Escuela Agrícola Panamericana, Zamorano

Agricultural Science and Production

B.S. in Agricultural Sciences

Special Graduation Project

Establishing a protocol for the artificial inoculation of *Verticillium dahliae* on eggplant (*Solanum melongena L.*)

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Honduras, October 2024

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Acknowledgements

I would like to express my deepest gratitude to the Eggplant and Tomato Breeding Program at Cornell University for their invaluable financial and mentoring support throughout this investigation. Additionally, I extend my heartfelt thanks to Grupo Jaremar. Your unwavering support through my scholarship over the years has been truly instrumental, and for this, I am endlessly grateful.

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Abstract

Verticillium wilt is a significant soilborne disease affecting eggplant (*Solanum melongena* L.) production worldwide. This study aims to establish a standardized protocol for the artificial inoculation of *V. dahliae* on eggplant to facilitate disease resistance screening in breeding programs. The research was conducted at the Guterman Greenhouse Complex, Cornell University, and involved multiple experiments to optimize inoculation conditions. Three *V. dahliae* isolates were tested for pathogenicity on two eggplant cultivars, and the effects of spore concentration, immersion time in the spore suspension, root cutting, and inoculation method (dipping vs. drenching) were evaluated. The study also compared the susceptibility of 24 eggplant varieties, including 12 *Solanum aethiopicum* varieties. Results indicated significant differences in disease severity and progression based on isolate virulence and interaction between genotype and isolate, as well as some inoculation parameters. It was concluded that 10^5 conidia mL^{-1} is a high enough concentration to produce severe symptoms, as well as 5 minutes of immersion in the spore suspension. Cutting the tips of the roots does not influence disease severity when performing artificial inoculations, and dipping the roots causes more severe symptoms in less time than root drenching. All the varieties inoculated were found to be highly susceptible to *Verticillium* wilt. The established protocol provides a reliable method for screening eggplant germplasm for resistance to *Verticillium* wilt, contributing to the development of resistant cultivars.

Keywords: Artificial inoculation, Disease resistance, Eggplant, Plant breeding, *Solanum aethiopicum*, *Solanum melongena*, *Verticillium dahliae*, *Verticillium wilt.*

Resumen

La marchitez por *Verticillium* es una enfermedad importante transmitida por el suelo que afecta la producción de berenjena (*Solanum melongena* L.) a nivel mundial. Este estudio busca establecer un protocolo estandarizado para la inoculación artificial de *V. dahliae* en berenjena, facilitando la evaluación de la resistencia en los programas de mejoramiento. La investigación, realizada en el Complejo de Invernaderos Guterman de la Universidad de Cornell, incluyó experimentos para optimizar las condiciones de inoculación. Se evaluaron tres cepas de *Verticillium dahliae* en dos cultivares de berenjena, y además se realizaron experimentos para determinar los parámetros adecuados para la concentración de esporas, el tiempo de inmersión, el corte de raíces y el método de inoculación (inmersión versus empapado). Se comparó la susceptibilidad de 24 variedades de berenjena, incluyendo 12 de *Solanum aethiopicum*. Los resultados mostraron diferencias significativas en la severidad y progresión de la enfermedad según la virulencia de la cepa, y en la interacción de la cepa con el cultivar, además de algunos parámetros de inoculación. Se observó que una concentración de 10^5 conidios mL^{-1} y una inmersión de 5 minutos son suficientes para causar síntomas severos. El corte de las puntas de las raíces no afecta la severidad de la enfermedad, y la inmersión de las raíces causa síntomas más severos y rápidos que el empapado. Todas las variedades inoculadas resultaron altamente susceptibles. El protocolo establecido proporciona un método confiable para evaluar el germoplasma de berenjena en busca de resistencia a la marchitez por *Verticillium*, contribuyendo al desarrollo de cultivares resistentes.

Palabras clave: Berenjena, Inoculación artificial, Marchitez por *Verticillium*, Mejoramiento de plantas, Resistencia a enfermedades, *Solanum aethiopicum*, *Solanum melongena*, *Verticillium dahliae*.

Introduction

Eggplant (*Solanum melongena L.*), commonly known as brinjal, aubergine, patlican, or guinea squash, is a member of the Solanaceae family, also known as the nightshade family, which includes other well-known crops such as potato, tomato, tobacco, and pepper. Eggplant is an herbaceous annual plant with white to purple-colored flowers, characterized by its bushy foliage; it typically reaches an average height of 60-95cm, and it is primarily cultivated during the warm season (Naeem & Ugur 2020). Eggplant has a high mineral content packed in few calories and ranks among the top ten vegetables with lowest calories ((Cao et al., 1996)). Out of all vegetables in the Solanaceae family, eggplant's pulp is the richest source of phenolic acids (Condurache et al., 2021). The phenolic compounds that eggplants possess have properties that make them beneficial against a variety of disorders, such as Metabolic syndrome, and to a series of cardio-metabolic disorders, such as obesity, insulin resistance, dyslipidemia, and hypertension. Eggplants also contain anthocyanins which have antidiabetic, anticancer, anti-inflammatory, antimicrobial, and anti-obesity effects. Anthocyanins can also play a role in the prevention of cardiovascular diseases (Khoo et al., 2017).

Due to its nutritional and culinary characteristics, eggplant is produced widely around the world. In 2022, eggplant was the fifth highest produced vegetable globally, with a production of 59.31 million metric tons (Food and Agriculture Organization of the United Nations, 2022). Its presence in the agricultural industry is especially high in Asia and Africa. China leads global production, accounting for over half of the 51 million tons produced, with India, Egypt, Turkey, and Iran following suit. Indonesia, Italy, Japan, Spain, and the Philippines round out the top 10 producers (Food and Agriculture Organization of the United Nations, 2018) Eggplant is also one of the most important vegetables for the Mediterranean region (Tani et al., 2018). In Honduras, eggplant is primarily produced in the Comayagua Valley. In 2023, production expanded to Danli, El Paraíso, due to strong national and international acceptance (Secretaría de Agricultura y Ganadería de Honduras, 2023). Additionally, Honduras is solidifying its position as the second largest supplier of eggplant to the U.S.

market (Fundación Hondureña de Investigación Agrícola, 2023). The production quantity in Honduras is an imputed value of 18962.53t (*Food and Agriculture Organization of the United Nations*, 2024). In the US, it is considered a specialty crop, as there are less than 7,000 acres in eggplant production annually. In 2019, New Jersey topped the nation in eggplant acres harvested, with 849 acres dedicated to this crop, surpassing California's 705 acres, Florida's 685 acres, and Georgia's 624 acres (Murphy & Fisher, 2019). In the state of New York, eggplant is commonly grown for direct sales in farm stands and farmer's markets (Eastern New York Commercial Horticulture- Cornell University - Cornell Cooperative Extension)

One of the biggest challenges of eggplant production in temperate climates, like that of New York, is Verticillium wilt. Verticillium wilt is a soilborne disease caused by two species of fungi, *Verticillium dahliae* and *Verticillium albo-atrum*. The former is most prevalent in temperate and Mediterranean climates and affects a broad range of over 300 plant species (Pegg & Brady, 2002). The pathogen grows optimally in temperatures from 75 to 85 ^{o}F and neutral to alkaline soil. It is a devastating disease because the pathogen produces microsclerotia that may survive up to 10 years in the absence of a host, also withstanding drought and cold temperatures (Stokes & Meadows, 2021). The symptoms of Verticillium wilt in eggplant are yellowing and wilting of leaves on a few branches or on the entire plant. The leaves of plants severely affected by infection become brown and desiccated. Plants infected at an early stage of the season may experience significant stunting, with their leaves becoming small and transitioning to a yellow-green color. Later, these plants retain dried leaves and shrunken fruits, ultimately leading to their death (Stapleton & Davis, 2010).

Management of Verticillium wilt is very difficult. Site selection or crop rotation are not consistently reliable management strategies, because not only is the host range for Verticillium wilt wide, but also, *Verticillium dahliae* has been found even in settings where non-susceptible hosts have been grown after becoming infested by susceptible crops, showing that these non-susceptible hosts have served as reservoirs for the pathogen (Berlanger & Powelson, 2000). Chemical fumigants are only

available for suppression of the disease , and the use of fumigation is costly and detrimental to the environment (Berlanger & Powelson, 2000). Grafting eggplant onto resistant tomato rootstocks is another effective way of protecting the crop (N. Liu et al., 2009). However, grafting is expensive, requires substantial labor, and the grafts might not always be successful due to several factors. The first and foremost recommendation for disease management in general for all crops is choosing a resistant cultivar. However, there are currently no commercial eggplant cultivars that are resistant to Verticillium wilt (UMass Center for Agriculture, Food, and the Environment, 2015; Wyenandt, 2020).

