Practical detection of fungicide resistance in the field

OTRF Progress Report (5 Jan 2021)
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Summary: The purpose of this project is to develop a field kit that turf managers can use to assess whether their dollar spot has resistance to DMI fungicides such as propiconazole. Funding for this project started 1 Sept 2020, and MSc student, Ed McNab, has been taken on for this work. During fall semester, 2020, the main objective was to develop a discriminatory concentration which could be used to distinguish between resistant and sensitive isolates of the dollar spot fungus. This objective has been achieved. In addition, other additions to the discriminatory medium were found to be needed since other organisms such as bacteria which are naturally resistant to DMI fungicides were also found on leaf blades. Continuing work in 2021 will be to test field samples (infected grass from the field) to see if other micro-organisms will grow, and to test if other measures are needed to develop this field kit. Field testing by turf managers may start in late summer 2021. So far, all funds spent have been directed toward support of the graduate student (around $6,000) plus some minor lab expenses for materials. We project that by summer 2022, a simple assay consisting of petri plates with selective media in them can be sent out to turf managers for them to test their dollar spot-infected leaf samples on their own, and see results in 2 or 3 days.

NOTE: These first two pages give background material from the original proposal. The Progress to date can be found on pages 3 to 11.

Background (as stated in original proposal)

During the past 25 years, the Hsiang Lab has been working on fungicide sensitivity of turfgrass pathogens in Canada, particularly Ontario. We have observed increasing levels of reduced fungicide sensitivity, and in some cases outright resistance to fungicides such as propiconazole. There have been increasing reports of decreased fungicide sensitivity among turfgrass diseases by turfgrass managers, usually loss of control or greatly reduced interval of control after fungicide application. Because most fungicides belong to a family defined by their mode action, then insensitivity to one fungicide (e.g. propiconazole in Banner MAXX) may result to insensitivity to other fungicides that share the same mode of action (e.g. myclobutanil in Eagle). A tool is needed by turf managers to be able to roughly assess their local levels of fungicide resistance. This research aims for the development of simple inexpensive kits that will allow a turf manager to test their own diseased blades of grass for resistance to fungicides such as propiconazole. Turf managers will be able to assess whether particular areas of turf harbour sensitive fungi or those which are no longer well controlled by particular fungicide applications. There have been no previous reports of development of such kits for field use. The first half year (fall 2020 through winter 2021) will be devoted to the development of proper discriminatory concentrations that can separate sensitive from moderately resistant isolates of the dollar spot fungus, and kits that can be used in the field. The initial kit will be field tested in summer 2020 by local staff as well as selected turf managers. With satisfactory progress, in the second year, the kit will be refined, perhaps for selection of other pathogens and other fungicides. These refined kits will be tested in summer 2021 and summer 2022. Funding after the first year will depend on satisfactory progress.

Objectives (as stated in original proposal)

OBJECTIVE 1 Find out a proper discriminatory concentration that can separate propiconazole sensitive isolates of *Clarireedia jacksonii* (cause of dollar spot on bentgrass) from resistant ones.
OBJECTIVE 2 Develop a field assay/kit that can be used by turfgrass managers to assess the level of fungicide sensitivity (propiconazole) in their local populations of *C. jacksonii.*

OBJECTIVE 3 Field testing of the plates with turf managers
OBJECTIVE 4 If this proof of concept works with the propiconazole - dollar spot system, then similar kits can be developed for other fungicides and other pathogens

Methods (as stated in original proposal)

OBJECTIVE 1 Discriminatory concentration

To determine a concentration of propiconazole which will inhibit sensitive isolates of *C. jacksonii* but not inhibit resistant isolates, a collection of isolates with known EC50 values will be used. In total, 10 isolates from courses in Ontario will be used, four resistant isolates with known EC50 values greater than 0.1 µg/ml, three isolates with moderate sensitivity, and three sensitive isolates with known EC50 values around 0.01 µg/ml. Chosen isolates will be sub-cultured from stock samples and grown on PDA plates for roughly 48-hours.

