# A Summer of Soybeans

Selecting Cultivars Resistant to Southern Stem Canker



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## **Personal Introduction**

My interest in the World Food Prize Foundation first began during my freshman year when another student had returned from the symposium in Des Moines and was quick to tell me about all of the inspirational experiences she had taken part in that week. I was amazed to not only hear about all of the speakers she had heard, but also by the research paper she had written and the solutions she had found to solve food insecurity in Cambodia. Since my parents are both immigrants from India, I've had the opportunity visit and see some of the incredible sights that a foreign country has to offer. However, I have also seen some of the more somber sights of poverty and food insecurity. Each time I visit India, I always think that I would like to do something to impact the lives of those individuals living in poverty. Therefore, as soon as I heard about the opportunity to find solutions to food security, I quickly jumped at the chance.

At this time, although I was interested in food security, I wasn't necessarily interested in the field of agriculture. Both of my grandfathers were agronomists, and my dad currently works in the field of food safety. My interest in agriculture, still, did not grow until I attended the Global Youth Institute in 2013. I was amazed when I heard about the research that Dr. Robert Fraley, Dr. Marc Van Montagu, and Dr. Mary-Dell Chilton had conducted on genetically modified organisms. The idea of examining particles as small as T-DNA fascinated me, and it caused me to look into the possibility of a career in genetics.

At the Global Youth Institute, I was also fascinated after hearing about the research opportunities and adventures the Borlaug-Ruan interns were able to experience. Upon my return home, I didn't even bother questioning whether I should or should not apply for a Borlaug-Ruan internship. I eagerly awaited the fall of my junior year so I could apply.

In the meantime, I took advantage of another opportunity and participated in an internship at the University of Nebraska-Lincoln. Over the course of this internship, I was able to participate in a research project on the assessment of the microbiological safety of cereal grains. Not only did I learn many important laboratory skills during this internship, but I also became more interested in applying for a laboratory project through the World Food Prize.

The year prior to my internship, I enrolled in AP chemistry. As the course proved to be very rigorous and challenging, it also ended up being the most influential class I had taken in high school. Not only did I extend my knowledge on conducting experimental procedures, which I was able to use throughout the course of my internship, but I also gained a true interest in research as well as science. I would work tirelessly in order to make sure that my lab reports were meticulous and I would spend many hours outside of class making sure that I was able to fully comprehend the material that I had been taught. As my interest in laboratory research grew over the course of AP chemistry, I became more eager to apply for the Borlaug-Ruan internship.

As soon as I sent in my application, I was filled with anticipation. Once I found out that I had been selected to participate in the interview phase, I was thrilled. After the interview phase, I was once again filled with anticipation and eventually received a letter saying that I had been selected to be a 2015 Borlaug-Ruan intern. Although I was more than excited to find out that I would be spending my summer conducting research abroad, my anticipation continued as I waited to find

out where I would be going. Soon enough, I found out that I would be spending my summer working at Embrapa Soybean in Londrina, PR, Brazil.

As soon as I found out I would be going to Brazil, I tried to find as much information on Londrina as possible. While doing this research, I came across the fact that Londrina has a population of approximately 800,000 residents. At that moment, I realized that living in Londrina would be a huge change from living in Shenandoah, Iowa, a town of 5,000 residents. However, once I landed in Londrina, I realized that the population difference was only a miniscule change from living in Shenandoah. Throughout the course of my internship, I would realize that the culture, language, and customs were different from those of both India and America-which made my experience truly memorable.

## Embrapa Soja

The Brazilian Agricultural Research Corporation (Embrapa) is a state-owned organization associated with the Brazilian Ministry of Agriculture. It consists of a total of 47 research centers devoted to different divisions of agriculture. Embrapa Soja, the research center in which I spent my summer internship, focuses its research specifically on soybeans. Embrapa Soja is located in Londrina, which is situated within the state of Paraná. Since Paraná is a part of Southern Brazil, the milder climates of the summer serve as an ideal location to grow soybeans. Embrapa Soja is also responsible for sunflower research for the entire nation as well as wheat research developed in partnership with Embrapa Wheat.

Due to Embrapa Soja's partnerships with several local universities, there are many undergraduate, master, and PhD students who work in its labs in addition to the employees who supervise them. Embrapa Soja also contains several buildings, including a library and an auditorium, which are often utilized by these students. Therefore, Embrapa Soja not only serves as a research facility, but also an ideal learning environment.