To develop a cultivar of eggplant resistant to Verticillium wilt, it is necessary to evaluate germplasm and select individuals possessing genes that confer resistance. To discern which individuals, hold the traits for disease resistance and cross them with others that have desirable qualities for yield, taste, etc., disease screening must be performed. Disease screening in plant breeding involves artificial inoculation with a virulent pathogen under conditions favorable for disease development so that we can be confident that the lack of symptoms in a particular accession is due to genetic resistance to the disease as opposed to unsuccessful inoculation. A standardized system to perform the disease screening is necessary to classify genotypes unambiguously over time (Gao, August/2004).

Therefore, the objectives of this study were: to establish a protocol for artificial inoculation of *Verticillium dahliae* on eggplant; to compare the virulence of three *Verticillium dahliae* isolates; to determine if there is an interaction effect between the variables isolate and genotype; to use the protocol to evaluate the susceptibility of 24 eggplant cultivars, including 12 *S. aethiopicum* cultivars

Materials and Methods

Study Location and Set-up

All the experiments presented in this study were performed at the Guterman Greenhouse Complex, Facility 1068K, Room 101C, which is part of the Cornell University Agricultural Experiment Station (Figure 1). It is in Ithaca, New York, United States of America. The greenhouse is made from concrete and glass, and Room 101C has eight metal rolling benches, of which four are 9ft x 6ft and the rest are sized 9ft x 5ft. Above each bench there are four lights, of which two are metal halide and the other two are high pressure sodium. Conditions are kept stable using Wadsworth and Argus Greenhouse Control Systems/Software. During the experiments, the greenhouses were maintained at a temperature of 71°F to 75°F (22°C to 24°C) during the day and 61°F to 65°F (16°C to 18°C) during the night, as well as a target relative humidity of 50-70% and a light period of 14 hours (7am to 9pm). Plants were watered as needed on weekdays with 200ppm of Jack's Classic 15-5-15 soluble fertilizer, and on the weekends, plants were watered as needed without fertilizer.

Figure 1

Location of the study - Map of Room 101C, Facility 1068K, Guterman Greenhouse Complex, Cornell University Agricultural Experiment Station

Fungal Isolates

The three isolates of *Verticillium dahliae* evaluated in this experiment were 23V01A, 23V03A, and 23V04A. 23V01A was isolated from a maple tree in NY in 2023 and provided by Sandra Jensen of the Cornell Plant Disease Diagnostic Clinic. Isolates 23V03A and 23V04A were collected from tomato plants in North Carolina and sent by Dr. Reza Shekasteband, NC State University. After getting the results for Experiment 1 Pathogenicity of Isolates, all the inoculations for the next experiments were performed using isolate 23V03A.

Inoculum Preparation

The inoculum was prepared the same way for all the experiments presented in this study. Cultures for inoculum preparation were prepared by transferring a single mycelial plug (5-7mm in diameter) to the center of potato dextrose agar (PDA) plates. We also tried growing them in potato dextrose broth, but even though the mycelium in all isolates started growing quickly, two of the isolates failed to sporulate within several weeks of growth in broth. Conidia were obtained from cultures by flooding a mixture of 17-, 24-, and 27-day cultures with water, dislodging fungal tissue with sterile flat blade scrapers, and filtering the resulting suspension over cheesecloth into beakers. The concentration of conidia in each suspension was measured using a hemocytometer and each suspension was then diluted with distilled water to bring to a volume of 1 liter with a concentration of 10^6 conidia mL^{-1}. This concentration was different only on Experiment 2 Spore Concentrations where that was the variable being tested.

Root-dipping Inoculation

The seedlings were inoculated 35 days after sowing to target plants with 3-4 true leaves. To prepare the seedlings for inoculation they were taken out of the trays, their roots were washed by rinsing in a bucket with tap water, and the tips of the roots were cut using a small pair of scissors. The plants for each treatment were then placed in a plastic container with enough inoculum to cover their roots. A different plastic container with unused inoculum was poured for each treatment to make sure

every batch was equally concentrated and to avoid mixing the varieties. The plants were kept in the inoculum for 10 minutes and then repotted into 6-inch plastic pots, and immediately watered. The inoculation method in each experiment presented in this study only differed according to the variable that was being tested on each of them.

Foliar Disease Symptoms and Disease Severity Evaluation

Disease ratings for the first experiment were collected starting 10 days after inoculation (DAI), the point at which disease symptoms first appeared for Experiment 1 Pathogenicity of Isolates. For Experiments 2-5, the disease ratings were collected starting 7 DAI, and for Experiment 6 Comparing Susceptibility of Varieties, the disease ratings were collected starting 9 DAI. Ratings were collected at 6 time points, with the final time point at 34 DAI for Experiment 1 Pathogenicity of Isolates, 24 DAI for Experiments 2-5, and 27 DAI for Experiment 6 Comparing Susceptibility of Varieties. A modified disease severity scale of 0-5 was used to rate all the plants, considering visual symptoms (Basay et al., 2011; Boncukçu et al., 2023; Namisy et al., 2019). A score of 0 was assigned for plants that showed no symptoms, 1 for plants that had one or two leaves wilting, 2 for those that had three or more leaves wilting, 3 for those that had at least one leaf yellowing, 4 for those that had at least one leaf that had completely desiccated or fallen off, and 5 for dead plants, as determined by having no more than two or three leaves overall that were also very small and desiccated (Figure 2) (Table 1).

Figure 2

Disease Rating Scale 0–5 Considering Visual Symptoms for Inoculation with Verticillium dahliae on Eggplant

Note. From left to right, 0 = no symptoms, 1 = one or two leaves wilted, 2 = three or four leaves wilted, 3 = minimum one yellow leaf, 4 = minimum one desiccated leaf, and 5 = all leaves desiccated and dead.

Table 1

Disease Rating Scale 0-5

	0	1	2	3	4	5
Symptoms	No symptom	One or two leaves wilted	Three or four leaves wilted	Minimum one yellow leaf	Minimum one desiccated leaf	All leaves desiccated and dead. Only 2-3 leaves total

Calculating the Area Under the Disease Progress Curve

The area under the disease progress curve (AUDPC) was calculated for every plant as a useful way to represent a quantitative summary of disease intensity over the six time points at which data was collected for each experiment Equation AUDPC Formula [1]. The trapezoidal method was used to

represent the time variable and to calculate the average disease severity between each pair of adjacent time points using the "AUDPC" function in the Agricolae package in R (Madden et al., 2017; Mendiburu, 2023) .

$$AUDPC = \sum_{i=1}^{n-1} \frac{y_i + y_{i+1}}{2} \times (t_{i+1} - t_i) \quad [1]$$

Note. Where Yi = severity of the disease at i-th date; ti = time at i-th observation; n = number of observations.

Experimental Design

Experiment 1 Pathogenicity of Isolates

The experiment was designed as a complete factorial design with two factors: plant variety and pathogen isolate/control. Two plant varieties were tested, Rosa Bianca and Black Beauty, and 5 different inoculation treatments including controls: isolate 23V01A, isolate 23V03A, isolate 23V04A, a non-inoculated control in which the roots were cut and another non-inoculated control in which the roots were not cut (Table 2). The factor of cutting the roots in the controls was included to discard the possibility that cutting the roots would cause symptoms that could be confused for Verticillium wilt. We included 10 replicates of each treatment combination. The experimental units were arranged after inoculation in a randomized complete block design.

Table 2

Treatment Combinations Experiment 1 Pathogenicity of Isolates

Cultivar	Isolate 1: 23V01A	Isolate 2: 23V03A	Isolate 3: 23V04A	Control: Water and roots cut	Control: Water and roots not cut
A. Rosa Bianca	S1-A	S2-A	S3-A	WC-A	WNC-A
B. Black Beauty	S1-B	S2-B	S3-B	WC-B	WNC-B

Experiment 2 Spore Concentrations

This experiment was performed to test the effect of spore suspensions with different conidia concentrations on disease development. It was set up in a completely randomized design, with one

independent variable, three treatments and three replicates. The treatments were the different spore concentrations: 10^5, 10^6, and 10^7 conidia mL^{-1}.

Experiment 3 Immersion Times

This experiment was designed to evaluate how the duration of root immersion in the spore suspension affects disease development. It was set up in a completely randomized design, with one independent variable, four treatments and three replicates. The treatments were the different durations during which the plants would be immersed in the spore suspension: 5min., 8min., 10min., and 15min. Spore concentration was kept the same as in Experiment 1 Pathogenicity of Isolates, at 10^6 conidia mL^{-1}.

Experiment 4 Cutting the Roots

Experiment 4 was designed to test the effect of cutting the tips of the roots before immersing them in the spore suspension. This experiment was set up in a completely randomized design, with one independent variable, 2 treatments and 5 replicates per treatment. The two treatments were cutting and not cutting the roots. Spore concentration was also kept the same as in the first experiment, at 10^6 conidia mL^{-1}.

