Determining the effect of juglone (5-hydroxy-1,4-naphthoquinone) on gut microbiota of *Diaphorina citri*

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ABSTRACT

Diaphorina citri is a vector of the harmful citrus greening disease huanglongbing (HLB), which is caused by bacteria in the genus *Candidatus* Liberibacter. Juglone is a natural chemical produced by walnut trees that has herbicidal, insecticidal, and bactericidal properties. This study tested the effects of juglone on the gut microbiota of *D. citri*. The digestive tracts of fifteen individuals were extracted and plated, producing two different kinds of bacteria and five different types of fungus. Ten individuals were crushed whole and plated for comparison. Each unique bacterial and fungal colony was transferred to a plate with three wells: one for a positive control (erythromycin for bacteria; tea tree oil for fungus), one for negative control (water), and one for the experimental treatment (juglone). Results were taken by measuring the radius of inhibition around each well. Both bacteria were inhibited by juglone, though not as strongly as they were by erythromycin. Two of the fungi were actually better inhibited by juglone than by the positive control, while the third one was not inhibited by juglone at all.

INTRODUCTION

The Asian citrus psyllid (ACP), *Diaphorina citri* (Figure 1), is an agricultural pest of citrus trees. It is particularly important because it is a vector of the citrus greening disease huanglongbing (HLB), which is caused by bacteria in the genus *Candidatus* Liberibacter. These bacteria reside in the phloem of their host tree, and the disease is spread when a psyllid feeds on an infected tree then flies to and feeds on a non-infected tree (CDFA, 2017). This disease has cost the citrus industry in Florida $4.5 billion in lost revenue between 2006 and 2011 (Hodges and Spreen, 2012). There is a method of biocontrol currently being applied to deal with the problem of ACP spreading HLB, and it involves using a natural predator of the ACP, *Tamarixia radiata*, which is a wasp that parasitizes ACP specifically. A female wasp will oviposit one or two eggs underneath an ACP nymph, and the wasp larvae feed on the nymph as they grow, eventually consuming all of the nymph's body contents, pupating, and flying away as an adult (Qureshi and Stansly, 2010). This method of biocontrol has had varying effects. In Florida, parasitism of ACP by *T. radiata* averaged 50% in the fall of 2009, while in Isabela, Puerto Rico, parasitism rates generally exceeded 50% and averaged 70% (Qureshi and Stansly, 2010). The research we are doing focuses on finding other methods of combatting the spread of HLB.

Juglone (5-hydroxy-1,4-naphthoquinone) is an allelopathic chemical known to inhibit the growth of nearby plant species, and it is commonly found in many members of the walnut family Juglandaceae (Kocaçaliskan et al., 2008). Though the use of juglone is mostly relegated to its use as a natural herbicide, it has the capability to function as an insecticide as well. When fourth instar Promethea silkmoth larvae, *Callosamia promethea*, were fed a diet supplemented with juglone, the results showed negative growth rates and decreased leaf consumption rates (Thiboldeaux et al., 1994). The toxicity of juglone has also shown an effect on *Lymantria dispar* L. in which contact and stomach toxicity increased the mortality rates of the larvae (Sun et al., 2007). This study seeks to understand the effects of juglone on the bacteria found in the ACP's gut, which can provide insight into its effects on *Candidatus* Liberibacter.

METHODS AND MATERIALS

To test the effect of juglone on the microbiota of the ACP, fifteen individual ACP from a sterile environment were dissected in order to extract the digestive tract. The digestive tracts were crushed and streaked onto Luria Broth (LB) agar plates using a sterile loop. These plates were labeled the "red" plates. From the same environment, ten more ACP were taken, crushed, and streaked onto LB plates, which were labeled the "black" plates. These plates were left alone for a week, allowing any resulting bacterial or fungal colonies to mature. After four days of growth, however, some of the plates with large bacterial colonies had to be placed in a refrigerator in order to slow their growth. At the end of the week, a sample of each unique bacterial and fungal colony from both sets of plates was transferred onto new plates. The bacteria were kept on LB agar plates, while the fungal colonies were transferred to Potato Dextrose Agar (PDA) plates infused with lactic acid for pH stabilization. The plates were divided into three sections, and using straws sterilized with 10% bleach, a 7mm hole was punctured in each section to create a well (Figure 3). For the bacterial plates, using a micropipette, one well was filled with deionized water (the negative control), one with erythromycin at a concentration of 5mg/mL (the positive control), and one with juglone at a concentration of 1mM (the experimental). A control plate was also made using *Bacillus subtilis* as the bacterial agent. These plates were allowed to develop for two days, after which the results were taken by measuring the zone of inhibition around the juglone well.

