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LÍNEAS DE INVESTIGACIÓN ENFOCADAS AL DESARROLLO DE ESTRATEGIAS PARA EL MANEJO INTEGRAL DE LA MARCHITEZ DEL LAUREL EN AGUACATE

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Resumen

La marchitez del laurel es una enfermedad vascular letal, causada por el hongo *Raffaelea lauricola*, que afecta especies de la familia Lauraceae. El hongo y su vector, el escarabajo ambrosial *Xyleborus glabratus*, fueron introducidos a EE.UU. en el año 2002. La enfermedad, ampliamente distribuida en las áreas productoras de aguacate en Florida, ha causado la perdida de aproximadamente 140 mil árboles. Puesto que aún no se han identificado materiales comerciales tolerantes o resistentes, el manejo se basa en el diagnóstico temprano y en la erradicación completa y oportuna. Nuestro grupo ha establecido líneas de investigación que buscan contribuir al manejo efectivo de esta enfermedad. Por ejemplo, desarrollamos una prueba de detección basada en PCR que reduce considerablemente el tiempo de diagnóstico y estamos desarrollando pruebas de detección temprana para ser utilizadas en el campo. Demostramos que estrategias culturales, como la tala parcial y solarización del muñón o tocón, son ineficientes y favorecen la propagación del patógeno. Estamos evaluando la eficacia del fungicida propiconazole (Tilt®) aplicado al suelo, y resultados preliminares sugieren que esta forma de aplicación es más eficiente para controlar la enfermedad y reducir los síntomas. Para identificar materiales genéticos resistentes o tolerantes que favorezcan un manejo sostenible, establecimos un protocolo de inoculación con plantas jóvenes, y a través de múltiples colaboraciones, estamos evaluando variedades comerciales, germoplasma silvestre y líneas mejoradas. Esta charla presentará los diferentes proyectos que están siendo desarrollados en nuestro equipo de investigación, los cuales contribuirán al manejo holístico de esta devastadora enfermedad.

Palabras claves: *Raffaelea lauricola*, Escarabajos ambrosiales, Detección rápida por PCR, Evaluación de germoplasma.

RESEARCH LINES FOCUSED ON THE DEVELOPMENT OF STRATEGIES FOR THE INTEGRAL MANAGEMENT OF LAUREL WILT IN AVOCADO

Abstract

Laurel wilt is a fatal vascular disease, caused by the fungus *Raffaelea lauricola*, which affects species of the Lauraceae family. The fungus and its vector, the ambrosial beetle *Xyleborus glabratus*, were introduced to the US in 2002. The disease, widely distributed in avocado-growing areas in Florida, has caused the loss of approximately 140,000 trees. Since tolerant or resistant commercial materials have not yet been identified, management is based on early diagnosis and complete and timely eradication. Our group has established lines of research that seek to contribute to the effective management of this disease. For example, we developed a PCR-based screening test that dramatically shortened diagnostic time, and we are developing early detection tests for use in the field. We show that cultural strategies, such as partial felling and solarization of the stump or stump, are inefficient and favor the spread of the pathogen. We are evaluating the efficacy of the fungicide propiconazole (Tilt®) applied to the soil, and preliminary results suggest that this form of application is more efficient in controlling the disease and reducing symptoms. To identify resistant or tolerant genetic materials that favor sustainable management, we established an inoculation protocol with young plants, and through multiple collaborations, we are evaluating commercial varieties, wild germplasm and improved lines. This talk will present the different projects that are being developed in our research team, which will contribute to the holistic management of this devastating disease.

Key words: *Raffaelea lauricola*, Ambrosial beetles, Quick PCR detection, Germplasm evaluation

Introduction

Laurel wilt (LW) is a vascular disease caused by the fungus *Raffaelea lauricola* T.C. Harr., Fraedrich and Aghayeva (Ophiostomatales) (Harrington et al., 2008) that infects species in the Lauraceae family. The fungus is a nutritional symbiont of the ambrosia beetle *Xyleborus glabratus* Eichhoff (Curculionidae: Scotylinae), native to Southeast Asia (Rabaglia et al. 2006). Both, the fungus and its vector, were introduced to the U.S.A in 2002 through Port Wentworth, GA but the disease was not reported until 2004 (Fraedrich et al., 2008). Laurel wilt has spread rapidly and extensively not only due to vector movement through susceptible hosts, but also due to the anthropogenic movement of infested wood (Ploetz et al., 2013). The first report of a laurel wilt infected avocado tree was in 2007, in a residential neighborhood in Jacksonville, FL (Mayfield et al., 2008). Five years after this report, the disease was detected in a commercial avocado orchard in Homestead, FL (Crane et al., 2020). Currently, the disease has been detected in 12 States affecting multiple forest species native to the U.S.A (Southern Regional Extension Forestry, 2021). In Florida, laurel wilt has become established, being present in all 67 counties, and it is considered ubiquitous in the avocado producing areas (Crane et al., 2020). To date, this disease has caused the loss of more than 140 thousand trees, threatening not only the livelihood of the farmers but also the State's economy, as the avocado industry has an overall economic impact of about $100 million per year (Evans and Lozano, 2010.)

