Taiwan Summer Internship

Modify Disease Screening Protocol and Evaluate Marker Assisted Selection for Black Leaf Mold Resistance in Tomato

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Abstract

The mycology department at AVRDC headquarters in Shanhua, Tainan, Taiwan has been working on a project involving black leaf mold on tomatoes. Black leaf mold is a condition where the plant becomes infected with *Pseudocercospora fuligena*, a pathogen that affects solanaceae plants.Mycology is specifically focusing on the effects on tomatoes using F1 generations crossed from a common tomato and an original tomato strain that was resistant to black leaf mold. Also, a major part of the research on this pathogen is finding molecular markers on chromosome one of the resistant variety of tomatoes. The purpose for locating the molecular markers is to discover the point on chromosome one where the genetic information that allows

the resistant variety to be resistant. The current F1 resistant variety contains a large amount of genetic material that does not pertain to traits that are sought after. So, to reduce excess material the genetic markers are located for future references when creating other resistant varieties.

The tomato breeders had already bread the F1 and F2 varieties prepared for experimentation by June 20th. The majority of preparation was mainly subculturing of P. fuligena of the 134a and 134a--2 strains. The smaller part of culturing of P. fuligena was multiplication of both strains through the use of V8 Broth and V8 Agar mediums. The use of two different mediums was to create a small experiment with the F1 testing to see which medium was more suitable for growing the pathogen. This was determined after inoculation. One final experiment involved was to find what concentration showed the most clear results, difference between susceptible and resistant. Two weeks after inoculation of F1 plants it was determined that 134a--2 in a V8 Broth at concentration of 1x10 ^{5} was the best for plant concentrations all plants were infected whether or not they were resistant. This was due to the thickness of the pathogen and in numbers of the pathogen that will eventually infect the plant. The 134a-2 strain was the fastest growing of the two strains and showed faster growth of colonies than most other strains of P. fuligena. V8 Broth is a mixture of water, V8 juice and antibacterial materials and is a liquid unlike the other mediums that solid like a jel. The broth showed better results than the agar plates because the broth was able to keep a higher number of the colonies alive and helped with making thicker colonies.

P. fuligena is a very slow growing pathogen that is easily overtaken in the plates by contaminates like penicillin. However, due to its unique traits and slow growth, it is easy to identify amongst others infecting a plant. This helps ensure the validity of the data by knowing that the pathogen samples are pure. The 134a--2 strain will help the process of finding molecular markers on chromosome one in the near future.

1 Introduction

1.1 Personal Introduction

I don’t believe that would have seen myself where I am now, two years ago. I grew up on a small three acre plot of land outside of Whittemore, Iowa. My family does not farm; however, I do practice farming techniques in our gardens, combined ten thousand square feet of garden land. I grew up with those gardens being filled with an extensive salsa garden collection, perennials, and wild flowers. Our family has had a very close connection with nature ever since my parents first moved to the acreage in the 80’s. With my parents influence, I grew to love plants and all that they have to offer. Due to our garden being an important part of our diet during the fall and winter, I started to focus mainly on the vegetable production instead of flowers.

My brother and I started looking into the world of hot peppers when I was about twelve, and ever since then my interest in plant varieties was sparked. The unique tastes and spice of the peppers was something that struck me as a kid but I had never really looked into it until that point when we planted the world hottest pepper at the time, Caribbean Red, in our garden. Since then, my brother and I tried to see what factors caused the peppers grow bigger and hotter than the others we had before. This later influenced me to start drying and selling my own peppers that were grown in my section of garden. With the money I got from the peppers, I was able to start tinkering with breeding of different peppers to get traits and observe the differences.

