The potential for trypsin inhibitor expression in leaves to convey herbivory deterrence in soybean

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Abstract

As soybean (*Glycine max)* cultivation expands into new areas, it is exposed to atypical pests like mammalian wildlife. The white-tailed deer (*Odocoileus virginianus*) is reported to cause the most yield losses out of all wildlife pests, and economic reports reflect that this is a worsening issue. Current crop protection strategies for wildlife are insufficient and outdated. In plant biotechnology, there are many examples of crop protectants being directly incorporated into the plant using genetic engineering. Such systems have been successful in deterring insect pests, and similar strategies could be employed to mammalian pests. Plant trypsin inhibitors show promise as a potential deterrent based on the established feeding strategies employed by deer. Newly developed lines of transgenic soybean with redirected expression of native trypsin inhibitors showed promise in deterring both insect and deer herbivory without any phenotypic differences from nontransgenic lines. Four lines of transgenic soybean expressing the KTi7 or BBi5 trypsin inhibitor genes under the control of the CaMV 35S or soybean RBCS-SRS4 green tissue promoter were selected for field testing against insects or deer, and the highest expressing line was examined in controlled deer feeding experiments. Two field experiments were designed to quantify transgenic trypsin inhibitor soybean lines' potential resistance defoliation from insects or deer. One field experiment was dedicated to growth characterization and quantifying herbivory resistance in ambient insect conditions. A separate field experiment was dedicated to quantifying herbivory resistance under ambient deer feeding conditions. Deer preference between transgenic trypsin inhibitor lines and nontransgenic soybean was also quantified in controlled deer feeding experiments in collaboration with the University of Georgia's Whitehall Deer Research Facility. No significant difference in growth characteristics were found between transgenic trypsin inhibitor lines and nontransgenic soybean grown in field conditions. Two of

the four transgenic trypsin inhibitor lines exhibited significantly reduced defoliation compared to nontransgenic soybean in ambient insect herbivory field conditions. In ambient deer herbivory field conditions, there was no difference in defoliation between nontransgenic and transgenic trypsin inhibitor soybean lines. In controlled feeding trials with tame captive deer, there was no significant difference between the amount of transgenic trypsin inhibitor and nontransgenic soybean consumed.

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Chapter 1

Literature review

Introduction

As an extensively grown crop, soybean (*Glycine max*) also faces damage from an extensive range of herbivorous pests. Effective strategies exist to manage damage from insect pests, but these options may not be sustainable and can result in harm to both humans and the environment. One pest without effective management strategies is the white-tailed deer (*Odocoileus virginianus*), an anomalous yet destructive pest to soybean in the Midwest, Midsouth, and Southeast United States. Damage from white-tailed deer appears to be a growing issue and has been quantified through several different methods. Current solutions to deer damage on soybean crops are insufficient in preventing yield loss and are far behind most agricultural pest management technology. A solution based on deer behavior and directly incorporated into the plant would prove far more successful. Deer prefer and seek forage rich in simple protein, and move to the next forage option once the best option is depleted. In many agroecosystems, soybean leaves are the most abundant and nutritionally valuable food source available. Soybean plants already contain some mechanisms to protect themselves from herbivory, but they are not naturally potent enough to protect the leaves from large herbivores. One example of these natural plant protectants is proteinase inhibitors, which are mainly concentrated in the plant's most valuable structures, such as seeds and storage organs. Many plant families are known to contain proteinase inhibitors. Legumes, such as soybean, contain high levels of serine and cysteine proteinase inhibitors. These proteinase inhibitors act as herbivory deterrents due to their antinutritional qualities. Proteinase inhibitors function by binding to proteinases, which are digestive enzymes that break down protein in the animal's gut and either destroy or inhibit the digestive enzyme. This prevents the animal from receiving the nutritional benefits of the protein, depletes the supply of proteinase enzymes in the animal's gut, and causes uncomfortable

digestion. While many studies have explored the use of soybean proteinase inhibitors for defense against insect herbivores, they may also prove useful in deterring large mammalian herbivores. Soybeans and other crops have also been genetically engineered to have redirected proteinase inhibitor expression in the leaves and other green plant tissue. Transgenic expression of trypsin inhibitors has already been proven to significantly reduce insect herbivory. Using these techniques and information, soybean plants that are genetically engineered to express proteinase inhibitors in their leaves could be considered as a strategy to reduce damage from a broad range of herbivores.

Need for sustainable wildlife pest control

The ever-broadening encroachment of soybean farming into new ecosystems subjects this valuable plant to a diverse range of biological sources of yield loss. (Pagano et al. 2016). Biotic constraints including weeds, pathogens, insects, and other herbivores cause soybean crop losses averaging 26–29% globally. There are many effective strategies to manage soybean yield loss from weeds, pathogens, and insects, but yield loss from other non-insect herbivores is often left unaddressed, despite causing 11% losses of attainable production (Heinrichs et al. 2018). Soybean experiences significant yield loss from a variety of larger animals including the common wood pigeon (*Columba palumbus*), European hare (*Lepus europaeus*), Canada geese *(Branta canadensis*), groundhog (*Marmota monax*), raccoons (*Procyon lotor*), squirrels (*Sciurus spp.*), wild hogs (*Sus scrofa*) and white-tailed deer (*Odocoileus virginianus*) (Lamichhane 2021; McKee et al. 2021; MacGowan et al. 2006; Duncan et al. 2023). The species responsible and severity of the resulting yield losses is dependent upon the location of the field and the growth stage of the soybean. In the United States, the white-tailed deer is the largest cause of wildlife-related damage to agricultural crops, including soybean (Conover et al. 1991). Higher rates of

deer defoliation have been shown to correlate with increased amounts of herbivorous insects, as well as allowing light to reach the soil, which increases weed pressure, in turn negatively impacting yield (VanGorder et al. 2021; McKee et al. 2021). Deer begin feeding on soybean plants in the earliest stages of their life cycle, which can decimate new growth in the first week after planting. While the plants may recover, damage during the first 1-5 weeks causes differences in maturation timing and results in the greatest reduction of yield. Differences in maturation group affect harvest timing, which can greatly hinder the careful planning required in large scale agricultural operations (deCalesta 1978).

Quantifications of soybean yield loss due to deer damage

A clear, quantitative view of soybean yield losses caused by wildlife can be seen in the data from indemnity payments. Farmers often choose or are even required to purchase insurance for agricultural loans due to the great financial risks associated with farming (McKee et al. 2021). When a crop yields below the amount it was insured for, the farmers' loss is then partially covered by an indemnity payment. Indemnity payments for wildlife-related soybean crop damage have been on the rise for the past two decades, likely due to loss of herbivore habitat and food sources (McKee et al. 2021; Duncan et al. 2023). In 2011, wildlife damage indemnity payments for soybean crops in Missouri, Kentucky, Arkansas, Tennessee, Mississippi, Alabama, and Georgia was $1 million. In 2022, payments had increased to $4.8 million. The wildlife damage reported in these indemnity payments is almost entirely from white-tailed deer, with some damage also resulting from wild hogs (Duncan et al. 2023). Another study on wildlife damage indemnity payments used data from 2015-2019 to create a large-scale model of the resulting crop losses. This model estimated that soybean crops suffered losses of approximately $323.9 million due to wildlife damage in 2018 (McKee et al. 2021). In a more direct approach to

quantification, a study by (Rosenberry et al. 2006) found that exclusion of deer results in significantly higher soybean yield. The study was designed to examine the efficacy of a new type of deer-proof fencing. This is one of the few studies that robustly quantifies the direct effect of deer herbivory on soybean yield in the field. Deer damage to soybeans was measured with the use of fenced plots to exclude herbivory, unfenced control plots, and measurement of the resulting yield. The protected plots resulted in greater yield (239.60±14.08 kg) compared to unprotected control plots (169.80±22.30 kg). This study was conducted in an area with high deer density comparable to agricultural regions that experience significant yield losses from deer herbivory (Rosenberry et al. 2006). Another relevant quantification of deer damage was achieved through a mail survey sent to Tennessee landowners with high levels of deer population and soybean production. This survey found that respondents identified white tailed deer as the largest cause of wildlife-related crop loss to soybean farmers. Results reflected that 55% of respondents reported wildlife damage, and 78% identified deer as the species causing the most damage. Of the farmers that took preventative measures against deer damage, 77% used regulated hunting with a depredation permit, which was ranked as the most effective method of control, followed by electric fences (Johnson et al. 2000).

Insufficiency of current management strategies

While depredation was ranked as the most effective form of control, it has several drawbacks and may even worsen the issue. White-tailed deer are some of the most fecund deer species in the world, meaning that their population numbers can change rapidly depending on the presence of forage or predation (McShea 2012). Since most of the agricultural land in the United States lacks large predators, deer populations are only limited by access to plant productivity. Hunting to reduce deer populations is often qualitative and does not ensure that populations are reduced

below the biological carrying capacity (McShea 2012). With plentiful food sources still available in the form of agricultural crops, deer population can quickly rebound from qualitative culls. This leads to unstable fluctuations in deer numbers, which puts further strain on crops as a food source. Fencing is the second-most common form of preventing deer damage to crops, but not all fencing is effective, and each fencing type has corresponding drawbacks (Rosenberry et al. 2006). Woven wire fences with high tensile strength are considered functionally deer-proof but are too expensive and difficult to move for application in large scale settings. Electric fences are cheaper and easier to move, they do not necessarily guarantee the absence of deer. Even with fencing materials that are inexpensive and effective at preventing deer presence, frequent movement is required for applications of pesticides and harvest. While fences are a commonly used management strategy, they are not always a feasible option for large scale applications (Rosenberry et al. 2006). Herbivory deterrents are another common form of deer damage management. These compounds must not harm the plant they are meant to protect, but cause deer to have adverse reactions sufficient to discourage or inhibit feeding. Such compounds are classified by their chemical mode of action, and the type of reaction they induce. Urine and hair of predator species have been studied for their deterrent effect of fear, and capsaicin for its deterrent effect of pain (de las Mercedes Guerisoli et al. 2020). Deer can also be deterred from feeding by unpalatable substances, such as hydrolyzed casein. This type of applied deterrent has several drawbacks: the compound may be expensive or difficult to extract, reapplication will become necessary as the deterrent breaks down in the environment, and effective repellents may be considered hazardous or unsanitary to humans, such as the use of blood, urine, or meat. Optical deterrents are also still used in many farms (de las Mercedes Guerisoli et al. 2020), but deer deterrent technology may finally progress past the use of scarecrows.

Need for sustainable insect pest control

While current strategies for managing deer damage to soybean are obviously flawed, the well-established strategies for managing insect damage to soybean lack long-term sustainability. Foliar applied pesticides have numerous harmful off-target effects due to their lack of specificity and mode of application. Foremost among these are dangers to human health from pesticide exposure, which can have negative effects on the skin, digestive system, cardiovascular system, lungs, reproductive organs, nervous system, and endocrine system (Nicolopoulou-Stamati et al. 2016). Runoff from agricultural areas can contain pesticides that make their way into aquatic ecosystems, harming invertebrates and amphibians (Baker et al. 2013; Sánchez-Bayo 2021; Mahmood et al. 2016). The cost of insect scouting, seed treatments, and applying foliar pesticides is also considerable, and must be upkept throughout the growing season (Musser er al. 2022). The transgenic expression of *Bacillus thuringiensis* (Bt) toxins as incorporated crop protectant provides a more precise approach to reducing insect herbivory. However, Bt's highly specific mode of action increases selective pressure and risks the development of resistance in insect populations (Tabashnik et al. 2013). An incorporated crop protectant with a more general mode of action and low selective pressure towards developing resistance may be more suitable.