Experiment 5 Dipping or Drenching

Experiment 5 was designed to determine which method of inoculation resulted in greater disease severity: dipping or drenching the roots. This experiment was set up in a completely randomized design, with one independent variable, 2 treatments and 5 replicates per treatment. The treatments were the methods of inoculation, in which one was repeating the dipping method performed in the first experiment, and the second one was simply pouring 25 mL of the 10^6-spore suspension over the soil of each plant.

Experiment 6 Comparing Susceptibility of Varieties

The objective of this final experiment was to compare the susceptibility of different commercial cultivars to the disease. The experiment was set up in a randomized complete block

design, with one independent variable, 24 treatments and 3 replicates. The treatments were the 24 cultivars that were inoculated to compare their susceptibility to Verticillium wilt. Inoculation method was the same as in the first experiment. 12 African eggplant (*S. aethiopicum)* and 12 eggplant (*S. melongena)* cultivars were inoculated (Table 3).

Table 3

Cultivars Inoculated In Experiment 6 Comparing Susceptibility Of Varieties

Variety name	Species	Vendor/Seed Source
Large Liberian Kittley	*S. aethiopicum*	Truelove
Liberian Green Pumpkin Bitterball	*S. aethiopicum*	Truelove
Comprido Verde Claro	*S. aethiopicum*	Thresh
Nigerian Garden Egg	*S. aethiopicum*	Truelove
Striped Garden Egg	*S. aethiopicum*	Truelove
Intore	*S. aethiopicum*	Truelove
Green Pumpkin Eggplant	*S. aethiopicum*	Truelove
Sweet Red Eggplant	*S. aethiopicum*	Seed Savers Exchange
Long Green Bitterball	*S. aethiopicum*	Truelove
Round Green African Eggplant	*S. aethiopicum*	Truelove
Brazilian Orange	*S. aethiopicum*	Smart Seeds Emporium
Morro Redondo	*S. aethiopicum*	Thresh
Orient Express	*S. melongena*	Johnny's
Fairy Tale	*S. melongena*	Johnny's
Hansel	*S. melongena*	Johnny's
Rosa Bianca	*S. melongena*	Burpee
Gretel Hybrid Mini Eggplant	*S. melongena*	Johnny's
Nigral	*S. melongena*	Johnny's
Petch Siam	*S. melongena*	Johnny's
Black Beauty	*S. melongena*	Burpee
Nadia	*S. melongena*	Seedway
Aretussa	*S. melongena*	Johnny's
Thanos	*S. melongena*	Johnny's
White Garden Egg	*S. melongena*	Truelove

Statistical Analysis

To focus on the effects of the experimental variables for Experiment 1 Pathogenicity of Isolates, the two control groups were excluded from the dataset since they consistently received a rating of 0 across all six time points. The variables for all the experiments were statistically analyzed

using analysis of variance (ANOVA), conducted using the “ANOVA” function in R. In Experiment 1 Pathogenicity of Isolates and Experiment 6 Comparing Susceptibility of Varieties that were RCBD, the blocks were considered as fixed effects, and the treatment main effects and their interaction as fixed effects. The “aggregate” function was used to calculate the means of each treatment for all experiments (R Core Team, 2023). Tukey's honestly significant difference test (Tukey's HSD) was performed using the function “HSD.test” from the Agricolae package in R to determine the least significant difference at $p<0.05$, which shows up labeled as minimum significant difference in the console output, and conduct pairwise comparisons for treatments in each experiment (Mendiburu, 2023).

Results and Discussion

Experiment 1 Pathogenicity of Isolates

In the experiment evaluating the pathogenicity of three isolates against two cultivars, there was a significant difference (p<0.001) for the main effect of isolate on AUDPC (Table 4). The results for the means of each treatment ranked from greatest AUDPC to least showed that isolate 23V03A inoculated on Black Beauty resulted in the highest disease overall and isolate 23V01A inoculated on Black Beauty resulted in the lowest disease overall (Table 5) (Figure 3). Lack of symptoms on either of the control treatments demonstrated that cutting the tips of the roots alone did not cause the effects shown in plants inoculated by any of the isolates. Whether the plant genotype was Rosa Bianca (Genotype A), or Black Beauty (Genotype B) did not have an effect on the response variable, showing that the average disease progression did not differ significantly across different genotypes overall.

However, there was a significant difference caused by the interaction between isolate and genotype. The significant interaction between isolate and genotype means that the effect of a specific isolate on disease severity was dependent on which plant genotype it was applied to. Whereas Black Beauty and Rosa Bianca were similarly susceptible to isolates 23V01A and 23V04A, isolate 23V03A caused a significantly greater level of disease on Black Beauty compared to Rosa Bianca (Figure 4). On a study published by Vallad in 2006, they inoculated lettuce using 26 isolates of *Verticillium dahliae*, and there were also interaction effects, so they used the information to classify the isolates as either race 1 or 2. They determined that concern over the durability of resistance to *V. dahliae* in commercial lettuce cultivars is important and will continue to be for developing a resistant cultivar.

Table 4

ANOVA Table for Experiment 1 Pathogenicity of Isolates

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	SD
Isolate	2	4929.25833	2464.62917	28.4987714	3.5577E-09	***
Genotype	1	36.0375	36.0375	0.41670548	0.52131916	
Isolate: Genotype	2	813.925	406.9625	4.70575104	0.01306455	*
Residuals	54	4670.025	86.4819444			

Note. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '; Df = degrees of freedom; Sum sq = sum of squares; Mean sq = mean of square

s; F value = F statistic; Pr(>F) = P-value

Table 5

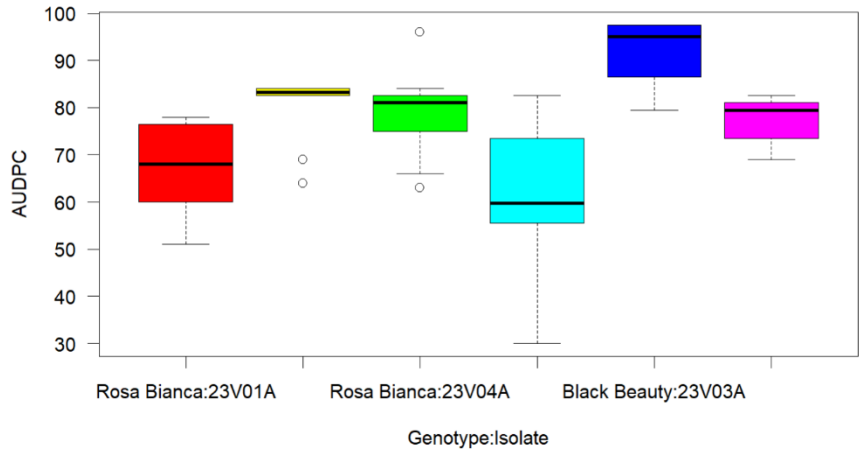
Means of Treatments Experiment 1 Pathogenicity of Isolates

Isolate	Genotype	AUDPC	n	se	group	LSD
23V03A	B	91.65	10	2.94	a	12.28736
23V03A	A	80.05	10	2.94	b	12.28736
23V04A	A	78.75	10	2.94	b	12.28736
23V04A	B	77.65	10	2.94	b	12.28736
23V01A	A	66.9	10	2.94	c	12.28736
23V01A	B	61.05	10	2.94	c	12.28736

Note. Means from greatest to least disease severity on each treatment determined by AUDPC value, where genotype A is Rosa Bianca and genotype B is Black Beauty. S1 = 23V01A, S2 = 23V03A, and S3 = 23V04A. n= repetition; se= standard error; LSD= least significant difference

Figure 3

Mean AUDPC for inoculation of different genotype: isolate treatments

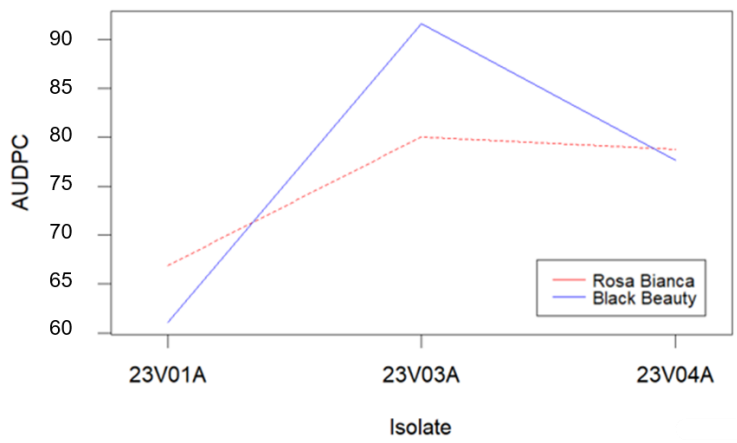


The significant difference found in between isolates confirms what has been described in the literature, in which it has been affirmed that plant pathogenic species such as *V. dahliae* are not a homogenous population, instead, they often show extensive genetic variation that may result in variation in pathogenicity, virulence and host range (McDonald & McDermott, 1993; Woudt, 1995). Many studies describe *V. dahliae* isolates to be host-adapted, rather than host-specific, meaning that they cause disease on a wide range of hosts but produce more severe symptoms on certain hosts, which explains that there was a difference on the virulence between the isolates from both crops used

in this study, tomato and maple tree, and also the interaction between isolate and genotype (Douhan & Johnson, 2001; Subbarao, 1995). In another investigation which inoculated eggplant using isolates collected from artichoke, bell pepper, cabbage, cauliflower, chili pepper, cotton, eggplant, lettuce, mint, potato, strawberry, tomato, and watermelon to test their pathogenicity on eggplant, stunting and wilting were caused by the tomato isolate, as was in this study, and only cabbage, cauliflower, and cotton were nonpathogenic, with the mint isolate being classified as less aggressive (Bhat & Subbarao, 1999).

