Title: Characterization of Fusarium Dry Root Rot in California Citrus Nurseries

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Introduction: Dry Root Rot (DRR), caused by *Fusarium solani*, represents a significant threat to citrus trees in California. The severity of this disease is exacerbated under conditions of tree stress or co-infection with other pathogens, such as *Phytophthora* species. Symptoms of DRR include stunted growth, dry and brittle roots, discoloration, and ultimately lead to leaf wilting and premature fruit drop, primarily due to compromised water and nutrient uptake. The symptoms of DRR are not only detrimental to the individual plants but also can lead to broader ecological and economic repercussions, including reduced yield and quality of citrus fruit. The importance of addressing DRR cannot be overstated. Citrus production is a vital component of California's agricultural economy, contributing billions of dollars annually. The spread of DRR could lead to significant losses, affecting not only farmers but also related industries such as distribution, retail, and food processing. With climate change and increased occurrences of tree stressors, the urgency to manage and mitigate the impacts of DRR is more critical than ever. Nurseries play a pivotal role in the prevention and management of DRR. They are often the first point of contact for new citrus trees entering the agricultural ecosystem. Ensuring that these trees are free from pathogens and are robust enough to withstand environmental stresses is crucial for maintaining healthy orchards. Nurseries can serve as a frontline defense, implementing best practices in plant health management and disease prevention. By focusing on the health of nursery stock, we can significantly reduce the incidence of DRR in established groves and prevent its spread to new areas. To effectively address the issue of Dry Root Rot, a comprehensive strategy is crucial. Conduct extensive research on the biology and characterization of *Fusarium solani* and its interaction with other pathogens. Regular monitoring of citrus nurseries and orchards for signs of DRR and co-infection will help identify emerging threats early. It is essential to implement and promote best practices in nursery management, including rigorous sanitation protocols, the use of disease-resistant rootstocks, and proper irrigation techniques to minimize tree stress. By adopting this comprehensive approach, we can

enhance the resilience of citrus trees against Dry Root Rot and ensure the sustainability of California's citrus industry.

Objectives: The primary objective of this study is to assess the prevalence of Dry Root Rot (DRR) caused by *Fusarium solani* and *Phytophthora* Root Rot in citrus trees across various nurseries in California. This includes isolating and identifying fungal pathogens from root and soil samples, determining the species-level classification of the isolates through morphological characterization and DNA analysis. The second objective is to test multiple water types associated with a recycled irrigation system at citrus nurseries for presence of *Fusarium* and other soil-borne pathogens like oomycete plant pathogens. Our third objective is to assess the susceptibility of currently used lemon rootstocks such as Carrizo, Trifoliate and C-35 to *Fusarium* dry root rot and to *Phytophthora* root rot diseases. Ultimately, this study aims to provide valuable insights that will inform effective management strategies to mitigate the impact of these pathogens on citrus health. In this progress report we will provide a comprehensive overview of our project's current status, key achievements, and future directions.

Materials and methods:

- **Soil, Root and Water sampling:**

Sampling was conducted through five nurseries in the Southern, Central and West Coast of California. Due to confidentiality agreements, we are unable to disclose the names and addresses of the nurseries from which samples were collected. From each nursery we got samples from water, soil and root. From nursery#1 we got 20 bags of soil/root and 2 water samples. From nursery#2 we got 33 bags of soil/root and 2 water samples. From nursery#3 we got 44 bags of soil/root and 2 water samples. From nursery#4 we got 45 bags of soil/root and no water samples. From nursery#5 we got 36 bags of soil/root and no water samples. Samples were kept in a cooler with ice and then transferred to the lab immediately for processing.

Fungal Isolation:

***Phytophthora* isolation from soil samples:**

To isolate *Phytophthora*, we employed the pear baiting method using D'Anjou pears. We began by washing pears with detergent and then surface sterilized them by 70% EtOH. Then weighing approximately 50 grams of soil and adding it to a Ziploc bag containing 250 ml of sterilized water and the pears. The bags were incubated in boxes at 25°C, ensuring they were open for air exchange. After 3 to 5 days, lesions developed on the pears. We excised small pieces from these lesions and placed them onto PARPH-V8 selective media. After 2 to 3 days, colonies had grown, which we then purified by extracting plugs using a cork borer and transferring them to regular V8 media.