The main mode of disease transmission is through beetle vectoring, but while *X. glabratus* is the primary vector of *R. lauricola* (Rl) in Asia and in natural forest of Southeastern U.S.A., this beetle species is seldomly found in avocado orchards (Carrillo et al., 2012). On the other hand, *R. lauricola* has been recovered from several other species of ambrosia beetles, demonstrating that alternative vectors have an important role in the avocado-laurel wilt pathosystem (Carrillo et al., 2014; Cruz et al., 2021; Ploetz et al., 2017). Besides beetle transmission, the pathogen can move to adjacent trees through root grafts (Ploetz et al., 2017a), spread pathway that has a direct and significant impact on the management of the disease.

Laurel wilt is a lethal disease that compromises the functionality of the vascular system, due to the obstruction of the sap flow by tyloses and gums produced by the tree in response to the colonization and multiplication of the pathogen (Inch et al., 2012). Therefore, external symptoms associated with the disease resemble those caused by drought stress. Symptoms start with turgor loss and consequently foliar wilting ("green wilting") which turns into brown-wilting within 2-3 weeks (Figure 1A). The location of the first symptoms depends on where the beetle attack has occurred, beetle-inoculated limbs will become symptomatic first. When the transmission is through root-to-root, symptoms tend to appear on the side facing the infected tree (personal observation) (Figure 1B). Brown desiccated leaves can stay attached to the branches for months (Kendra et al., 2013). Stem and limb dieback can also be observed, but less often. Internal symptoms include a blackish-blue-stained streaking of the sapwood, which can be also observed surrounding the entry hole leading to the beetle's galleries (Kendra et al., 2013). Depending on the inoculum concentration and tree diameter, infected trees can succumb to the disease within two to eight weeks (Ploetz et al., 2016).

Tolerance or resistance to laurel wilt has not been observed yet, neither in potted avocado plants nor in mature trees of any of the 47 commercial cultivars evaluated so far (Castillo-Argaez et al., 2021; Crane et al., 2020a; Ploetz et al., 2012). Thus, disease management is based on cultural practices, including scouting, early diagnosis, complete removal of infected trees, and appropriate destruction of infected material. This work summarizes the control strategies recommended by researchers from the Tropical Research and Education Center (University of Florida/IFAS) to manage laurel wilt of avocado in South Florida, as well as several of the research lines our group is working on towards developing a timely, efficient, and sustainable management of the disease.

Scouting and Sanitation

Frequent scouting of avocado orchards is crucial to detect potential infected trees. Since the disease spreads quickly, the identification of trees in an early stage of disease progression is necessary to reduce the dissemination of the pathogen to adjacent trees through ambrosia beetle vectoring or root graft transmission (Crane et al., 2020a). If laurel wilt has not been previously detected in the orchard, sapwood samples should be collected and sent for a confirmatory analysis (Crane et al., 2015; Crane et al., 2020b). Affected trees must be rogued, including the uproot and destruction of the tree by chipping/shredding, to avoid root-to-root transmission to adjacent trees and reproduction of the vector (Crane et al., 2020a). In addition, contact insecticides should be applied to the chipped/shredded wood to avoid multiplication of the vector in infested wood (Crane et al., 2020a). A rigorous sanitation strategy includes the removal of the adjacent asymptomatic trees. Although unpopular, this practice has been shown to be highly effective in reducing the spread of the disease (Figure 1C).

