Comparison of the amended agar assay vs. a microplate absorbance assay for assessing propiconazole sensitivity of turfgrass pathogen *Clarireedia jacksonii*

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Introduction

Clarireedia jacksonii is the causal agent of dollar spot disease of turfgrass (Smiley et al. 2005). The golf industry has low tolerance for this highly destructive fungal pathogen as it causes aesthetic damage and pits (Fig. 2). Turfgrass superintendents rely heavily on fungicides to manage yearly epidemics of dollar spot disease (Smiley et al. 2005) and use several applications per year. The commonly used fungicide, propiconazole, belongs to the family of sterol inhibitors which interfere with fungal cell membranes (Price et al. 2015). Frequent use of fungicides select for fungal genotypes which are less sensitive to the applied fungicide and disease damage may become severe even with fungicide use. Many research groups have identified this phenomenon for the dollar spot pathogen (Hsiang et al. 2007, Van Den Nieuwelaar and Hsiang 2014, Golembiewski et al 1995, Bishop et al. 2008).

Figure 1: Symptoms of dollar spot disease caused by *C. jacksonii* as sunken tan patches in creeping bentgrass (*Agrostis stolonifera*) maintained by putting green height. Picture courtesy of T. Hsiang.

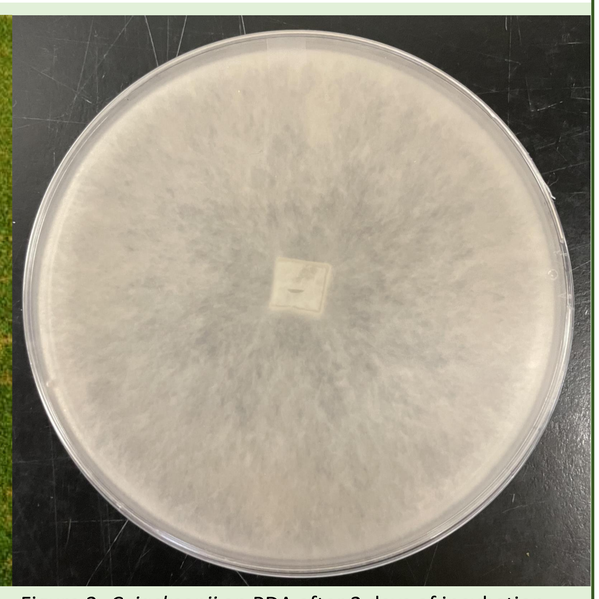

Figure 2: *C. jacksonii* on PDA after 2 days of incubation

The amended agar assay has long been used to measure fungicide sensitivity of plant pathogens *in vitro.* The microplate absorbance assay is commonly used to measure drug sensitivity of bacteria but has recently been considered for fungi. Fungal spores can be harvested and used for absorbance sensitivity assays in lieu of bacterial spores, but this is not possible for *C. jacksonii*, which does not produce spores. The purpose of this study was to develop a microplate absorbance assay for *C. jacksonii* and compare it to the traditional amended agar assay. A benefit of the microplate assay is higher throughput processing of more isolates with less labour and materials.

Methods

Selection of Isolates: Thirty isolates of *C. jacksonii* were selected for both the amended agar assay and the microplate absorbance assay. Isolates were chosen for both tests based on their previously measured sensitivities to propiconazole. Isolates were revived from refrigerated stock composed of hyphal PDA (potato dextrose agar) plugs in water.

Strip Agar Assay: Media was prepared by pouring molten PDA amended with propiconazole to concentrations of 0, 0.01, 0.1, 1.0, and 10 µg/ml into 10-cm-diameter petri dishes. Solidified agar was cut using a 6-blade stencil and excess agar was removed to create separated strips (Fig. 3). Hyphal agar plugs were cut with a 5-mm-diameter cork borer and placed in the center of an agar strip. Radial growth was marked at 24 and 48 hours (Fig. 5). The growth difference of 48 h – 24 h was used to calculate an EC_{50} value using SAS Proc Probit (SAS version 9.1, SAS Institute, Cary NC), and each experiment was repeated three times.