Isolation from water samples:

Isolation of *Phytophthora spp* from water samples, followed by "Detection of Phytophthora spp."the same protocol as we used for soil samples. We began by washing pears with detergent

and then surface sterilized them by 70% EtOH. Then add ~250 ml of sterilized water into a Ziploc bag containing 250 ml and put three pears for each water sample in a separate box. The boxes were incubated at 25°C, ensuring they were open for air exchange. So far, we haven't retrieved any pathogen from water samples, but we lyophilized the water sample for further DNA extraction. Lyophilization, also known as freeze-drying, is a process used to remove moisture from a material, typically food or pharmaceuticals, while preserving its structure and properties. The process involves freezing the material, reducing the pressure, and then removing the ice through sublimation. This technique helps to extend the shelf life of products by preventing microbial growth and chemical reactions that require moisture. Lyophilized products can be rehydrated easily, making them convenient for storage and transport. Lyophilization process was done by Dr. Philippe Rolshauson's lab using a freeze dryer machine.

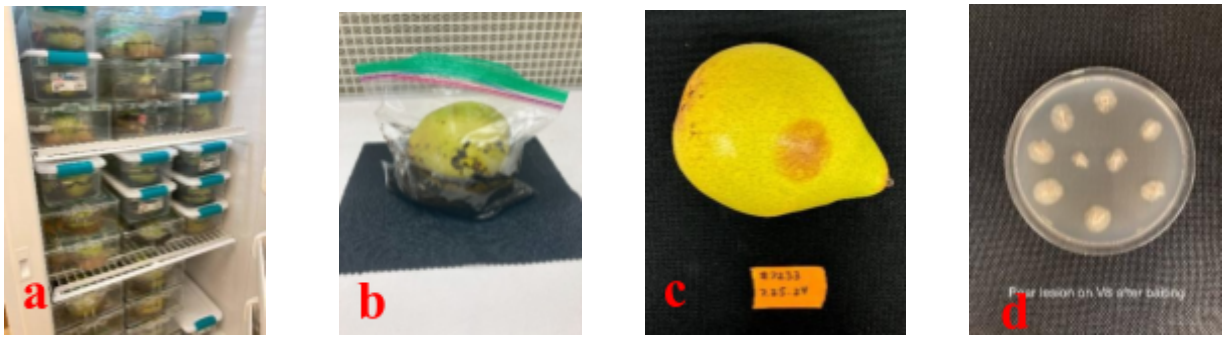


Figure 1. Pear baiting method for *Phytophthora* isolation out of soil samples **(a)** Boxes of pears with soil and water kept inside incubator **(b)** One bag of pear containing soil and water **(c)** Lesion appeared on pear following 3-5 days incubation **(d)** Tiny pieces of lesion on PARPH-V8 selective media after successful pear baiting

***Fusarium spp* isolation from soil and root**:

To isolate *Fusarium spp.* out of root samples, the procedure based on that described in Murugan et al began by soaking approximately twenty small pieces of roots in sterilized deionized (DI) water. Following this, the roots were treated with 70% ethanol for about 2 minutes, then immersed in 10% bleach for another 2 minutes, and finally rinsed with sterile DI water. The disinfected root pieces were placed on a KimWipe to dry in the fume hood. Subsequently, the dried root pieces were plated on Komada's media, a selective medium for *Fusarium spp.*, and incubated in the dark at 25°C. After 3 to 5 days, when the colonies had sufficiently grown, fungal colonies exhibiting the characteristic morphology of *Fusarium spp*. were purified using a cork borer to extract small pieces of fungal mycelium, which were then subcultured onto new PDA plates.

To isolate *Fusarium spp.* out of soil samples we collected four samples from each soil bag and performed two-fold serial dilutions (10^1, 10^2). We then plated the 10^2 dilution soil on Komada's media, a selective medium for *Fusarium spp.*, and incubated in the dark at 25°C. After 3 to 5 days, when the colonies had sufficiently grown, fungal colonies exhibiting the characteristic

morphology of *Fusarium spp*. were purified using a cork borer to extract small pieces of fungal mycelium, which were then subcultured onto new PDA plates. The purified isolates were stored in 20% glycerol at -80°C for future use.

