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Cover Page Footnote

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FIU Undergraduate Research Journal

In Vitro Efficacy of Fungal Endophytes and Silver Pyrazolate Against Raffaelea Lauricola, Causal Agent of Laurel Wilt of Avocado

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The South Florida avocado industry is being severely impacted by laurel wilt disease. Laurel wilt disease of avocado is caused by the fungal pathogen, *Raffaelea lauricola* (RL) and is vectored by ambrosia beetle, *Xyleborus glabratus*. Treatments options are limited, economically not sustainable, and require reapplication fungicides every couple of years. There is a crucial need for developing multiple modes of control using novel biological and chemical agents. The ambrosia beetle associated pathogenic fungi are known to outcompete other microorganisms by taking advantage of ethanol produced by the pathogen and the stressed tree. Endophytes, which reside inside the host plant tissue are part of the plant microbiome represent source of new potential biological control agents. In this study, three ethanol tolerant endophytic fungal species, isolated from avocado bark, were evaluated using in vitro dual culture assay and colonization tube (packed with bark/sapwood shaving) against RL. The endophytic isolates *Tricoderma crissum*, *Tricoderma simmonsii*, *Lasiodiplodia theobromae* were found to be highly capable of suppressing the mycelial colony growth of RL. The results suggest that combined abilities of ethanol tolerance and competitive colonization can provide useful criteria for identifying potential biocontrol agents. In vitro anti-RL activity of silver pyrazolate compound was assessed in both agar and liquid medium. Silver pyrazolate at levels of 30 and 45 ppm were found to be highly effective against RL. Further in planta research is needed to study the effects of endophytic fungal isolates and silver pyrazolate to assess their potential as additional tools for management of laurel wilt.

Keywords: *endophytes*, *avocado laurel wilt*, *ambrosia beetle*, *Raffaelea lauricola*, *silver pyrazolatece*, *Aedes aegypti*

Laurel wilt has been a devastating disease of avocados in Southern Florida, and has since become a potential threat to the California avocado industry. Laurel wilt is caused by *Raffaelea lauricola* (RL), a fungal pathogen transmitted by the insect vector, the invasive ambrosia beetle *Xyleborus glabratus*. The fungus attacks the vascular system of trees in the Lauraceae family. Since its discovery in 2004, this fungus has killed thousands of trees in the southeastern United States (Hughes et al., 2015). The insect vector carries the fungal pathogen, RL in special pouches near the mouthpart called the mandibular mycangia. As the insect vector bores into the bark of an avocado tree, it forms tunnels, known as galleries, in which the fungus spores are introduced. The host tree responds to this reaction by blocking the flow of water and nutrient in the xylem, causing wilt (Hughes et al., 2015). Fungicide treatment is expensive, time-consuming, and must be repeated every two to three years (Hughes et al., 2015). Therapeutic control methods for the laurel wilt disease in Red Bay, a native tree species related to avocado have been studied using root-flare injections of the fungicide Propiconazole; however, this control method demonstrated limited duration of efficacy and requires reapplication every two to three years (Mayfield III et al., 2008). It has been found that no avocado cultivars are resistant to laurel wilt disease (Pérez Martínez et al., 2018).

Alternative methods of disease control such as biological control are favorable due to the use of beneficial plant microbiomes potentially reducing harm to non-target organisms (Rabiey et al., 2019 and Pérez-Martínez et al., 2018). Endophytes are fungi that are part of the plant microbiome and do not cause any apparent disease. Endophytic microorganisms can play a key role in protecting the plant host by directly attacking the pathogens (Khare et al., 2018). Avocado endophytes capable of antagonistic activity against RL can potentially be used as an environmentally friendly mode of control for laurel wilt (Shetty et al., 2016). The commercially available fungus *Beauveria bassiana* also proves to be antagonistic to RL *in vitro* (Zhou et al., 2018), in addition to being highly effective against the beetle vector *X. glabratus*.