The effectiveness of alternative cultural practices to manage LW, such as "tree stumping", "tree stump and bagging", and the "barrier method" have shown conflicting results and could favor the spread of the disease. The "tree stump and bagging" method consists of cutting down the LW-infected tree to 3 to 5 ft high, covering it with a 10 mm-thick clear plastic bag and sealing it to the ground with soil for 2 to 4 months (Figure 1D). This strategy is based on a study that reported that the *in vitro* growth of *R. lauricola* is impaired at high temperatures (>32°C) (Zhou et al., 2018). In the "barrier method" the symptomatic tree is rogued, whereas the adjacent trees are stumped (sometimes they are also bagged). Even though, temperatures inside the stump reach the desire temperature to stop the growth of the fungus, the pathogen inoculum remains viable and active in the root system (Gazis, unpublished). The canopy of the sprouts of stumped and stumped-and-bagged trees appears healthy for few months; however, sprouts eventually succumb to LW (Figure 1E). Even worse, our group has consistently shown that *R. lauricola* can be recovered from roots of stumped and stumped-and-bagged trees (Figure 1F and 1G). The viability and multiplication of *R. lauricola* in roots and stems of the re-sprouting stumps represent a risk for the orchards' health.

Rapid Detection of the LW Pathogen from Sapwood Samples

Since early diagnosis of LW is critical in facilitating the timely implementation of proper management practices, and therefore in stopping the spread of the disease within the orchard, the development of a rapid diagnostic method is a priority. The conventional detection method requires the isolation of *R. lauricola* from sapwood chips or slivers (a detailed sampling guidelines in Crane et al. 2020b), using a semi-selective media (CSMA: 1 % malt extract and 1 % agar, supplemented with 200 ppm cycloheximide and 100 ppm of streptomycin sulfate), followed by DNA extraction and the amplification of two specific microsatellite markers (Dreaden et al., 2014). The complete process can take up 10 days. To reduce the diagnosis time, our group developed a PCR-based method to detect *R. lauricola* directly from symptomatic sapwood (Figure 2A), reducing the diagnosis time to 24 hours (Parra et al., 2020). In this direct test, DNA is isolated using a commercial kit (QIAamp Fast DNA Stool mini kit, QIAGEN) from sawdust collected from drilled symptomatic sapwood (Figure 2B, 2C) or sapwood slivers. DNA is then used as template to amplify the two *R. lauricola* specific microsatellites primers IFW and CHK (Dreaden et al., 2014), through conventional PCR. The specificity of both primers was assessed by testing cross amplification against a diverse set of fungal strains, representing close relatives of the pathogen and fungi that are often found colonizing avocado sapwood and phloem in commercial orchards. The amplification of the IFW region was found to be more consistent and reliable with a detection rate close to 90 %, when symptomatic tissue was used (Parra et al., 2020). In our laboratory, IFW primers are routinely used to detect *R. lauricola* in sapwood samples from avocado orchards and experimental plants (Figure 2D).