Figure 4

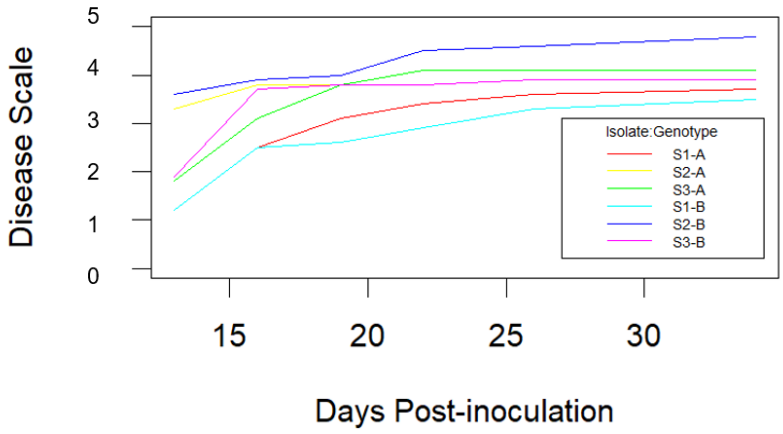
Interaction Plot for Verticillium dahliae Isolates with Eggplant Cultivars



By visualizing the disease progression over time, it was clear that the greatest difference between treatments was apparent at 17-20 DAI, and that there was little further disease progression following 22 DAI (Figure 5).

Figure 5

Disease Progression for Experiment 1 Pathogenicity of Isolates



The higher disease severity caused by isolates 23V03A and 23V04A over 23V01A may be due to the origin of the isolates, since both were isolated from tomato plants, which are more closely related to eggplant than maple trees. Host switching requires genetic change in order for the pathogen to adapt to the new host (Woolhouse et al., 2001), so in this case, infecting eggplant might not have required as much adaptation by the pathogen due to its higher genetic similarity to tomato.

Experiment 2 Spore Concentrations

The results for the spore concentrations experiment did not show significant difference overall, meaning that the rate and extent of disease progression was not affected by the spore concentrations tested (Table 6). Any of these concentrations could be used to inoculate for disease screening new accessions to the germplasm (Table 7) (Figure 6).

Table 6

ANOVA Table for Experiment 2 Spore Concentrations

Df	Sum Sq	Mean Sq	F value	Pr(>F)	SD
2	52.6666667	26.3333333	0.23326772	0.79880131	
6	677.333333	112.888889			

Note. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Df = degrees of freedom; Sum sq = sum of squares; Mean sq = mean of squares; F value = F statistic; Pr(>F) = P-value; SD = significant Difference

Table 7

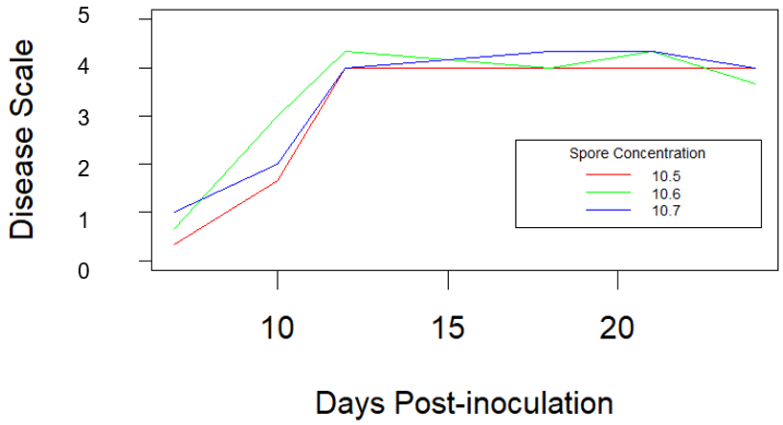
Means of Treatments Experiment 2 Spore Concentrations

trt	audpc	n	se	LSD	group
10.5	56.6666667	3	6.134299	26.61792	a
10.6	62.3333333	3	6.134299	26.61792	a
10.7	61	3	6.134299	26.61792	a

Note. n= repetition; se= standard error; LSD= least significant difference

Figure 6

Disease Progression for Experiment 2 Spore Concentrations



The results for Experiment 2 Spore Concentrations align well with the literature. A study inoculating eggplant with a Verticillium isolate found significant differences in disease response with different incubation temperatures but not with different spore concentrations (Gao, 2004). Gao concluded that the study suggests that if environmental conditions are kept the same for all the plants, slight difference in inoculum concentration does not cause a significant difference. The plants in this study were all kept in the same greenhouse environment so the transportation of fungal conidia and production of bud spores in the xylem should be as active across all plants. Other studies, for example, have performed successful inoculations using 3 x 10^6 conidia mL^{-1} (Alconero R. et al., 1988) and 1 x 10^7 conidia mL^{-1}(COLAK ATES et al., 2019)

Experiment 3 Immersion Times

The results for the immersion times experiment did not show significant difference overall, meaning that 5 minutes is the minimum time that the plants could be immersed in the suspension to achieve severe disease symptoms among the treatments evaluated with isolate 23V03A (Table 8) (Table 9) (Figure 7).

Table 8

ANOVA Table for Experiment 3 Immersion Times

Df	Sum Sq	Mean Sq	F value	Pr(>F)
3	97.4166667	32.4722222	1.57441077	0.26982531
8	165	20.625		

Note. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; Df = degrees of freedom; Sum sq = sum of squares; Mean sq = mean of squares; F value = F statistic; Pr(>F) = P-value; SD = significant difference

Table 9

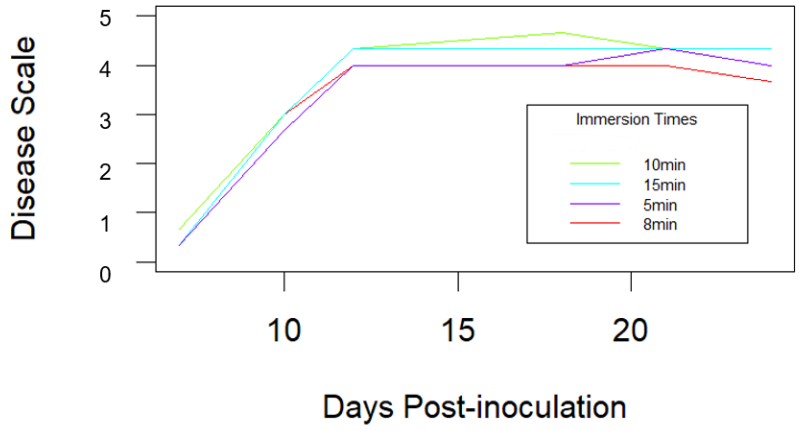
Means of Treatments for Experiment 3 Immersion Times

Treatment	AUDPC	n	se	LSD	group
5min	64.3333333	3	2.622022	11.87464	a
8min	60.1666667	3	2.622022	11.87464	a
10min	59.5	3	2.622022	11.87464	a
15min	66.3333333	3	2.622022	11.87464	a

Note. n= repetition; se= standard error; LSD= least significant difference

Figure 7

Disease Progression for Experiment 3 Immersion Times



The lack of significant differences in this experiment was expected as a possible result considering how much this parameter varies in the literature. A study was conducted evaluating the effect of seedling size and length of time of dipping, comparing 10 minutes and 1 minute among two cultivars, and it was determined that extending the duration of root dipping in a Verticillium wilt resistance test heightens the severity of disease symptoms and decreases the variation in disease severity within the treatment, however, the author also called for the need of more time points to be included in future studies to find a minimum immersion time required for severe and uniform screening (Gao, 2004). Other studies that have detailed descriptions of their inoculation procedures mention as little as 2min, 4-5min, and as high as 15min. as evaluated in this experiment (Boncukçu et al., 2023; Çolak Ates, 2020; J. Liu et al., 2015; Xu et al., 2013).