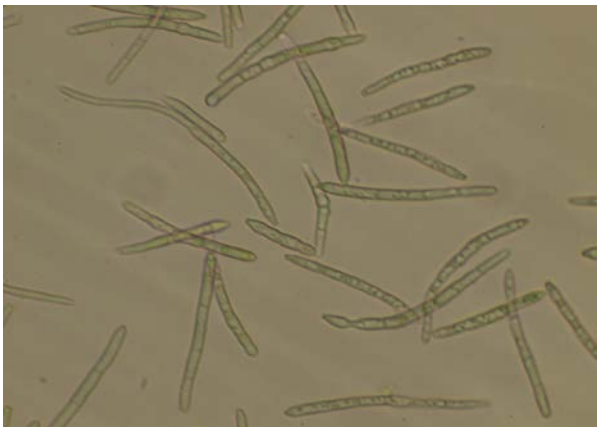
With the new found interest in plant breeding, I decided my college majors for college were going to work towards a future career in plant breeding or plant genetics. I enrolled at South Dakota State before my internship in Taiwan. Lisa Fleming and AVRDC chose a field I would have an interest in and be somewhat successful in as well. The work I was given was almost entirely based off plant genetics and research, which is exactly what I was interested in.

1.2 Tomato Background

Tomatoes are part of the Solanaceae family, nightshade, and are in the same area as plants like peppers and tobacco. The wild tomato plants bear little resemblance to the commercial types of tomato that are being used today. The wild species of tomato have thick leaves and are more vine-like in that they are more of a ground coverer. The wild tomato was found in Central and South America, but the origin is thought to be in Peru (Planet Natural). When the tomato was first brought to Europe, people thought that fruit was poisonous like the other plants in the nightshade family. So, the tomato virtually had no market in Europe, and the tomato was not widely distributed. The tomato seeds, however, were thought to be aphrodisiacs by the french (Planet Natural). This was the first way that the tomato was starting to be on the market for fruit production.

1.3 History of Black Leaf Mold

Figure 1: *Pseudocercospora fuligena* magnified by 400x



Black Leaf Mold, commonly referred to as BLM and scientific name pseudocercospora fuligena, is a semi-common plant disease in Southeast Asia, Ivory Coast and Florida. The history of BLM starts in the Philippines in 1938 (Hartman). The farmers noticed a drop in yield was due to a field wide disease that showed symptoms of a new type of tomato sickness. The plants decayed at a rapid rate and a vast majority of the crops died. The fields looked gray due to the symptom of BLM being dark dots of mold on leaves(Plantwise).

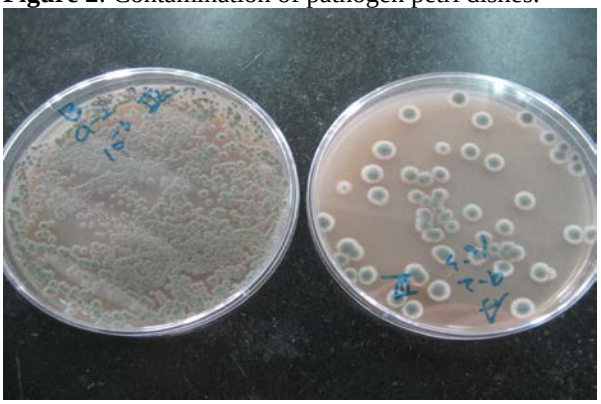
Black Leaf Mold quickly spread to other areas around Southeast Asia due to improper containment of the plant material. The seeds and plants were used and disposed of in an improper way, which led to the distribution of the spores of Black Leaf Mold to the Ivory Coast and Florida as well.

Black Leaf Mold is slow growing fungus that is perfectly suited for the tropical weather or even greenhouse conditions. This particular type of mold yields highly in humid conditions at about 90-95% humidity. Temperature for the spores is also an important element for the longevity of the mold and requires a 28-30^{o}C environment. These conditions are ideal for Black Leaf Mold and are also the conditions of Southeast Asia, Ivory Coast, and Florida.

2 Research

1.1 Research Background

Figure 2: Contamination of pathogen petri dishes.