Mechanisms of plant proteinase inhibitors

Proteinase inhibitors are a form of anti-nutritional protein found in a wide variety of plants and were first discovered as a type of regulatory element involved in stress response, plant growth, and defense against herbivores (Edwards & Singh 2006). There are many different types of proteinase inhibitors, which researchers have classified into families such as mustard, squash, rapeseed, serpine, cereal, potato I, potato II, Bowman-Birk, and Kunitz (Laskowski & Qasim, 2000; Leo et al. 2002). These families are determined by the proteinase inhibitors' plant of

original discovery, molecular structure, and active site. Due to its prevalence as a crop, soybean proteinase inhibitors and their applications have been studied thoroughly. Soybean contains a type of serine proteinase inhibitor known as a trypsin inhibitor, named because it inhibits the digestive enzyme trypsin. Serine trypsin inhibitors are classified as Bowman-Birk inhibitors (BBIs) or Kunitz trypsin inhibitors (KTIs) based on structural differences and protein binding sites (Laskowski & Kato, 1980). BBis have one polypeptide chain, two reactive sites, and seven disulfide bridges formed from fourteen cysteine residues. KTis have one or two polypeptide chains, one reactive site, and two disulfide bridges formed from four cysteine residues (Birk 2003). Subtle differences between different types of proteinase inhibitors can impact how they interact with the digestive enzymes in an animal's gut (Leo et al. 2002). Proteinase inhibitors can bind with proteinases in the gut two different ways, known as reversible and irreversible reactions. In a reversible reaction, the inhibitor binds to the active site of the proteinase, and then the inhibitor either degrades the proteinase or stays bound to the proteinase in a complex structure. In an irreversible reaction, when the inhibitor binds to the active site of the proteinase, the inhibitor and proteinase undergo structural changes that render them both non-functional (Sultana et al. 2022).

Transgenic expression of plant proteinase inhibitors for insect defense

Plant proteinase inhibitors have already been extensively researched, and their antinutritional effects on insects are well-documented. A study examining the effects of crude extract of soybean trypsin inhibitor (a type of proteinase inhibitor) applied to the diet of *Spodoptera littoralis* larvae caused significant reduction of weight, pupation, and emergence compared to larvae receiving the control diet (Ibrahim et al. 2020). While these results demonstrate the potential applications of proteinase inhibitors, other research has gone a step farther and

manipulated the expression of proteinase inhibitors at the genetic level. By isolating proteinase inhibitor genes from plant genomes, the resulting genes of interest can be manipulated to increase or redirect expression in a plant, as well as being transformed into different plant species. A 2023 study used *Agrobacterium*-mediated transformation to overexpress several types of native trypsin inhibitors found in soybean. The resulting transgenic soybean plants were tested against *Helicoverpa zea* larvae in detached leaf and whole plant feeding assays. In the detached leaf assays, two of the transgenic soybean lines showed significant reduction in larval weight. Next, whole plant feeding bioassays were performed using the two significant lines from the detached leaf assay. Each plant was inoculated with 10 corn earworm larvae, and defoliation was measured after 10 days. All transgenic lines showed significant reductions in defoliation compared to the wild type (Sultana et al. 2023). There are many benefits to selecting a plant's native proteinase inhibitor genes for overexpression, but some studies find that expressing proteinase inhibitor genes from one plant in another plant show additional advantages. For example, another study examining the transgenic expression of soybean serine proteinase inhibitors in potato (*Solanum tuberosum)* and tobacco (*Nicotiana alata*) found striking reductions in defoliation, larval weight, and larval viability. *Agrobacterium*-mediated transformation was used to incorporate the soybean serine proteinase inhibitor genes into tobacco and potato plants. The resulting transgenic plants were used in detached leaf feeding assays with *Spodoptera littoralis* larvae. In the transgenic tobacco feeding assays, larvae death was often observed after only two days of feeding. In the transgenic potato feeding assays, larvae death was not frequently observed, but the best transgenic plants resulted in a 50% reduction of larval weight compared to the control. The difference in larvae response in tobacco and potato was likely due to the nutritional differences in the leaves (Marchetti et al. 2000). Yet another study

examined the coexpression of potato type I (StPin1A) and II (NaPi) proteinase inhibitors in cotton (*Gossypium spp.*). In this study, researchers chose to express more than one proteinase inhibitor because the tobacco proteinase inhibitor (NaPi) inhibits both trypsin and chymotrypsin but has potential for the evolution of insect resistance. The addition of the potato type 1 inhibitor (StPin1A) negates the potential for the development of resistance. Cotton plants were individually transformed using *Agrobacterium* to express either StPin1A or NaPi proteinase inhibitors. After stable homozygous transgenic plants had been achieved for both lines of transformation, the two lines were crossed to produce cotton plants expressing both StPin1A NaPi proteinase inhibitors. In cotton leaf feeding assays with *Helicoverpa armigera* larvae, the addition of NaPi resulted in 50% reduced weight, StPin1A resulted in 40% reduced weight, and the combination of NaPi and StPin1A resulted in 90% reduced weight compared to the control larvae. Insect resistance was also observed in field trials using transgenic cotton expressing NaPi and StPin1A. Cotton expressing NaPi and StPin1A had a significantly higher yield of cotton bools and plant weight compared to non-transgenic cotton (Dunse et al. 2010). These studies clearly exhibit the antinutritional effects of proteinase inhibitors on herbivores, and the well-researched biotechnology applications of soybean proteinase inhibitors. The many options for increasing expression, transforming new proteinase inhibitors into plants, and engineering plants to express multiple proteinase inhibitors allow for a variety of approaches in novel adaptations to other issues, such as wildlife herbivory deterrents.

Deer feeding behaviors

Deer feed by preference, consuming all the most nutritious food available before moving on to their second choice. Their natural food source consists mostly of browsing young twigs, leaves, and forbes that grow at the edge of forested areas (McShea 2012; deCalesta 1978). In the

absence of an ecosystem that can provide enough of this preferred forage, deer will consume the next best thing in terms of accessibility and energy requirements, which is often row crops such as wheat or soybean. In some agroecosystems, row crops can comprise up to 77% of a deer's diet (Colligan et al. 2011). Deer are also known to cause the most damage to crops at the edges of fields, where yield is already reduced due to edge effects. (deCalista 1978). Deer are conencrete selectors, which means they require diets that are easily digestible and high in protein due to their short digestive retention time and limited gut volume. Deer optimize their feeding efficiency by identifying and seeking nutritionally preferable forage, then remembering and seeking them in the environment. Consequently, deer also identify nutritionally poor and unsuitable forage, and avoid their consumption, as well as the areas where they are located (Gillingham & Bunnell 1989).

Potential applications as broad range herbivory deterrent

The impact of deer damage to soybean yield and the feeding strategies behind this interaction are well documented and can be reduced to nutrition and availability. Management attempts to reduce the availability of soybeans to deer have proved ineffective. Instead, removing the nutritional appeal of soybean leaves may be a far more practical solution. The technology to accomplish this is already well-researched and has been pioneered in soybean. If genetically engineered soybean plants can express sufficient levels of proteinase inhibitor in their leaves, the resulting antinutritional effects would likely deter both deer and insect herbivory and potentially provide yield protection. If additional protectant is needed, multiple proteinase inhibitor genes could be transformed or crossed into soybean plants until the leaves become entirely unappealing. Since deer's feeding preference prioritizes the availability of easily digestible protein, consuming proteinase inhibitor soybean would go against their innate behavioral

patterns. Compared to the expensive, labor intensive, unreliable, and archaic methods of deer deterrent currently in use, genetically engineered proteinase inhibitor soybean could prove to be a sustainable solution. As an incorporated plant protectant, transgenic trypsin inhibitor soybean could prevent yield loss caused by herbivory damage without requiring farmers to purchase, install, or apply any additional management tools.

The goal of my thesis research was to characterize the performance of transgenic trypsin inhibitor soybean in field conditions and assess its potential to deter herbivory from insects and deer. The objectives were to characterize transgenic trypsin inhibitor soybean lines' growth parameters in field conditions, quantify pest deterrence in ambient insect field conditions, quantify deer deterrence in ambient herbivory field conditions, and quantify deer deterrence in controlled feeding trials with tame deer.

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Chapter 2

Insect herbivory and growth characterization in field conditions

Abstract

Plant incorporated protectants against insect herbivory that exhibit significantly decreased defoliation in controlled feeding assays do not always translate into ambient herbivory field conditions. Transgenic soybean (*Glycine max*) lines expressing the KTi7 and BBi5 trypsin inhibitor genes under the control of the CaMV 35S or soybean RBCS-SRS4 green tissue promoters were shown to significantly reduce insect defoliation against corn earworm (*Helicoverpa zea*) in detached leaf feeding bioassays and whole plant greenhouse feeding bioassays. The growth parameters of plant height, dry aboveground biomass, and seed weight did not significantly differ between transgenic trypsin inhibitor soybean lines and wild type soybean grown in greenhouse conditions. A field site was designed to characterize insect defoliation and growth parameters in ambient herbivory field conditions. There was no significant difference in plant height, aboveground dry biomass, or seed weight between transgenic trypsin inhibitor lines and wild type grown in field conditions. Two of the transgenic trypsin inhibitor lines exhibited a significant difference in insect defoliation from wild type in ambient herbivory field conditions.

Introduction

Soybean (*Glycine max*) farmers go to great lengths to protect their crops from insect herbivores, but pest control can be expensive, labor intensive, ineffective, or lead to unintended consequences (Mahmood et al. 2016; Nicolopoulou-Stamati et al. 2016; Isenring 2010). However, the damage caused by herbivorous pests is too costly to ignore. In 2021, yield loss due to insects cost Tennessee soybean farmers $35,000,720 (Musser et al. 2022). Attempts to mitigate this damage through pesticide application is also expensive, with the cost of insect scouting, seed treatments, and foliar treatments totaling $26,130,700 for Tennessee soybean farmers in 2022 (Musser et al. 2022). Beyond preventing insect herbivory, pesticide application

can affect the environment in unintended ways, such as pesticides leaching into water and soil, as well as causing harm to non-target organisms (Mahmood et al. 2016). Pesticide exposure is also dangerous to human health, and can cause issues such as hypertension, kidney failure, Alzheimer's, and cancer (Nicolopoulou-Stamati et al. 2016). An incorporated plant protectant could mitigate these off-target effects caused by pesticides. Soybean that has been genetically engineered to express *Bacillus thuringiensis* (Bt) toxins (Stewart et al. 1996) could offer more direct protection, but due to its highly specific mode of action, there is a greater risk for insects to evolve a resistance (Tabashnik et al. 2013). One promising candidate for an incorporated plant protectant in soybean are plant proteinase inhibitors. Soybean plants naturally contain proteinase inhibitors as an herbivory defense mechanism but are mainly expressed in the seeds (Kunitz 1946; Birk 2003; Palacios et al. 2004). Nonetheless, low levels of some proteinase inhibitors can be found in the plant's other tissues as well (Sultana et al. 2022). By using genetic engineering to overexpress proteinase inhibitors in the leaves and other green tissue, proteinase inhibitors could protect the plant from insect herbivory and prevent yield loss due to defoliation (Sultana et al. 2023). A study by Sultana et al. (2023) produced transgenic soybean plants expressing the KTi1, KTi2, KTi3, KTi5, KTi7, and BBi5 trypsin inhibitor genes under the control of either the CaMV 35S and soybean RBCS-SRS4 promoters. In these lines the goal was to express trypsin inhibitor genes in shoots to deter herbivores. Potential herbivory deterrence of F3 transgenic trypsin inhibitor soybean lines was quantified in greenhouse feeding bioassays utilizing corn earworm (*Helicoverpa* zea) larvae (Sultana et al. 2023). In most transgenic events, there was significant reduction in larval weight and total leaf defoliation compared to nontransgenic soybean. In greenhouse conditions, the trypsin inhibitor soybean shows no difference in yield and growth characteristics compared to wild type soybean (Sultana et al. 2023). The objective of the current

study is to determine whether greenhouse findings translated to ambient insect herbivory field conditions.

Materials and methods

Plants

Field experiments used homozygous T5 seeds from four transgenic events of redirected expression of the BBi5 and KTi7 genes under control of the CaMV 35S and soybean prbcS-SRS4 promoters from (Sultana et al. 2023), which were the top-performing lines from that greenhouse study. (**Table 2.1**). The original germplasm was the soybean cultivar 'TN15-5007' (Pantalone et al. 2020).