Experiment 4 Cutting the Roots

The results for Experiment 4, testing the effect of cutting the roots on disease severity, did not show significant difference overall (Table 10) (Table 11). The plants with and without their roots cut followed almost the same disease progression over the six time points at which data was taken (Figure 8).

Table 10

ANOVA Table for Experiment 4 Cutting the Roots

Df	Sum Sq	Mean Sq	F value	Pr(>F)
1	4.225	4.225	0.14772727	0.71073191
8	228.8	28.6		

Note. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 Df = degrees of freedom; Sum sq = sum of squares; Mean sq = mean of squares; F value = F statistic; Pr(>F) = P-value; SD = significant Difference

Table 11

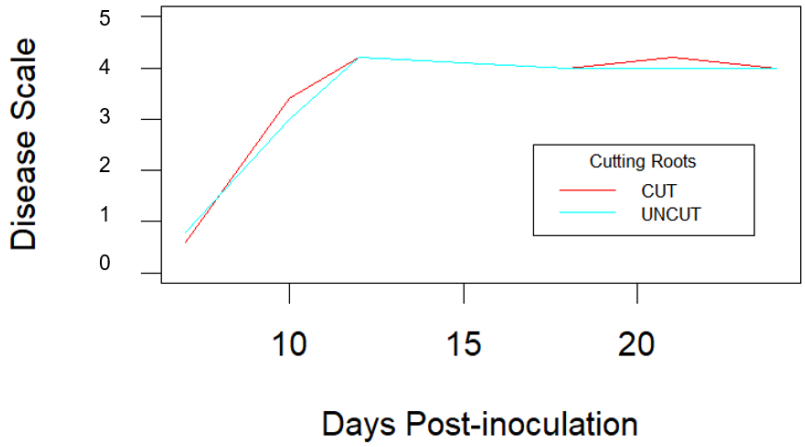
Means of Treatments for Experiment 4 Cutting the Roots

trt	audpc	n	se	LSD	group
CUT	62.8	5	2.391652	7.799614	a
UNCUT	61.5	5	2.391652	7.799614	a

Note. n= repetition; se= standard error; LSD= least significant difference

Figure 8

Disease Progression for Experiment 4 Cutting Roots



The results for this experiment help make a useful conclusion as to the effect of cutting the tips of the roots before inoculating with *Verticillium dahliae*, as there seems to be no consensus on whether it is necessary or not. Some authors do not specify in their methodologies having cut the roots (Gao, 2004; Xu et al., 2013; Yu et al., 2016), whereas others do (Alconero R. et al., 1988; Boncukçu et al., 2023; Çolak Ates, 2020; J. Liu et al., 2015). Removing this step from a protocol can save more time as more plants are screened.

Experiment 5 Dipping or Drenching

The experiment where dipping and drenching were compared as inoculation methods showed significant difference in AUDPC ($p < 0.05$) between the two treatments, providing strong evidence that dipping the roots results in greater disease severity than drenching (Table 12) (Table 13). For the first 15 days, the drenching method showed less disease. By around day eighteen, the severity of disease caught up with that of the plants whose roots were dipped (Figure 9).

Table 12

ANOVA Table for Experiment 5 Dipping or Drenching

Df	Sum Sq	Mean Sq	F value	Pr(>F)	SD
1	342.225	342.225	7.64108289	0.02451332	*
8	358.3	44.7875			

Note. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 Df = degrees of freedom; Sum sq = sum of squares; Mean sq = mean of squares; F value = F statistic; Pr(>F) = P-value; SD = significant Difference

Table 13

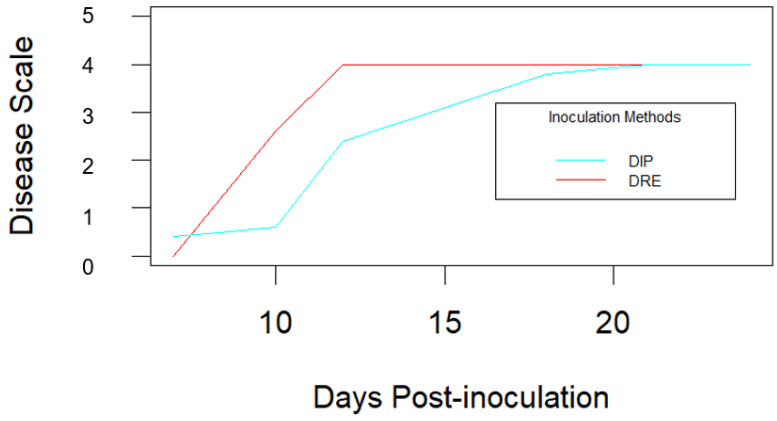
Means of Treatments for Experiment 5 Dipping or Drenching

trt	AUDPC	n	se	group	LSD
DIP	58.5	5	2.992908	a	9.76042
DRE	46.8	5	2.992908	b	9.76042

Note. n= repetition; se= standard error; LSD= least significant difference

Figure 9

Disease Progression for Experiment 4 Cutting Roots



The method of dipping the roots is the one used most extensively in literature and was expected to pose some advantages in disease severity or uniformity. However, the time saved by simply drenching the roots, even if it required a higher volume of inoculum, warranted confirmation to determine if the difference was significant and justified the more complex procedure. Although the disease severity caused by the drenching method eventually caught up with that of dipping, the initial lag in manifestation of symptoms would elongate the time screened plants would spend at the

greenhouse, which also entails more resources spent in maintaining the plants. A similar study performed using Ralstonia *solanacearum* isolate to inoculate eggplant rootstocks had similar results. The authors cut 1 cm of the root tips and poured on the soil 10 mL of inoculum at a concentration of 3.8×10^4 CFU ml−1 on the first day of inoculation. The next day they poured another 15 mL, and compared this method to dipping the roots for 2min. after cutting the tips, but still found root-dipping to cause more severe symptoms in less days, settling it as the more efficient method (Kumbar et al., 2021). Another study performed in Korea also compared methods of inoculation of *V. dahliae* in eggplant including dipping for 10 minutes and drenching with 15 mL of inoculum, and inferred that dipping methods show higher incidence levels due to the direct contact with the roots, whereas the drenching method may reflect difficulties in fungal invasion and colonization (Kim, 2000).

Experiment 6 Comparing Susceptibility of Cultivars

There was no significant difference in AUDPC among any of the 24 cultivars that were inoculated for Experiment 2.5 (Table 14). All the cultivars showed a high susceptibility to Verticillium wilt (Table 15) (Figure 10).

Table 14

ANOVA Table for Experiment 6 Comparing Susceptibility of Cultivars

Df	Sum Sq	Mean Sq	F value	Pr(>F)
23	1752.46875	76.1942935	1.50879789	0.11412441
48	2424	50.5		

Note. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 Df = degrees of freedom; Sum sq = sum of squares; Mean sq = mean of squares; F value = F statistic; Pr(>F) = P-value; SD = significant
Difference

Table 15

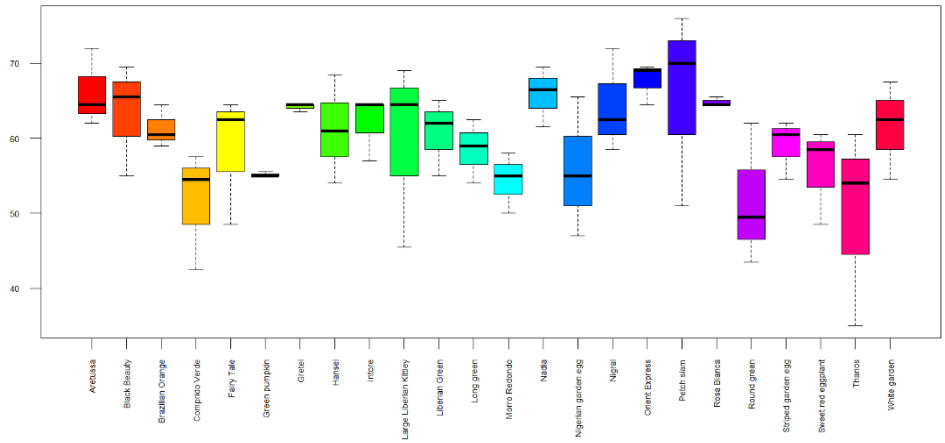
Means of Treatments for Experiment 6 Comparing Susceptibility of Cultivars