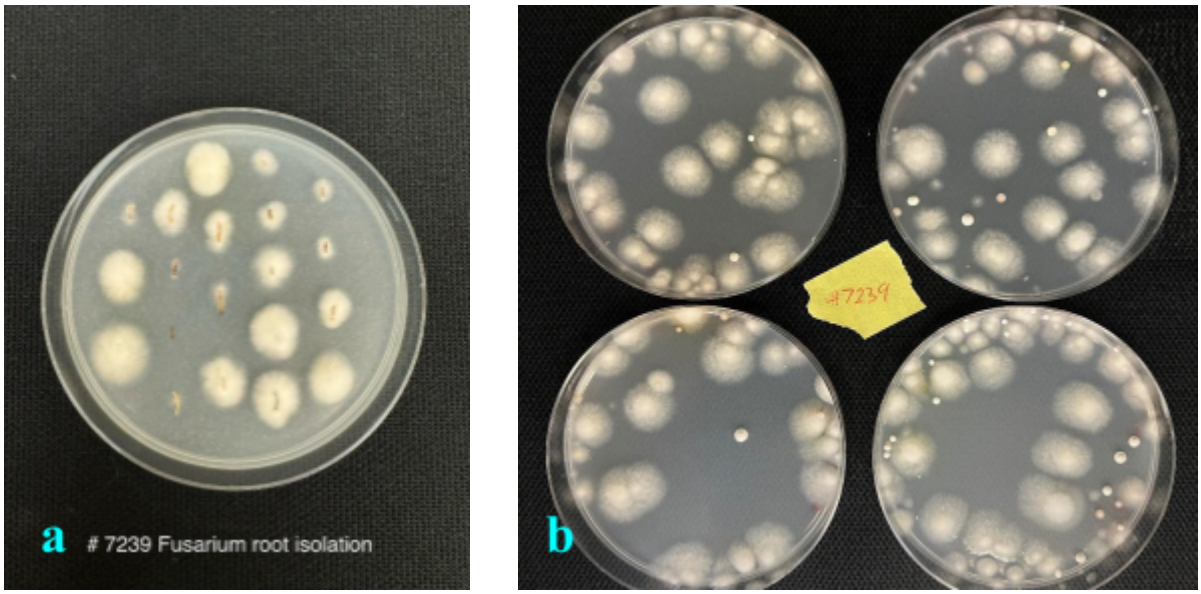


Figure 2. Isolation of *Fusarium spp* from root and soil samples **(a)** fungal cultures grown from root on Komada's media after 2–3 days at 25 °C **(b)** fungal cultures grown from soil on Komada's media after 2–3 days at 25 °

- ***Fusarium spp.*** **DNA Extraction & PCR:**
  After examining morphology for randomly 17 selected isolates from four different nurseries we prepared them for DNA extraction. To extract total DNA, all isolates were incubated on potato dextrose agar (PDA) covered with sterile cellophane sheets before inoculation, to facilitate collection of the mycelium. After 7 days Mycelia were harvested and crushed by mortars and pestles using liquid nitrogen. Later ~250 mg of the finely powdered mycelium was used for DNA extraction using Qiagen, plant mini kit. Later the quality of DNA checked on 1% agarose gel stained with sybr™ safe DNA gel stain and and visualized in Bio-Rad GelDoc Imaging System (Bio-Rad, USA) (Figure 6)
  DNA samples were stored at − 20 °C for further use.
  Genomic DNAs of *Fusarium* isolates were PCR amplified ITS region using the primers ITS1 and ITS4, *F. oxysporum* using species specific primer pairs FOF1/FOR1 and *F.solani* using the primers TEF-Fs4f and TEF-Fs4r to amplify the fungal translation elongation factor 1-alpha (TEF1*a*) gene. The description of the DNA regions and primers used for molecular identification is shown in Table 4. The PCR amplification was carried out in a 40 µL reaction volume consisting of 20 µL of DreamTaq Green PCR master mix 2X (Thermo Scientific™ K1081), 1.20 µM of each primer set (forward and reverse), 15.6 µL of nuclease free water and 2 µL of DNA template. PCR amplification was performed in a T100 Thermal Cycler (Bio-Rad, USA) with 3 min of pre-denaturation

at 95 C, 35 cycles of denaturation at 95 C for 30 s, 35 cycles of annealing at 55 C for 30 s, 30 s of extension at 72 C, and 10 min of renaturation at 72 C for ITS1/ITS4 and TEF-Fs4f/TEF-Fs4r and for FOF1/FOR1 thermal cycler machine programmed for initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. PCR amplicons were resolved on 1% agarose gel stained with sybr™ safe DNA gel stain and and visualized in Bio-Rad GelDoc Imaging System (Bio-Rad, USA)