PDA plates amended with propiconazole at 0, 0.01, 0.1, 1.0 and 10 µg/ml will be made from a Banner Maxx stock solution. Fungicide treatment concentrations used in this study are determined based on previous EC50 values and consultation with Dr. Tom Hsiang. PDA amended with fungicide will then be cut into three strips to allow for multiple isolates to be grown in the same plate. Isolates will be sub-cultured from the active mycelium edge on the un-amended PDA plates and placed on the plates amended with various fungicide concentrations mentioned above. After inoculating the amended plates, they will be incubated at 22ºC for 48-hours. After 24-hours of incubation, the edge of mycelium will be marked, and repeated after 48-hours. The difference between the two measurements will be used to calculate the isolate's EC50 value. The difference between the measurements will be used rather than just the 24- or 48-hour measurements because some isolates may have difficulty establishing, therefore taking the difference removes that confounding variable. The EC50 value for each isolate will be determined by a probit regression using SAS (Hsiang et al., 1996). By examining the results, a potential discriminatory concentration of propiconazole will be chosen.

Objective 2 Methodology: (field assay/kit)

PDA plates with the inhibitory concentration of propiconazole and appropriate levels of antibiotics (to inhibit bacterial growth) will be used to test field samples of dollar spot disease. Four leaf blades (up to 2 cm long) bearing dollar spot lesions will be placed on these test plates and incubated for up to four days. The dollar spot fungus grows very quickly and results should be visible within 2 or 3 days. The fungicide and antibiotic should inhibit other organisms and only allow resistant dollar spot isolates to grow (unless there are other microbiota which have resistance to these chemicals).

Objective 3 Methodology: (turf manager testing)

Once the plates amended with the discriminatory concentration are made, the amended plates can be distributed to golf course managers. A small instruction manual will be made to help managers collect samples properly and avoid contamination. A manager will take a sample of infected leaf tissue from their course and place it on the amended PDA plate. They will monitor it over the following days and if mycelium does not grow, they have a sensitive population and can continue to apply propiconazole because it will continue to provide control. On the other hand, if mycelium does grow, they have a resistant population and should not continue to apply propiconazole because it does not provide control unless applied at a high rate. This will be done systematically with plates sent to at least 10 turf managers (to get at least 10 results).

Objective 4 Methodology: (other fungicides & pathogens)

If this proof of concept works with the propiconazole - dollar spot system, then similar kits can be developed for other fungicides and other pathogens using the methods stated above.

Research Progress

The funding for this research started 1 Sept 2020, and MSc graduate student, Ed McNab, was taken on for this work at this time. Because of shutdown conditions at the University of Guelph, research time and lab and field access has been more limited compared to normal times. However, we have been able to achieve Objective 1, development of a discriminatory concentration and medium that can select between sensitive and resistance isolates of *Clarireedia jacksonii.* The details follow:

Test selected isolates of the dollar spot pathogen, *Clarireedia jacksonii,* towards propiconazole, using strip agar assays

The purpose was to select and confirm the sensitivity of select isolates of *Clarireedia jacksonii*. These were isolates stored in the lab which had been collected for previous studies, and isolates showing resistance were targeted. In addition, fresh isolates from turfgrass in and around Guelph were collected also.

Among stored isolates, 10 were retrieved. As well two new isolates were collected from the Guelph Turf Institute (GTI). Growth of each isolate was then assessed using a strip agar assay and compared to previously reported values. The growth rate of the mycelium was then marked at 24 and 48 hours. Figure 1A shows an example of the strip-agar assay to illustrate how less agar can be used and the hyphal plug placement. Figure 1B shows the same assay after 48 hours of growth on unamended the top strip contains a resistant isolate, the middle strip contains a reduced sensitive isolate and the bottom strip has a sensitive isolate.

PDA. The 12 isolates were also assessed using PDA amended with propiconazole at concentrations of 0.01, 0.1, 1.0, and 10 µg/ml (Figures 1C, D, E, F). Inhibitory concentration (EC_{50} values) for each isolate were then calculated using SAS (statistical analysis system) with the probit algorithm. Based on these values, each isolate was assigned a sensitivity towards propiconazole of sensitive, moderately sensitive or resistant. Results for each isolate were then compared to those of the previous study and differences noted. The results from these trials generally matched the original designations and are reported in Table 1 below. The resistance levels for the two isolates gathered in 2020 were assigned using the same methodology.