Field design

A field experiment was designed to characterize the performance of transgenic trypsin inhibitor soybean lines compared to wild type lines in field conditions at the East Tennessee AgResearch and Education Center, in Knoxville, Tennessee, USA. A randomized block design was chosen to account for field variability. The total planted area of this field was 83.57 m², and the total area

Table 2.1. Soybean lines that were used in the present study.

Name used in paper	**Promotor**	**Transgene**	**Line from (Sultana et al., 2023)**
35S-KTi7	35S CaMV	KTi7	Line 1
35S-BBi5	35S CaMV	BBi5	Line 2
GTP-KTi7	prbcS-SRS4	KTi7	Line 2
GTP-BBi5	prbcS-SRS4	BBi5	Line 1
Wild type (WT)	-	-	-

including the 15.24 m fallow border was 278.89 m². The field contained a total of 480 soybean plants: 312 transgenic and 168 wild type. The field was divided into two sections, the left side was dedicated to observation and data collection, and the right side was used to produce plant material for other experiments and seed bulk. There was a 3.05 m unplanted border between the two sections. Both fields combined contained eighty plots, and each plot contained six plants. The spacing between rows was 76.2 cm, and the spacing between columns was 22.86 cm (**Figure 2.1**). Seed were planted by hand June 6, 2023. No irrigation, insecticide, or fertilizer was used at any time during the growing season and all maintenance was done using hand tools. Two weeks after the field was planted, any seeds that failed to germinate were replaced with new seeds. The plants were regularly monitored and allowed to reach maturity. The field was harvested by hand, completed on October 28, 2023, then treated with herbicide to terminate the trial.

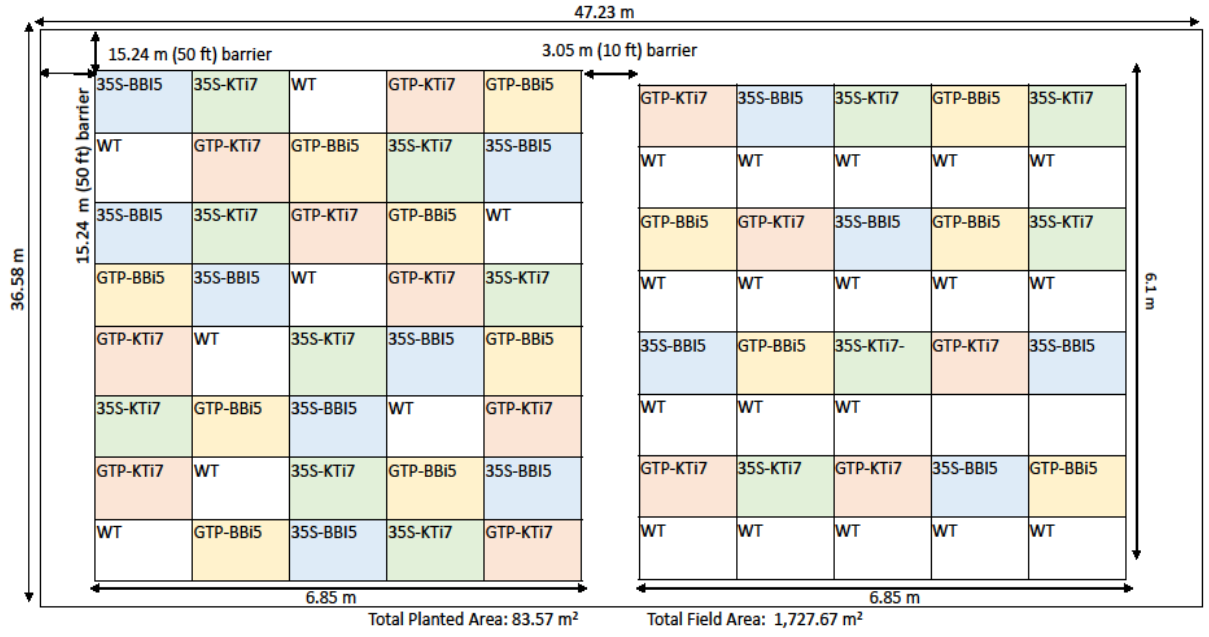


Figure 2.1. Layout of the field site. The right side was used for growth characterization and insect herbivory quantification. The left side was used for producing plant material for other experiments and seed bulking.

Insect herbivory

To confirm and quantify insect pest damage in the field, two approaches were used. Defoliation ratings were used to score insect damage to plants, and insect population surveys were used to confirm the presence of common soybean pests. Defoliation ratings were taken on three time points: July 13, 2023, August 1, 2023, and August 15, 2023. Each plant was visually scored by selecting a representative leaf from the top, middle, and bottom of the plant. The most and least defoliated leaves were not considered, and the remaining leaf was used for rating. The defoliation rating was determined using a reference sheet with leaf area removed quantified as a percentage. The reference sheet was generated with ImageJ and specific to soybean (Ortega et al. 2016). Defoliation measurements were averaged for each construct across the three measurement time points. Population surveys were performed on four time points: July 7, 2023, August 8, 2023, August 22, 2023, and September 5, 2023. The top and bottom of each leaf, petiole, and the stem were carefully observed on each plant, and all herbivorous insects were recorded.

Results and discussion

Growth Characterization

The plants were allowed to mature in the field, and then the plant height was recorded for each individual plant. Once the plants were fully dried, individual plants were either stripped of pods or cut at the base and placed into a pre-labeled bag. The harvested material was taken back to the laboratory and processed by hand. Average seed weights and dry aboveground biomass were recorded for each line. The height measurements from forty-eight plants from each line were used for statistical analysis. All analysis was performed using SAS. Growth parameters were analyzed using an analysis of variance (ANOVA). According to the ANOVA, there were no significant differences among plant heights of any transgenic trypsin inhibitor lines and wild

type. Seed weights were measured and statistically analyzed using the same method. There was no significant difference in seed weights among transgenic trypsin inhibitor lines and wild type. Total dry aboveground plant biomass was collected from two plants in each plot, for a total of sixteen measurements per line. There was no significant difference between aboveground dry biomass of any transgenic trypsin inhibitor lines and wild type. This is consistent with the growth parameter differences recorded in (Sultana et al. 2023) for plants grown in greenhouse conditions.

Insect Herbivory

The herbivorous insect species observed in the four conducted population surveys were mostly consistent with common insects that cause soybean yield loss. For representation in this paper, they are grouped by damage type. Pod feeders and piercing-sucking insects were observed in adult form and defoliators were observed in larval form. Pod feeders include stink bug

Table 2.2. Seed weight per plant average, plant weight average, and plant height average for the five transgenic trypsin inhibitor soybean lines and wild type grown in field conditions

Line	**Seed weight average (g)**	**Plant weight average (g)**	**Plant height average (cm)**
35S-BBi5	35.5	70.6	70.3
35S-KTi7	41.3	93.4	76.7
GTP-BBi5	43.5	98.7	72.3
GTP-KTi7	39.7	79.3	75.1
WT	39.2	82.6	75.4

(*Hemiptera: Pentatomidae*) and bean leaf beetle (*Cerotoma trifurcate*). Piercing-sucking include scale (*Coccoideas spp*.), leaf hoppers (*Cicadellidae spp*.), and kudzu bugs (*Megacopta cribraria*). Defoliators include corn earworm (*Helicoverpa zea)*, bean leaf beetle (*Cerotoma trifurcata*), and cabbage looper (*Trichoplusia ni*). Aphids (*Aphis* spp.) were also observed consistently across all lines. (Musser er al. 2022; Wilkerson et al. 1986; Beckendorf et al. 2008; Potter & Held 2002; Biddle et al. 1992). All the herbivorous insects with more than ten observations over the course of the population surveys were included (**Table 2.3**). Defoliation ratings were averaged for each line across the three time points: July 13, 2023, August 1, 2023, and August 15, 2023. Relevant descriptive statistics for the defoliation data from each line are represented in (**Table 2.4**). Data was analyzed in SAS using a mixed model analysis of variance (ANOVA) and log transformed to meet normalcy assumptions, with a Dunnett post-

Table 2.3. The total count of herbivorous insect species observed ten or more times on each transgenic trypsin inhibitor soybean line and wild type across population surveys performed on July 7, 2023, August 8, 2023, August 22, 2023, and September 5, 2023

Line	**Pod feeders**	**Piercing-sucking**	**Defoliators**	**Grand total**
35S-BBi5	8	87	50	147
35S-KTi7	11	154	42	213
GTP-BBi5	7	140	38	192
GTP-KTi7	10	141	37	191
WT	13	245	45	304
Grand Total	49	767	212	1047

hoc if the ANOVA results were statistically significant at the 0.05 level. The defoliation of the 35S-KTi7 and GTP-KTi7 transgenic trypsin inhibitor lines were significantly less compared to wild type, with p values of 0.0236 and 0.0343, respectively. The other two transgenic trypsin inhibitor lines were insignificantly different from wild type by tenths or hundredths of a decimal place; the p value for 35S-BBi5 was 0.0544 and the p value for GTP-BBi5 was 0.0891. Relevant descriptive statistics are shown in the table below (**Table 2.4**). While two lines did show a significant reduction in defoliation compared to wild type, the overall defoliation observed at this field site was low. In the raw data, only a few plants approached 10% defoliation. The observed defoliation was far below the economic threshold for yield loss (Turnipseed 1972). Distinctly low defoliation in both transgenic trypsin inhibitor soybean lines and wild type indicates that there may have been other ecological interactions responsible for reducing defoliation throughout the entire field.

Table 2.4. Descriptive statistics for insect defoliation across transgenic trypsin inhibitor lines and wild type soybean

Line	Mean	Std Dev	Std Error	Median	Minimum	Maximum
35S-BBi5	1.7188	0.3907	0.1381	1.6667	1.0000	2.1667
35S-KTi7	1.6792	0.6551	0.2316	1.5000	1.0000	3.2000
GTP-BBI5	1.7646	0.3710	0.1312	1.8167	1.1667	2.3333
GTP-KTi7	1.6854	0.5033	0.1779	1.5833	1.2000	2.7500
WT	2.4875	0.8817	0.3117	2.3333	1.3333	4.4000

The boxplot below shows defoliation for the transgenic trypsin inhibitor lines and wild type under ambient insect herbivory conditions (**Figure 2.2**). The large fallow region required in the release permit may have hindered insect movement into the field, as the total areas of the fallow zone was wider than the field on all sides, and its total area was much larger than the planted area. The fallow region was also kept generally free of any plants or ground cover, and mainly consisted of bare soil. However, the insect population surveys confirmed the consistent presence of common soybean insect pests throughout the growing season.