Treatments	AUDPC	n	se	LSD	Group
Thanos	49.8333333	3	4.1	22.36463	a
Comprido Verde Claro	51.5	3	4.1	22.36463	a
Round Green African Eggplant	51.6666667	3	4.1	22.36463	a
Morro Redondo	54.3333333	3	4.1	22.36463	a
Green Pumpkin Eggplant	55.1666667	3	4.1	22.36463	a
Nigerian Garden Egg	55.8333333	3	4.1	22.36463	a
Sweet Red Eggplant	55.8333333	3	4.1	22.36463	a
Fairy Tale	58.5	3	4.1	22.36463	a
Long Green Bitterball	58.5	3	4.1	22.36463	a
Striped Garden Egg	59	3	4.1	22.36463	a
Large Liberian Kittley	59.6666667	3	4.1	22.36463	a
Liberian Green Pumpkin Bitterball	60.6666667	3	4.1	22.36463	a
Hansel	61.1666667	3	4.1	22.36463	a
Brazilian Orange	61.3333333	3	4.1	22.36463	a
White Garden Egg	61.5	3	4.1	22.36463	a
Intore	62	3	4.1	22.36463	a
Black Beauty	63.3333333	3	4.1	22.36463	a
Gretel Hybrid Mini Eggplant	64.1666667	3	4.1	22.36463	a
Nigral	64.3333333	3	4.1	22.36463	a
Rosa Bianca	64.8333333	3	4.1	22.36463	a
Petch Siam	65.6666667	3	4.1	22.36463	a
Nadia	65.8333333	3	4.1	22.36463	a
Aretussa	66.1666667	3	4.1	22.36463	a
Orient Express	67.6666667	3	4.1	22.36463	a

Note. Means of disease severity of cultivars are ordered from least to greatest. n= repetition; se= standard error; LSD= least significant difference

Figure 10

Boxplot of Means Experiment 6 Comparing Susceptibility of Cultivars



No difference was detected between the *S. aethiopicum* cultivars and the *S. melongena* cultivars neither in terms of virulence nor manifestation of symptoms. Although *S. aethiopicum* had been used as rootstock for commercial eggplant productions throughout Asia, the first report of disease caused by V. dahliae on *S. aethiopicum* in Washington was published (Johnson et al., 2013). A study based in Italy also found *S. aethiopicum* to be susceptible to a *V. dahliae* isolate and presenting the same symptoms known for the infection in *S. melongena* (Camele et al., 2006). Most recently, a study performed in 2015 also did not find a decrease in the disease severity in eggplants grafted with *S. aethipicum* rootstocks when compared to the non-grafted control (Miles et al., 2015). Given the small sample size for this experiment, it cannot be determined whether any of these cultivars could become tolerant. In addition, it was observed that despite having the same symptoms, some cultivars grew their foliage more than others, but this was not recorded as part of the disease scale but could be considered for a new experiment. The results reaffirm the need to work on developing eggplant cultivars with resistance to this devastating disease.

Conclusions

It was proven in Experiment 1 Pathogenicity of Isolates that all the *Verticillium dahliae* isolates evaluated are highly pathogenic on eggplant. Isolate 23V03A was the most virulent overall and should be used to inoculate additional germplasm in the Cornell University Eggplant Breeding Program. Although both Black Beauty and Rosa Bianca proved to be highly susceptible to strain 23V03A and the rest of the isolates, we conclude that Black Beauty inoculated by isolate 23V03A should be used as reference of susceptibility, as it showed the highest mean of AUDPC over time.

There was an interaction between isolate and genotype, where Black Beauty was more susceptible than Rosa Bianca for isolate 23V03A, but not for the other isolates.

It was found in Experiment 6 Comparing Susceptibility of Cultivars that there is no significant difference in the severity of symptoms manifested by the cultivars inoculated. All the cultivars inoculated were highly susceptible to Verticillium wilt.

Recommendations

To test for several strains of *Verticillium dahliae* once resistance is found to ensure horizontal resistance.

To compare other disease scales in accuracy and ease of rating, perhaps putting into consideration the size of the foliage.

To perform a new experiment combining all the parameters evaluated to test whether there is interaction between them: spore concentration, immersion time and cutting the tips of the roots before dipping them.

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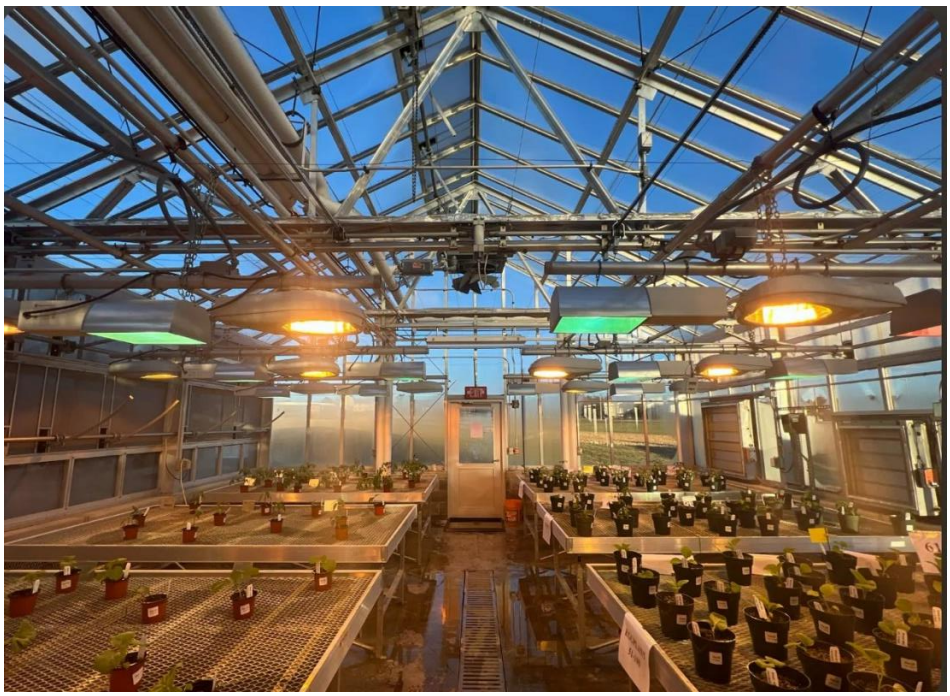
Appendixes

Appendix A

Room 101C



Appendix B

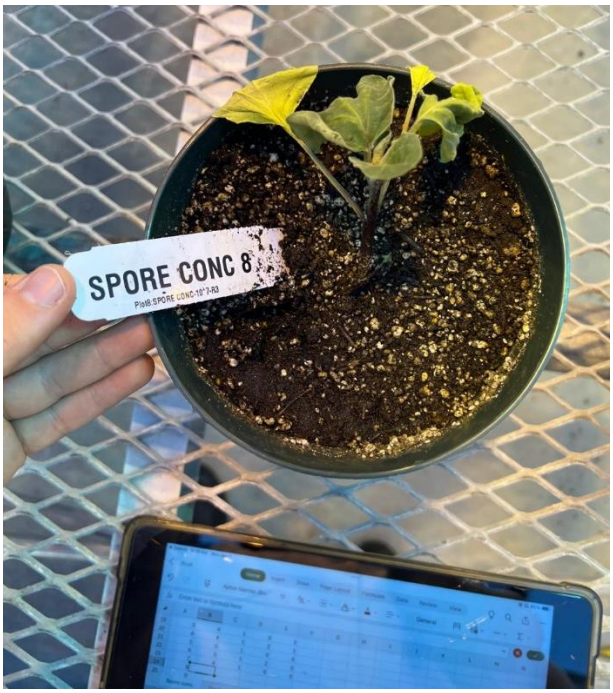
Experiment 1 Pathogenicity of Isolates Inoculation