Within Embrapa Soja, there are numerous facilities devoted to specific aspects of soybean research. For example, Embrapa Soja has labs for soil, seeds, microbiology, pathology, and many other areas of focus. I spent my internship in the plant biotechnology lab, which has a focus on molecular biology and genetics. Although I spent a majority of my internship in this lab, I was also able to spend time in the microbiology and pathology labs due to my specific research project.

## Research

Stem canker caused by the fungus *Diaporthe* spp. is a very devastating disease in soybeans worldwide and is particularly prevalent in the United States of America as well as Brazil. In 1994, stem canker, compared to the other various diseases and pests of soybean, caused the second highest estimated yield loss at 1.9 million metric tons collectively in seven of the top ten soybean producing countries, which includes Brazil (Wrather et al., 1997).

My internship consisted of conducting research on isolates of *Diaporthe phaseolorum* var. *Meridionalis* under the direction of Dr. Francismar Correa Marcelino-Guimaraes and a Masters

student Bruna Bley Brumer. Dr. Marcelino-Guimaraes completed a majority of her studies in Brazil; however, she also spent a year studying at Iowa State University during the course of her post doctoral work. Under the direction of Dr. Marcelino-Guimaraes, Bruna's main project aims to morphologically and molecularly analyze 27 isolates of *Diaporthe/Phomopsis* complex collected in different regions of the world. She is also responsible for analyzing the reaction of soybean genotypes when they are inoculated with DPM, the causal agent of stem canker. She will use this analysis to identify and genetically map resistant genes. Eventually, the findings will be used to develop molecular markers that will be used in marker-assisted selection aiming to develop soybean varieties with enhanced resistance to stem canker.

My individual project involved analyzing the genetic diversity of the ITS1 and ITS2 regions of 27 isolates in the *Diaporthe/Phomopsis* complex. Internal Transcribed Spacer (ITS) regions are the most commonly sequenced regions of DNA since they contain a higher degree of variation compared to other regions. Since the objectives of the overall project involved identifying genetic variations, analysis of these regions is ideal. Additionally, this project also involved analyzing the reaction of soybean genotypes when inoculated with three isolates of DPM. The objective of this portion of the project was to evaluate the different levels of severity the inoculation presented in soybean genotypes with the genes Rdm1, Rdm2, and Rdm?.

In addition to working on this specific project, I was also provided the opportunity to work with several other students and researchers on their projects. Many of the procedures the other students conducted were similar to the ones Bruna was using for her project. This provided me with more experience and also allowed me to gain a more complete understanding of the procedures before I was allowed to conduct any on my own. For example, I was able to observe Valeria Lopes Caitar, a PhD student, as she extracted DNA from nematodes during the first week of my internship. During the third week of my internship, I was able to assist Bruna as she extracted DNA from fungal mycelium.

Some other procedures also exposed me to different biotechnology tools and aspects of plant science. For example, I worked with Marcia Kuwahara, a technician at Embrapa, and Kenia, a post doctorate student, as they used a gene gun in a transient plant transformation. In addition, I was able to assisted Suellen Silva by plating, planting, and counting *Arabidopsis*. Although *Arabidopsis* does not have much agronomic significance, it serves as a model plant because it reacts to stress and disease the same way as many plants.

Since this was my first time working in a biotechnology lab, I spent the first few weeks of my internship observing Bruna and other students. I was also able to observe Bruna in the microbiology lab as she used the Castellani method to preserve the isolates of the fungal mycelium. As my internship progressed, I was able to practice other protocols independently. I was able to make agarose gel, practice electrophoresis, and prepare polymerase chain reaction (PCR) mixtures. I was also able to learn many important skills such as using BioEdit, creating and interpreting phylogenic trees, and identifying plants resistant or susceptible to stem canker. All of these new methods and skills I learned allowed me to work on the research project detailed in the following presentation.

