5 µm, 250 x 4.6 mm, with a Metaguard column (GL Sciences, Torrance, CA,
USA). Original setting for phenolic analysis was 20% methanol and 0.025% TFA in water with a 1 ml/min flow rate. The effluent was monitored at 280 nm on the variable wavelength detector of the UV–Vis detector. Following completion of injection of a ~25 µl sample, the column was kept at the original setting for 2 min, then moved to 100% methanol and 0.025% TFA in a linear gradient over 50 mins. Using pure standards both prepared and purchased commercially, standard curves developed by nmol injected were made.

Using an LC-ESI high-resolution MS, isoflavones and glyceollin were confirmed. The samples were then run on a Thermo Electron LTQ Orbitrap Discovery MS (a linear ion trap coupled to a high precision electrostatic ion trap MS) (LTQ XL; Thermo Scientific Waltham, MA, USA), using an Ion Max ESI source (Thermo Scientific), under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software. Using a standard calibration mixture, which is recommended by Thermo Scientific, the mass spectrometer was calibrated as needed weekly. Signal detection was optimized by running the necessary auto-tune software feature. Source inlet temperature was 350°C and the ESI probe in the negative mode. 35 arbitrary units was the setting for sheath gas rate, 2 arbitrary units was the setting for the auxiliary gas rate, and 2 arbitrary units was the setting for sweep gas rate. Maximal mass resolution was 30,000, and spray voltage was 3.0kV. Tube lens was –100V and the column used was Inertsil ODS-3 reverse phase C-18 column (3 µm, 150 × 3 mm) (GL Sciences). Again, effluent was monitored at 280nm on a PDA. The column was kept at the initial conditions for 2mins; initial condition being 90% water, 10% methanol, and 0.1% formic acid with the flow rate 0.25 ml/min. The column was then advanced with a binary gradient up to 100% methanol and 0.1% formic acid over 50mins.
4.3 Results and Discussion

4.3.1 Comparison of Glyceollins Producing Abilities of Different Soybean Varieties

Glyceollins content of different soybean varieties showed wide variation after the fungal incubation (Fig. 4.1 and Table 4.1). All varieties started with no detectable amount of glyceollins prior to fungal inoculation (0 mg/g at 0 hr). Although at low levels, glyceollins synthesis was induced in soybeans by 24 hr of fungal inoculation. The glyceollins accumulation was peaked in about 4 to 5 days (Fig. 4.1 and Table 4.1) for almost all the varieties tested, but further increase in incubation period to (up to 336 h) had no positive impact on glyceollins synthesis (data not shown). The concentration of glyceollin ranged from .02 to 2.077 mg/gram. The MN01050628 variety produced the highest level of glyceollin while the Kouri variety produced the lowest. Although there was slight difference between the glyceollins levels of soybean varieties at different sampling times, there was no significant difference between the varieties at 120 hr. The results obtained in this study clearly indicated that the amount of glyceollins accumulated after fungal infection is greatly influenced by the soybean variety.
**Figure 4.1:** Total glyceollins produced by different soybean varieties during several stages of fungal incubation.
<table>
<thead>
<tr>
<th>Soybean Variety</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tankuro</td>
<td>0.067± .133ABb</td>
<td>0.717± .103ABb</td>
<td>0.733± .133ABa</td>
<td>0.803± .002ABa</td>
<td>0.717± .023Aa</td>
<td></td>
</tr>
<tr>
<td>Brookings</td>
<td>0.023± .023Ba</td>
<td>1.087± .504Aa</td>
<td>0.686± .449ABa</td>
<td>0.633± .909ABa</td>
<td>1.164± .717Aa</td>
<td></td>
</tr>
<tr>
<td>Kouri</td>
<td>0.030± .03Ba</td>
<td>0.031± .016Ba</td>
<td>0± 0Ba</td>
<td>0.020± .011Ba</td>
<td>0.005± .009Aa</td>
<td></td>
</tr>
<tr>
<td>Codington</td>
<td>0.029± .017Bb</td>
<td>0.460± .288ABab</td>
<td>0.760± .641ABab</td>
<td>0.812± .427ABab</td>
<td>1.00± .508Aa</td>
<td></td>
</tr>
<tr>
<td>Davison</td>
<td>0.09± .01ABa</td>
<td>0.67± .32ABa</td>
<td>0.70± .65ABa</td>
<td>0.56± .94ABa</td>
<td>0.61± .85Aa</td>
<td></td>
</tr>
<tr>
<td>Deuel</td>
<td>0.005± .010Bc</td>
<td>0.098± .010Bbc</td>
<td>0.125± .107Babc</td>
<td>0.193± .121Bab</td>
<td>0.271± .057Aa</td>
<td></td>
</tr>
<tr>
<td>Dixon</td>
<td>0.069± .069ABa</td>
<td>0.495± .358ABa</td>
<td>0.320± .229ABa</td>
<td>0.301± .258ABa</td>
<td>0.515± .264Aa</td>
<td></td>
</tr>
<tr>
<td>NE1318382</td>
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<td>0.63± .3ABa</td>
<td>0.69± .3ABa</td>
<td>0.80± .48ABa</td>
<td>0.40± .23Aa</td>
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</tr>
<tr>
<td>MN01050628</td>
<td>0.168± .054Ab</td>
<td>0.461± .326ABb</td>
<td>1.148± .261ABab</td>
<td>1.832± .766Aa</td>
<td>1.092± .614Ab</td>
<td></td>
</tr>
<tr>
<td>MN1806CN</td>
<td>0.024± .02Ba</td>
<td>1.087± .504Aa</td>
<td>0.686± .449ABa</td>
<td>0.633± .909ABa</td>
<td>1.164± .717Aa</td>
<td></td>
</tr>
<tr>
<td>27130</td>
<td>0.047± .006ABa</td>
<td>0.184± .097Ba</td>
<td>0.507± .149ABa</td>
<td>0.546± .561ABa</td>
<td>0.529± .309Aa</td>
<td></td>
</tr>
</tbody>
</table>