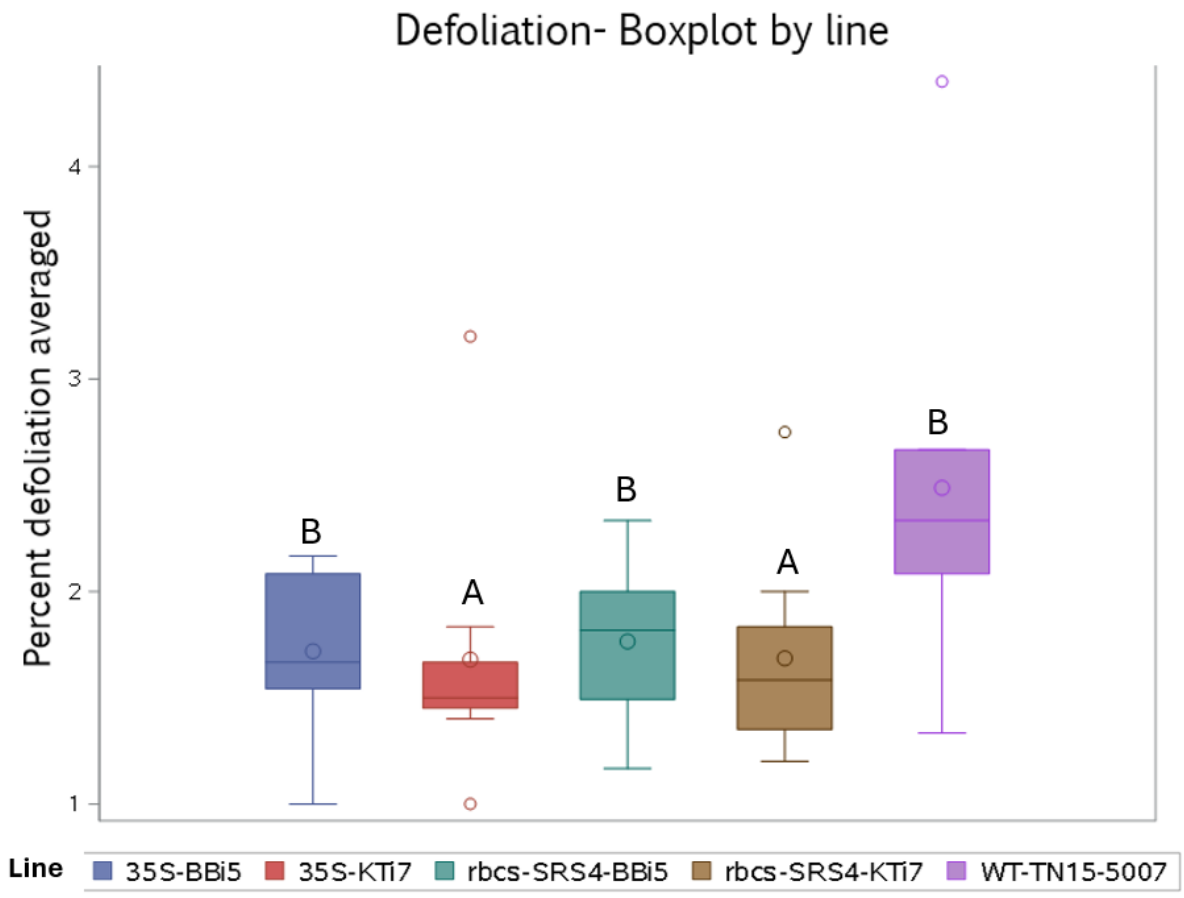


Figure 2.2. Defoliation averaged across three time points for each transgenic trypsin inhibitor line and wild type (WT) Significantly different lines are marked with different letters.

Transgenic trypsin inhibitor soybean exhibits significantly reduced levels of defoliation compared to wild type soybean, but because all lines including wild type exhibited distinctly low levels of defoliation, there may be more to this interaction in an ecological perspective. In the herbivorous insect population surveys, the consistent presence of other notable species was recorded. A population of four to five gray treefrogs (*Hyla chrysoscelis*) were observed in each of the four surveys. Other predatory species such as assassin bugs (*Reduviidae spp.*) ladybugs, (*Coccinellidae spp*.) and spiders including jumping spiders (*Salticidae spp.),* crab spiders (*Thomisidae spp.),* and lynx spiders (*Oxyopidae spp.*) were noted in multiple population surveys. The consistent presence of predatory species may partially explain the low levels of defoliation across transgenic lines as well as wild type. Many foliar pesticides increase mortality of both pest and predator species. Moreover, after predatory species are lost in an agroecosystem, fecund pest populations can rapidly repopulate (Sánchez-Bayo 2021). Diversity of predatory species provides compounded biocontrol of insect pests, which can result in yield protection (Cardinale 2003). Transgenic expression of trypsin inhibitors in soybean has shown significant reduction of lepidopteran larval weight and delay development in insect feeding bioassays but does not usually lead to insect mortality (Sultana et al. 2023; Marchetti et al. 2000; Dunse et al. 2010; De Leo & Gallerani 2002). Crops containing antinutrients that hinder digestion and impede development of herbivorous insects leave them in lifecycle stages that are vulnerable to predators for longer periods of time, potentially increasing their risk of becoming prey (Groot 2002; Loader & Damman 1991; Benrey & Denno 1997). Transgenic trypsin inhibitor soybean could reduce insect herbivory in field conditions not only by deterring feeding and weakening pest insects, but also by retaining conditions that support predatory species. This potential multi-level interaction would need to be examined in further research. Transgenic trypsin inhibitor soybean

could be a solution for providing protection from insect herbivores in sensitive ecosystems. Unlike foliar pesticides that can result in harm to off-target organisms (Mahmood et al. 2016), transgenic trypsin inhibitor soybean only affects insects that are actively engaging in herbivory. Transgenic trypsin inhibitor soybean would be well suited for fields located near aquatic ecosystems such as wetlands, rivers, and lakes (Baker et al. 2013). In this setting, transgenic trypsin inhibitor soybean could significantly reduce defoliation by insect pests without harmful off-target effects to aquatic ecosystems associated with the application of foliar insecticide sprays (Sánchez-Bayo 2021, Mahmood et al. 2016)

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Chapter 3

Characterization under ambient deer herbivory conditions

Abstract

Currently, incorporated biotechnology approaches to crop protection are focused on defense against diseases and insect pests. However, important crops like soybean (*Glycine max*) face other pests in some areas of the world, such as the white-tailed deer (*Odocoileus virginianus*) in the Southeast, Midsouth, Midwest, and Northeast United States. Current methods of controlling deer damage to soybean are insufficient. Trypsin inhibitors, an incorporated plant protectant strategy that have already been pioneered for defense against insects, were considered as potential deer deterrent. Four lines of transgenic soybean expressing the native trypsin inhibitor genes KTi7 or BBi5 under the control of the CaMV 35S or soybean green tissue RBCS-SRS4 promoters were used for a field study to determine if any resistance to deer damage was exhibited. A field trial was designed to quantify ambient deer herbivory across the four transgenic trypsin inhibitor soybean lines and wild type. To ensure deer activity, a site with a history of prolific deer herbivory was selected at the West Tennessee AgResearch and Education Center. There was no significant difference between deer herbivory on the four transgenic trypsin inhibitor lines and wild type.

Introduction

Deer damage to soybean has been ascertained as a significant source of yield loss using economic data, field studies, and surveys sent to farmers (McKee et al. 2021; Rosenberry et al. 2006; Johnson et al. 2000). This is a multimillion-dollar issue; economic reports from indemnity payments (crop insurance for potential yield loss) indicate that soybean yield losses from deer damage have increased consistently for the past twenty years and will likely continue worsening (McKee et al. 2021; Duncan et al. 2023). Current methods of control are haphazard and outdated. Popular methods of reducing deer herbivory include depredation hunting, electric fences, applied

herbivory deterrent compounds (e.g. blood, urine, putrid egg solids), and optical deterrents such as scarecrows (Johnson et al., 2000; McShea 2012; Rosenberry et al., 2006; Ward & Williams 2010; de las Mercedes Guerisoli et al. 2020). Soybean has already been a successful subject of incorporated biotechnology approaches for crop protection such as herbicide resistance and expression of *Bacillus thuringiensis* (Bt) toxin (Bonny 2008, Stewart et al. 1996). However, the burgeoning problem of soybean yield loss due to deer herbivory lacks a similarly systemic solution. While plant incorporated protectant strategies such as Bt toxins and RNAi are prized to endow resistance to insect pests (Bramlett et al. 2020; Bravo et al. 2011), these modes of action would be unacceptable for larger animals like deer. Trypsin inhibitors could be a suitable option for an incorporated plant protectant against deer herbivory. Trypsin inhibitors are proteinase inhibitors: a type of molecule produced in a wide variety of plants as a stress response, growth regulator, and herbivore defense strategy (Edwards & Singh 2006) Proteinase inhibitors prevent the digestion of protein and deplete the supply of digestive enzymes in an animal's gut (Laskowski & Qasim, 2000). Transgenic expression of trypsin inhibitors has been thoroughly studied as an incorporated crop protectant against insects, and their effects are largely anti-nutritional instead of abiotic while still significantly reducing pest damage (Ibrahim et al. 2020; Marchetti et al. 2000; Dunse et al. 2010). Soybean plants naturally contain trypsin inhibitors, but their protective properties are mostly limited to the seeds (Kunitz 1946; Birk 2003; Palacios et al. 2004). Transgenic soybean plants were produced by Sultana et al. (2023) that expressed redirected trypsin inhibitor genes in green tissue with the intent to reduce herbivory. In greenhouse bioassays performed with corn earworm (*Helicoverpa* zea) larvae, most transgenic trypsin inhibitor events exhibited significantly reduced total leaf defoliation and larval weight compared to nontransgenic soybean. The proven herbivory deterrent mechanism of trypsin

inhibitors' anti-nutritional effects may translate into protection from deer damage. White-tailed deer are highly selective in the balance of their diets, carefully maximizing nutrients and minimizing toxins. If a source of forage has antinutritional or toxic aspects, deer will strive to keep their intake of that forage below the threshold of toxicity (Dykes et al. 2020). Due to their morphology, deer prefer a diet high in easily digestible protein, and they will identify and seek the location of such suitable forage, as well as avoiding the locations of unsuitable forage (Gillingham & Bunnell 1989). In many agroecosystems soybean, wheat, or other crops are the optimal source of nutrients, and can compose over three-fourths of deer's diets (Colligan et al. 2011). Based on these critical feeding strategies, deer may systematically avoid feeding on transgenic trypsin inhibitor soybean if the levels of proteinase inhibitor are sufficiently detrimental to the overall nutrient content of the soybean leaves. This hypothesis was evaluated in a field experiment consisting of a randomized block design containing four lines of transgenic trypsin inhibitor soybean from (Sultana et al. 2023) and wild type in a site with history of deer damage.

Materials and methods

Plants

Field experiments used homozygous F5 seeds from four transgenic events of redirected expression of the BBi5 and KTi7 genes under control of the CaMV 35S and soybean prbcS-SRS4 promoters from (Sultana et al. 2023), which were the top-performing lines from that greenhouse study (Table 3.1). The original germplasm was from the soybean cultivar 'TN15-5007' (Pantalone et al. 2020).

Table 3.1. Soybean lines that were used in the present study.

Name used in paper	Promotor	Transgene	Line from (Sultana et al., 2023)
35S-KTi7	35S CaMV	KTi7	Line 1
35S-BBi5	35S CaMV	BBi5	Line 2
GTP-KTi7	prbcS-SRS4	KTi7	Line 2
GTP-BBi5	prbcS-SRS4	BBi5	Line 1
Wild type (WT)	-	-	-

Trial design and planting

An experimental field site at the University of Tennessee West Tennessee AgResearch and Education Center in Jackson, TN, USA was selected based on a history of consistent and destructive deer herbivory to soybean during prior growing seasons. Deer had been observed to enter the field from the edge of the forest and browse plants throughout the entire field. The field was planted with homozygous T6 seeds harvested from a field trial performed the previous year. A randomized block design was used for the distribution of the four lines and wild type in the field site. Each of the five lines, including wild type, was represented in eight plots, for a total of 40 plots. Each individual plot measured 3.05 by 9.14 m, and contained four rows of 240 seeds, planted at a density of twenty-four seeds per meter. The total area of the field was 1113 square meters. The field was oriented with block one aligned to face the forest, and block eight facing an open field. The other two sides of the field faced cotton and corn. The field was bordered by a fifteen-foot fallow region as required in the USDA APHIS BRS environmental release permit (**Figure 3.1**).