In response to abiotic and biotic stress, trees produce ethanol (Ranger et al., 2018; McPherson et al., 2008). Previous studies have shown that the ambrosia beetle prefers stressed trees to support the fungal pathogen's growth because the ethanol produced suppresses the growth of competing microorganisms inside the beetle gallery, promoting growth of ambrosia associated fungi (Ranger et al., 201; Lehenberger et al., 2021). This tactic ensures that the fungal pathogen can out-compete other microorganisms susceptible to the antimicrobial potency of ethanol. Inhibition of ethanol susceptible microbial competitors is advantageous to ambrosia associated fungi (Ranger et al., 2018). The introduction of ethanol improves the chances of the fungal pathogen establishing itself on the galleries formed by the insect vector; however, ethanol tolerance of RL has not been studied. An ethanol tolerant antagonistic endophyte would be an ideal biocontrol candidate for management of laurel wilt disease.

Chemical control is one of the many aspects of integrated pest management (IPM) that has been effective at preventing losses due to plant disease (Hirooka & Ishii, 2013). Silver nanoparticles have been found to inhibit the growth of fungal pathogens due to their strong antimicrobial properties with multiple modes of action (Kim et al., 2009). The growth of oak wilt pathogen *Raffaelea* sp. in the presence of silver nanoparticles was significantly inhibited in a dose dependent manner (Kim et al., 2009). Therefore, use of

silver-based compounds may expand potential chemical treatment options for the management of laurel wilt.

In the present study, fungal endophytes from avocado bark and silver pyrazolate were evaluated for *in vitro* impact on RL growth. A research study hypothesized that tolerance to ethanol could help antagonistic endophytes gain advantage against RL within the host tissue. Thus, ethanol-tolerant endophytes were subsequently assessed for their impact on RL growth and colonization under in vitro conditions.

Materials and Methods

Sample Collection and Endophyte Isolation from Avocado Bark

Cultures of *R. lauricola* strains #180256 and #180254 were provided by Dr. Romania Gazis-Seregina, of the University of Florida Tropical & Research Education Center in Homestead, Florida. The RL fungal cultures were grown on malt extract agar (MEA) amended with cycloheximide (100 ppm) and maintained at 4 °C. Avocado sap wood shavings were also collected from the USDA Sub-Tropical Center and stored at 4 °C.

Avocado tree bark samples were collected from Possum Trot Tropical Fruit Nursery (25° 32'7" N; 80° 28' 37" W) located in Miami's Redland Agricultural District, Florida. The four avocado trees chosen showed no indications or external symptoms of disease or insect damage. Samples were placed in individual plastic bags and immediately transferred to the lab. The bark samples were first washed using a mild soap solution and tap water, then submerged individually in a sterile petri dish containing a 10% bleach solution for three minutes. The bark samples were then rinsed three times in a sterile beaker containing sterile deionized water (DI water). Using a sterile scalpel, bark samples were cut into 3 mm to 5 mm pieces. Individual pieces were placed on malt extract agar (MEA) plates amended with 5% ethanol and gently pressed into the agar. The agar plates were incubated at room temperature in the dark. Observations for fungal growth were made every two days for a total of 15 days. Pure isolates were sub-cultured onto MEA agar plates.

Ethanol Tolerance

Ethanol amended MEA media was prepared using two concentrations of filter-sterilized ethanol, 2.5% and 5% and sterile distilled water was used for the control. The ethanol amended agar plates were stored at 4 °C for one day to preserve the ethanol-amended media. A mycelial plug (3 mm diameter) of a two-week old fungal pathogen and individual fungal endophyte isolates were transferred onto the center of each replicate plate. Inoculated plates were incubated at room temperature, observed, and photographed every 2 days.

Dual-Culture Assay

The *in vitro* dual-culture assay was carried out based on the methods previously described by Rahman et al. (2009) and Pérez-Martínez et al. (2018) with some modifications. A total of eight fungi were collected from endophytic isolation agar plates and were tested against RL. Two-week-old MEA culture plates of RL and potential endophytic fungi were used for this experiment. Samples were incubated at room temperature for a total of two weeks; antagonistic fungal growth interaction observations were recorded and photographed every week for a total of two weeks. Of the eight isolates, only three demonstrated strong competitive

interaction against RL: GF-1, 4C-WC, and 3A-BC. The identity of the three isolates were confirmed by polymerase chain reaction (PCR) amplification and analysis of the internal transcribed spacer (ITS) region from genomic DNA sample extracted from a 10-day-old culture grown on PDA at Genewiz Service Lab (Azenta Life Sciences), MA, USA. PCR-amplified ITS sequences were searched using the NCBI BLAST (http://www.ncbi.nlm.nih.gov) against those in the GenBank database (**Table 2**).