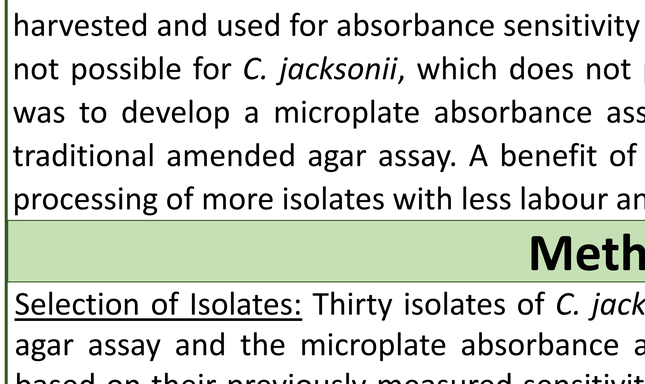

Figure 3: Solidified agar cut with a 6-blade stencil with excess agar removed

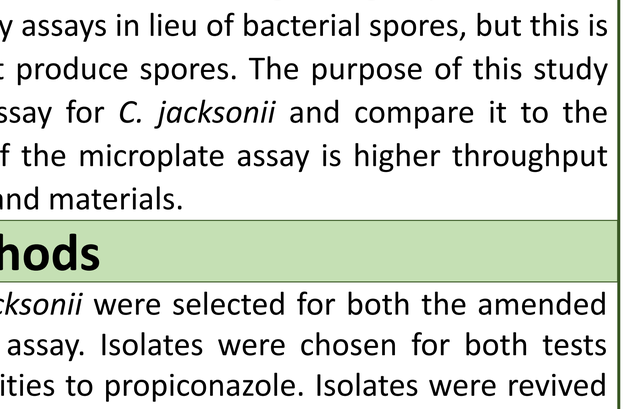

Figure 4: Cutting of a 1-mm-hyphal plug from a culture of *C. jacksonii* (A). The 1 mm hyphal plug ejected from the pipette tip (B).

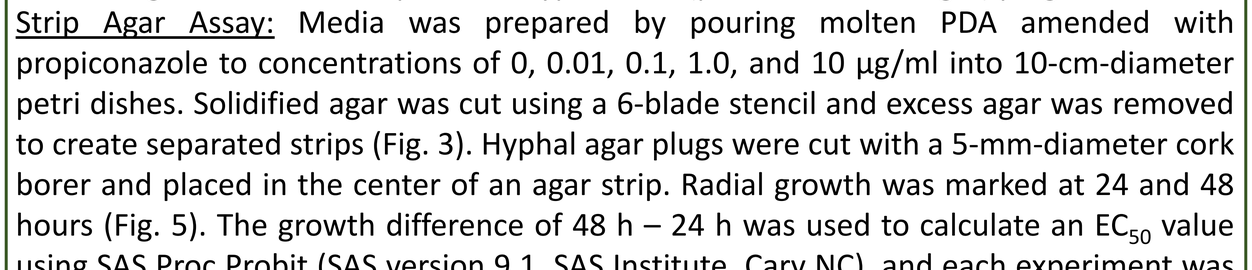

Figure 5: Radial growth of *C. jacksonii* after 48 hours of incubation. Hyphal plugs were placed in the center of each agar strip and radial growth was marked at 24 and 48 hours. Isolates had low inhibition of growth at lower fungicide concentrations (B-C) and higher inhibition of growth at higher fungicide concentrations (D-E). The isolate 19145 is more resistant to propiconazole than SH15 or SH25 by having lower inhibition at 1.0 µg/ml (D).

Microplate Absorbance Assay

Potato Dextrose Broth (PDB) was amended with propiconazole to the concentrations 0, 0.01, 0.1, 1.0, and 10 µg/ml. Microplates (Thermofisher, Waltham, MA) were filled with amended PDB with 8 wells per concentration and 2 isolates per microplate (Fig. 6). A 1-mm-diameter hyphal plug was obtained from agar plates by stabbing a standard 1000 µl pipette tip (Global Scientific, Mahwah, NJ) into the growing margins of the culture, and ejecting the micro-plug into each well (Fig. 4). Absorbance was measured at 24 and 48 hours using an MBI AMR-100 microplate reader. Files were transferred to a computer using ReaderIt software (MBI, Montreal, QC). Absorbance at 48 h – 24 h was used to calculate an EC_{50} value for each isolate using SAS Proc Probit, and each experiment was repeated three times.