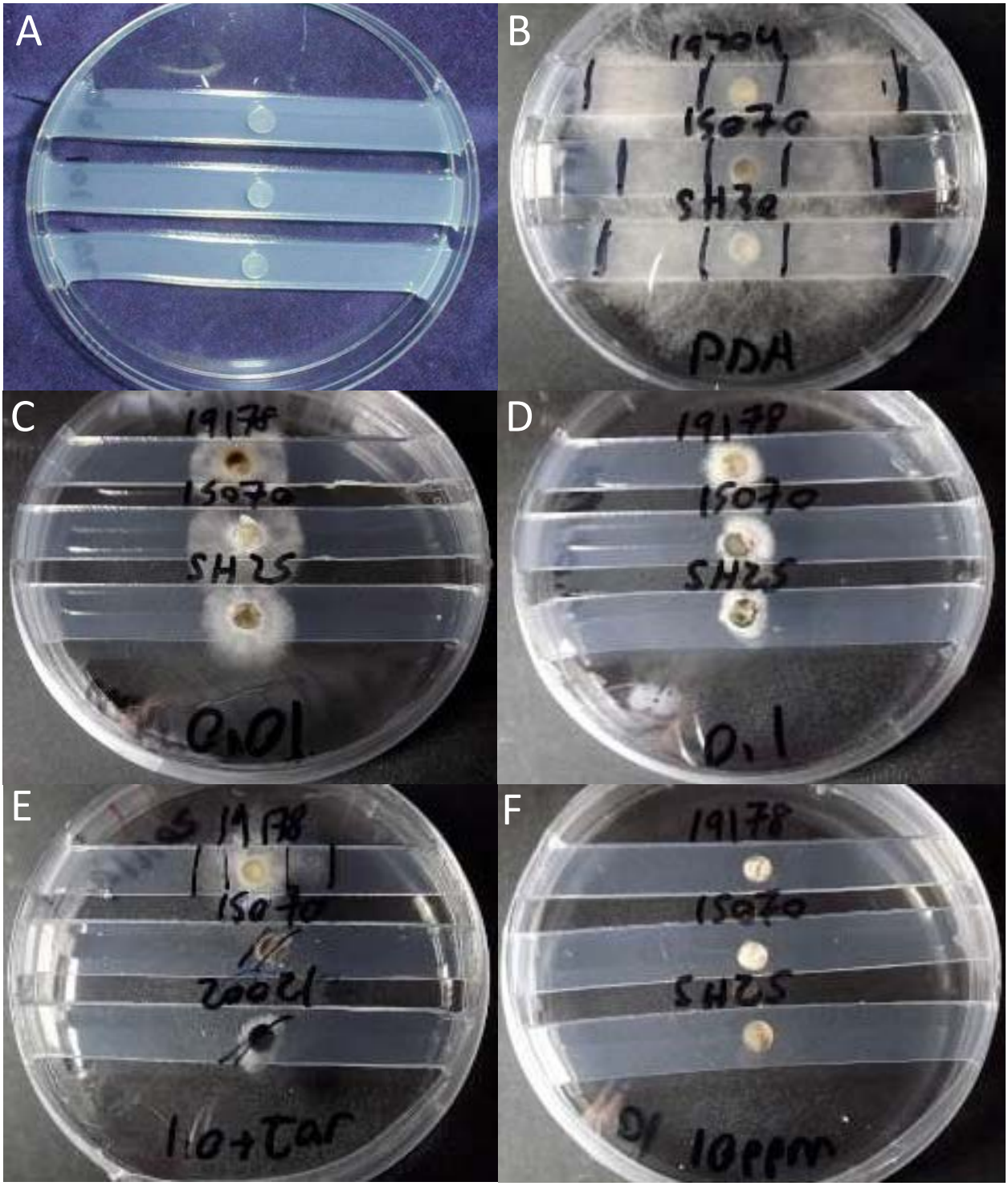

Figure 1: Strip agar assays to illustrate comparative growth rates of *Clarireedia jacksonii* isolates on PDA amended with increasing concentrations of propiconazole. (A) Strip agar assay showing agar strips cut from regular 9 cm diameter plate and the placement of hyphal plugs. (B) Growth on potato dextrose agar plate without any propiconazole added after 48 hours. Growth after 48 hours on plates amended with propiconazole: (C) 0.01 µg/ml, (D) = 0.1 µg/ml, (E) = 1.0 µg/ml, and (F) = 10 µg/ml. In each plate,

Table 1: Results from isolate sensitivity assessment experiment, showing calculated EC_{50} values and assigned sensitivities for the current (2020) study and the previously documented values (2015).