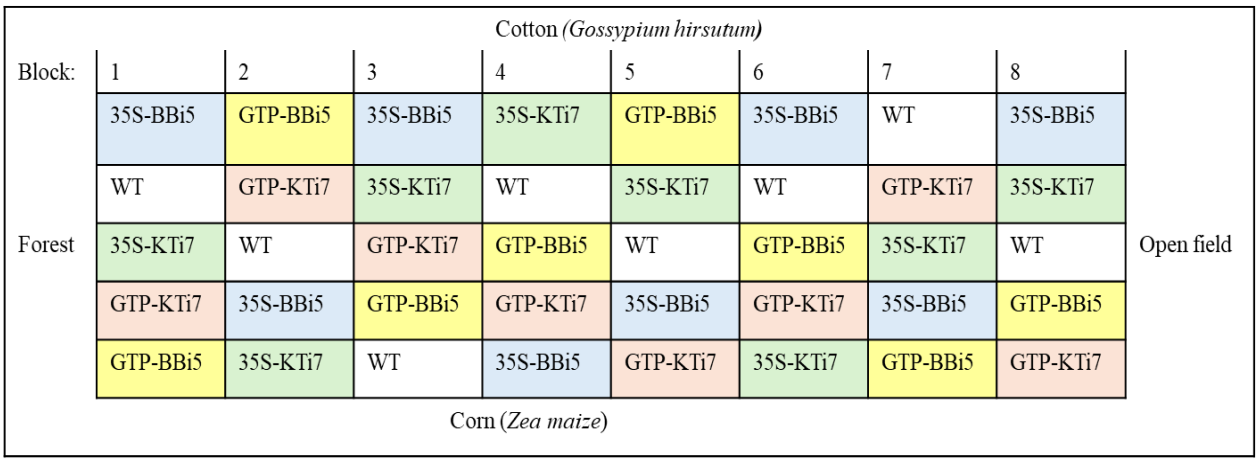


Figure 3.1. Map of soybean lines location in the field and orientation in relation to other crops and environment

The field was planted on June 14, 2024, treated with pre-emergent, herbicide, and plots were marked with stakes after emergence. All non-target pests were controlled following the University of Tennessee's recommendations. Two game cameras (Browning Spec Ops Elite HP5) were placed on the outer edges of the field and set to capture ten-second videos when motion was detected for the duration of the experiment to observe deer feeding behavior. Plants were allowed to mature in the field and then mechanically harvested. The total weight of seeds from each plot was recorded and analyzed.

Data collection

Deer defoliation was measured twice during the growing season: on June 28, 2024, two weeks after planting, and on July 8, 2024, approximately three weeks after planting. Deer predation was measured by counting the number of emerged soybean plants that were defoliated per six row meters in each plot. Deer feeding in the early stages of soybean growth is visually distinct (**Figure 3.1.**) and can be easily identified. This method of quantifying deer defoliation to soybean is frequently used in other studies (Hinton et al. 2017; VanGorder et al. 2021; Mullins 2014).

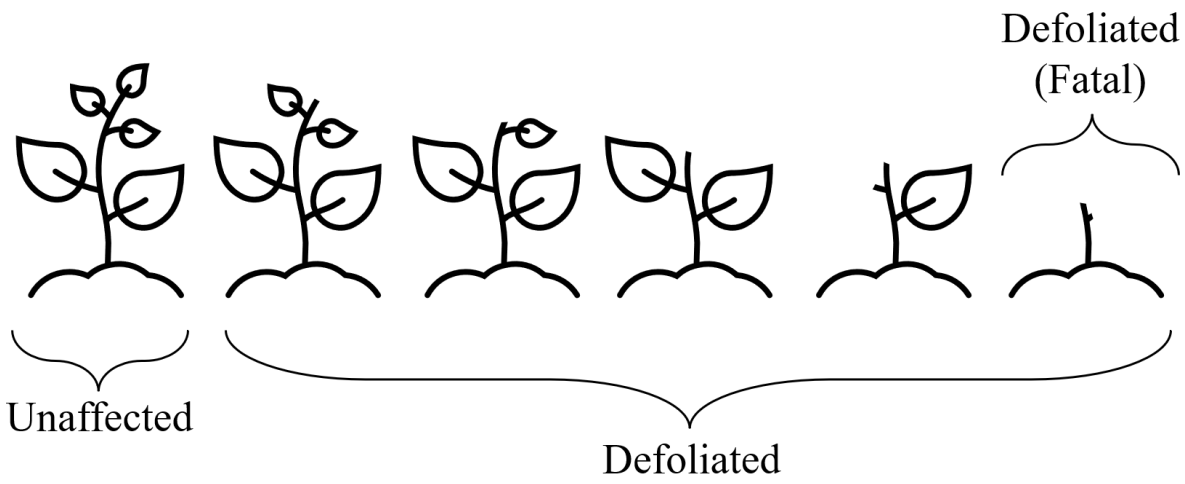


Figure 3.2. Detail of damage phenotypes recorded as deer defoliation during data collection

The data was collected by placing a three-meter measuring tape between the two middle rows of each plot and counting the number of defoliated soybean plants in both adjacent rows. The percentage of defoliated soybeans out of the total emerged was used for statistical analysis.

Game Cameras

From the planting to the end of the R6 growth stage, the two motion activated game cameras captured twenty ten-second videos of deer activity in and around the field out of 1,500 total videos captured. The game cameras were able to capture motion at a range that encompassed the entire length of the field.

Results and discussion

Game camera footage

Most of the videos captured the motion of birds in the field, as well as motion caused by weather, and farm operations in the background. Out of the ~1,500 videos captured, only twenty videos featured deer, and only four videos feature deer consuming soybean. Twenty-four days after planting, a group of deer was observed browsing the center of the soybean field at night. This

instance was captured by both cameras. Another video of a single deer browsing the center of the field was captured two months and twenty days after planting. This instance was captured by both cameras. The other sixteen videos show deer walking though the field, moving around the edges of the field, or browsing on other adjacent plant material. For example, twenty days after planting, when the soybean were in the V3 growth phase, two videos capture a group of deer grazing on the grass in the fallow region surrounding the field in the midmorning. Another video two months later shows deer browsing in the same location. Deer feeding behavior recorded on the game cameras was consistent with deer feeding behavior to soybean documented in literature.

Yield

The total weight of seeds harvested from each plot was analyzed in SAS using an ANOVA. Yields were low due to the planting date and lack of rainfall during the growing season. No significant difference between the yields of the transgenic trypsin inhibitor soybean lines and wild type (**Table 3.2**).

Table 3.2. Average yield per plot for each transgenic trypsin inhibitor soybean line and wild type

Line	**Average yield per plot (g)**
35S-BBI5	1112.435
35S-KTI7	1276.296
GTP-BBI5	1005.841
GTP-KTI7	1432.218
WT	1410.105

Defoliation measurements

Data from the two timepoints, June 28, 2024, and July 8, 2024, were analyzed in SAS using a randomized block design with repeated measures. None of the transgenic trypsin inhibitor soybean lines exhibited a significant effect on the percent of soybeans that were defoliated by deer compared to wild type, with a p value of 0.5928 for the effect of the treatment of the percent of plants defoliated (**Table 3.3**).

Deer feeding behavior recorded in the field was consistent with the literature. The majority of herbivory was observed within the first two weeks after emergence, and leveled off as the plants matured, as established by (deCalesta 1978). Deer readily and repeatedly consumed transgenic trypsin inhibitor soybean plants. One explanation for this continued consumption is the category of deterrent that trypsin inhibitors fall into. Trypsin inhibitors would be considered a conditioned-aversion repellent. Conditioned-aversion repellents cause feelings of illness, digestive discomfort, and post-consumption malaise (Ward & Williams 2010). Avoidance behavior is learned by association of sickness after consumption of the plants, which may take multiple instances of feeding (Ward & Williams 2010). This explanation would be congruent with the two instances of early-season herbivory by a group of does followed by a lack of deer activity recorded on the game cameras. This behavior may represent deer learning the effects of the transgenic trypsin inhibitor soybean and avoiding it for the rest of the growing season. However, because the deer did not differentiate between plots, this potential avoidance behavior is not reflected in the data. The deer likely did not experience the deterrent effects of the trypsin inhibitor soybean quickly enough to potentially learn the difference between different lines in the field, and considered the entire field as the same treatment.

Table 3.3. Total percent defoliation, and percent defoliation on June 28th, 2024, and July 8th, 2024

Percent of soybean lines defoliated on June 28 and July 8						
Line	**Date**	**Mean**	**Std Dev**	**Median**	**Minimum**	**Maximum**
GTP-KTi7	June 28	0.19	0.23	0.09	0	0.56
	July 8	0.22	0.21	0.16	0	0.48
35S-KTi7	June 28	0.12	0.22	0	0	0.62
	July 8	0.18	0.23	0.07	0	0.62
35S-BBi5	June 28	0.21	0.22	0.15	0	0.72
	July 8	0.16	0.25	0	0	0.53
GTP-BBi5	June 28	0.11	0.15	0.05	0	0.42
	July 8	0.17	0.22	0.11	0	0.56
WT	June 28	0.13	0.17	0.12	0	0.57
	July 8	0.09	0.16	0	0	0.41

The location of the plots in the field was more distinctly related to the percentage of plants defoliated by deer (**Table 3.2, Figure 3.2**) Blocks one and eight represent the outer edges of the field, where deer damage is known to be highest (deCalesta 1978). The only notable difference in deer feeding behavior on soybean in this field site was the lack of stand loss. Stand loss occurs when deer defoliate the entire shoot portion of a young soybean plant, leaving only a short section of hypocotyl. This form of herbivory is fatal to the soybean plant, and the main cause of yield loss due to deer damage (deCalesta 1978). Young soybeans can partially recover from defoliation above the cotyledonary nodes, which usually results in stunted growth, delayed maturity timing, and lower yield (Hanley et al. 2007). Soybean stand loss due to deer damage has been consistently observed at this field site in years past, but did not occur during this experiment.

A different field design could be more suitable for quantifying transgenic trypsin inhibitor soybeans' potential as a deer deterrent. Randomized block designs are a standard method for examining insect herbivory on multiple lines of transgenic crops compared to nontransgenic crops in field conditions (Álvarez-Alfageme et al. 2011, Stewart et al. 1997, Johnson & Gould 1992). Randomized block designs have also been used in field trials to quantify deer herbivory and preference between forage options (Ward & Williams 2010, Dykes et al. 2020, Mullins 2014). However, due to the high similarity of the transgenic lines, delayed mode of action for deterrence, and overall low percentage of nontransgenic soybean in the field trial (one-fifth of total plants), deer may not have been able to differentiate between the effects of transgenic trypsin inhibitor soybean and nontransgenic soybean in a randomized block design.

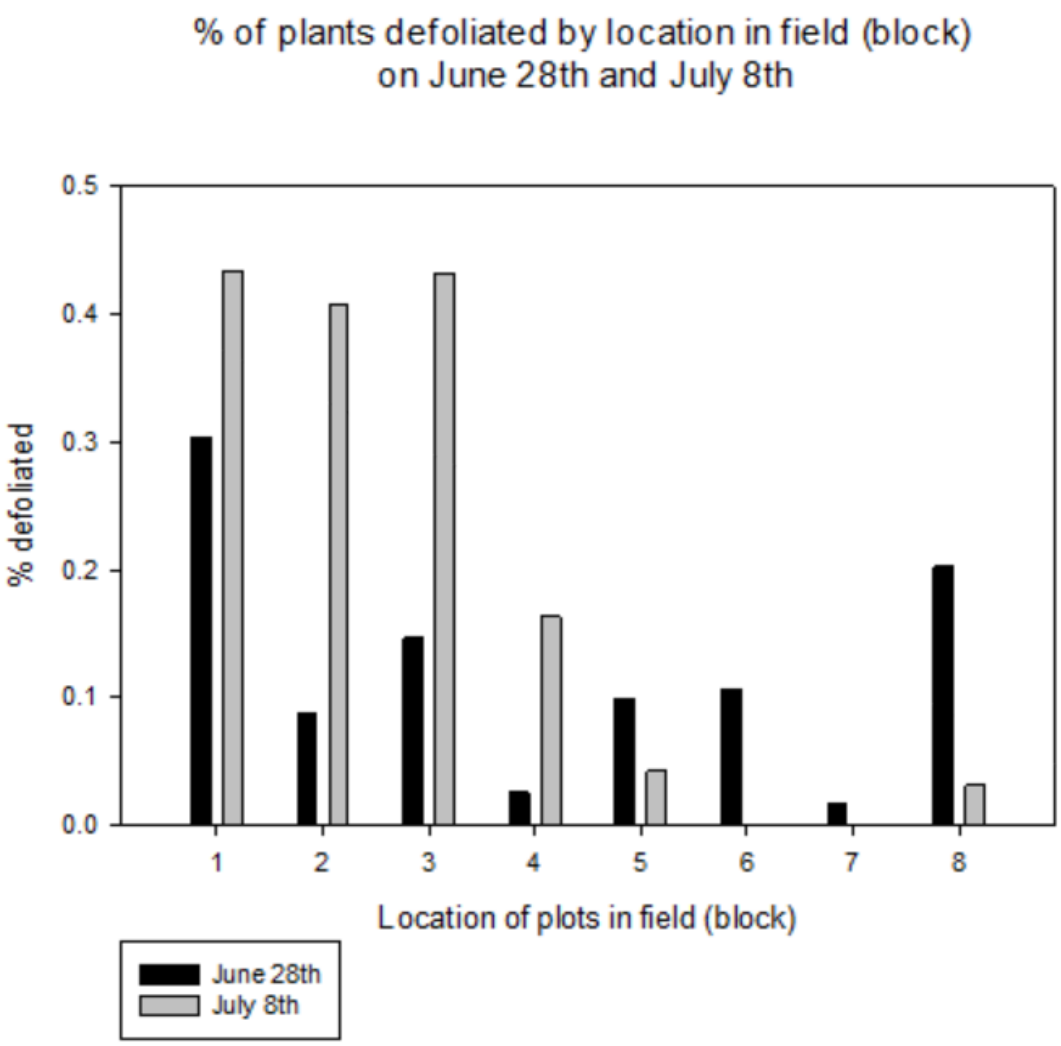


Figure 3.3. Percent of soybean plants defoliated by their location in the field



Figure 3.4. Location of blocks in the field in relation to the surrounding environment.