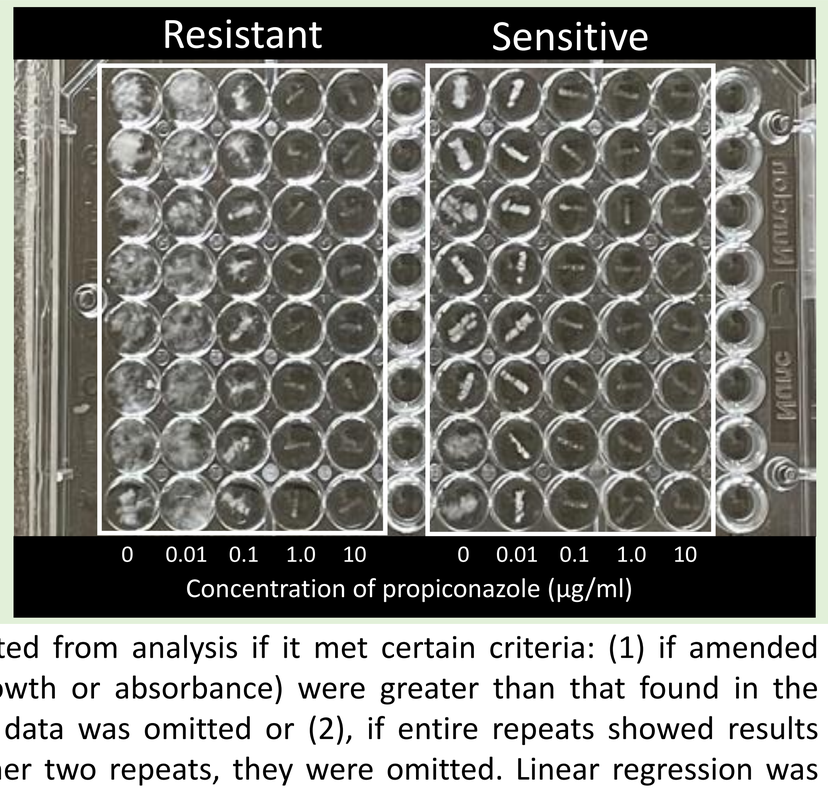

Figure 6: Growth of *C. jacksonii* in a 96-well-microplate after 48 hours. Hyphal micro-plugs were cut and ejected into wells. Absorbance was measured at 24 and 48 hours. Resistant isolate (Resistant) had growth at 0.1 µg/ml compared to sensitive isolate (Sensitive) which did not show any growth at 0.1 µg/ml.

Some of the data was omitted from analysis if it met certain criteria: (1) if amended medium measurements (growth or absorbance) were greater than that found in the non-amended medium, the data was omitted or (2), if entire repeats showed results vastly different than the other two repeats, they were omitted. Linear regression was used to compare EC_{50} values between strip agar assays and microplate absorbance assays for non-transformed and log transformed values.

Results

Strip Agar Assay vs. Microplate Absorbance Assay

The EC_{50} values from the microplate absorbance assay showed a significant relationship ($p = < 0.0001$) with their counterparts from the amended agar assay, but the correlation was low ($R^2 = 0.06$) (Fig. 7). Log transformation of both amended agar EC_{50} values and microplate absorbance EC_{50} values revealed a higher correlation ($R^2 = 0.47$) and was also highly significant ($p = < 0.0001$) (Fig. 8).

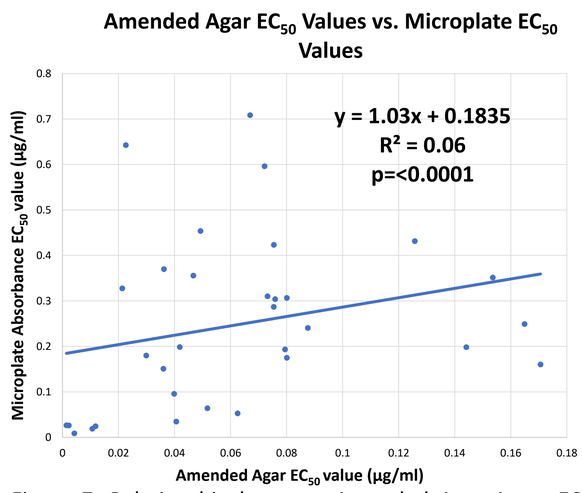

Figure 7: Relationship between Amended Agar Assay EC_{50} and Microplate Absorbance Assay EC_{50} values. The relationship is highly significant ($p = <0.0001$) but is of low correlation ($R^2 = 0.06$).

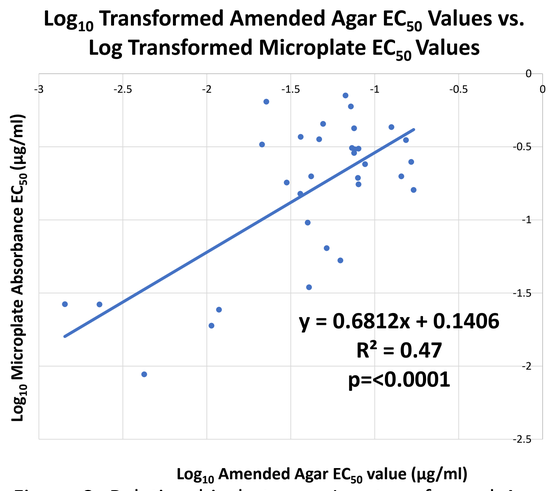

Figure 8: Relationship between Log transformed Amended Agar Assay EC_{50} and Log transformed Microplate Absorbance Assay EC_{50} values. The relationship is highly significant ($p = <0.0001$) and is of higher correlation ($R^2 = 0.47$) compared to the non-transformed relationship.

Conclusion

The microplate assay method for measuring fungicide resistance of *C. jacksonii* was developed to increase the speed with less expense and labour compared to the amended agar assay. The results showed that linear comparisons of the two sets of results were significant but of low correlation ($R = 0.25$, $p = <0.0001$). A transformation (log base 10) of the results greatly increased their correlation values ($R = 0.69$, $p = <0.0001$). These results imply that the current microplate absorbance assay cannot directly replace the amended agar assay for calculation of specific EC_{50} values, but future developments in the methods may ultimately allow for such use.

References:

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