Isolate	Year collected	EC_{50} (µg/ml)		Sensitivity[1]	
		2015	2020	2015	2020
SH25	1994	0.01	0.01	Sensitive	Sensitive
SH30	1994	0.03	0.01	Sensitive	Sensitive
SH15	1994	0.03	0.01	Sensitive	Sensitive
15067	2013	0.04	0.04	Moderately sensitive	Moderately sensitive
15070	2013	0.05	0.04	Moderately sensitive	Moderately sensitive
15069	2013	0.06	0.16	Moderately sensitive	Resistant
19185	2013	0.31	0.23	Resistant	Resistant
19178	2013	0.28	0.41	Resistant	Resistant
19204	2013	0.27	0.13	Resistant	Resistant
19145	2013	0.34	0.28	Resistant	Resistant
20020	2020	n/a	0.83	n/a	Resistant
20021	2020	n/a	0.01	n/a	Sensitive

[1] Sensitivity categories were defined using calculated EC_{50} values: isolates with an $EC_{50} > 0.1$ µg/ml are considered resistant, $0.04 < EC_{50} > 0.1$ µg/ml are moderately sensitive, and $EC_{50} < 0.04$ µg/ml are sensitive.

Determining a concentration of propiconazole to distinguish sensitive vs. resistant isolates of *Clarireedia jacksonii*, under lab conditions.

The goal was to identify a concentration of propiconazole that when amended to PDA would completely inhibit mycelial growth of sensitive and moderately sensitive isolates but allow for some growth of resistant isolates. This would eventually allow for simple visual assessment of assay results in a field setting (e.g. in the hands of a turf manager). Using the sensitivity levels in Table 1, the experiment was conducted to determine a concentration of propiconazole that best inhibits moderately sensitive and sensitive isolates of *Clarireedia jacksonii*. The same series of propiconazole concentrations used in the previous experiment were assessed for this selectivity. Figure 1C-F shows the effect of 0.01, 0.1, 1.0 and 10 µg/ml. In Figure 1E specifically, the concentration of 1.0 µg/ml inhibited both the sensitive and moderately sensitive isolates, while the resistant isolate still displayed noticeable growth. These results suggest a concentration of 1.0 µg/ml propiconazole is suitable for identifying resistant isolates of *Clarireedia jacksonii* using pure isolates in a lab environment. Going forward PDA amended with 1.0 µg/ml propiconazole will be referred to as the discriminatory media or medium.

Using lab grown grass samples as an infected field sample analogue to test the discriminatory media.

To simulate field samples, tubes of sand were seeded with Penncross (*Agrostis stolonifera*) and grown in a lab setting, as seen in Figure 2. After 2-3 weeks of growth, these tubes were inoculated with *Clarireedia jacksonii* isolates of varying sensitivity. Inoculation with a hyphal agar plug is shown in Figure 3A and with ground seed inoculum in Figure 3B. Once inoculated, tubes were monitored for disease development, Figure 4 depicts a healthy (right) and an infected (left) cone-tainer with creeping Penncross five days after inoculation. Once abundant mycelium and foliar lesions were observed, leaf segments (< 2 cm) could be cut and placed on discriminatory media to assess growth of the fungus. Only isolates which were resistant should be able to grow on this selective medium.

Figure 2: Cone-tainers seeded with creeping bentgrass (*Agrostis stolonifera*), allowed to grow at 22 °C for 2.5 weeks with 12 hour light cycle. Prior to inoculation each Cone-tainer is trimmed to within 3 cm of the cone-tainer rim.