#### Abstract

The most successful method to minimize the risk of stem canker involves the use of resistant cultivars, containing resistance genes *Rdm1*, *Rdm2*, *Rdm3*, *Rdm4*, and *Rdm?*. The genomic regions of these genes are present in these genotypic differentiators: D85-10404, D85-10412, Crockett, Dowling and Hutcheson, and PI 398469, respectively. Embrapa Soja has a collection of isolates that exhibit differentiating soybean genotypes, which are used in order to identify isolates capable of differentiating genes resistant to stem canker. Thus, the analysis of the genetic diversity of the *Diaporthe/Phomopsis* complex, through sequencing of the ITS1 and ITS2 regions, allows the identification of species within the complex as well as discriminating DPM isolates, which will be used for phenotypic evaluation. And therefore, with the addition of molecular analysis, an understanding of the interaction between DPM and its host will contribute to the development of molecular markers for use in selecting DPM resistant soybean cultivars, and thus, improving the soybean yield.

The objectives of this experiment are: (i) to analyze genetic diversity by sequencing the ITS1 and ITS2 regions of the genome of the 27 isolates of *Diaporthe/Phomopsis* complex (ii) to identify the isolates of DPM that will be used for phenotypic evaluation based on polymorphism, collection and collection year and (iii) to phenotypically characterize 3 isolates of DPM and identify genotypes with resistant genes *Rdm1*, *Rdm2*, and *Rdm?* for the validation of molecular marks.

#### Introduction

Stem canker is categorized into two distinct diseases, northern stem canker and southern stem canker. The two common variations of *Diaporthe phaseolorum* that cause northern and southern stem canker are *caulivora* (DPC) and *meridionalis* (DPM), respectively. DPC and DPM remain within the *Diaporthe/Phomopsis* complex, which also includes the species *Phomopsis longicolla* and *Diaporthe phaseolorum* var. *sojae*. Due to severe reduction in soybean yields worldwide, the most devastating disease within this complex is stem canker.

The ideal temperature for growth of *Diaporthe phaseolorum* var. *caulivora* is 25°C, whereas the ideal growth temperature for *Diaporthe phaseolorum* var. *meridionalis* is between 28°C and 30°C (Costamilan et al., 2008). Thus, the warmer temperatures of Brazil's soybean growing regions are more ideal for the growth of *Diaporthe phaseolorum* var. *meridionalis*.

The first symptoms of DPM occur as small, reddish-brown lesions near the leaf node during the reproductive stage. The lesions expand longitudinally to form cankers as the growing season progresses. Older lesions may appear dark with a grayish-brown center and a reddish-brown margin (Fernandez et al., 1999).

The dead tissue of the stem blocks the flow of water through the tissue. Therefore, the seedbearing portion of the plant becomes water-stressed and dies before the plant's full yield potential is reached (Hildebrand, 1952). This premature plant death causes a reduction in the quantity and size of the seeds. Also, since symptoms of stem canker do not appear until the reproductive stages, growers often spend valuable resources on infected plants that will most likely not reach their full growth potential (Fernandez et al., 1999).

There are at least five different dominant resistance genes that condition cultivar resistance to stem canker. In 1994, the germplasm lines of D85-10404 (Rdc1) and D85-10412 (Rdc2) were released with canker resistance (Kilen and Hartwig, 1994). Tracy-M was the source of resistance for these two germplasms, which were both developed from the cross of Tracy-M x J77-339 (Kilen et al., 1985). Additional resistant genes have been found in Crockett (Rdc3) and Dowling (Rdc4) (Bowers et al., 1993). PI 230976 and PI 398369 also have single dominant genes that condition resistance to stem canker, but the genes are at different loci than Rdc1, Rdc2, Rdc3, and Rdc4. A gene symbol has yet to be assignment since it is not known if the genes in these plant introductions are unique (Tyler, 1995). Therefore, this gene is referred to as Rdc?.

Through the processes of phenotypic characterization and sequencing, molecular markers can be developed. Identifying molecular markers associated with genes that condition resistance to stem canker will eventually aide soybean growers in maximizing the yield of soybeans.

#### Materials and Methods

In order to sequence the ITS1 and ITS2 regions of the 27 isolates within the *Diaporthe/Phomopsis* complex, we followed two distinct protocols.

The first part involved amplifying the target regions. These regions were then purified. This process involved DNA extraction from fungal mycelium through the use of CTAB protocol with modifications. Polymerase Chain Reaction (PCR), with the use of primers ITS4 and ITS5, was used to amplify the ITS1, 5.8S rDNA, and ITS2 regions. The PCR product was then purified.