Appendix C

Eggplant at 3-4 Leaf Stage

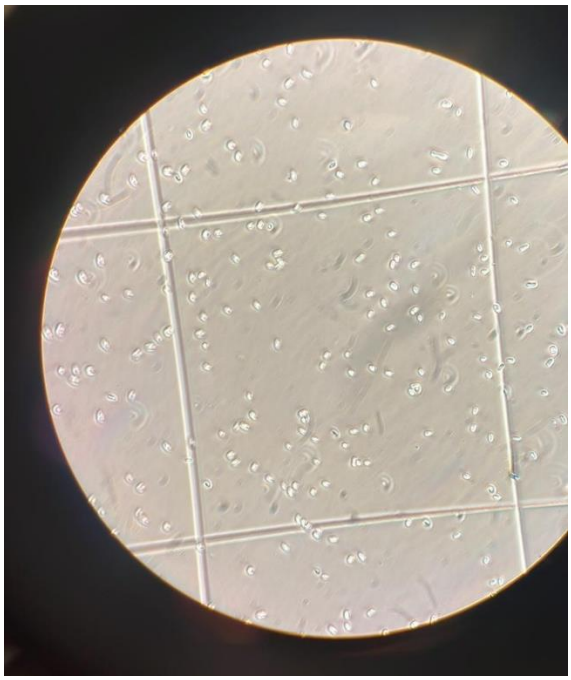
Appendix D

Experiment 2 Spore Concentrations Experimental Unit 8



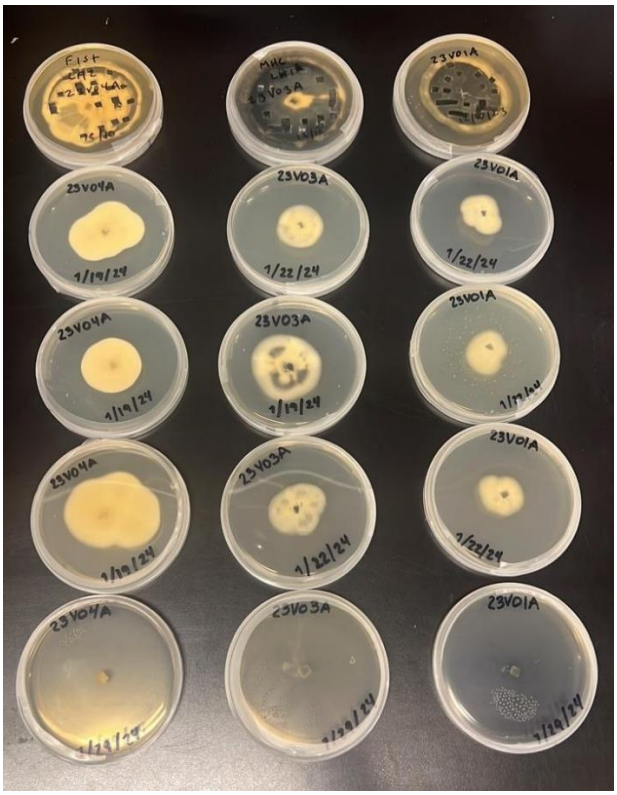
Appendix E

Verticillium dahliae spores view under the microscope



Appendix F

PDA Plates Inoculated with Verticillium Isolates



Appendix G

Actual Protocol completed

Internal protocol for artificial inoculation of Verticillium dahliae into eggplant.

Ivanna Castillo, April 15, 2024

Application

To cause Verticillium wilt in eggplants in a controlled, efficient way, to evaluate for resistance or tolerance traits in their genome. This protocol is designed to inoculate 40 plants. Amounts can be increased accordingly to meet demand, and at the end of the document is a table with examples of number of materials for different quantities of plants to be inoculated.

- **Preparing the media and plates**

Materials

-39g of PDA

-40 standard sized Petri dishes

-1L of distilled water

-1L flask

-1L beaker

-graduated cylinder

-Lab scoop

-precision balance

-weighing boat

1. Prepare 1L of PDA, which is enough to fill about 40 standard sized petri dishes.

The PDA powder is measured in a weighing boat over any precision balance. Make sure that the balance is calibrated and to tare the weighing boat before measuring the PDA. After measuring the PDA, we want to dissolve it well in distilled water. It is easier to dissolve the powder in a small amount of the distilled water with a lab scoop in a beaker, and once it is well dissolved, passing it onto the flask with the rest of the distilled water. Next, the opening of the flask must be covered with aluminum foil but leaving a small gap to allow for gas to exit. After putting the flask in a container that can be autoclaved, we put in the autoclave and set it for the liquid cycle. *

2. To pour the plates, turn on the laminar flow chamber and wipe all the flat surfaces (except the one where the air comes from), using tissue and 70% ethanol. Everything in the laminar flow chamber should be kept at least three fingers inward, away from the edge to make sure they are being kept sterile. We find it to be easier to stack the plates in groups of three, and pour the one on the bottom first, then the second, and finally the one on the top. Once the liquid

   bounces back from the edge of the plate it's enough. After pouring all the plates, you can turn off the hood and leave them to thicken. Once the media has gelatinized, they should be placed in the same bag that they came in and the bag sealed with tape, labeled with the type of media, the date they were poured and your initials.

*If the media must be prepared days ahead because of time restraints, it can be left in the refrigerator after it has cooled down from the autoclave, still covered by aluminum foil, and be returned to liquid state the day that it will be poured by boiling it in the water bath at 100°C for about an hour. You can check if it has melted completely by lifting it using the autoclave gloves and checking that it is clear.

- **Fungal culture transfer**

Materials

-17 fresh PDA petri dishes

-tissues

-70% ethanol spray

-super permanent ink marker

-alcohol burner lamp

-matches

-scalpel

-forceps

-95% ethanol

-glass jar to dunk scalpel

-parafilm

-agar punch*

1. The plates must be inoculated 28 days before scraping them to inoculate the plants. At this point each plate will have about 10^8 spores. Start preparing the plates by labeling them with a super permanent ink marker. They should be named on the edges of the plate, not too big so that they don't cover too much surface, and on the side that contains the agar, not the lid, to make sure they don't get confused.
2. Make sure to leave at least 2 plates to be used as mother cultures for the next time you inoculate, that way you can use more of the leading edge instead of the middle, which is the part of the fungus in the plate that has more active growth, and to make sure you can put uniform sizes of plugs across the plates.
3. To make 1 L of inoculum you need ten plates at least. Make 5 extra in case they get contaminated, or they produce less spores than expected.
4. To inoculate the plates, prepare the laminar flow chamber the same way as when pouring the plates, gather the materials and place the glass jar with the 95% ethanol to the left of the burner to avoid dunking it back after putting it over the fire, 95% ethanol is highly flammable and putting a flame on it could cause an accident. Sterilize the scalpel before using it, and any time after it placed on the table, also after you finish inoculating the plates. It is not necessary to sterilize it in between plates, so long as you are still using the same fungus. After putting the scalpel over the flame, dunk it in the agar that does not have fungi a few times to make sure it is no longer hot so that it does not kill the piece of fungus you will cut.
5. To inoculate a plate, cut a square of about 6mm in diagonal and put it in the middle of the plate. You may use the forceps to take it out of the plate if necessary.
6. It is best to wrap the edge of the plates with parafilm so that they do not dry too much, but it is not necessary. Do make sure to wrap the ones to be used as mother cultures as these may be left unused for a longer time.

*An agar punch is not necessary but could be used alternatively to cutting squares, as that would ensure a more uniform size and make the procedure slightly quicker.

- **Inoculum preparation**

Materials

-1L beaker

-cheesecloth

-rubber band

-sterile flat blade scraper

-1L glass container with cap

-hemocytometer

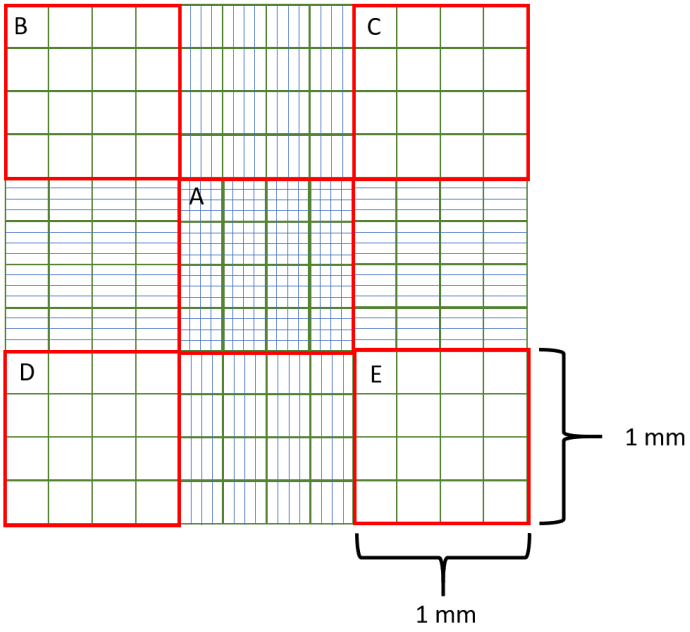
-100P micropipette

-wash bottle with distilled water

-funnel

1. Place the beaker over the counter and cover the opening with cheesecloth, held by a rubber band. Using the wash bottle, splash some water over a plate and scrape the mycelium from the agar and pour the liquid into the beaker. Repeat for all the plates.
2. Take a sample of 100 µL using the micropipette and put it on the hemocytometer. Shake the liquid a bit before doing this to make sure the spores had not settled in the bottom of the beaker.
3. Count the spores in five large squares of the hemocytometer, the four on each corner and the one in the middle. Record the number of spores for each large square and calculate the average. It is best to use Excel to record this to automate calculations and reduce errors in calculations. You may use the spreadsheet I created to do this; it is on the lab's Google Folder.