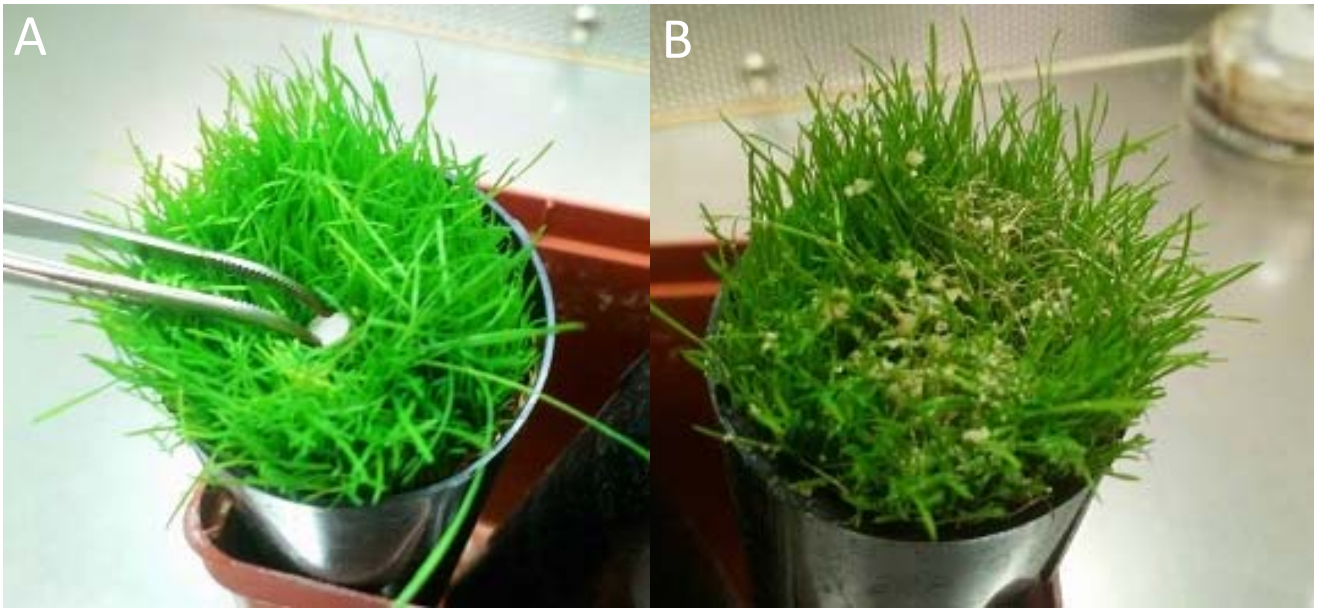

Figure 3: Inoculation of a cone-tainer with *Clarireedia jacksonii* isolate, using (A) 5 mm agar plugs and (B) ground seed inoculum. Both tubes were trimmed to within 3 cm of the cone-tainer rim, and inoculated after 2.5 weeks of growth.

Figure 4: Comparison between creeping bentgrass (left) with and (right) without inoculation of *Clarireedia jacksonii*. The (left) infected tube shows hyphal masses and yellowing of the leaf blades.

To ensure the mycelium present within the leaf blade was being assessed, a surface sterilization step was introduced. An infected grass blade sample would be dipped in a mild sodium hypochlorite solution (diluted bleach) for a pre-determined period of time to kill any remnants of inoculation material (seed or agar) present on the leaf surface. A series of treatments ranging from 0.25% sodium hypochlorite for 1 second to 1% sodium hypochlorite for 3 seconds were examined. From these samples it was shown dipping in 0.25% sodium hypochlorite for 1 second was enough to kill any remaining inoculum. Infected leaf samples were placed on the discriminatory media and growth marked at 24 and 48 hours. Results could then be compared previous strip agar tests.

It was found that fungi from infected samples started growth later compared to hyphal plugs. This could represent the time that a fungus requires to growth out of the leaf blade and onto the agar and start feeding on the agar for further growth. First signs of hyphal growth could be observed after 24 hours at room temperature, but the hyphae were easier to observed at 72 hours. Regardless of the delay in establishment, the discriminatory media still produced a visible distinction between sensitive and resistant isolates. An example of these results can be seen in Figure 5.

Testing of Discriminatory Medium with Field Samples

Samples of creeping bentgrass and of Kentucky bluegrass were collected for testing with the discriminatory concentration. The leaf blades were cut or torn to small pieces (< 0.5 cm lengths) and placed on PDA amended with 1.0 µg/ml propiconazole. Some of these results revealed that there are other organisms on field samples that are resistant or show a natural tolerance toward the 1.0 µg/ml propiconazole. Figure 6 shows examples of plates presenting such organisms. The identity of these other organisms, particularly fungi, is being examined by DNA extraction and sequencing, but this is still in progress. We also noticed that bacteria would sometimes grow on these samples, so additions to the media are needed to deter bacterial growth.