Different letters indicate that means are statistically different at p<0.05  
Capital letters: comparing the significance of different varieties  
Lowercase letters: comparing the significance of fungal incubation period (0 to 120 h)
There are several reports on glyceollins production by soybeans using different microbial elicitors such as *Aspergillus sojae*, *Aspergillus oryzae*, *Rhizopus oligosporous*, and physical elicitors such as UV irradiation (S. M. Boué et al., 2000; Jeon et al., 2012; M. R. Lee, Chun, et al., 2010; I. S. Park et al., 2017). The reports have suggested that glyceollins synthesis varies significantly depending on the soybean cultivars and elicitors used. For example, Park et al., (2017) studied 60 different varieties of Korean soybean cultivars and reported that glyceollins level ranged from 0.1 to 9 mg/g after 72 hr of incubation with *Aspegillus sojae* and *Rhizopuz oligosporous*. Our results corroborated with the findings of Park et al., (2017).

### 4.3.2 Total Isoflavones and Daidzein Content

Isoflavones content of the soybean is known to have several benefits to human health and hence, remain as one of the widely studied topics on soybean (Huber & Genazzani, 2016; Larkin et al., 2008; Tansaz & Boccaccini, 2016; Thorat, 2018). Among vegetables fit for human consumption, soy is the sole one which has a high content of isoflavones (Liu, 2004). These isoflavones are divided into four groups and 12 different forms. Aglycones is one of the groups which contains three different fractions (daidzein, genistein, and glycine) (Liu, 2004). In this study, we measured these three fractions of isoflavones and summed them to determine the total isoflavones content (Table 4.1).

Total isoflavones content of soybean varieties ranged from 1 to 6 mg/g at the beginning of incubation (0 h, Table 4.1). Although isoflavones content of soybean cultivars varied significantly from each other’s, no significant change in isoflavones level was detected during the fungal incubation and germination period (comparing 0 to 120 hr within the variety), with exception of the Davison variety. There was significant reduction in the
isoflavones content of Davison variety after 48 h. It has been previously reported that isoflavones levels in soybean cultivars depend largely on many factors, such as climate, planting location and cropping year (Berger et al., 2008; Tsukamoto et al., 1995; Wang & Murphy, 1994), pathogen load (Wegulo et al., 2005), genotype, and factors such as the type of soy product being tested i.e. sprout, seed, meal, etc. (Aussenac et al., 1998; Devi et al., 2009; Kudou et al., 1991). A similar pattern was observed in this study where wide variation was observed in the isoflavones levels of soybean. Total isoflavones contents of the soybean varieties obtained in this study were higher than those reported by Devi et al. (2009). For example, the average total isoflavones content of soybean cultivars reported by Devi et al., (2009) were in the range of 335 to 986 mg per kg of soybean for different varieties.