As shown in Figure 3, the PDA plates received the same treatment as the LB plates, except the positive control used was tea tree oil, instead of erythromycin, because of its antifungal properties (Peèiulytë, 2005). A control plate was also made using *Pleurotus djamor* as the fungal agent. These plates were allowed to develop for three days, after which the results were taken.

Figure 1. *Diaphorina citri.*

Figure 2. Mark (left) and Omar (right) making LB plates.

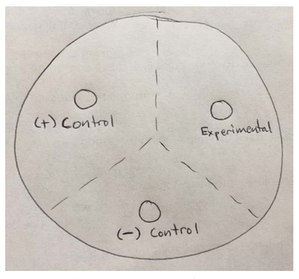

Figure 3. Schematic of plate division into three sections with a well in each one.

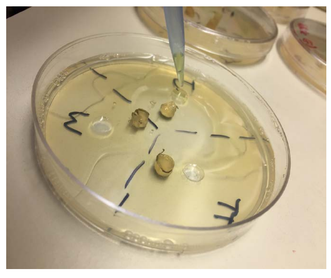

Figure 4. Experimental well of a PDA plate being filled with juglone.

RESULTS

There were two strains of bacteria found, a yellow one (Figure 5) and a white one (Figure 6). They were found on both, red and black, plates, and they were always found together (Table 1). Two of the red plates had no bacterial growth (Table 1). The bacterial control plate (Figure 7) showed a 1.5cm radius of inhibition around the positive control well and a 0.5cm radius of inhibition around the experimental well (Table 2). The experimental plate with the yellow bacteria (Figure 8) showed a 2.1cm radius of inhibition around the positive control well and a 0.7cm radius of inhibition around the experimental well (Table 2). The experimental plate with the white bacteria (Figure 9) showed a 1.5cm radius of inhibition around the positive control well and a 0.7cm radius of inhibition around the experimental well (Table 2). As expected, all three plates showed no inhibition around the negative control well (Table 2). There were three different types of fungus found: yellow, black, and dark green. All three fungi were found on both the red and black plates. Two of the black plates and two of the red plates had no fungal growth (Table 1). The fungal control plate (Figure 10) showed a 1.6cm radius of inhibition around the positive control well and a 1.4cm radius of inhibition around the experimental well (Table 3). The experimental plate with the yellow fungus (Figure 11) showed a 0.4cm radius of inhibition around the positive control well and no inhibition around the experimental well (Table 3). The experimental plate with the black fungus (Figure 12) showed a 0.3cm radius of inhibition around the positive control well and a 0.6cm radius of inhibition around the experimental well (Table 3). The experimental plate with the dark green fungus (Figure 13) showed a 0.4cm radius of inhibition around the positive control well and a 0.7cm radius of inhibition around the experimental well (Table 3). As expected, all four plates showed no inhibition around the negative control well (Table 3).

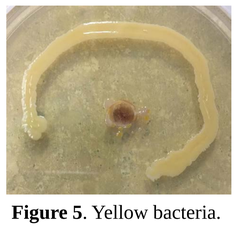

Figure 5. Yellow bacteria.

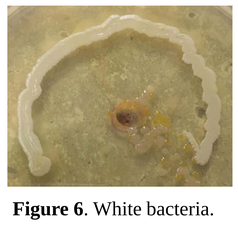

Figure 6. White bacteria.

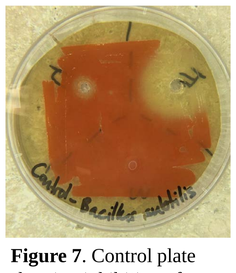

Figure 7. Control plate showing inhibition of *Bacillus subtilis*.