***In Vitro* Interaction in Colonization Tube**

In vitro interactions in artificial media between RL and fungal endophytes were assessed using colonization tubes packed with fine bark/sapwood shavings. Avocado bark/sapwood shavings were collected from the USDA-ARS Horticultural research lab, Homestead, FL and stored at 4 °C. The colonization tubes were made of plastic (2 cm in length and 2 cm inner diameter) and tightly packed with avocado bark/sapwood shavings. The tubes were covered with aluminum foil, then sterilized twice with a 24-hour gap between each cycle. Two-week old MEA culture plates of RL and endophyte fungal strains (a) GF-1, (b) 4C-WC, and (c) 3A-BC were used as inoculum. For inoculum preparation, 4 ml of sterile DI water was pipetted onto a fungal pathogen culture plate and the fungal growth was gently scraped using a sterile spreader then transferred into a sterile beaker. Thirty µL of the inoculum was pipetted into one end of the tube (bark filled tube). The experimental set up consisted of four treatments: RL, RL + GF-1, RL + 4C-WC, and RL+3A-BC. Each treatment received 30 µL of respective inoculum. The pipette tubes were kept inside sterile petri dishes and incubated for two weeks at room temperature in the dark. After two weeks, three pieces of shavings were collected from the opposite end of each tube and placed onto a single malt extract agar (MEA) plate. The plates were incubated at room temperature in the dark for another two weeks, and fungal growth observations were recorded and photographed.

Sensitivity to Silver Pyrazolate

The silver pyrazolate compound (**Figure 4)** was synthesized in Dr. Raphael Raptis's lab at Florida International University (Raptis et al., 2018, 2020, and 2021). Silver pyrazolate was found to have antimicrobial properties and is light-sensitive; thus, all laboratory work was performed under low light conditions. A stock solution of silver pyrazolate was prepared using sterile DI water before amending it to sterile ME broth flasks and MEA agar. The concentrations tested were 0 ppm, 15 ppm, 30 ppm, and 45 ppm. Three-week-old MEA culture plates of RL strains 180256 and 180254 were used as inoculum sources for the experiment. For RL inoculum preparation, 4 ml of sterile DI water was pipetted onto a culture plate and the fungal growth/spores were gently scraped using a sterile spreader. The liquid inoculum from the plates was collected into a sterile beaker and 0.5 mL was transferred to each of ME broth flask and MEA plates. For the MEA plates, 50 mL of inoculum was pipetted onto the agar plate surface, evenly spread using a sterile spreader, and allowed to dry for 5 minutes inside the biological safety cabinet. A single cylindrical hole (6 mm diameter) was punched out using a sterile corkborer and 50 µL of silver pyrazolate stock solution was introduced into each well. All flasks and plates were wrapped in aluminum foil. The flasks incubated at 28 °C for 7 days in an orbital shaker set at 125 rpm. After 7 days of incubation, inhibition diameter (mm) zones on MEA plates and turbidity in ME liquid culture were recorded. The ME liquid culture flasks showing no

growth were tested for RL viability by inoculating 100 µL of culture onto MEA agar plates and monitored for fungal growth.