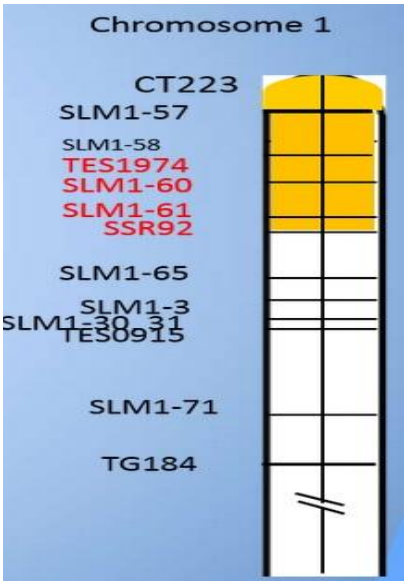
It was discovered that Black Leaf Mold resistance was associated with the first chromosome of the tomato. The purpose of the research was to fine pick through past papers to gather information on differences of side effects between Black Leaf Mold and other similar types of common mold that may contaminate the materials used in the experiment. There was a case in the lab where all of the petri dishes that were growing Black Leaf Mold started to become contaminated with an unknown mold that took over the the entire plate. There were nearly 250 plates that needed to be thrown out, and this meant that new trays had to be started. The reason why it was so easy to see that it was contamination is because the contaminate grew at a very quick rate, and it was known that Black Leaf Mold takes several days to even show up.

Research also showed that the mold spores also could grow easily on mineral rich, organic material. This meant that the media for the petri dishes needed to be made of some sort organic liquid, such as V8 juice. This also means that Black Leaf Mold can also thrive in conditions where it could be transported to other areas by fruit, even though the mold does not infect the fruit of the plant itself.

2.2 Experiment Background

The previous experiments done with Black Leaf Mold proved that the resistance was associated with chromosome one of the tomato, but the exact location of the resistance is unknown. The main purpose of the experiment was to pinpoint the exact location of the resistance. The way that resistance was found was by breeding the susceptible commercial type of tomato with the resistant wild type of tomato that comes from Brazil. When the two were bred, it was noticed that chromosome one was affiliated with the resistance, but the area of genetic material on chromosome one was mostly wild genes. This mean that the wild species of tomato had expressed more genes than the specific sequence needed for Black Leaf Mold resistance. In order to limit the wild genes, research needed to be done in order to take the large portion of wild genes from chromosome one out and narrow it down to just resistance. This is just to ensure that resistance is the only trait that would be expressed.

Table 3: Chromosome one, showing markers



The wild gene was marked with introgression lines that stood as an indication of the section of the chromosome which held the resistance to Black Leaf Mold. For this experiment, there were four specific markers, SLM1-60, SLM1-61, SSR92, and TES1974, that were going to be used. The four lines were to indicate specific sections of the wild gene to locate the section where the resistance was expressed so that selection could be tested for the next year. The process of locating a specific gene by marker selection is a long and tedious process. The research that was done in 2014 was not the last step in discovering the answer. It was actually just meant to refine the search to a specific location. The four markers, LA3920, TS19, and LA1777, were used for the modified screening protocol. The modified screening protocol was to test and see which isolate, Pf134a and Pf134a-2, was more suitable for the main experiment.

The main experiment used the introgression lines LA3920, CLN3078C, L2110, and TS19.

3 Experiment

3.1 Modified Screening Protocol

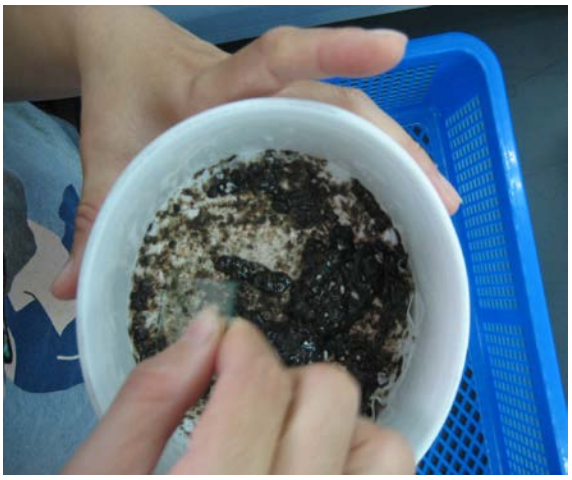
The purpose of modified screening protocol is to test to see which pathogen shows the clearest signs of infection. The clarity of the infection is important when grading the plants in future