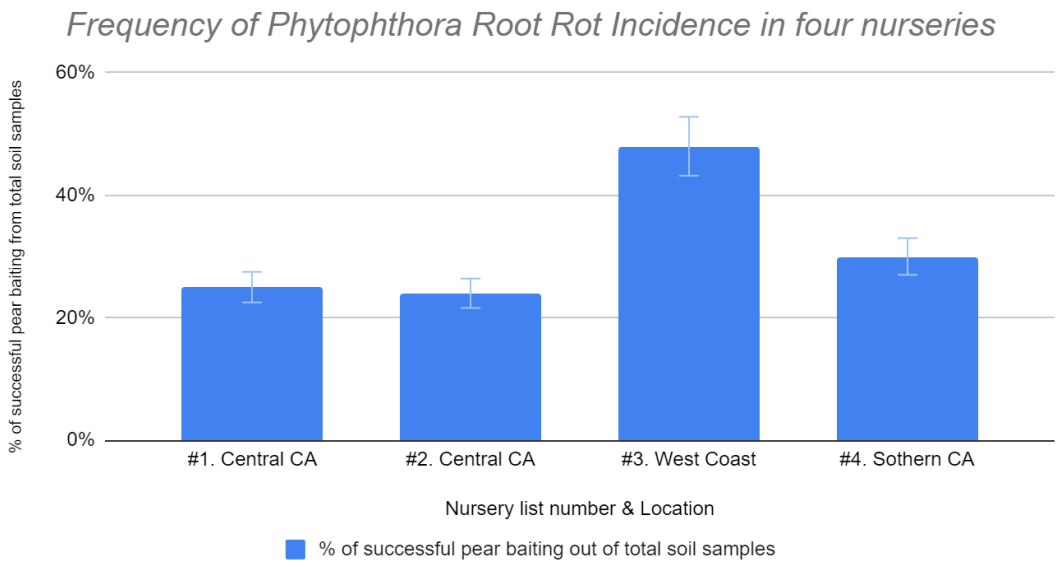
Table 4. List of primers used for characterization of *F. solani* and *F. oxysporum*

S. No	Primer name	Sequence (5′-3′)	Target	Reference
1.	FOF1	ACATACCACTTGTTGCCTCG	ITS	P.K. Mishra et al.
2.	FOR1	CGCCAATCAATTTGAGGAACG		
3.	ITS1	TCCGTAGGTGAACCTGCGG	ITS	White et al.
4.	ITS4	TCCTCCGCTTATTGATATGC		
5.	TEF-Fs4f	ATCGGCCACGTCGACTCT	TEF1*a*	Arif et al.
6.	TEF-Fs4r	GGCGTCTGTTGATTGTTAGC		

- ***Phytophthora spp.* DNA Extraction:**
  After examining morphology for randomly 10 selected isolates from four different nurseries we prepared them for DNA extraction. Isolates were grown in 10% V8C broth (Ribeiro et al, 1978) at 25°C in the dark for 5 to 7 days. Mycelia were collected on the cheesecloth and crushed by mortars and pestles using liquid nitrogen. Later ~100 to 200 mg of the finely powdered mycelium was used for DNA extraction using Qiagen, plant mini kit. Later the quality of DNA checked on 1% agarose gel stained with sybr™ safe DNA gel stain and visualized in Bio-Rad GelDoc Imaging System (Bio-Rad, USA) (Figure 7). DNA samples were stored at − 20 °C for further use.

- **Data Analysis:**
  So far, among the four nurseries examined, F.W nursery has the highest frequency of *Phytophthora* root rot. The result aligned with our expectations since the nursery has kept their pots in direct contact with the soil. This practice likely contributed to the higher incidence of *Phytophthora* root rot observed in this specific nursery.