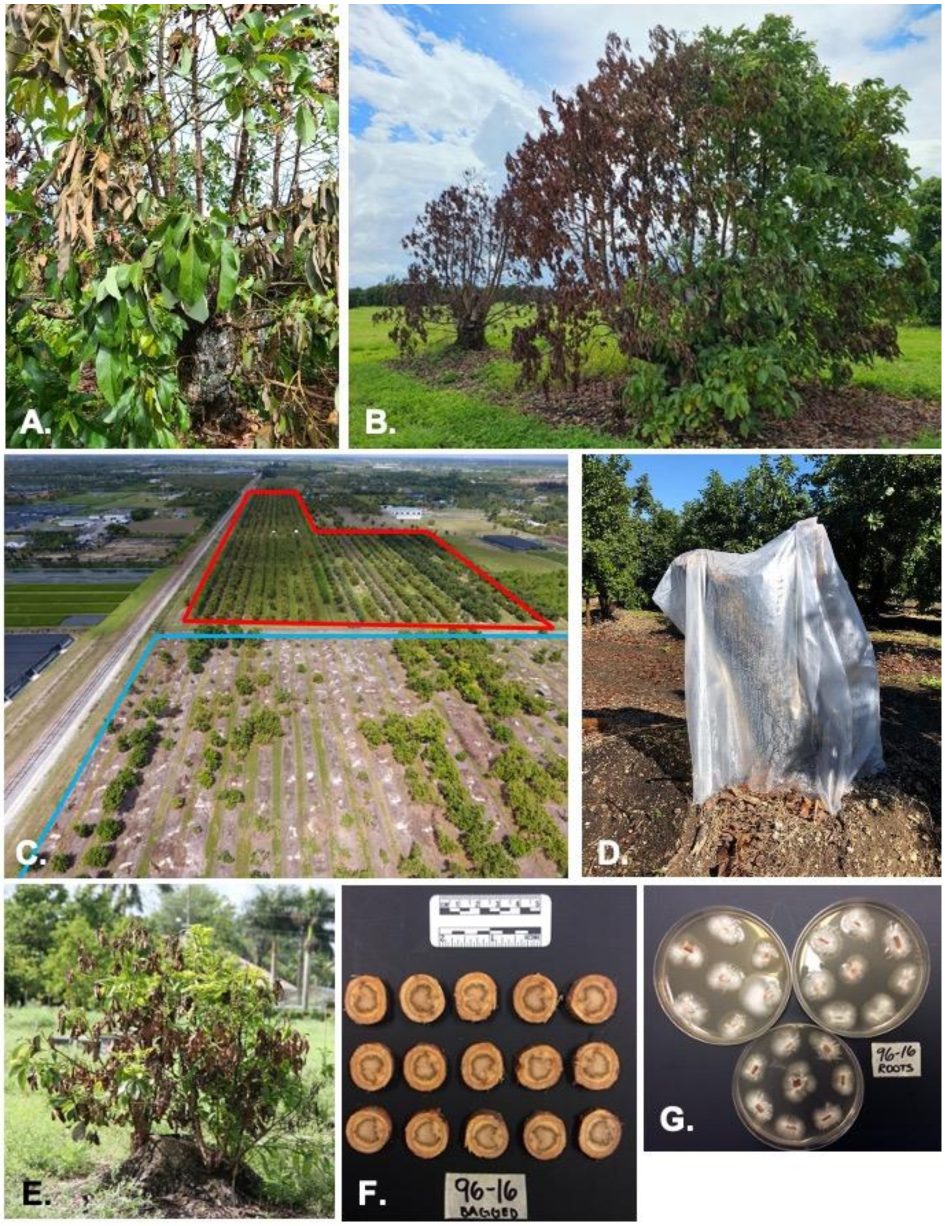

Figure 1**.** Green-leaf-wilting and brown-wilting (desiccation) in an avocado tree infected with the laurel wilt (LW) pathogen (A); root-to-root transmission in a mature avocado planting (B); aerial photo of two avocado orchards showing the outcome of contrasting management strategies: one showing "extreme sanitation" with intense scouting and early complete removal of LW symptomatic trees [planting within the red box], and another one in which multiple management practices have been implemented, including tree stumping and tree bagging [planting within the blue box] (C); example of a stumped-and-bagged tree (D); LW symptoms in re-sprouts coming from a stumped tree (E); cross sections of roots from a LW-infected tree ("tree 96-16") that was stumped and bagged for ~5 months, showing staining of the xylem (F) and *R. lauricola* growing out of the plated root pieces from the same tree (G).

Currently, our efforts are focused on the development of rapid, field-deployable assays that can facilitate the diagnosis of LW in situ. Genomic sequencing efforts will allow the identification of new specific markers which will be used to develop primers and probes for RPA (Rapid Isothermal Amplification) and LAMP

(Loop-mediated isothermal amplification) assays. In addition, we are working on the identification of *R. lauricola* early secreted proteins that can be used as detection markers in paper-based biosensors. These point-of-care diagnostic assays do not require DNA purification or sophisticated and expensive equipment, favoring quick decision making.

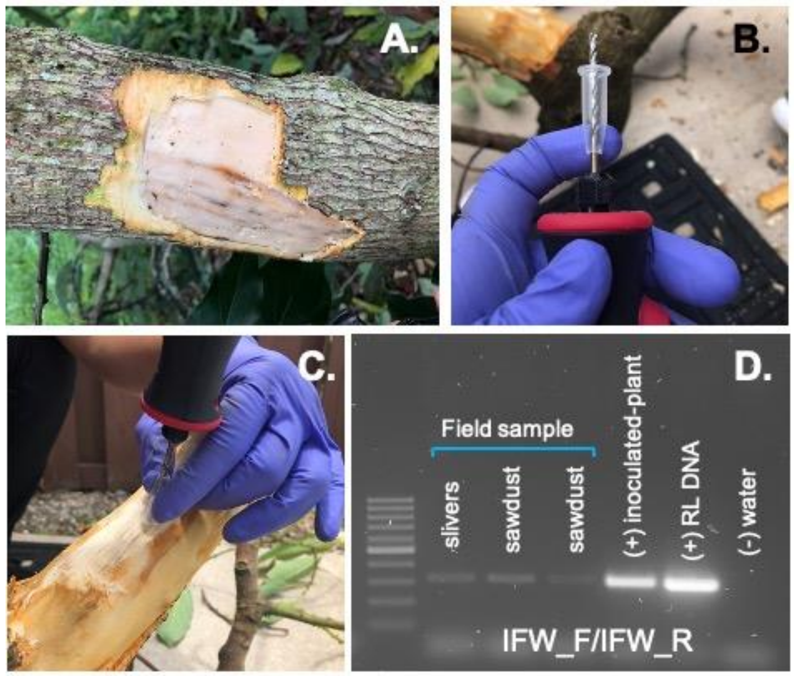

Figure 2. (A) Characteristic sapwood discoloration (streaking) in laurel wilt affected avocado trees; (B) and (C) collection of sawdust from symptomatic sapwood using a mini-drill and a microcentrifuge tube, (D) Detection of *Raffaelea lauricola* (RL) from field samples (slivers and sawdust) using the rapid PCR-based method developed by our research group (Parra et al. 2020).

