In Vitro Assay Development to Screen Early and Late Leaf Spot Pathogens for SDHI, DMI, and QoI Resistance Management

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ABSTRACT

Fungicide resistance in plant pathogens poses a significant threat to global food security. Early leaf spot, caused by Passalora arachidicola, and late leaf spot, caused by Nothopassalora personata, are major diseases of peanuts (Arachis hypogaea). In vitro studies of these slow growing pathogens have been limited. This study optimized two assays: a microtiter plate assay for measuring biomass in liquid medium and a resazurin assay for detecting fungal metabolism, with a focus on incubation periods and inoculum concentrations. We performed a fungicide resistance study comparing germination assays, biomass inhibition assays on solid media, and optimized assays. Isolates were exposed to various concentrations of SDHI, QoI, and DMI, and the effects of different inoculum types (conidia and homogenate) on biomass inhibition assays were examined. Our optimization study revealed that for the microtiter plate assay, the ideal incubation periods were 6 days for P. arachidicola and 8 days for N. personata. In the resazurin assay, optimal incubation was 2 days for P. arachidicola and 3 days for N. personata. In the assay comparison study, the assays generally did not differ in their ability to detect sensitivity to effective fungicides ($P \geq 0.59$), except for N. personata with pydiflumetofen ($P = 0.033$). Significant differences were found between assays for ineffective fungicides ($P \leq 0.006$), except for P. arachidicola with azoxystrobin ($P = 0.239$). Four out of six cases for effective fungicides showed that the inoculum did not affect results, while three out of five instances indicated it did matter for ineffective fungicides. Consequently, the choice of in vitro assay and inoculum can impact conclusions. The resazurin assay is more sensitive in detecting resistance than the other assays, while the homogenate shows more resistance than the conidia.

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Chapter I:

Literature Review

Peanut, *Arachis hypogaea L.,* is a leguminous plant grown worldwide in subtropical and tropical regions. In the United States, peanuts are a popular crop used primarily for peanut butter and confections. Georgia is the leading producer in the United States, with an estimated annual value exceeding $600 million (Brannen et al., 2019). Multiple biotic stresses threaten peanut production, of which the two foliar fungal diseases, early leaf spot (ELS) caused by *Passalora arachidicola* (Hori) U. Braun (syn. *Cercospora arachidicola*) and late leaf spot (LLS) caused by *Nothopassalora personata* (Berk. & M.A. Curtis) U. Braun, C. Nakash., Videira & Crous (syn. *Cercosporidium personatum*), often predominate.

The disease symptoms of both species are similar (Cantonwine et al., 2008a; McDonald et al., 1985; Porter et al., 1997). They manifest as necrotic leaf spots ranging from 2 to 5 mm in width, with or without a yellow halo. In the case of *N. personata*, conidia measuring 20–70 × 49 µm emerge primarily from the underside of the lesions. Conversely, *P. arachidicola* produces conidia that are 35–110 × 3–6 µm primarily on the upper side of the (Jenkins, 1938; McDonald et al., 1985; Meswaet et al., 2021). In high disease-pressure situations, the diseases can cause complete defoliation (Nutter & Shokes, 1995) and up to 70% yield loss (Anco et al., 2020b; Mohammed et al., 2018; Shokes & Culbreath, 1997) if not managed. In Georgia, a total cost of 44.05 million dollars was reported for damage and control related to these diseases in 2019 alone (Brannen et al., 2019).

In the southeastern United States, both diseases often occur together in most fields (Cantonwine et al., 2008b). However, the severity of each epidemic varies by field and season. While some fields may primarily feature ELS and other LLS, the predominance of each tends to fluctuate over time (Fulmer et al., 2019).

The onset of early leaf spots occurs between 1 and 2 months after planting, while late leaf spots emerge about two weeks later (Shokes & Culbreath, 1997; Smith, 1980). In recent years, LLS has increased in prevalence in the southeastern region and has been reported as the dominant foliar disease in Georgia (Fulmer et al., 2019). However, ELS has reemerged in some fields as the dominant disease (A.K. Culbreath, personal communication).

Management practices, such as planting partially resistant varieties and using crop rotation, can reduce the emergence and spread of both diseases (Brown et al., 2005; Cantonwine et al., 2006, 2007; Jordan et al., 2019). However, frequent fungicide applications are usually still required (Cantonwine et al., 2008b; Culbreath et al., 2020, 2023). The first fungicide application is recommended before primary infections are established, with additional sprays at 2- to 3-week intervals until harvest (Culbreath et al., 2002, 2006; Grichar & Woodward, 2016). Repeated application is needed to replace degraded and weathered chemicals and to protect new plant growth (Damicone & Smith, 2009). Fungicide spray programs typically employ a combination of multisite fungicides such as chlorothalonil and single-site fungicides, including demethylation inhibitors (DMIs) (Fungicide Resistance Action Committee [FRAC] Code 3), which targets the enzyme lanosterol 14α-demethylase (CYP51), Succinate dehydrogenase inhibitors (SDHIs) (FRAC Code 7), which target the succinate dehydrogenase (SDH) complex, and quinone outside inhibitors (QoIs) (FRAC Code 11), which target the quinol outer binding site of the cytochrome bc1 complex (Complex III). Frequent use of a single class of single-site fungicides may lead to

the development of resistance in pathogens (Avenot & Michailides, 2010; Massi et al., 2021; Woodward et al., 2015).