experiments based off of the percentage of the plant that is infected. The rating is used to compare amongst the introgression lines. So, clarity is of the utmost importance. The way that the two pathogens, Pf134a and Pf134a-2, were tested was by using susceptible lines, LA3920 and TS19, and the resistant line, LA1777, to determine which pathogen was best suited for the main experiment.

The two isolates, Pf134a and Pf134a-2, were first cultured in petri dishes that contained a solution that was composed of V8 agar or V8 broth. The reason for the two different medias was to test to see which cultured the pathogen better. The V8 agar was a gel substance that was composed of V8 juice and agar, which is a common type of media used for culturing molds. In order to spread the mold onto the surface of the agar, the solution must be allowed to set up. Then a water and pathogen, used from an original culture from the field, suspension is pipetted onto the surface and is spread by the use of an l-shaped utensil. The V8 broth was composed of V8 juice and a mineral water. The V8 broth was a liquid media where no setting up is necessary. The water and pathogen suspension is pipetted directly into the liquid media where it disperses.

The pathogen is allowed to grow for about two weeks before it is used for making an inoculate. An inoculate is a suspension that contains a concentration of the pathogen and is mixed with water. After two weeks, the plates of inoculate are taken out of the incubator that was set at optimum temperature, 28^{o}C, and taken to be cleaned. The cleaning process consists of scraping out all of the V8 broth suspension that contains the colonies of Black Leaf Mold, and then the suspension is run through a filtered vacuum where all of the liquid is drawn through the filter. The purpose is to as much of the media out of the suspension in order to leave behind only the colonies of the pathogen. The trays are dumped into the filtered vacuum and rinsed with distilled water. Then, the pathogen is scraped off of the filters and put into a blender with water. The blender is to segment the colonies in order to make a more uniform suspension with the water. Also, Black Leaf Mold can grow from individual segments; so, the breaking up of the colonies ensures a greater chance of uniform infection as well.

Table 4: Vacuum filter where the pathogen is rinsed of the media.



After the the suspension has been made with the blender, the concentration needs to be determined in order to make the inoculates. The inoculates were set at different concentrations as a secondary test to see which concentration shows the clearest infection. The V8 broth concentrations were set for $1x10^6$, $5x10^5$, $1x10^5$, and $5x10^4$ segments per milliliter, but the V8 agar did not have a high enough initial concentration to make a $1x10^6$ concentration.

Table 4: Inoculate being prayed on the screening

The inoculate was then sprayed onto the

protocol trial plants

tomato plants by a high pressure sprayer. The sprayer evenly sprays on the inoculate on both the bottom and top of the leaves. The greenhouse workers were the sprayers and the caretakers of the tomato plants. Once the plants were sprayed, they were taken to a sealed off room where the conditions were set to 100% humidity, 28^{o}C, and no light for the first 24 hours. After 24 hours, the conditions were changed to 80-95% humidity, 28^{o}C, and 12 hours of light per day. The reason for the 24 hours darkness is to let the pathogen take hold on the plants because light is damaging to mold and could majorly affect the results.

3.2 Detached Leaf

The detached leaf experiment comes from the concept that plant tissues can continue to live when cut from the main plant just as long as the life functions, like sunlight and water, are still present. The purpose to this experiment is to see if detached leaves are a viable method for Black Leaf Mold trials. The detached leaf method uses less space and can come to the same results as a full plant method, providing that the pathogen grows on the leaves. The detached leaf experiment took place at the same time as the modified screening protocol.