Results and Discussion

Ethanol Tolerance

In order to overcome competition from RL, it is advantageous for an endophytic fungus to be tolerant to ethanol. Although RL strains showed reduced growth compared to the control plates, they did demonstrate tolerance to ethanol (**Figure 1** and **Table 1**). There was a distinct difference in ethanol tolerance between the two *Raffaelea lauricola* strains tested. At 5% ethanol, both strains showed reduced growth compared to the control. There were stark differences between the two RL strains in their growth response to ethanol concentration. The growth of RL strain 180256 was found to be less affected by ethanol than strain 180254 and its growth at 2.5% ethanol was comparable to that of control plates. Given that in nature, RL is tolerant to the presence of ethanol inside host tissue (Lehenberger et al., 2021), 5% ethanol was chosen for the remainder of the experiments. The effect of ethanol at 5% on all three fungal endophytes, *Tricoderma crissum* (GF-1), *Tricoderma simmonsii* (4C-WC), *Lasiodiplodia theobromae* (3A-BC) was minimal, showing slightly reduced growth compared to the MEA control plates (**Fig. 1**). These results are consistent with the findings of Ranger et al., (2018) and Lehenberger et al., (2021), highlighting the benefits of ethanol tolerance to mutualistic ambrosia beetle fungi and its defensive role against competing microorganisms.

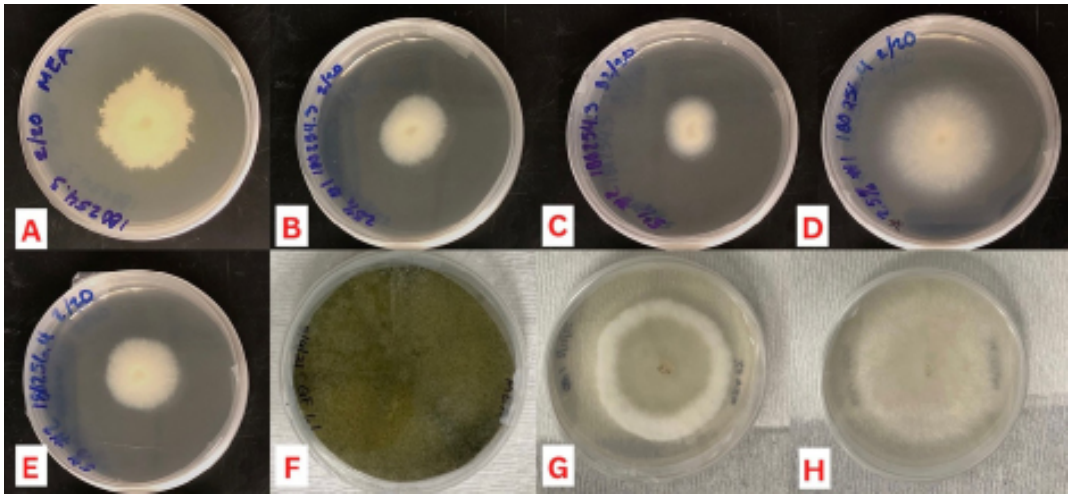

Figure 1. Effect of ethanol on growth of *Raffaelea lauricola* strains and fungal endophytes on MEA medium containing indicated concentrations of ethanol. Experiments were performed in triplicate and repeated twice. Images were taken at 14 days post-inoculation. (A) RL strain 180254, (B) RL strain 180254 on 2.5% ethanol, (C) RL strain 180254 on 5% ethanol, (D) RL strain 180256 on 2.5% ethanol, (E) RL strain 180256 on 5% ethanol, (F) *Tricoderma crissum* (GF-1) on MEA, (G) *T. crissum* on 5% ethanol, (H) *Tricoderma simmonsii* (4C-WC) on 5% ethanol amended MEA.

	Strain 180254	Strain 180256	GF-1	4C-WC	3A-BC
Ethanol 2.5 %	+	++			
Ethanol 5.0 %	+	++	++	+−	−+

Table 1. Ethanol tolerance of *Raffaelea lauricola* strains and fungal endophytes *Tricoderma crissum* (GF-1), *Tricoderma simmonsii* (4C-WC), *Lasiodiplodia theobromae* (3A-BC) grown on 2.5% and 5% ethanol amended MEA medium. Experiments were performed in triplicate and repeated twice. + indicates moderate fungal growth; ++ indicates good fungal growth.