Phytophthora detection in four nurseries		
Nursery list #	Location	% of successful pear baiting out of total soil samples
1	Central CA	25%
2	Central CA	24%
3	West Coast	48%
4	Southern CA	30%



- **Results and conclusions:**
  We successfully isolated *Phytophthora* and *Fusarium* species from soil and root samples. A total of 112 *Phytophthora* isolates were obtained through the pear baiting method, 7 isolates from nursery#1, 22 isolates from nursery#2, 60 isolates from nursery#3, 23 isolates from nursery#4. We were unable to obtain any isolates from the water samples. We believe this is because the water collected from the nursery was not in direct contact with the soil.We could retrieve over 400 isolates of *Fusarium spp.* were collected using the root and soil isolation technique. From nursery#1, 12 isolates retrieved from soil and 47 isolates from root. From nursery#2, 4 isolates retrieved from soil and 55 isolates from root. From nursery#3, 101 isolates retrieved from soil and 123 isolates from root. From

nursery#4, 26 isolates retrieved from soil and 48 isolates from root. The morphological examination of these isolates indicated distinct characteristics corresponding to each genus. Although we have made significant progress in isolating these pathogens, further work is required. Specifically, we need to perform DNA extraction for more isolates and conduct molecular analyses to confirm species identification and assess genetic diversity. This next phase will provide deeper insights into the prevalence and distribution of these pathogens in the sampled environments.

After conducting the PCR, all isolates tested with the ITS primer set for *Fusarium spp.* displayed amplification, producing band sizes between 500-540 bp (Figure 8). The species-specific primers TEF-Fs4F/R, used to identify *F. solani*, resulted in an amplification band size of approximately 658 bp (Figure 9). Additionally, the species-specific primer set for detecting *F. oxysporum* showed amplification with a band size around 340 bp (Figure 10).

In conclusion, this study has successfully isolated both *Phytophthora* and *Fusarium* species, indicating their presence in the sampled soil and root tissues. The findings highlight the potential risks these pathogens pose to citrus health in California. However, to fully understand their impact and develop effective management strategies, additional molecular analyses are necessary. Future work will focus on DNA extraction and genetic characterization of the isolates.

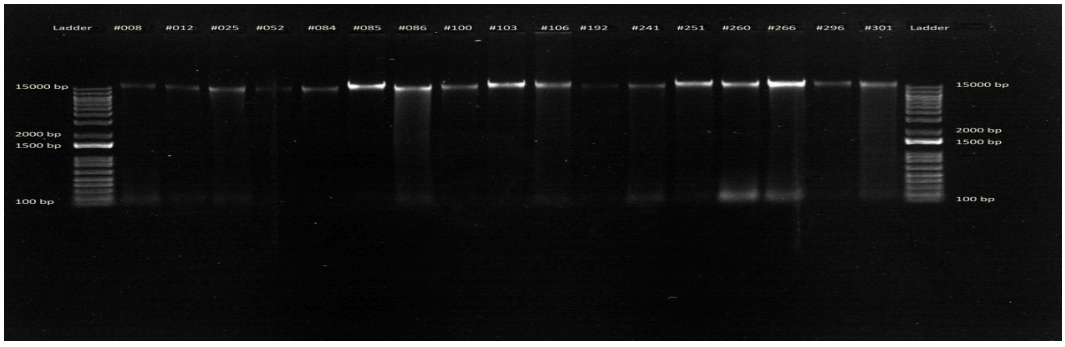


Figure 6. *Fusarium* DNA Extraction of selected isolates Image Checked on 1% agarose gel stained with sybr™ safe DNA gel stain