According to the metabolic pathway of glyceollins synthesis (Ng et al., 2011; Yu et al., 2003), isoflavone fraction daidzein is a direct precursor to glyceollins (T. Graham et al., 1990). This could mean that high amount of daidzein could lead to the high amount of glyceollins in soybean, hence; daidzein content was analyzed separately (Table 4.2) with the aim of understanding the potential relationship or correlation between the two. As mentioned in the section above, levels of daidzein and other isoflavones contained in soybeans depends on several factors and hence it could vary significantly. This was prominent with the daidzein content (ranged from 0.413 to 2.74 mg/g at time 0 hr) of the soybean varieties used. Daidzein levels for varieties have more variation at the 0 hr then the subsequent sampling times. The Dixon variety was consistently a high producer of daidzein and other isoflavones reaching around 3.169 mg/g and 7.064 mg/g for each respectively at the 48 h. In contrast the Tankuro and Kouri varieties were consistently
among the low-end producers for both daidzein and total isoflavones produced (Table 4.1 & 4.2). Irrespective of the soybean variety used, the fungal incubation and germination period did not have any impact on the overall daidzein content except for the Davison variety. A trend of slight increase followed by eventual decrease can be seen when comparing values of most varieties, but not to significant levels (Table 4.2). The Davison variety showed some noted difference among values. Differences among values of Davison show an apparent decrease of daidzein production in the later sampling times with those of the higher earlier sampling times. Similar differences can be seen in the total isoflavone values for Davison. The Codington variety also showed some small differences between earlier and later time points in daidzein and total isoflavone production.
Table 4.2: Total isoflavone content of different soybean varieties during fungal incubation for production of glyceollins.

<table>
<thead>
<tr>
<th>Soybean Variety</th>
<th>Incubation (hrs)</th>
<th>Total Isoflavones (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Brookings</td>
<td>1.996±.094^Ga</td>
<td>2.328±.26^DEFa</td>
</tr>
<tr>
<td>Cocington</td>
<td>3.775±.091^Da</td>
<td>3.693±.124^BCa</td>
</tr>
<tr>
<td>Davison</td>
<td>2.79±.15^EFa</td>
<td>3.34±.26^BCDa</td>
</tr>
<tr>
<td>Kouri</td>
<td>1.29±.18 ^Hb</td>
<td>1.460±.261^EFa</td>
</tr>
<tr>
<td>NE1318382</td>
<td>2.32±.34^FGa</td>
<td>2.55±.23^CDEa</td>
</tr>
<tr>
<td>MN01050628</td>
<td>4.038±.147^CDa</td>
<td>5.981±.747^AAa</td>
</tr>
<tr>
<td>MN1806CN</td>
<td>3.053±.128^EAa</td>
<td>3.71±.52^BCa</td>
</tr>
<tr>
<td>Tankuro</td>
<td>1.001±.17^HAa</td>
<td>1.153±.162^FAa</td>
</tr>
<tr>
<td>27130</td>
<td>4.735±.315^Ba</td>
<td>6.464±1.12^AAa</td>
</tr>
</tbody>
</table>

Different letters indicate that means are statistically different at P < 0.05
Capital letters: comparing the significance of different varieties
Lowercase letters: comparing the significance of fungal incubation period (0 to 120 hr)
Table 4.3: Daidzein content of different soybean varieties during fungal incubation for production of glyceollins.

<table>
<thead>
<tr>
<th>Soybean Variety</th>
<th>Incubation (hrs)</th>
<th>Daidzein (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Brookings</td>
<td>1.354±.067Eab</td>
<td>1.643±.183CDEa</td>
</tr>
<tr>
<td>Codington</td>
<td>1.821±.051Da</td>
<td>1.846±.103CDA</td>
</tr>
<tr>
<td>Davison</td>
<td>0.97±.04Gab</td>
<td>1.26±.16EA</td>
</tr>
<tr>
<td>Deuel</td>
<td>2.305±.062Ba</td>
<td>2.489±.243Ba</td>
</tr>
<tr>
<td>Dixon</td>
<td>2.74±.022Aa</td>
<td>3.057±.337Aa</td>
</tr>
<tr>
<td>Kouri</td>
<td>0.532±.087Ha</td>
<td>0.620±.150FGa</td>
</tr>
<tr>
<td>NE1318382</td>
<td>0.96±.04Ga</td>
<td>1.13±.07EFA</td>
</tr>
<tr>
<td>MN01050628</td>
<td>2.055±.066Ga</td>
<td>2.178±.209BCa</td>
</tr>
<tr>
<td>MN1806CN</td>
<td>1.147±.018FA</td>
<td>1.504±.086DEa</td>
</tr>
<tr>
<td>Tankuro</td>
<td>0.413±.081Ha</td>
<td>0.473±.093Ga</td>
</tr>
<tr>
<td>27130</td>
<td>2.054±.117Ca</td>
<td>2.665±.359ABa</td>
</tr>
</tbody>
</table>

Different letters indicate that means are statistically different at P < 0.05
Capital letters: comparing the significance of different varieties
Lowercase letters: comparing the significance of fungal incubation period (0 to 120 hr)
The daidzein content as obtained are in a similarly fluctuating range with those reported in the literature. For example, the Kudou et. al., (1991), using Maple Arrow soybean seeds recorded a daidzein level around 1.3 mg/g with a total isoflavone level at 2.47 mg/g where another study by Devi et al., (2009), reported a daidzein level of .26 mg/g and a total isoflavone content of around .75 mg/g.