Dual-Culture Assay

Of the eight endophytic isolates tested, only three isolates demonstrated to be strong capabilities to combat RL. All three isolates, *Tricoderma crissum* (GF-1), *Tricoderma simmonsii* (4C-WC), *Lasiodiplodia theobromae* (3A-BC) displayed antagonistic effects against RL on the MEA agar plate (**Figure 2**). The endophytic fungal isolates *Tricoderma crissum* (GF-1) and *Tricoderma simmonsii* (4C-WC) completely overgrew the pathogen in a short period of time. The endophytic fungal isolate *Lasiodiplodia theobromae* (3A-BC) demonstrated competitive antagonism against RL on the MEA agar plate. Endophytic fungi can directly attack pathogens by producing antifungal compounds which inhibit the growth of pathogens (Rabiey et al., 2019), thereby limiting the effects of the fungal pathogen on host plant growth (Thambugala et al., 2020). These results demonstrate potential for expanded discovery and development of novel ethanol tolerant endophytic fungi antagonistic to RL.

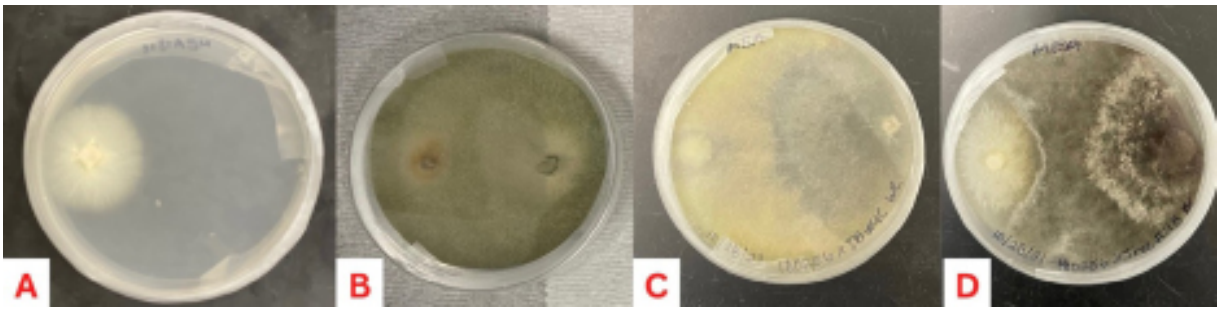

Figure 2. Dual culture plate assay between some endophytic fungi against the pathogen *Raffaelea lauricola*. (A) *R. lauricola* grown separately in MEA plate, *R. lauricola* was grown in MEA plates together with (B) *Tricoderma crissum* (GF-1), (C) *Tricoderma simmonsii* (4C-WC), and (D) *Lasiodiplodia theobromae* (3A-BC). Experiments were performed in triplicate and repeated twice. The plates were incubated for 14 days at room temperature.

***In Vitro* Interaction in Colonization Tube**

At the end of the experiment, recovery of RL colonies on MEA (from control tubes) demonstrated successful colonization and spread of RL. Recovery of the inoculated endophyte lacking RL from tubes receiving co-inoculation of endophytic fungal isolates and RL, indicates suppression of RL colonization and spread through the avocado bark/sapwood shavings medium (**Figure 3**). Recovery of RL from

co-inoculated tubes were tried again using cycloheximide amended MEA, but no RL colony growth was observed. The survival of the ambrosia beetle is dependent on the overall growth of the fungal pathogen symbiont (Menocal et al., 2018). The discovery of an endophytic isolate capable of competitively limiting the growth of the fungal pathogen, and eventually limiting the food supply of the ambrosia beetle, can be a potential strategy for effective biological control. The possibility of endophytes producing antifungal properties can attribute to that discovery (Thambugala et al., 2020). Pérez-Martínez et al., (2018) observed that avocado endophytic fungi showing significant in vitro antagonistic activities against RL did not prevent the development of laurel wilt in plants because the endophytic fungi were unable to systemically colonize the vascular tissue. Additional efforts towards discovering diverse collection of antagonistic endophytes capable of systemic colonization of avocado needs to be considered. The potential application of bark endophytes on bark surface, and the opportunity for systemic colonization through the ambrosia beetle mediated entomovectoring (Smagghe et al., 2012), and its effect on RL growth inside the beetle tunnel need to be explored further.