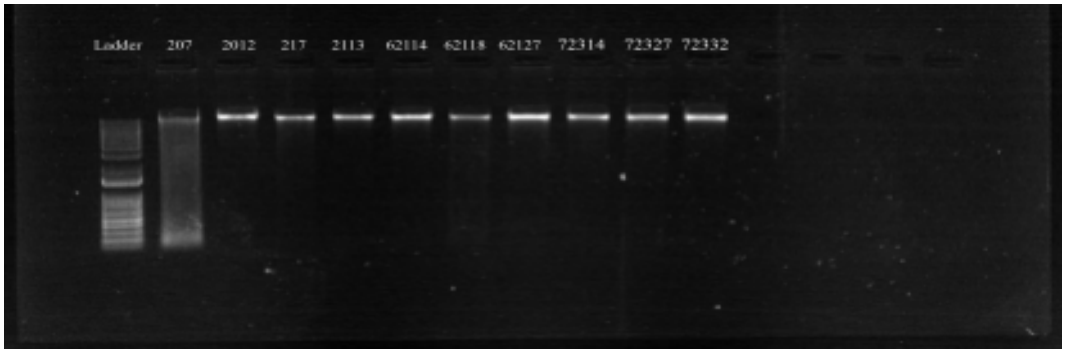


Figure 7. *Phytophthora* selected isolates DNA extraction Image Checked on 1% agarose gel stained with sybr™ safe DNA gel stain

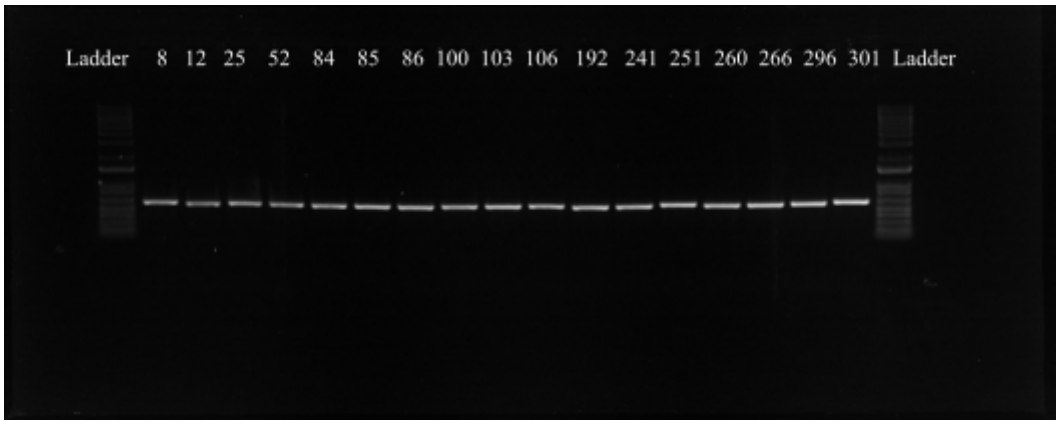


Figure 8. PCR Imaged checked using ITS primer sets, target gene ITS

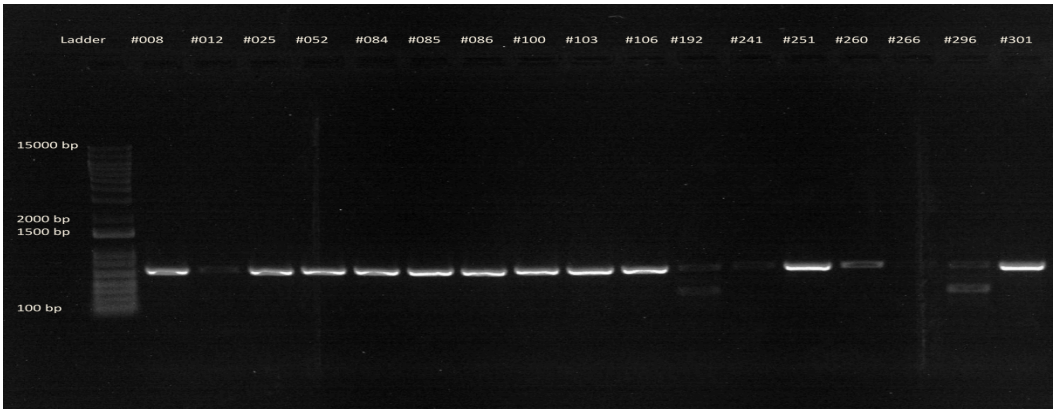


Figure 9. PCR Imaged checked using TEF-Fs4 primer sets, target gene TEF1*a* for Amplicons exhibiting amplification implies *F. solani*