Contrary to our anticipation, results showed no direct correlation between the level of daidzein and glyceollins accumulation in the soybean (Figure 4.1 and Table 4.2). Our findings agree with the findings of Park et al., (2017) where authors studied 60 different varieties of Korean soybean cultivars and showed no direct correlation between daidzein and glyceollins levels. Glyceollins synthesis is rather dependent on the soybean genotype and elicitors used.

4.4 Conclusion

Different soybean varieties have the different levels of isoflavones content and their abilities to produce glyceollins varies significantly. Although initial increase in incubation time results in some increased accumulation of glyceollins, not much increase is noted beyond 72 and 96 hr with very little to no improvement after 120h. Variety MN01050628 was the highest glyceollin producer when using T. reesei as the fugal stimulator, while variety Dixon was the highest producer of daidzein and total isoflavones. No direct correlation between glyceollin accumulation and initial daidzein content is observed. The study findings suggested that soybean varieties and elicitors are the two key role players in glyceollins synthesis, hence wide range of soybean cultivars and elicitors need to be considered during process optimization for glyceollin synthesis.
CHAPTER V – SUMMARY AND CONCLUSIONS

The value of soybean may be highly dependent on its protein concentration (Brumm & Hurburgh, 2006; Hurburgh Jr et al., 1990), but the total market success also stems from its versatile uses: in food products (e.g. tofu, soybean sauce), edible vegetable oil, biofuel, and especially its use as a major protein source in livestock feeds (Boerema et al., 2016). The major role it plays in livestock feed is a what gives soybeans a great jump start to being a potential answer to reducing the perpetual continuation and growth of antibiotic resistance in food animals due to use of feed additives.

The main limitation of using soybean glyceollin as an antimicrobial additive to feedstock is sustaining a cost-effective level of glyceollin produced by the soybeans.

Approximately 3 mg/g is the level which would need to be achieved to comfortably use glyceollin enriched soybeans as an antibiotic alternative. The fluctuation of production levels varies greatly among varieties and elicitors used and with no isoflavone correlation it can be difficult to pinpoint which varieties will be the best performers.

Adjusting various aspects of the fungal stimulation and incubation process slightly enhanced production of glyceollins (Chapter III). Surface sterilized beans which were soaked overnight displayed the greatest concentration results for glyceollins with ~1 mg/g in the Davison variety and ~.8 mg/g in the Brookings variety; compared to the results from the nonsterile beans which were ~.6 mg/g and ~.5 mg/g for each variety respectively. Inoculation using either the dropwise or by spread method showed results of glyceollins concentration which were all favorable. Due to an increased lag phase when using a spore inoculation method; the mycelial inoculation method produced higher
levels of glyceollin at each of the sampling times. In addition, incubation of 96 to 120 hr at 30°C in dark conditions produced the highest concentrations when comparing sampling hours.

Levels of glyceollins are significantly influenced by soybean variety and can be successfully increased by fungal infection. This is contradictory to the idea that concentration of total isoflavones or daidzein was correlated with collection of glyceollins in reply to fungal stimulation. (I. S. Park et al., 2017). Different soybean varieties produced showed capacities for producing glyceollins in response for fungal stimulation from T. reesei (NRRL-3653) independent of the isoflavone levels produced, most notably independent from the glyceollin precursor isoflavone daidzein (Chapter IV). Soybean variety MN01050628 was the highest producer of glyceollins with an average peak ~1.8 mg/g at the 96 hr while the Dixon variety produced the highest levels of daidzein peaking at ~3.17 mg/g at the 48 hr. In contrast the soybean variety with the lowest glyceollin levels was Kouri, its average peak being ~.03 mg/g at the 48 hr. Kouri and Tankuro varieties were the lowest producers of daidzein with an average peak of ~.620 mg/g at the 24 hr and .623 mg/g at the 72 hr respectively.

Future trials should be conducted with other fungal strains, such as Aspergillus species, and maintain a large variety of soybeans types. Research into seeds of different grades and growing areas should also be investigated. Duration of soybean storage after harvesting and before processing should also be investigated between varieties for effects on levels of glyceollins. Other elicitors, such as UV irradiation and application of biologicals or chemicals, with potential to induce production of glyceollins should also be explored in combination with fungal infection. It may also prove beneficial to try
adjusting various aspects of processing with several soybean varieties and comparing resulting levels. Once sufficient accumulation levels of glyceollins are achieved and sustained, ~3 mg/g, work to test effectiveness on various microbial infectors can be done. Glyceollins enriched soybeans should then be tested for incorporation into the diets of food animals.


Steele, C. L., et al. (1999). Molecular characterization of the enzyme catalyzing the aryl migration reaction of isoflavonoid biosynthesis in soybean. *Archives of Biochemistry and Biophysics, 367*(1), 146-150.