To simplify any potential future field tests, only the highest expressing line, 35S-KTi7, as determined by Sultana et al. (2023) should be considered going forward. An improved experimental design could involve two or more nonadjacent sites with a history of consistent deer damage. By alternating plantings of transgenic trypsin inhibitor soybean and wild type soybean between the sites for several growing seasons, transgenic trypsin inhibitor soybeans' potential to deter deer herbivory could be distinctly quantified through the most important metric: yield. One limiting factor in this line of research is the natural fluctuations of both deer populations and soybean yields from year to year in relation to other environmental factors. To accurately represent the potential effect of an incorporated herbivory deterrent on interactions between deer damage and soybean yield, a study would need to be replicated over a minimum of three years, up to seven years. Quantification of this novel interaction needs to be further refined, but the potential for transgenic expression of plant protectants to deter deer herbivory in soybean should not be dismissed. Currently, no other study has examined transgenic expression of a plant protectant to deter mammalian feeding on a major crop. Choosing the correct experimental design and data collection method for quantifying deer herbivory to soybean is not straightforward, and the literature examining this subject does not agree on a singular approach to quantify this issue. Reducing the nutritional appeal of soybean through transgenic expression of proteinase inhibitors addresses the issue of deer herbivory at a deeper level than any current management strategy, but this novel approach will require further research to accomplish the desired results. The addition of multiple proteinase inhibitors may be necessary to achieve effective protection due to soybean leaves' high nutritional content. The transgenic expression of a distinct flavor along with proteinase inhibitors could increase plant protection by allowing deer to more easily differentiate between transgenic deer-deterrent soybean and wild type.

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Chapter 4

Controlled deer feeding trials

Abstract

White-tailed deer (*Odocoileus virginianus*) herbivory in field conditions is notoriously difficult to quantify due to its overlap with other variables such as edge effects and insect damage. Preferences between different feed options are most thoroughly examined by controlled feeding studies. A new line of transgenic soybean (*Glycine max*) overexpressing a native trypsin inhibitor gene exhibited significant reduction of defoliation by insects in greenhouse whole plant feeding bioassays using corn earworm, and in ambient herbivory field conditions. The mode of action for this herbivory protection is the antinutritional effects of trypsin inhibitors, which obstruct the digestion of protein and deplete the supply of digestive enzymes in the gut of herbivores that consume this molecule. Feeding strategies employed by deer are methodical, with the goal of keeping toxins and antinutrients in their diet under harmful thresholds while consuming the maximum amount of limited essential nutrients. Because soybean is widely grown and contains prioritized nutrients for deer, soybean damage due to deer herbivory often leads to yield loss. The transgenic expression of native trypsin inhibitors in soybean may cause deer to avoid this food source due to the antinutritional effects of transgenic trypsin inhibitor expression in the leaves. Deer's preference between transgenic trypsin inhibitor soybean and wild type was tested using cafeteria-style feeding trials with tame deer in a controlled environment. No significant difference was found between the amount of transgenic and wild type soybean consumed by deer in the trials.

Introduction

The multifaceted issue of soybean yield losses due to deer herbivory can be examined through the perspective of ecology, agriculture, wildlife management, cultural practices, or biotechnology. The complex interactions at play in this agroecological system are difficult to

quantify, and small changes in study methodology can lead to wildly different results, then opposing conclusions on the impact of deer herbivory to soybean. However, thorough studies from multiple disciplines have determined that deer damage causes significant yield loss in soybean (McKee et al. 2021; Rosenberry et al. 2006; Johnson et al. 2000). The significance of this yield loss is an economic burden on soybean farmers. From 2011-2022, Tennessee farmers' indemnity payments reported total losses of $5.65 million due to wildlife damage (Duncan et al. 2023). Soybean is a sought-after food source for deer due to its highly desirable nutrients and availability. In particular, soybean leaves are rich in crude protein, which is a limiting factor in deer's diets (Dykes et al. 2020; Dostaler et al. 2011; Colligan et al. 2007). Attempts to protect soybean from deer damage include depredation hunting to reduce deer population, herbivory deterrent sprays to reduce appeal, and fencing to reduce access (Johnson et al. 2000; McShea 2012; Rosenberry et al. 2006; Ward & Williams 2010). Deer are intelligent, methodical and capable animals, so management attempts to reduce the availability of soybean crops to deer only see limited success in the best cases. A more direct approach to protecting soybeans from deer damage would be to negate the nutritional appeal of the leaves. Herbivory protection by means of concentrated antinutritional molecules is already naturally at work in soybean seeds, which contain trypsin inhibitors (Kunitz 1946; Birk 2003; Palacios et al. 2004). Redirected transgenic expression of plant trypsin inhibitors for protection against insect herbivory is well-documented and has already been achieved in soybean (Sultana et al. 2023). To determine if transgenic soybean plants expressing native trypsin inhibitors in their shoots could deter deer herbivory, cafeteria-style feeding trials with captive tame deer were designed. Cafeteria trials present multiple choices of feed to an animal at the same time to discern and quantify preference. In interest of simplicity, the transgenic trypsin inhibitor soybean line with the highest expression

level of the transgene expression was selected from the study by Sultana et al. (2023) to be compared to wild type soybean in controlled deer feeding trials. The transgenic line expressed the trypsin inhibitor gene KTi7 under control of the CaMV 35S promotor and is referred to as 35S-KTi7 in previous chapters.

Materials and methods

This trial was designed to quantify the level of herbivory protection provided the increased expression of trypsin inhibitor genes in soybean leaves while in a controlled environment. In collaboration with the University of Georgia's Whitehall Deer Research Facility, cafeteria-style trials were carried out with tame deer that were presented a choice between transgenic trypsin inhibitor soybean and wild type soybean. A total of two feeding trials were performed following the same methods.

Production of plants

Homozygous T5 transgenic trypsin inhibitor soybean seeds from Sultana et al. (2023) were bulked in a field experiment. The resulting T6 seeds were used to produce the plants for these experiments. The soybean plants used in this experiment were grown in greenhouse conditions and harvested at the VN phase. Once the necessary plant mass was established, the soybean plants were cut at the base and packed tightly into large sealable freezer bags, then flash frozen and devitalized using liquid nitrogen. The freezer bags were then packed into Styrofoam coolers with dry ice and shipped overnight to Athens, Georgia, the site of the feeding trials. The frozen leaves were immediately stored in a -20C freezer upon arrival.

Feeding trial design

For the feeding trials, a doubly repeated randomized block design was used. The trials were conducted between 7:00 a.m. and 12:00 p.m., and hay/regular feed was removed between

approximately 6:00-8:00 p.m. the night before each trial, allowing for twelve hours of fasting between each trial. The fasting period was included to encourage involvement and curiosity toward the soybean leaves. Hay/normal feed was replaced after each trial.

Acclimatization phase

First, twelve tame deer participated in a three-day acclimation trial to select suitable subjects and familiarize the deer with the experimental procedure. During this acclimation trial, the deer were presented with one pan containing 100 g of wild-type soybean for 90 minutes. At 30-minute intervals, the pan was weighed, the amount of soybean consumed was recorded, and the pan was refilled to 100 g. After this three-day acclimation trial, the deer were given a two-day rest period in compliance with the animal use permit conditions. From the data collected in the acclimation trials, the ten tame deer that were the most amenable to experimental procedures were selected out of the original twelve for the choice trials. After the two days of rest, the choice trials began.

Trial implementation

The choice trials followed the same feed and fasting schedule as the acclimation trials and took place over five consecutive days. Each day, each of the ten deer were presented with two pans of soybean: one pan containing 300 g of transgenic soybean, and one pan containing 300 g of wild-type soybean for a 90-minute period. At 30-minute intervals, the pans were weighed, the amount consumed from each pan was recorded, and each pan was refilled to 300 g. The pans were refilled at 30-minute intervals to ensure there would be a remainder for statistical analysis. Total reduction in soybean from each pan over the 90-minuite period was measured for each of the ten deer for the five consecutive days of the trials. The location of the pans in the stall was randomized each day to avoid any placement bias. During the second trial, the last of the three reduction measurements during the 90-minute period were not recorded for deer W198 and

Bambam on day four. The sum of the first two reduction measurements fell within the normal variation of the data, and thus was used for statistical analysis.

Results and discussion

Total reduction in wild type and transgenic soybean was summed from the 30-minute interval measurements, and this total was recorded for each deer each day of the feeding trials. The resulting data was analyzed in SAS using a repeated measures ANOVA. There was not a significant difference in feeding preference between wild type and transgenic soybean for either trial. The p value for the effect of the treatments for the first trial was 0.6025, and 0.7582 for the second trial. There was a very high amount of variation in feeding among different deer and different days of the trials. Relevant descriptive statistics for the feeding trials can be seen in the tables below. (**Table 4.1, Table 4.2**). When the data is broken down into the amount of total wild type and transgenic consumed on individual days, the variation and lack of preference between options is distinct.

Within the individual days of the first feeding trial, the descriptive statistics show that on four out of the five days, on average deer consumed more wild type than transgenic soybean. However, on day four the average transgenic soybean consumed was nearly quadruple that of wild type (**Figure 4.1**). No abnormal conditions or behaviors were observed on day four of the feeding trial that would explain this reversal in trend, suggesting that this variation in preference between the two feed choices is normal and could be observed in repetitions of this experiment. To confirm that this feeding behavior observed in the first feeding trial did not reflect a pattern that was disturbed by an unknown factor, a second feeding trial was performed following the same methods as the first. The second feeding trial ascertained that the feeding behavior observed in the first trial was normal and did not represent any significant trends. During the

Table 4.1. Average reduction in transgenic and wildtype soybean options per day and related descriptive statistics for the first feeding trial.

Day	Treatment	Mean	Std Dev	Lower 95% CL for Mean	Upper 95% CL for Mean	Median	Minimum	Maximum
1	**Transgenic**	135.36	140.72	34.6951	236.0249	85.1	8.8	361.9
	WT	157.48	141.512	56.2484	258.7116	116.1	19.8	417.5
2	**Transgenic**	121.85	121.497	34.9361	208.7639	62	17	361.5
	WT	217.32	158.379	104.0227	330.6173	249.8	27.1	446
3	**Transgenic**	107.96	119.384	22.5581	193.3619	74	3.5	388.5
	WT	160.39	143.559	57.6943	263.0857	189.75	10.5	429.9
4	**Transgenic**	207.74	170.695	85.6322	329.8478	255.45	8.9	462.5
	WT	61.28	94.5375	-6.348	128.908	15.5	12.5	314.5
5	**Transgenic**	94.65	87.9217	31.7546	157.5454	72.25	20	276.5
	WT	133.95	116.183	50.8376	217.0624	101.25	24	304

Table 4.2. Average reduction in transgenic and wildtype soybean options per day and related descriptive statistics for the second feeding trial.