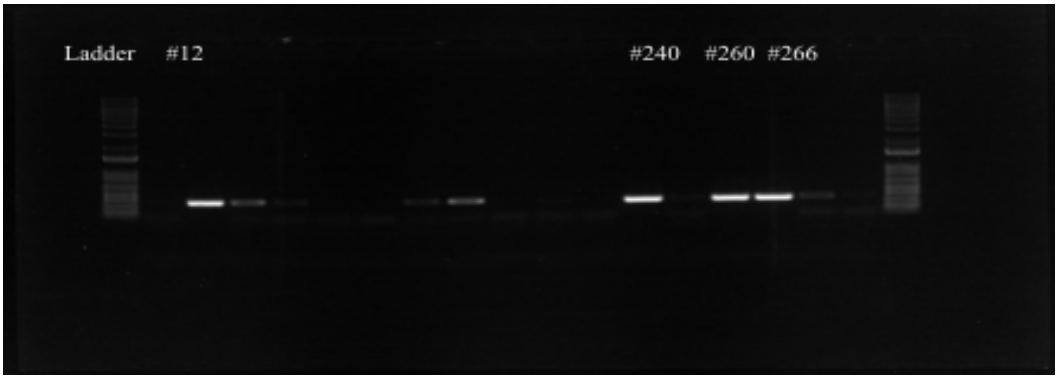


Figure 10. PCR Imaged checked using FOF1/FOR1 primer sets, target gene ITS Amplicons exhibiting amplification implies *F. oxysporum*

- **Visuals:**
    - *Phytophthora* Morphology Characteristics:

We examined the morphology of isolates visually on the sixth day of growth. According to Van Tran et al we could identify isolation that could possibly be *P. nicotianea,* and *P. citrophthora*. *P.nicotianea* is white, slightly cottony with a fuzzy texture on reg-V8 media and *P. citrophthora* has a velvety texture, smooth, slightly radiate on reg-V8 media. (Figure 3). Further molecular study is required to confirmed those species.

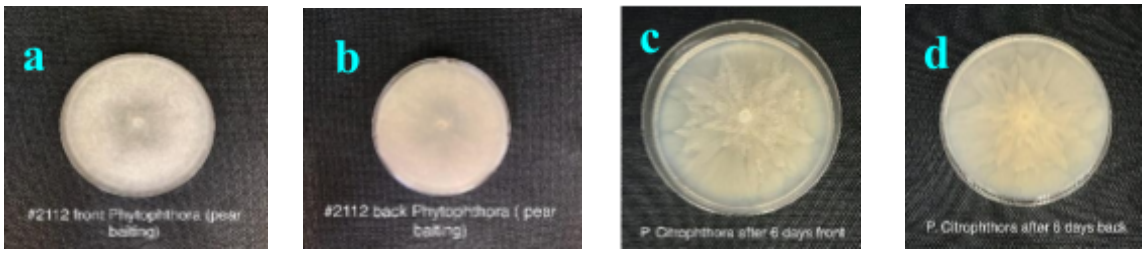


Figure 3. Culture characteristics and appearance on reg-V8 media, **(a)(b)** Front and back appearance of *P.nicotianea* isolate in pure culture on reg-V8 media on day sixth of growth; **(c)(d)** Front and back appearance of *P. citrophthora* isolate in pure culture on reg-V8 media on day sixth of growth

- *Fusarium* Morphology Characteristics:

We examined the morphology of isolates visually on the sixth day of growth and checked the spore under the microscope. According to Ezrari et al, *F. solani* has a creamy color, fluffy texture and *F. oxysporum* has pink to purple color with smooth texture.