During the second part, the target regions were cloned. There are two methodologies, PCR product and cloning, that could have been used to sequence the target regions. In this case, cloning was preferable since it sequences a specific portion more completely. After cloning, miniprep was used to remove the bacteria from the target region and the vector.

In addition, the following methods were also used for DNA sequencing, pathogenic analysis, and phenotypic analysis.

#### DNA Sequencing

After cloning, a plate was prepared in order to sequence the 27 isolates. The primer M13 was used in the DNA sequencing reaction. The PCR reaction mixture consisted of  $3.0\mu$ l of DNA with a concentration of 200-300ng,  $3.2pmol/\mu$ l of the forward and reverse M13 primers,  $1.5\mu$ l of Big Dye and  $3.0\mu$ l of the Big Dye buffer. This formed a total volume of 20 µl. The cycling conditions were 1 minute at 96°C, 35 cycles of 15 seconds at 96°C, 15 seconds at 50°C, 4 minutes at 60°C and a final temperature at 4°C. The plate was then stored in a freezer overnight. 2 µl of 7.5 M ammonium acetate and 6.5 µl of absolute ethanol were added to the samples and were mixed using the vortex. These samples were then centrifuged for 45 minutes at 23°C with a speed of 4,000 rpm. This caused the DNA to precipitate. Then sample was decanted. Next, 150 µl of 70% ethanol was added to the wells. After centrifuging for 25 seconds at a rate of 300 rpm and a

temperature of 23°C, the plate was decanted once again. After isolating the DNA, the samples were sequenced.

#### **Phylogenic Analysis**

The ITS1 and ITS2 regions were sequenced in both directions and then edited with BioEdit, which was used to remove the 5.8S ribosomal DNA region as well as the forward and reverse M13 primers. The ITS1 and ITS2 sequences of all of the isolates were compared with sequences present in the NCBI database, using the option nBLAST. Next, these sequences were aligned through the use of the program MEGA6.0. The phylogenic tree was built with the same program through the use of the bootstrap method. Bootstrap support values with 1000 replicons were calculated for tree branches in both methods.

#### Pathogenic Analysis

150 soybean genotypes, including several differentiating genotypes, were used for pathogenic analysis. Specific genotypes were chosen based on the presence of a known resistance gene. Twenty-six seeds of each of these genotypes were used. Three specific isolates of *Diaporthe phaseolorum* var. *meridionalis*, identified as isolates 4, 8, and 11, were used. In the experiments conducted by Bruna, the Rdm 1 and Rdm2 genes were selected from all three isolates. The Seedlings were inoculated once they were 12-14 days old through the use of the toothpick method (Yorinori, 1996). The plants were kept at a relatively high humidity (90-100%) for the first 72 hours following the inoculation. The reaction of the plants to the fungus was then measured through the percent of dead plants (%DP). The %DP was measured again after thirty days of inoculation.

Plants were determined to be susceptible, resistant, or dead based on appearance. Susceptible plants often exhibited the symptom of less inflammation surrounding the toothpick. Other Susceptible plants had a reddish brown lesion with a length of 1 inch or greater (Figure 1). The leaves of these plants also showed discoloration 60 days after inoculation (Figure 2). Resistant plants were identified based on the presence of a resistant callus surrounding the toothpick (Figure 3 and Figure 4).



Figure 4



Figure 2

Yorinori proposed five categories for classifying the soybean genotype reaction when inoculated with stem canker-producing isolates. In this scale, genotypes were considered resistant if they

Figure 3

had a 0 to 25%DP. However, Pioli's more stringent categories were used for this project (Table 1) (Pioli et al.).

Table 1: Pio	li's Categories
Incompatible	0-14.9%
Compatible	15-49.9%
Very Compatible	50-84.9%
Highly Compatible	85-100%

#### Results

#### **Phylogenetic Tree**

Through the use of the phenotypic analysis method detailed above, a phylogenetic tree was developed (Figure 6). The numbers located on the branches of the tree represent the number of matching replicons between certain genotypes. For example, there were 39 replicon matches between genotypes JQ697848 DPS and JQ697846 DPS. Genotypes are placed on the tree based on their sequential similarities with one another. The tree allowed easier interpretation of the relationships between the different genotypes more easily. Building a phylogenic tree (using matching replicons) and using its analysis allows identification of soybean genotypes that are resistant to the fungus *Diaporthe phaseolorum* var. *meridionalis*. Verifying for the markers in the soybean genome prior to sowing may be a good method to test for fungal resistance to minimize the risk of fungal infection, and lower soybean yields.