Day	Treatment	Mean	Std Dev	Lower 95% CL for Mean	Upper 95% CL for Mean	Median	Minimum	Maximum
1	**Transgenic**	156.8	138.628	57.6315	255.9685	137	7	428
	Wild type	93.65	137.3888	-4.632	191.932	45.75	0	453.5
2	**Transgenic**	183.36	165.874	64.7009	302.0191	133.45	2.2	417.9
	Wild type	214.59	163.3809	97.7144	331.4656	179	12.2	502.1
3	**Transgenic**	206.47	163.9775	89.1676	323.7724	235.1	5.9	518.8
	Wild type	230.39	154.9317	119.5586	341.2214	209.95	84.5	601.2
4	**Transgenic**	268.29	131.2354	174.4098	362.1702	294.45	9.2	422.3
	Wild type	244.46	170.1891	122.7141	366.2059	184.3	74.7	490.1
5	**Transgenic**	222.13	181.179	92.5223	351.7377	158.15	8	507.3
	Wild type	284.15	146.2187	179.5514	388.7486	297.95	13.1	561.5

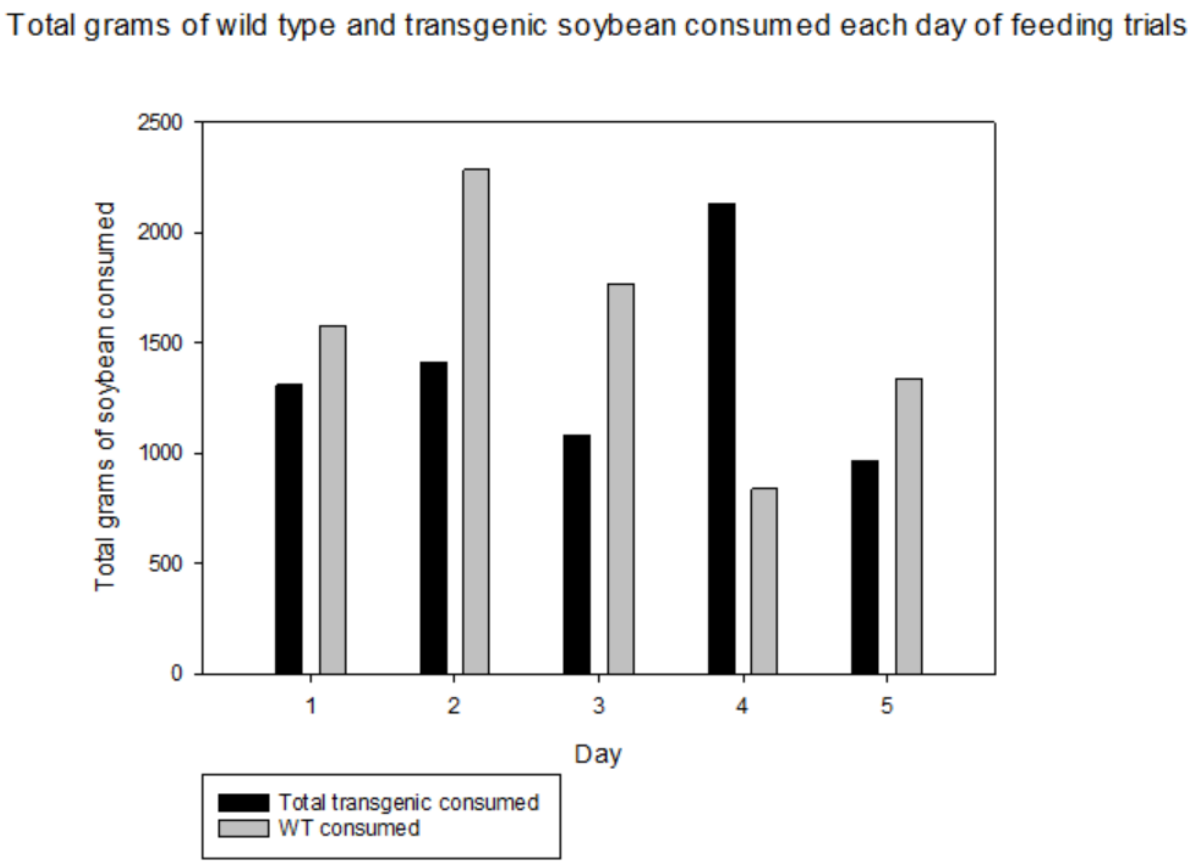


Figure 4.1. Deer consumption of transgenic and wild type soybean leaves by ten animals on each day of the first feeding trial. No statistically significant differences were observed.

indicate that it would likely be more beneficial to consider alternative deterrents rather than continue to examine trypsin inhibitors.

second feeding trial, the deer consumed more wild type than transgenic soybean on three of the five days, and more transgenic soybean on two of the five days (**Figure 4.2**). The total amount of each option consumed during each day was more consistent during the second trial compared to the first trial. No clear preference between transgenic and wild type soybean can be discerned from the total amount of each option consumed per day in the feeding trials.

Individual deer also showed high levels of variation between which feed option was preferred in both feeding trials. During the first feeding trial, three deer consumed more transgenic soybean than wild type soybean total over the five days: Red metal tag (RMT), Y66, and Presley. Three other deer consumed only a small fraction of both transgenic and wild type soybean compared to other deer in the first feeding trial: W55, Paris, and Y199 (**Figure 4.3**). During the second feeding trial, half of the deer consumed more wild type soybean overall, and the other half consumed more transgenic soybean overall. The total amount of soybean consumed varied greatly among individual deer, but the amount of each option consumed by each deer was mostly equivalent (**Figure 4.4**). The lack of statistical significance and consistency in the data indicates doubly that transgenic trypsin inhibitor soybean does not deter deer herbivory of soybean plants in a controlled setting. Deer readily and repeatedly consumed transgenic trypsin inhibitor soybean leaves in both feeding trials. If deer preference between these transgenic trypsin inhibitor lines and wild type is not discernable in a simple two-option controlled feeding study, it may not be suitable for preventing deer damage in agronomic fields. Further research may be beneficial to clarify the results of these trials. Live plants may have better reflected deer feeding

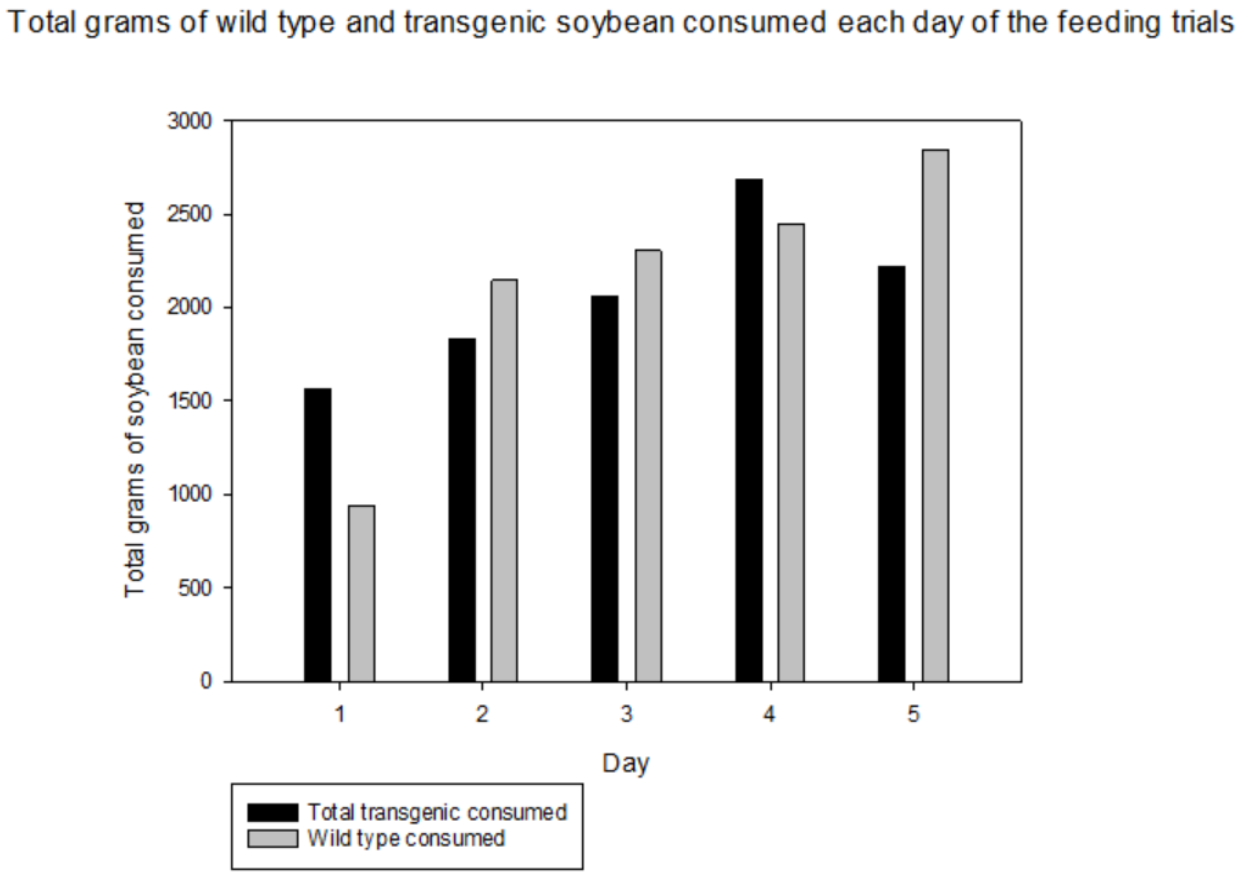


Figure 4.2. Deer consumption of transgenic and wild type soybean leaves by ten animals on each day of the second feeding trial. No statistically significant differences were observed.

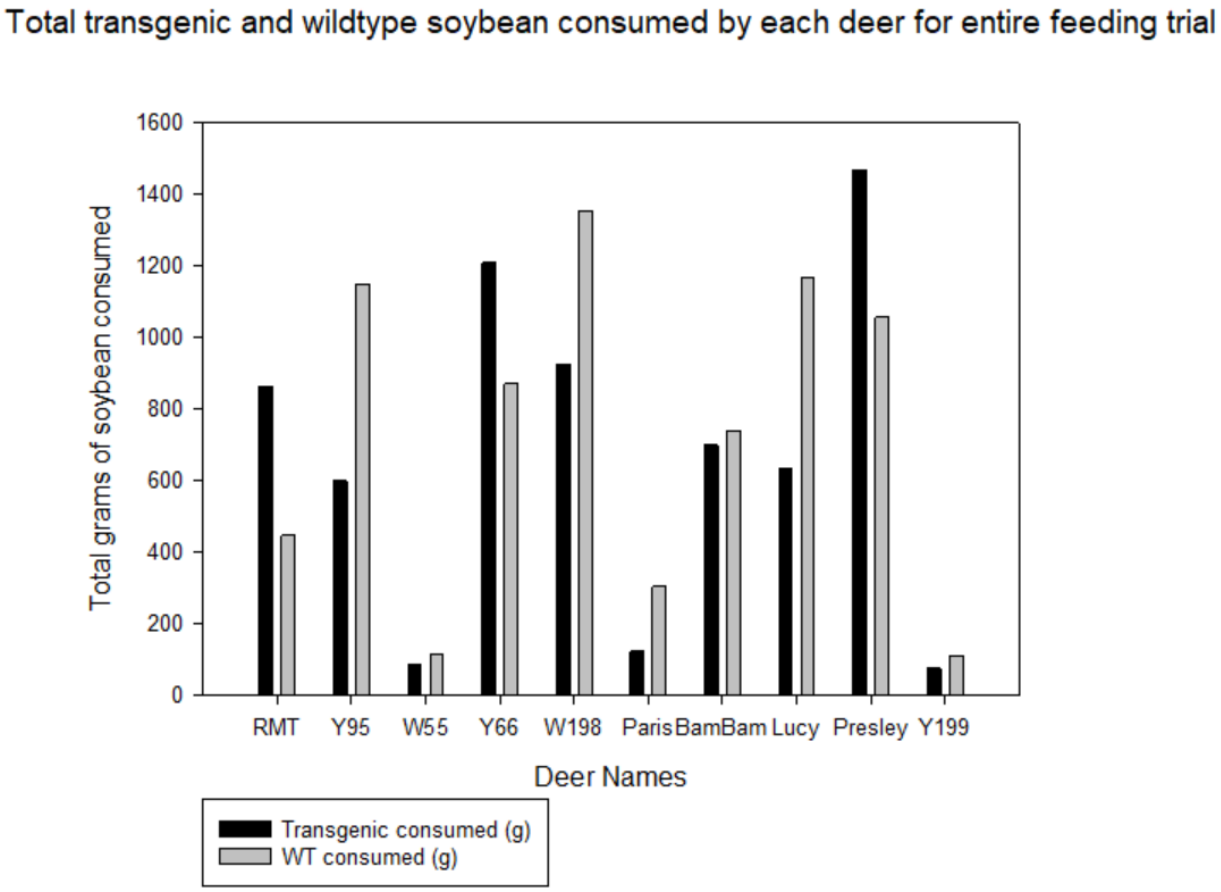


Figure 4.3. Consumption of transgenic and wild type soybean leaves by individual deer across five days of feeding for the first trial. No statistically significant differences were observed.