Figure 1: Large squares in hemocytometer



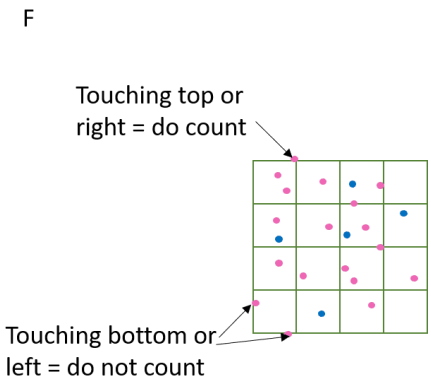
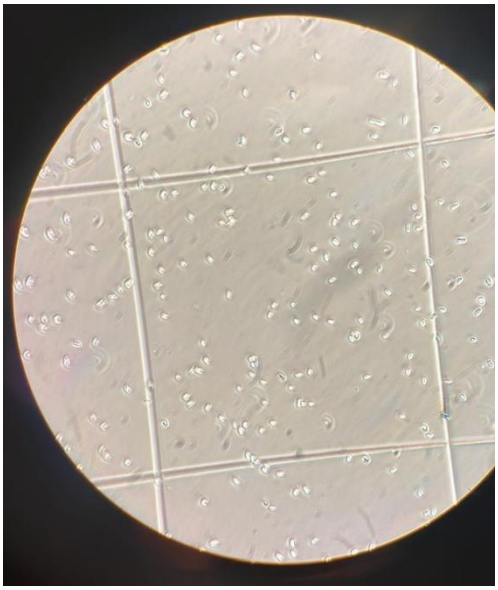


Figure 2: Verticillium spores under the microscope



4. Use this formula to calculate the concentration:

$$\frac{(1000\ x\ Average\ of\ spores\ per\ large\ square)\ /\ 0.1}{\text{Total number of spores in 1 mL}}$$

Example:

Large square 1: 126
Large square 2: 136
Large square 3: 109

Large square 4: 132
Large square 5: 122

Average: 125

$$\frac{1000\ x\ 125}{0.1} = \ 1250000\ spores\ per\ mL\ or\ 1.25E + 06\ concentration$$

For the protocol we have chosen to use a concentration of 10^6 because experiments have shown that you can cause severe disease symptoms with it by dipping as little as five minutes. A concentration of 10^5 takes a bit longer to show symptoms and 10^7 is just too time-consuming when preparing the inoculum because it means that you'd have to count an average of 1000 spores per large square, and it's not worth it because we get good enough results with 10^6.

Tip: To save time, before I start counting, I use a paper to estimate the concentration of the inoculum. Just put the paper with some writing in permanent marker behind the liquid. If the writing is clear as water it is probably at 10^5, so avoid adding too much water before it gets too clear, and if the writing looks very blurry, it is too concentrated. Add water as necessary to make it be not too blurry, but don't make it too clear either because if you don't have extra plates you will not be able to increase the concentration. This will become easier to distinguish with practice but once you get it, it will save a lot of time of diluting:

Figure 3: Comparison of how clear the inoculum looks at different concentrations

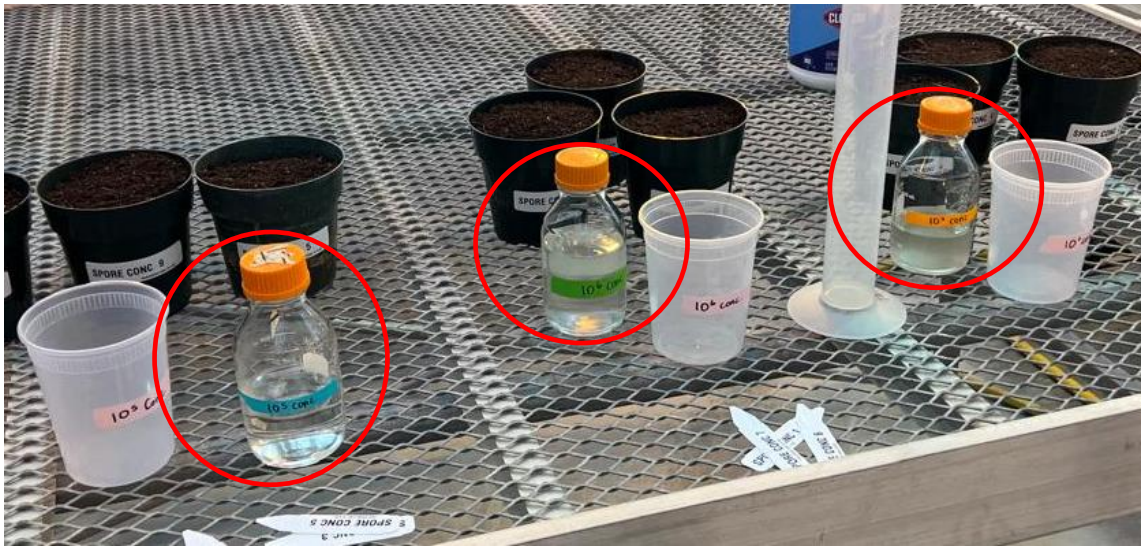


Figure 4: Rough look at how blurry the inoculum should look to count the spores.

5. Once you've reached the targeted concentration of 10^6, get the glass container, label it with tape and pour the inoculum from the beaker to the container using a funnel and seal it well.

- **Root-dipping inoculation**

Materials

-4 plastic containers

-small pair of scissors

-bucket

-45 6-inch pots

-1 L of inoculum

-graduated cylinder

-gloves

-40 plants with 3-4 true leaves

Figure 5: How the plants should look like for inoculation, 3-4 leaves

1. How many days before the plants should be sown will depend on the cultivars. For commercial cultivars used in the US, like Rosa Bianca and Black Beauty, 35 days before has been good. For one of our experiments in which we tried 12 African eggplant cultivars and 12 commercial cultivars, I inoculated at 22 days, and they were too small. When they are too small they are harder to work with because the leaves fall into the inoculum, they are more delicate and that makes them harder to handle. Additionally, on this case they started showing symptoms a bit later in comparison.
2. Making the labels and organizing the greenhouse.

   I would recommend making two types of labels: stickers and stakes. The stickers are easier to look at from far away, and the plot number should be bold, big, and visible from far away to make your life easier when collecting the data. Details for the treatments should be a bit smaller, not only so that the plot number stands out, but also to prevent bias when rating the plants. The stakes are useful especially for taking images of the plants. You can move the stake to either side, and also put it face up so that you can take pictures of the plant from above and still look at the label. The plots should be put in order in the greenhouse and randomized within the order.

   Example:

Plot 1: Var. Pink	Plot 6: Blue
Plot 2: Var. Red	Plot 5: Yellow
Plot 3: Var. Green	Plot 4: Orange

Plot 7: Var. Red	Plot 12: Pink
Plot 8: Var. Yellow	Plot 11: Orange
Plot 9: Var. Green	Plot 10: Blue

3. We prepare the pots with Cornell soil mix. I would recommend preparing 5 more than the plants you will actually inoculate in case pots fall, so that you can have some soil to fill in the pot, and also to leave a few control plants.
4. Put all the pots that are of the same cultivars together for now, we will distribute in a random order when we are done. Paste the sticker labels but don't put the stakes yet.
5. To inoculate a plant, take it out of the tray, dunk its roots in a bucket with tap water and remove the soil.
6. Trimming the roots is not a crucial step to show disease, but it can be done to show the most symptoms when comparing cultivars that seem to have resistance.
7. Dunk 10 plants in a plastic container with enough inoculum to cover the roots, which is about 250mL. The containers I prefer are the ones that are tall so that the plants stand upright. In the case of inoculating just two or three plants together, plastic cups would suffice. Plastic containers are great because they won't break when moving them from the lab to the greenhouse and are cheap so they can be discarded.

Figure 6:

8. One of our experiments has shown that dipping for as little as five minutes is enough to show severe disease symptoms with a 10^6 concentration.
9. After the 5 minutes are up, just repot the plants and when you are done put the stake labels.
10. Distribute the plots in order, having them evenly spaced throughout the greenhouse.

- **Disease scale**

A score of 0 was assigned for plants that showed no symptoms, 1 for plants that had one or two leaves wilting, 2 for those that had three or more leaves wilting, 3 for those that had at least one leaf yellowing, 4 for those that had at least one leaf that had completely desiccated or fallen off, and 5 for dead plants, as determined by having no more than two or three leaves overall that were also very small and desiccated.

Figure 7:

Note. From left to right, 0 = no symptoms, 1 = one or two leaves wilted, 2 = three or four leaves wilted, 3 = minimum one yellow leaf, 4 = minimum one desiccated leaf, and 5 = all leaves desiccated and dead.

Table 1:
Disease Rating Scale 0-5

	0	1	2	3	4	5
Symptoms	No symptom	One or two leaves wilted	Three or four leaves wilted	Minimum one yellow leaf	Minimum one desiccated leaf	All leaves desiccated and dead. Only 2-3 leaves total