In this method, the leaves are cut from the plants and are separated and logged to know which leaf belonged to which introgression line. The leaves were then put into glass plates with cotton around the base of the cut stem. The cotton was saturated with a solution that prolongs the life of a cut leaf by providing nutrients. The plates were then sprayed with the same concentrations as the full plants from the modified screening protocol, but the prayer was a smaller sprayer. the leaves were coated on both sides with the inoculate. When the plates were prayed, the top and bottom halves were sealed with paraffin wax and put into an incubator. The incubator was set to 28^{o}C and with 12 hours of light. For this method, the leaves had to be checked every other day to ensure that the cotton stayed wet so that the leaves didn't die.

Figure 5: Detached leaf method after praying.

3.3 Locate Molecular Markers (Final Trial)

The final trial, which was the main trial, was to use the results from the modified screening protocol to have more precise results. The trial used the main introgression lines to be tested that came from the CLN3868 F2 population which came from the cross between LA3920 and CLN3078C. The resistant species, LA3920, was crossed with the susceptible species, CLN3868C, to make two introgression lines, TS19 and L2110.

Table 6: CLN3868 F2 population in climate controlled room.

With this experiment, only the V8 broth was used because it grew the pathogen better. Also, only the concentration $5x10^5$ segments per milliliter was used because it was determined from the modified screening protocol that it showed the best contrast. The rest of the conditions were kept the same as the screening protocol.

Also, in this experiment, leaf samples were taken from all 480 leaves. The new leaf growth at the top of the plants were taken and put into 96 well blocks with glass beads. The purpose of this is to use the leaves for the genotyping, genetic traits. The glass beads emulsify the leaves into a pulp so that it can be used for the PCR, polymerase chain reaction, process. PCR is used to replicate specific sections of DNA by the use of markers. PCR takes about two hours of heating to initiate the three additives, tag, buffer, and the markers. Each well of DNA is put into four different 96 well plates relative to the original's location. The reason for the four different wells is because the markers cannot be mixed, otherwise the data will be ruined. Also, the tag is highly sensitive to temperature and needs to be kept near 0^oC so it doesn't activate and ruin the DNA samples.

After the PCR, the samples are taken to a room that is dedicated to gel electrophoresis.Gel electrophoresis is the process in which DNA is put into gel slots and an electrical current is run through a buffer solution that surrounds the gel sheet that holds the DNA. DNA is negatively charged so that means that the larger bands of DNA will be pulled further down the gel sheet, towards the positive charge, than the smaller bands. This difference in movement is how the difference in genes in the individual plants is determined. The four markers are then read, and associated with the plants that the DNA came from, to set up a statistical analysis that would use both the genotyping and the phenotyping results to come up with a percentage of genetic association with each marker and the resistance to Black Leaf Mold.

Phenotyping of the plants takes place 20 days after inoculation. By that point, the pathogen had taken hold of the plant and has developed to a point where differences in susceptibility are obvious. The rating of the severity and coverage of the leaves is based off of a 1-11 scale. This scale was developed by Barratt and Horsfall. The purpose of the scale is to reduce the amount of human error in determining the amount of coverage by using varying percentages to make a 1-11 decision.

4 Results

4.1 Modified Screening Protocol Results

The variables being tested, isolate, culture method, and inoculate concentration, were determined in order to prepare for the main experiment. The isolate that was found to be the best was 134a-2, which was the most virulent of the two isolates and infected the plant easier than the 134a. Of the two culture methods, it became apparent that the V8 broth was the best choice for the future experiment with Black Leaf Mold. The V8 broth allowed the pathogen to be immersed in the nutrients need to grow. The agar didn't even have enough segments to make all of the

concentrations needed for the experiment to take place. With the V8 broth selected, it was also determined that the concentration of $5x10^5$ segments per milliliter was the best choice. $5x10^5$ showed the best contrast between the susceptible and the resistant plant. Even though a plant may be resistant to the mold, it will still show symptoms due to the amount of pathogen applied to the leaves where the mold can live off of itself for a short time before dying.