Something to consider is that all these samples were collected in mid to late fall 2020, and the leaf surface microflora differs considerably from that which would be found in late spring and summer and even early fall when dollar spot is normally seen. So, we need to test grass samples from these time periods to see what else might grow, which we propose to do in 2021

Figure 5: Leaf segments placed on PDA amended with 1.0 µg/ml propiconazole and 1.0% tartaric acid which can distinguish between resistant (19178 and 19185), moderately sensitive (15070), and sensitive (SH15) isolates of *Clarireedia jacksonii* after 48 hours of growth at 22 °C.

Using acidified media as an alternative for antibiotics, potentially adding additional selective pressures.

While DNA analysis is being conducted an experiment involving the efficacy of acidifying the media was pursued. Acidified media is a potential alternative to antibiotics for selecting against bacteria and may provide additional selective pressure. To ensure *Clarireedia jacksonii* was not negatively impacted by the presence of the acid isolate growth was assessed across multiple concentrations of two acids. Tartaric and lactic acids are commonly used as amendments to PDA for bacterial inhibition. These were evaluated through in a series of concentrations amended to PDA: tartaric acid ranging from 0.05% to 0.15%, and lactic acid ranging from 0.015 to 0.75%. Growth rates were assessed using the strip agar test and marked at 24 and 48-hours and compared to the growth of the same isolates on unamended PDA. Tartaric acid presented reduced growth at 0.15%, thus a concentration up to 0.1% is suitable for use with this fungus. Lactic acid showed no impact on growth at any concentration tested. A comparison between using antibiotics and tartaric acid can be seen in Figure 7, this shows no difference between the two media for selection purposes given no other organisms are present.

Finally, asymptomatic grass samples collected from the University of Guelph, Halton Hills, and Parry Sound, were assessed on media containing 1.0 µg/ml propiconazole and 0.1% tartaric acid, Figure 8. This shows no contaminating organisms growing within 72-hours. The addition of tartaric acid was also successful at inhibiting one of the non-target organisms encountered at GTI, specifically the one shown to detoxify propiconazole. Figure 9 shows a strip agar test of this organism in the presence of lactic acid (Figure 9A) or tartaric acid (Figure 9B). While the presence of lactic acid appeared to have