Chemical Control

The active ingredient in Tilt® (propiconazole) has been shown to be effective in stopping the growth of *R. lauricola in vitro* and *in planta* (Ploetz et al., 2011; Ploetz et al., 2017c). Even though its efficacy has not been proven through a robust replicated experiment, Tilt® is currently being used to prevent the infection of field trees (prophylactic) and it is registered for the management of LW when used as injection and infusion (Crane et al., 2015). Main drawbacks of using Tilt® injections is that it is expensive and labor intensive, and the product cannot be homogenously delivered throughout the active xylem. Previous studies showed a reduction in LW symptoms when Tilt® was used as a drench in potted plants (Ploetz et al., 2011; Ploetz et al., 2017c), but field trees were not tested. Our group's preliminary results support Ploetz et al. (2011, 2017c) observations, with no external symptom development in treated potted plants artificially inoculated with a suspension of propagules at high concentration (1 million) (Figure 3A and 3B). Preliminary field tests, using few field trees (4), have shown a delayed onset of symptoms and localized branch dieback. Currently, we are conducting a field experiment with a replicated design where different Tilt® concentrations (1X label concentration, 2X and 4X) are being evaluated. Our goal is determining if Tilt®, applied as a drench, is efficacious in reducing disease progression and severity in artificially inoculated field trees and establish a baseline for its use in avocado orchards.

Figure 3. Clonal avocado plants (Toro Canyon cultivar, Mexican race) artificially inoculated with a spore suspension of *R. lauricola* (RL4 strain, 1 million CFUs). Three weeks after inoculation, plants drenched with Tilt® (at a rate of 0.25 oz/inch) did not show symptoms (A), whereas un-treated control plants showed wilting and desiccated leaves (B).

Screening of Avocado Germplasm to Identify Tolerance/Resistance

The use of cultivars with tolerance or resistance is the most sustainable and environmental-friendly alternative to the long-term management of LW. Although tolerance/resistance has not been formally observed in commercial cultivars, our research team has identified few trees in commercial orchards that exhibit tolerance to natural infections. These surviving trees, often called "escape trees", have been confirmed to be infected with the pathogen and have developed mild LW symptoms, but have recovered and remained productive. We believe that a genetic component (for example, the ability of the plant to quickly compartmentalize the pathogen) could be involved. To evaluate the response of a wide variety of materials to LW, our group developed a protocol to artificially inoculate young potted plants, saving resources such as time, space, and supplies for plant management. Briefly, a hole is drilled at about 10 cm from the groundline (or ~ 10 cm from the graft) using a conical drill bit (0.7 x 0.4) at 45° angle, and a second hole is made on the opposite side, with a minimum distance of 3 cm between both holes. In each hole, 10 µl of the inoculum (20 µl total per plant, 10^6 propagules per plant) is injected into the new xylem and the stem is wrapped with parafilm to prevent the inoculum from drying. The protocol is highly reproducible and characteristic LW symptoms are observed 10 to 14 days after inoculation. Previous studies indicated that LW progresses more slowly in avocado cultivars of Mexican genetic background, compared with West Indian derived cultivars due to their smaller xylem vessel diameter (Castillo-Argaez et al., 2020; Beier et al., 2020). Through collaboration with researchers from Mexico, we have partnered with the Fundación Salvador Sánchez Colín-CITAMEX, S. C. and are currently growing twenty germplasm accessions for future screening against *R. lauricola*.

Summary

Laurel wilt disease is a serious threat to the Florida avocado industry and will likely reach other avocado producing areas. Currently, disease management relies on the timely identification and proper eradication of affected trees. Our group is working on the development of detection tests that can shorten diagnosis time and therefore facilitate rapid decision-making. We are also assessing the effectiveness of drench-applied Tilt® (propiconazole) as a tool to delay disease progression in field trees. Long-term goals include the establishment of an avocado-laurel wilt resistance/tolerance screening program at the University of Florida, Tropical Research and Education Center.

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