4.2 Detached Leaf Results

After about two weeks in the incubator, the leaves started to show signs of mold; however, the mold quickly turned into rot. The plates had built up a high humidity inside of the sealed container, and this caused the lesions to start to rot. It was determined that the detached leaf method is not a viable method to be used for Black Leaf Mold because the mold's nature to burst through the surface of the leaves allows it to be susceptible to rotting in high humidity.

4.3 Locate Molecular Markers (Final Trial) Results

Using the results from the modified screening protocol, we were able to get the most reliable results. The 134a-2 was virulent enough to infect the plants with the greatest of ease. However, some of the plants were so severely affected that they rotted all of the way down the plant and had to be thrown out. This may have been due to location of the plants and spacing. The control group was also noticed for having signs of the mold on the leaves and it was separated from the infected plants. It doesn't necessarily show that the mold is airborne, because it is not. The transfer of the pathogen may have been from some other method like human contact or insects. The resistant group showed the expected results of very little infection. The susceptible plants were mostly nine and above ratings on the Barratt-Horsfall scale.infected plants. It doesn't necessarily show that the mold is airborne, because it is not. The

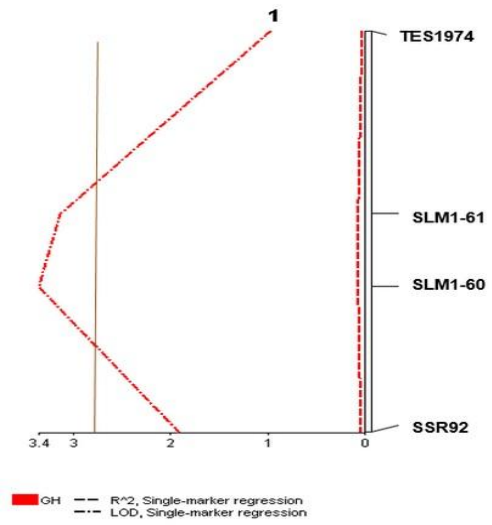
Table 7: F2 Population rating statistics.

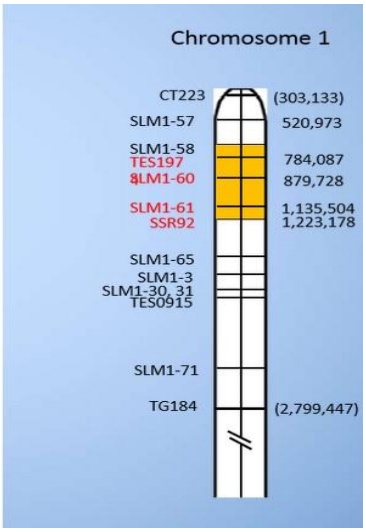
Genotype	Avg. Score	Avg. Percentage
F2 Population (200 Plants)	3.7 (0-7)	20.7 (1.2-81.3)
LA3920 (R)	0.08	1.4
CLN3078C (S)	3.5	14.8
L2110 (MSC)	3.3	14.8
TS19 (SC)	5.4	47.4

transfer of the pathogen may have been from some other method like human contact or insects. The resistant group showed the
expected results of very little infection. The susceptible plants were mostly nine and above ratings on the Barratt-Horsfall scale.

Table 8: Statistical chart of marker's relation to Black Leaf Mold Resistance

The phenotyping results were then compared to the genotyping results to form a statistical percentage of each marker's association with Black Leaf Mold resistance. It was





determined that the SLM1-61 and SLM1-60 were closely associated with the resistance to Black Leaf Mold. The resistance is between the two marker, but the SLM1-61 marker has a closer relation to the resistance meaning that the target allele is closer to the SLM1-61 marker than the SLM1-60 marker.

5 Bibliography

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