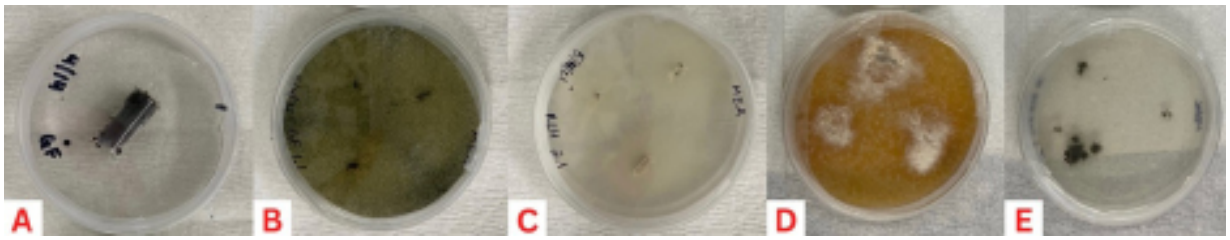

Figure 3. *In vitro* interaction in colonization tubes and recovery of inoculated fungi. (A) plastic tube packed with avocado bark/sapwood shavings, (B) recovery of *Raffaelea lauricola* (RL) from control tube, (B) recovery of *Tricoderma crissum* (GF-1) alone from a tube inoculated with RL+GF-1, (C) recovery of *Tricoderma simmonsii* (4C-WC) alone from a tube inoculated with RL + 4C-WC, and (D) *Lasiodiplodia theobromae* (3A-BC) from tube inoculated with RL +3A-BC. Experiments were performed in triplicate and repeated twice.

Sensitivity to Silver Pyrazolate

Antifungal activity of silver pyrazolate against RL was assessed in both solid and liquid media. Inhibition zone diameters were measured for all agar plates (**Figure 4**); the turbidity of the liquid cultures was measured using the baseline of no turbidity (complete inhibition of RL), moderate turbidity (some inhibition of RL), and complete turbidity (no inhibition of RL). As expected, the 0 ppm control showed no visible zone of inhibition and the liquid culture showed complete turbidity, indicating maximum RL growth. The silver pyrazolate was found to be more effective in inhibiting the growth of RL only at higher concentrations levels (30 and 45 ppm). At 15 ppm, the silver pyrazolate caused minimal inhibition of RL growth on agar and no inhibition of growth in liquid culture. A very strong growth inhibition of RL on agar plates was observed at 30 ppm and 45 ppm. The 30 ppm treatment resulted in a clear zone inhibition zone (diameter) of 60 mm. At the highest tested concentration, 45 ppm, the agar plate results demonstrated significant zone of

inhibition of 70 mm. In liquid culture the RL growth was completely inhibited at 30 ppm and 45 ppm of silver pyrazolate. These results indicate that at a silver pyrazolate concentration of 30 or 45 ppm is capable of completely inhibiting the growth of RL pathogen. The absence of an enhanced level of inhibition of RL growth on the agar plates at these concentrations, compared to liquid culture, may be due to the binding of silver molecules by agar, restricting its availability and movement. Viability of RL from 30 and 45 ppm culture flasks at the end of the experiment were tested on MEA, and the results were negative. Silver nanoparticles have been found to inhibit the growth of fungal pathogens resulting in developmental damage (Kim et al., 2009). Silver ions can produce reactive oxygen which causes damage to the proteins, lipids, and nucleic acids within the cells (Kim et al., 2009). At a higher concentration, this process can be effective compared to lower concentrations. Although the results are encouraging, further studies using silver pyrazolate treatments need to be conducted *in-planta*, to evaluate its anti-RL activity and any phytotoxicity effects.