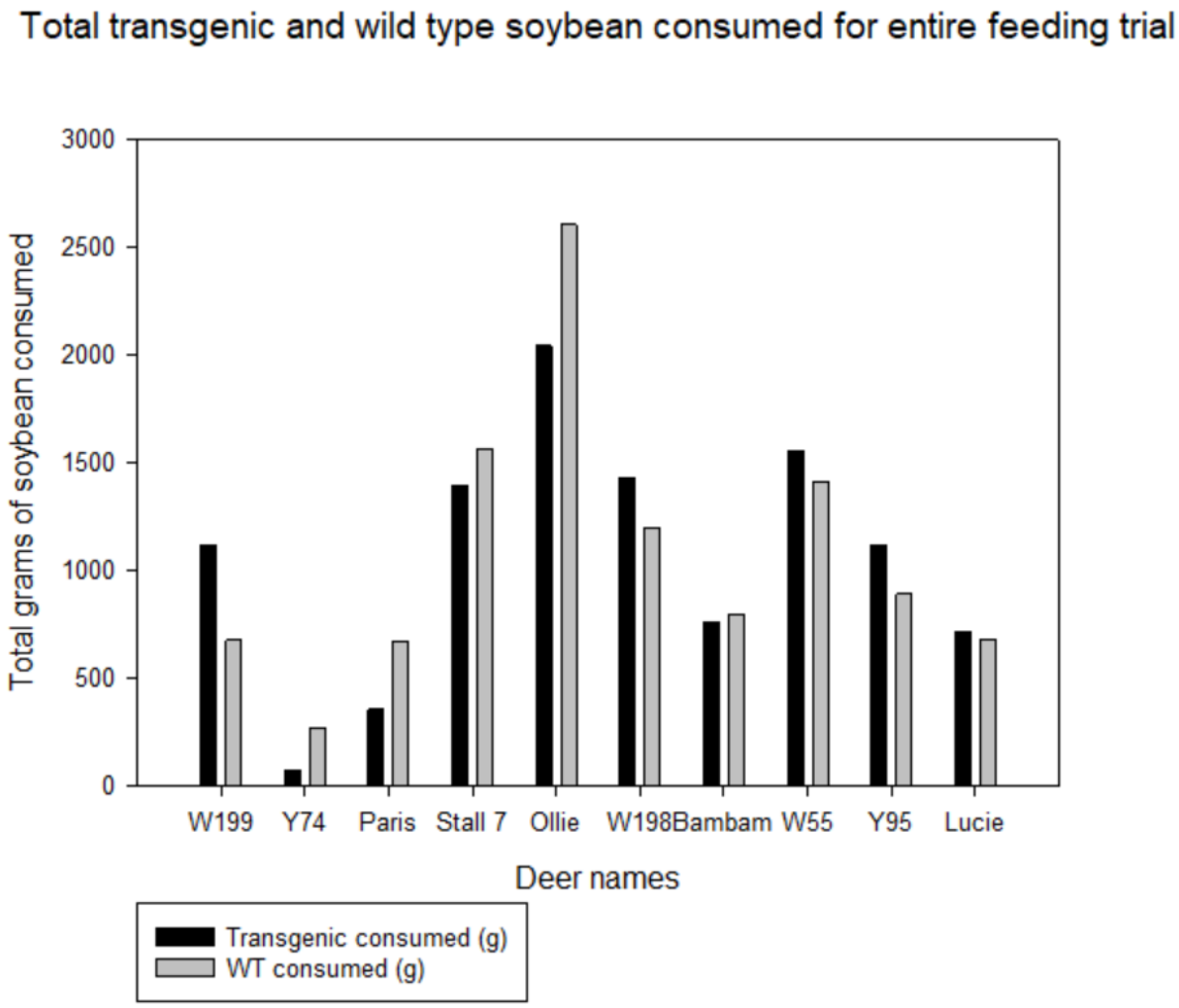


Figure 4.4. Consumption of transgenic and wild type soybean leaves by individual deer over five days of feeding for the second trial. No statistically significant differences were observed.

behavior to trypsin inhibitor soybean opposed to frozen and thawed leaves. Unfortunately, this was not an option for this study. A longer trial period in an environment that the deer are more accustomed to may also yield more accurate behavioral results. However, the results of this trial Despite the lack of significant results, this study could represent useful progress towards the development of transgenic crops with incorporated protectants for deer deterrence. Controlled deer feeding studies could be used to identify other candidates for incorporated plant protectants without the need for the protectant to be transformed and expressed in the plant beforehand. Deer feeding assays using wild type soybean that is externally treated with a potential deterrent could be performed to observe and quantify the deterrent's efficacy before attempting genetic transformation of soybean for transgenic expression of the deterrent. These assays could also be used to determine the necessary concentration of the deterrent or examine the additive effects of multiple deterrent compounds acting together. Pre-screening potential plant protectants using treated soybean in controlled deer feeding studies would reduce the cost, time, and labor required to develop transgenic deer-resistant soybeans. Controlled deer feeding studies also allow the close monitoring of animals to assess any behavioral changes in response to deterrents. In future research examining potential transgenic expression of plant protectants to deter deer herbivory, it would be logical to use controlled deer feeding studies to select a suitable protectant before transformation, then test the effectiveness of the transgenic soybean expressing the protectant in further controlled deer feeding trials before environmental release.

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Chapter 5
Conclusion and recommendations

Insect herbivory deterrence of transgenic trypsin inhibitor soybean in the field

Two of the four transgenic trypsin inhibitor soybean lines exhibited reduced defoliation by insects compared to nontransgenic soybean. These two lines expressed the KTi7 gene, which is a Kunitz trypsin inhibitor (Kunitz 1946). The two transgenic lines that did not show significant reduction in insect defoliation compared to nontransgenic soybean expressed the BBi5 gene, which is a Bowman-Birk trypsin inhibitor (Birk, Y. 2003). Kunitz and Bowman-Birk trypsin inhibitors are differentiated by their molecular structure (Laskowski and Kato, 1980). Both types of soybean trypsin inhibitors were effective in reducing defoliation from the lepidopteran corn earworm larvae in greenhouse whole plant feeding bioassays by Sultana et al. (2023), but only the soybean expressing Kunitz trypsin inhibitor transgenes were effective in significantly decreasing ambient insect defoliation in a field setting, indicating that the structure of the KTi7 trypsin inhibitor may be more effective against a broad range of insects, and the BBi5 trypsin inhibitor is more specific to lepidopteran insects. It could also be beneficial to make crosses between the transgenic trypsin inhibitor lines to incorporate multiple transgenic trypsin inhibitor genes into the genome. Expression of multiple trypsin inhibitor transgenes has been shown to increase herbivory deterrence (Marchetti et al., 2000; Dunse et al. 2010). The overall low levels of defoliation seen in the transgenic trypsin inhibitor lines and nontransgenic soybean under ambient insect herbivory was also noteworthy. Beyond the expected herbivory deterrent effects of the transgenic trypsin inhibitor soybean, the low overall defoliation could be explained by the large fallow border hindering insect movement into the field, or the delayed development of herbivorous insects increasing their chances of becoming prey to predatory species. The multi-trophic interactions present in transgenic trypsin inhibitory soybean field conditions would benefit from further research. Transgenic expression of a crop protectant that provides

compounded insect resistance through ecological synergies could represent an interesting path in applied biotechnology towards sustainable agriculture. Areas where pesticide runoff from soybean cultivation is particularly detrimental to the environment could benefit from planting transgenic trypsin inhibitor soybean (Sánchez-Bayo 2021; Mahmood et al. 2016). Soybean fields near wetlands and in important watersheds would be top candidates for transgenic trypsin inhibitor soybean to provide protection from insect pests without the need for foliar insecticide sprays that can leach into runoff and cause harm to sensitive aquatic ecosystems (Baker et al. 2013). Large-scale field trials to assess the herbivory deterrence properties of transgenic trypsin inhibitor soybean more thoroughly in an ecological context would be an important next step.

Potential of transgenic trypsin inhibitor soybean as a deer deterrent

While the reasoning behind transgenic trypsin inhibitor soybean potentially deterring deer herbivory was sound, ambient deer herbivory field studies and controlled deer feeding trials indicated that trypsin inhibitor expression in transgenic soybean plants was ineffective to significantly deter deer herbivory. In both experiments, deer were observed to consume transgenic trypsin inhibitor soybean readily and repeatedly. The only notable indicator for deer deterrence was the lack of stand loss in the ambient deer herbivory field trial. Transgenic trypsin inhibitor soybeans' potential herbivory deterrence could be more successfully quantified by using a more suitable field design in a multi-year study. Incorporation of multiple trypsin inhibitor genes by crossing different transgenic trypsin inhibitor lines may also increase the effectiveness of the deterrent properties. However, the current experiments reveal a considerable setback in the use of trypsin inhibitors as a deer deterrent. Trypsin inhibitors are a conditioned-avoidance deterrent, requiring at least one, but likely several feeding sessions before deer learn to associate the deterrent with feelings of digestive discomfort (Ward & Williams 2010). Most of

the deer damage to soybean happens in the first two weeks after emergence, when a single bite from a deer can result in plant death, leading to stand loss (deCalesta 1978). It is not advantageous to rely on deer learning to avoid transgenic trypsin inhibitor soybean by trial-and-error during the most vulnerable growth phases for soybean. Deer damage to soybean remains a growing issue and transgenic expression of a plant protectant could still provide an efficient solution. Other plant-based deterrents with more immediate modes of action would be worthy of consideration. Plant-based pain inducing deer deterrents such as capsaicin and peppermint may be more practical choices (Ward & Williams 2010). A distinct pain-based component in addition to the conditioned-avoidance deterrent of the trypsin inhibitor may provide twofold protection by allowing deer to associate distasteful flavor with uncomfortable digestion, expediating the learning process and increasing protection to young soybean plants.

One consideration in moving forward in the development of transgenic deer-resistant soybean is the root cause of this issue: lack of population control and suitable forage options. If a successful deer-deterrent soybean is developed and implemented in the field, deer will seek the next best forage option. Due to habitat loss from expanding urbanization and agriculture, the next forage choice for deer will likely be a valuable plant for humans, such as another row crop or ornamental landscape plant. Thus, the issue of deer damage cannot be addressed completely by deterrents, only redirected. A successful deer-resistant soybean should be implemented with a suitable alternative forage option and comprehensive population control measures. Otherwise, deer-resistant soybean will simply necessitate the development of more deer-resistant plants.

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Vita

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