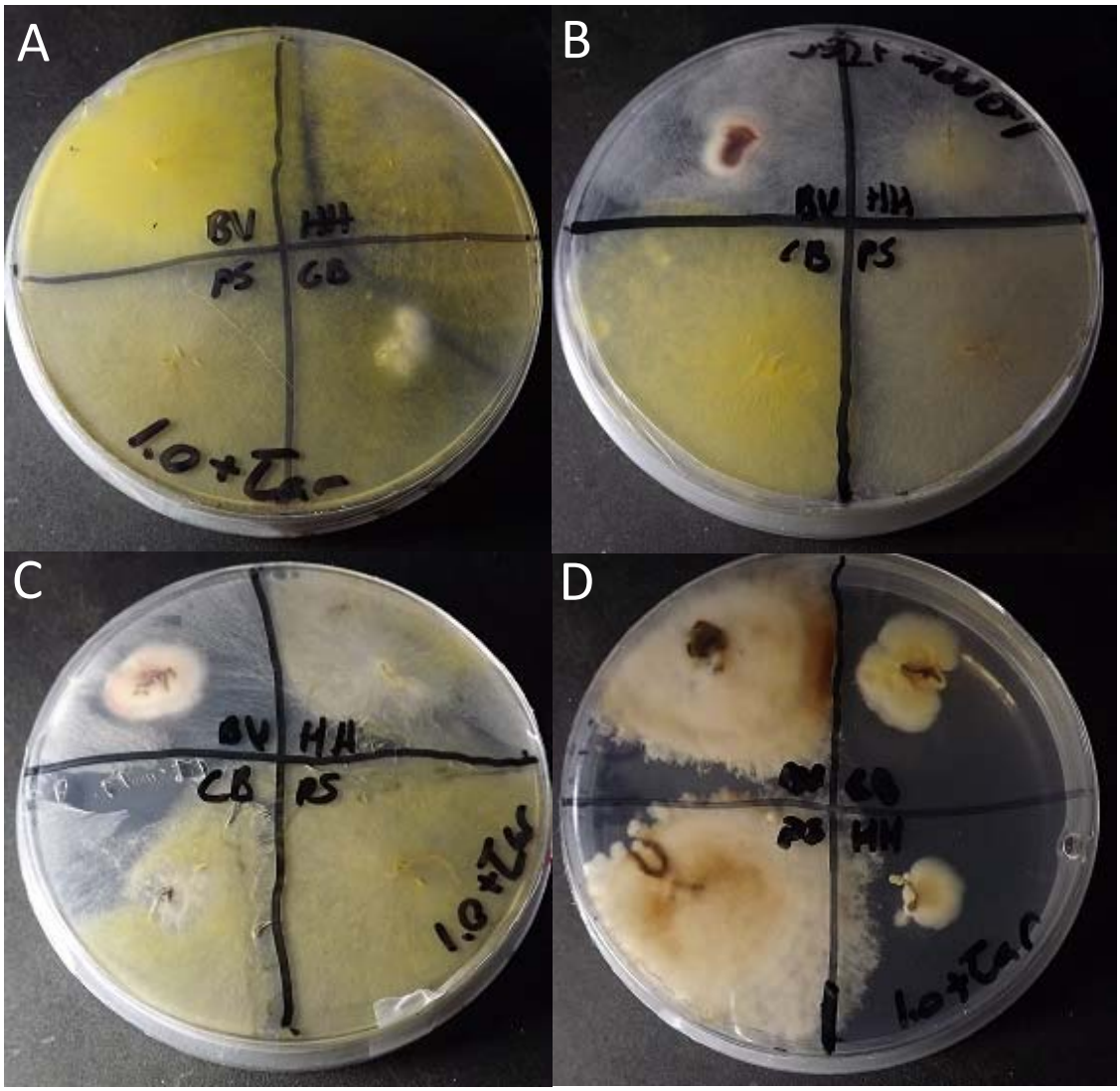

Figure 6: Asymptomatic field samples evaluated on PDA amended with 1.0 µg/ml propiconazole. Plates A, B, C, and D show organisms not sensitive to 1.0 µg/ml propiconazole. Plates were incubated for 48 hours at 22°C, growth was marked and 24 and 48 hours.

little to impact on the growth of the organism, the presence of tartaric acid greatly reduced the rate of growth. However, it is to be noted that this test was conducted in mid-November, and while this medium containing propiconazole and tartaric acid was successful in selecting for *Clarireedia jacksonii* at this time, the microenvironment will change with the seasons. This may present with additional non-target organisms that could interfere with the assay. As such, this experiment is ongoing.

Current state of the discriminatory media and future steps:

Currently two media are suitable for preliminary use; PDA amended with 1.0 µg/ml propiconazole with either 100 µg/ml tetracycline and streptomycin or 0.1% tartaric acid amendments can select for resistant isolates of *Clarireedia jacksonii* in a lab setting. Both also show potential for selection of field samples in the late fall, but further selection may be required as the seasons progress. Going forward, the continued identification of non-target organisms and additional amendments will further refine the discriminatory medium and increase its chances of success. In the coming spring and summer season field trials will be conducted to evaluate the efficacy of the medium for use in the field. Pending field trial success, a small distribution can be undertaken to assess the success of the field kits in the hands of the target consumers.