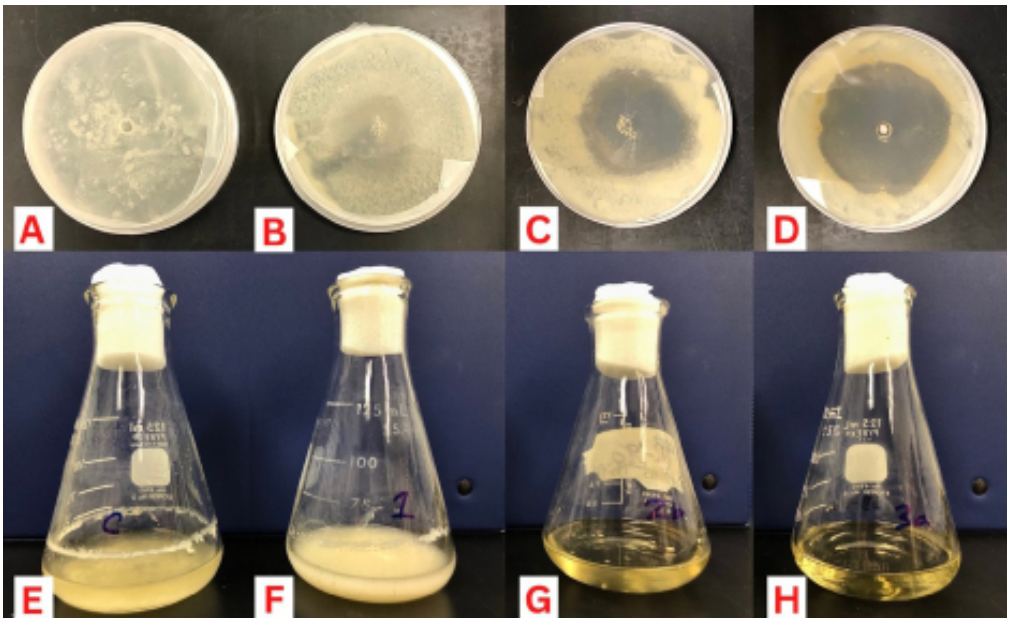

Figure 4. Effect of silver pyrazolate on growth of *Raffaelea lauricola* on ME agar and in ME broth medium containing different concentrations of silver pyrazolate, 0 ppm (A and E), 15 ppm (B and F), 30 ppm (C and G) and 45 ppm (D and H). Experiments were performed in triplicate and repeated twice.

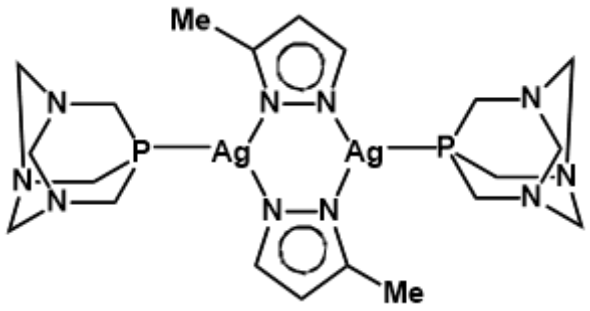

Figure 5. Structure of silver pyrazolate (Silver(I)-(m-3-methylpyrazolido)-1,3,5, triaza-7-phosphaadamantane).

Scientific Name	Accession Number [1]	Max identity (%)
Trichoderma crassum	NR_134370.1	97.73%
Trichoderma simmonsii	NR_137297.1	99.83%
Lasiodiplodia theobromae	NR_111174.1	99.20%

Table 2. Putative taxonomic affinities of the isolated fungal endophytes from avocado barks. The closest matched accession numbers were included after BLAST searches of Internal Transcribed Spacer (ITS) sequences of fungal endophytes.[1] GenBank accession numbers of closest fungal sequence of ITS.[1]

Conclusion

The isolation of ethanol tolerant endophytic fungi from avocado bark and evaluation of endophytes using dual culture and colonization tube assay led to three potential biocontrol agents *Tricoderma crissum*, *Tricoderma simmonsii*, and *Lasiodiplodia theobromae*. Our findings showed these fungal endophytes were highly effective against RL growth and spread under *in vitro* conditions. Initial *in vitro* studies demonstrated the antifungal activity of silver pyrazolate to be highly effective against RL. Further application of these results and the use of silver pyrazolate in laurel wilt should continue to be researched in order to maximize management.

[1] GenBank accession numbers of closest fungal sequence of ITS.

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