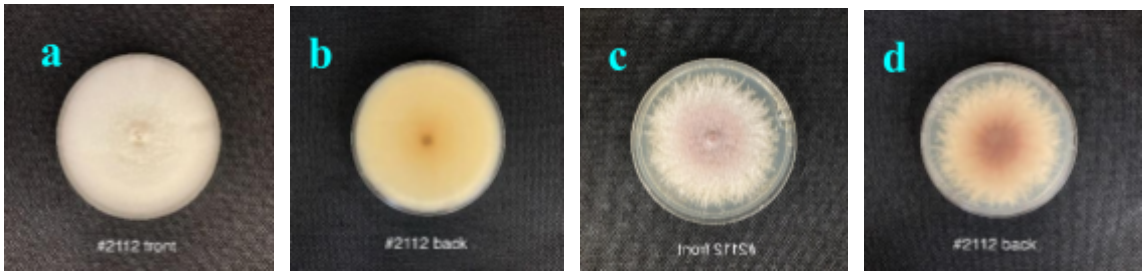


Figure 4. Cultural and morphological characteristics of *Fusarium spp.* associated with dry root rot. Colony aspect on PDA **(a)(b)** Front and back appearance of *F. solani* isolate in pure culture on PDA media on day sixth of growth; **(c)(d)** Front and back appearance of *F. oxysporuum* isolate in pure culture on PDA media on day sixth of growth

- *Fusarium* spore Characteristics:

The *Fusarium spp.* macroconidia is sickle-shaped, multiseptate, and have a hooked apical end and it's often produced by monophialides or by sporodochia. (Ezrari et al., 2022). Both species has a sickle-shaped spore, but the spore for *F. oxysporum* has a sharp end on each side.

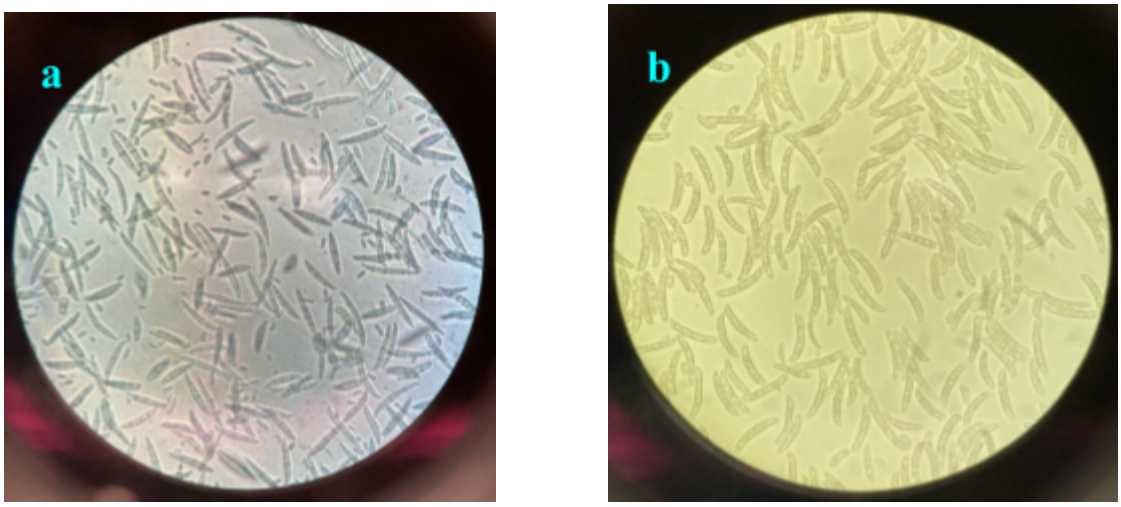


Figure 5. *Fusarium spp.* spore characteristic, **(a)** *F. oxysporum* spore (40X); **(b)** *F. solani* spore (40X)

- **Remaining objectives for Proceeding Project:**
    - We will conduct more sampling through Southern California nurseries if we can get permission from the citrus manager easily to go for sampling.
    - Perform DNA extraction for more *Fusarium* isolates and use different primer sets to identify *F. solani and F. oxysporum.*
    - Perform DNA extraction for more *Phytophthora* isolates and use ITS as a universal primer sets and species-specific primer to identify *P. nicotianae and P. citrophthora.* We will use (Pn5BF/Pn6R) primer set for *P. nicotianae* detection, and (Pc2BF/ Pc7R) primer set for detection of *P. citrophthora.*
    - We will send the PCR amplicons for sequencing to confirm fungi species and then analyzing data and creating phylogenetic tree.
    - Once the fungal species is confirmed, we will move on to the second phase of the project, which involves assessing rootstock susceptibility. Identifying resistant rootstocks that are not vulnerable to Dry Root Rot is crucial. The results of this assessment will aid the citrus industry in increasing the rootstock variability within their orchards. We will also need to request funding to initiate this second phase of the project.

References

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