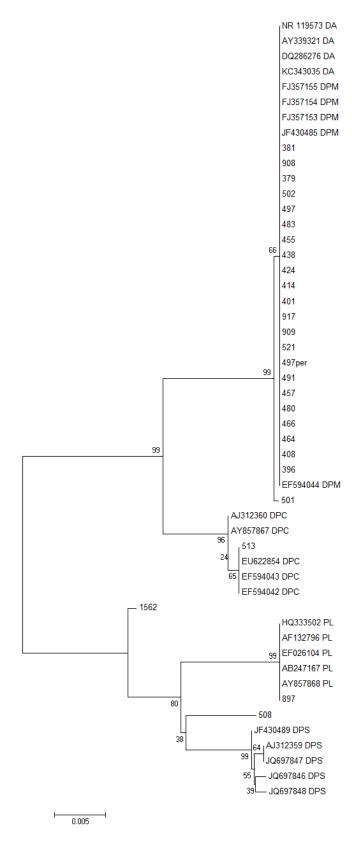


Figure 1 Phylogenetic Tree. DPM: Diaporthe phaseolorum var. meridionalis, DA: Diaporthe aspalathi, DPC: Diaporthe phaseolurm var. caulivora, DPS: Diaporthe phaseolorum var. sojae, PL: Phomopsis longicolla.

#### **Pathogenic Evaluation**

Plants were examined in order to identify whether they were resistant or susceptible to the fungi through the use of the pathogenic analysis methods mentioned previously. The results of the reactions of 14 soybean differentials are shown in the chart below with values for total plants (TP), dead plants (DP), susceptible plants (SP), resistant plants (RP), and %DP.

The isolate collected in the earliest year showed the greatest number (8) of genotypes with the resistant genes *Rdm1*, *Rdm2*, and *Rdm?* Mutations in the soybean genome are most likely responsible for the loss of resistance to *Diaporthe phaseolorum* var. *meridionalis* fungus as evidenced by loss of resistance in subsequent years.

Isolate 4 CMES 480 - Brasil/ GO Rio Verde/ 2001							
Genotypes	ТР	DP	SP	RP	%DP		
BRSMT Uirapuru	13	0	0	13	-		
	12	0	0	12			
CD 201	13	0	0	13	-		
	13	0	0	13			
Conquista_16	11	0	0	11	-		
	13	0	0	13			
Doko_17	13	0	0	13	-		
	13	0	0	13			
Emgopa 301_18	9	8	1	0	85.00		
	11	6	5	0			
FT Abyara_19	8	5	3	0	73.53		
	9	3	6	0			
FT Cristalina_20	12	10	2	0	82.00		
	13	6	7	0			
IAC 8_21	13	0	0	13	-		
	13	0	0	13			
IAS 5_22	11	4	7	0	63.04		
	12	2	10	0			
NA 5909 RG_23	6	0	0	6	-		
	8	0	0	8			
P98Y11_24	13	0	0	13	-		
	13	0	0	13			
Paraná_25	13	4	9	0	68.00		
	12	5	7	0			
Sta Rosa_26	12	4	8	0	72.92		
	12	7	5	0			
VMaxRR_27	13	0	0	13	-		
—	13	0	0	13			
Forrest_28	8	3	5	0	66.67		
	7	2	5	0			

OCEPAR10_144	11	6	5	0	78.26
	12	8	3	1	
PI437654_44	2	0	2	0	85.71
—	5	5	0	0	

GenotypesTPDPSPRP%DPBRSMT Uirapuru_141013617.39130310127.691303101010CD 201_15130013-130013-10Ooko_171300131.92Emgopa 301_18110385FT Abyara_191000104.76110297FT Cristalina_20110471305834.0014X 5_221201201AC 8_211305834.0014X 55_2212012050.0011011011015.2212012066Paraná_2511011015.2212021013.04-130013140471512021013.041511011015.2213001314019041512021013.0411011013.04 <t< th=""><th>Isolate 8_CMES 502 - Brasil/ Ponta G</th><th>rossa/</th><th>2007</th><th></th><th></th><th></th></t<>	Isolate 8_CMES 502 - Brasil/ Ponta G	rossa/	2007			
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$\begin{array}{c ccccccccccccccccccccccccccccccccccc$		13	0	3	10	
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	CD 201_15	13	0	1	12	7.69
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#### **Impact on Food Security**