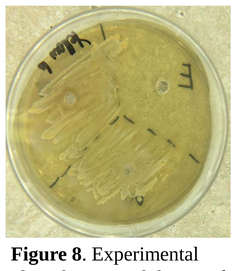

Figure 8. Experimental plate showing inhibition of yellow bacteria.

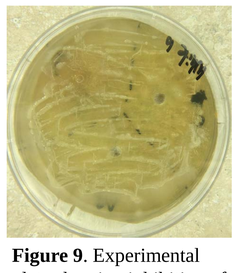

Figure 9. Experimental plate showing inhibition of white bacteria.

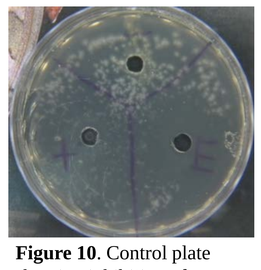

Figure 10. Control plate showing inhibition of *Pleurotus djamor.*

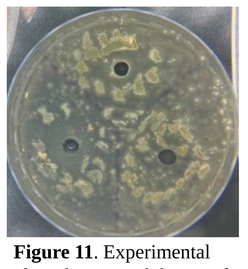

Figure 11. Experimental plate showing inhibition of yellow fungus.

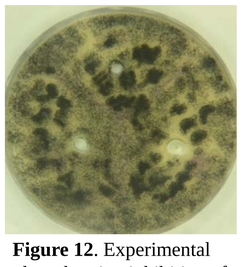

Figure 12. Experimental plate showing inhibition of black fungus.

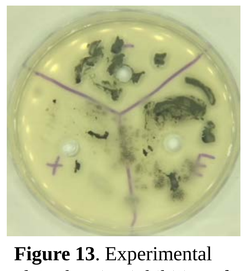

Figure 13. Experimental plate showing inhibition of dark green fungus.

Table 1. The number of different types of bacteria and fungi found on each red and black plate.

Red plate #	Fungi	Bacteria	Black plate #	Fungi	Bacteria
1	0	2	**1**	2	2
2	0	2	**2**	0	2
3	3	0	**3**	0	2
4	1	2	**4**	2	2
5	3	2	**5**	2	2
6	2	2	**6**	3	2
7	2	2	**7**	1	2
8	3	2	**8**	3	2
9	3	2	**9**	3	2
10	2	0	**10**	2	2
11	1	2	-	-	-
12	1	2	-	-	-
13	3	2	-	-	-
14	3	2	-	-	-
15	3	2	-	-	-

Table 2. Zones of inhibition on the bacterial plates, expressed as radius measurements.

Specimen	Negative control (cm)	Positive control (cm)	Experimental (cm)
Control	0	1.5	0.5
Yellow bacteria	0	2.1	0.7
White bacteria	0	1.5	0.7

Table 3. Zones of inhibition on the fungal plates, expressed as radius measurements.

Specimen	Negative control (cm)	Positive control (cm)	Experimental (cm)
Control	0	1.6	1.4
Yellow fungus	0	0.4	0
Black fungus	0	0.3	0.6
Dark green fungus	0	0.4	0.7

DISCUSSION

Only two types of bacteria were found in the gut of *D. citri*, and the lack of any other types of bacteria on the black plates, which had a whole ACP crushed on them, indicates that these bacteria come from the gut. Juglone did inhibit the growth of these bacteria, but it was not as significant in its inhibition as erythromycin, the positive control. A higher concentration of juglone could be more effective, and further research would need to be done to test this.

The amount of different fungi, compared to bacteria, found in the gut of *D. citri* was unexpected. However, studies show that many insects develop symbiotic relationships with fungi, and fungi harbored in an insect's gut can help break down large food molecules, as well as help with nutrient acquisition and uric acid metabolism (Vera-Ponce de León et al., 2016). The yellow fungus was not inhibited by juglone at all, while the other two fungi were actually better inhibited by juglone than the positive control. These results could be significant, but since we do not know the exact relationship between these fungi and the ACP, we cannot be sure. Further research on this relationship would provide insight on the significance of these results and determine the future direction of this project.

The unique properties of juglone are significant in research that attempts to utilize biocontrol over the use of commercial pesticides. Results from the experiment show that juglone may be an alternative in attempts to control the population and spread of ACP. However, it remains to be seen whether juglone can be effectively used while limiting any potential harmful effects.

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