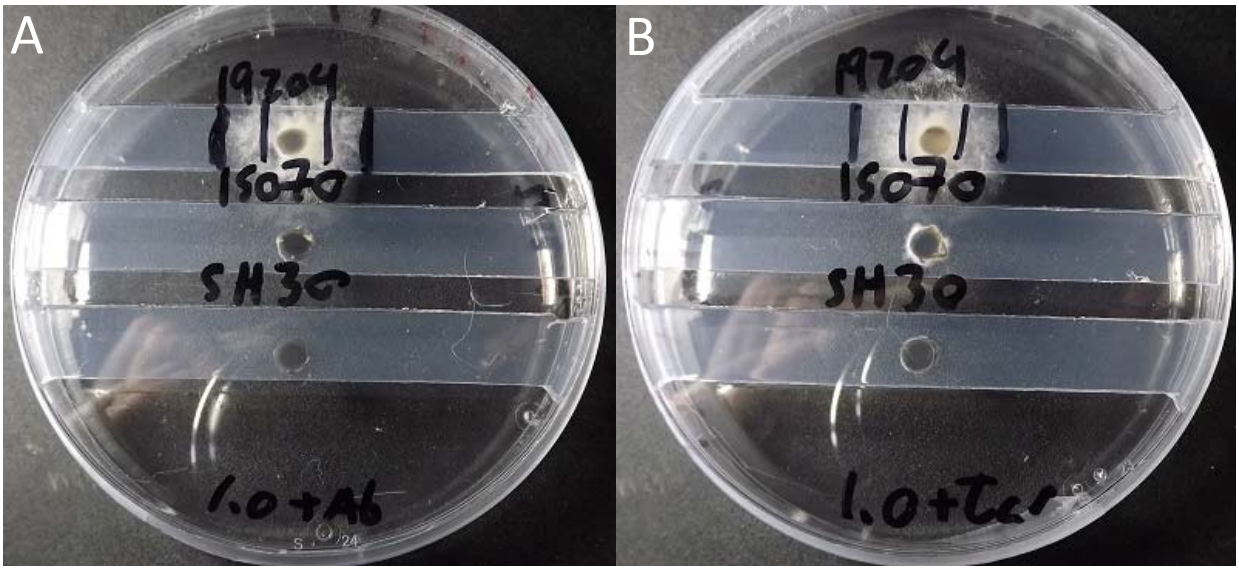

Figure 7: A comparison of the impact of using (A) 100 µg/ml tetracycline and streptomycin to (B) 1.0% tartaric acid in a basal PDA medium amended with 1.0 µg/ml propiconazole in selecting for resistant isolates of *Clarireedia jacksonii* after 48 hours.

Figure 8: Asymptomatic field samples evaluated on PDA amended with 1.0 µg/ml propiconazole and 1.0% tartaric acid. Plate was incubated for 72 hours at 22°C.

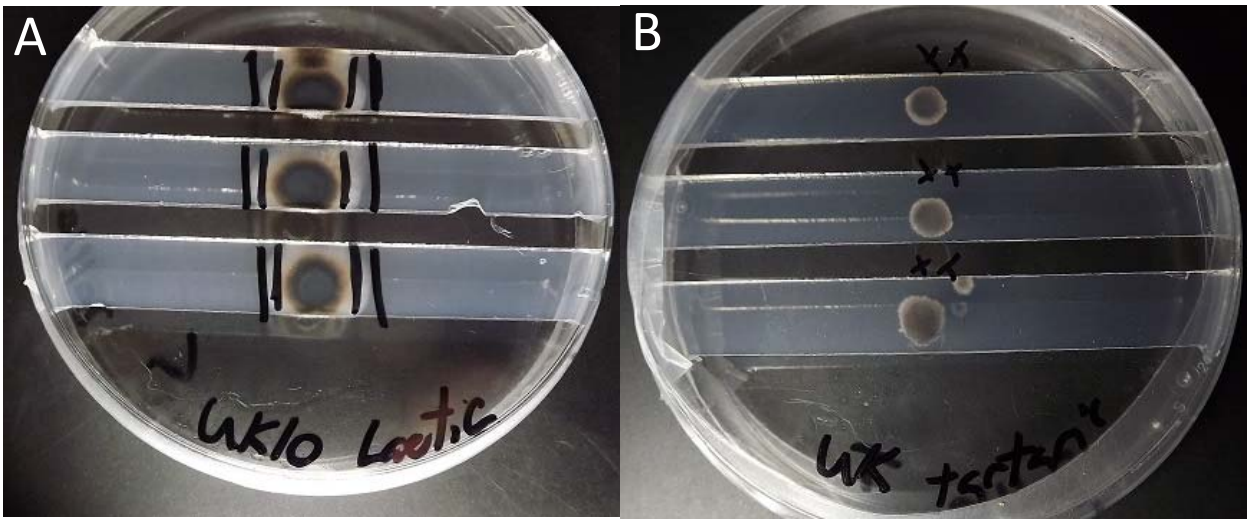

Figure 9: Unidentified organism growing on PDA amended with (A) 0.075% lactic and (B) 1.0% tartaric acid. Plates were incubated for 24 hours at 22°C, growth was marked at 24 and 48 hours.