Resistance to stem canker decreased from the year 2001 through 2006 and 2007. Since southern stem canker is often undetected until later in the reproductive process of plant growth, it is essential to select soybean cultivars resistant to this disease. By sequencing the ITS1 and ITS2 regions of the genome of 27 isolates, resistant genes were identified. This allowed the development of a phylogenetic tree, which can be used to determine which soybean isolates (cultivars) contain the resistant genes. The phylogenetic tree can be used to develop and validate new molecular markers in order to allow the selection of resistant cultivars when sowing the crop. The 27 isolates examined over the course of this study contribute to enhancing resistance to southern stem canker in soybean cultivars though the selection of the resistant cultivars.

## Reflection

#### A Global Career Path

As soon as I boarded my flight from Atlanta to Sao Paulo, I noticed that there would be a language barrier. Everyone within a 10-meter radius of my seat spoke only "Portuglish" at best. Although I had tried to learn as much Portuguese as possible prior to my arrival, I was still nowhere near fluent. Although I had traveled to China in the past, I didn't notice much of a language barrier during that trip since I traveled with a group of people, including some native speakers. Therefore, the idea of traveling to another country alone suddenly seemed daunting.

By the end of my first week in Brazil, however, I realized that the language barrier really was not as big of an issue as I had thought it to be. Although my host mother did not speak English, she introduced me to all of her friends as her "American-Indian daughter." My host sister and all of her friends welcomed me into their friend group by adding me to their WhatsApp group and eagerly practicing their English with me. By the end of my first two days, I had already received hundreds of hugs and translated messages from people wishing me the best throughout my stay in Brazil. I was invited to traditional celebrations, such as Festa Junina and my host family even surprised me with a traditional party for my own birthday. By the end of my stay in Brazil, I learned that language is only a mere factor, shared interests are what truly connect people-a lesson that proved to be very important in the decision of my career path. My shared interest in learning about culture with my new Brazilian friends and my shared interest in science with the students at Embrapa made my experience truly unforgettable and life changing. Although this language barrier had initially intimidated me, it ended up becoming one of the most transformative aspects of my internship.

The welcoming nature of everyone I met in Brazil made me a more open person. As soon as I saw my friends upon my return, I greeted them with warm and friendly hugs. Considering that I would awkwardly shy away from any sort of physical human interaction prior to leaving for Brazil, my friends knew that I had become a completely different person. Once school began, I quickly introduced myself to our new foreign exchange student as well as any other new students and was eager to learn about them. This new excitement about meeting new people ended up influencing my career path.

Experiencing the Brazilian culture and realizing that language barriers aren't as intimidating as they seem made me want to pursue a global career. As I had the previous conception that I wanted to pursue a career in medicine, I was able to add my interest in global affairs by deciding to pursue a minor in global health.

#### **Food Security**

Although I was not exposed to poverty during my summer in Brazil, I was amazed by everyone's awareness of food insecurity. The employees at Embrapa would take monthly trips to impoverished areas in Londrina and educate the residents on successful agricultural practices. Although I never had the chance to participate in one of these trips myself, I was amazed by the stories I would hear from students. They would tell me about how all of these families they visited would prepare big meals for them in order to thank them for the visits. Considering that most of these families were living in hunger, I was truly inspired after hearing about the hospitality they showed during these visits.

Prior to my experience in Brazil, food security had only been a mere, and distant, career option. After my summer, however, I realized that there are many different ways to make an impact on the rest of the world, including public health and medicine. Access to healthcare is one of the most pressing issues for many third world countries. My internship in Brazil gave me the inspiration I needed in order to pursue a global career in medicine. Although medicine does not directly affect food security, it can have a great effect on those suffering from food insecurity, which is the reason I have chosen to pursue this path and continue the fight against food insecurity that Dr. Borlaug greatly advanced.

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