Fungicide Resistance

Fungicide resistance occurs when a pathogen develops reduced sensitivity to a specific fungicide (Deising et al., 2008; Lucas et al., 2015).

Resistance development is influenced by several critical factors, including the fungicides' mode of action, the cropping systems used, the patterns of fungicide application, and the biology of pathogens ((Damicone & Smith, 2009; Deising et al., 2008; Lucas et al., 2015; Massi et al., 2021). Fungi utilize various mechanisms to help them resist (Hirooka & Ishii, 2013; Thind, 2021). These include alterations in the fungicide's target site, the production of alternative enzymes that can replace the targeted enzyme, overexpression of the fungicide's target, active expulsion of the fungicide from the cell, decreased uptake of the fungicide, and the metabolic degradation of the fungicide (Cannon et al., 2009; Damicone & Smith, 2009; Hirooka & Ishii, 2013; M. Hu & Chen, 2021).

Before 1970, all fungicides used to control plant pathogens were multi-site inhibitors that acted as disease protectants (Hirooka & Ishii, 2013; Thind, 2021). Despite being widely used, resistance to these protectant compounds failed to occur or developed slowly (Damicone & Smith, 2009). In contrast to protectant compounds, the introduction of systemic compounds has faced significant challenges due to the rapid development of resistance, as their single-site mode of action makes them more vulnerable to fungal adaptation (Damicone & Smith, 2009; Hahn, 2014). Fungicide resistance in phytopathogenic fungi is a significant concern for crop protection. Single-site fungicides are more likely to develop resistance than multi-site fungicides (Sierotzki

& Scalliet, 2013). This is because even a single mutation in a binding or target site can render fungi resistant to those chemicals.

SDHI: Mode of Action and Mechanisms of Resistance

SDHI fungicides target the succinate dehydrogenase (SDH) complex, a part of the complex II electron transport system known as the ubiquinone oxidoreductase respiratory chain, by binding to the ubiquinone-binding site of mitochondrial complex II (Avenot & Michailides, 2010; Sierotzki & Scalliet, 2013). The SDH complex has four subunits: A, B, C, and D (Sierotzki & Scalliet, 2013). Resistance to SDHI fungicides can occur through several mechanisms linked to mutations in the SDHB, SDHC, and SDHD genes (Avenot & Michailides, 2010; Scalliet et al., 2012; Sun et al., 2022).

For example, there are reports of a point mutation in the FsSdhC in Fusarium solani (Mao et al., 2024), H252L in Ustilago maydis (Keon et al., 1991) H267Y in Mycosphaerella graminicola (Skinner et al., 1998), H132R in Botrytis cinerea ((Leroux et al., 2010), D132R in Sclerotium sclerotiorum (Glättli et al., 2009). D123E and D133R in Alternaria alternata (Avenot et al., 2008), and the T901 in Aspergillus oryzae (Shima et al., 2009).

Indirect mechanisms of resistance have also been reported in the SDH fungicides. These include increased efflux of the fungicide, observed in Sclerotinia homoeocarpa (Sang et al., 2015) and B. cinerea (Kretschmer et al., 2009), and metabolic detoxification of the product (B. Zhang et al., 2024). Field resistance to SDHIs can occur quickly. Resistance to the broad-spectrum carboxamide boscalid was apparent in some fungi populations less than a decade after its introduction (Avenot et al., 2008; Miyamoto et al., 2010; Shima et al., 2009; Stammler et al., 2011).

The first SDHI to be widely used for leaf spot control in peanut was penthiopyrad, registered in 2012. Penthiopyrad was initially more effective than chlorothalonil (Culbreath et al., 2009), with curative activity up to 13 days after infection (Johnson & Cantonwine, 2014). By 2020, penthiopyrad for early and late leaf spot control was no longer effective (Culbreath et al., 2020). There is a report of potential cross-resistance of *N. personata* population between SDHI active ingredients penthiopyrad, pydiflumetofen, and bixafen (Renfroe-Becton et al., 2024).

QoI: Mode of Action and Mechanisms of Resistance

QoI fungicides were derived from natural sources of β-methyl acrylic acid, specifically from compounds like oudemansin A and strobilurin A, which are produced by the basidiomycetes *Strobilurus, mycena*, and *Oudemansiella* ((Fernández-Ortuño & Torés, 2008; Kraiczy et al., 1996). Strobilurin A was the first QoI fungicide produced from liquid cultures of *Strobilurus tenacellus* (Anke et al., 1977; Fernández-Ortuño & Torés, 2008).

QoI inhibits fungal respiration by blocking electron transfer at the "quinone outside" site of the cytochrome *bc1* complex within the mitochondrial electron transport chain (Fouché, 2021; Kovacevik et al., 2024; Musso et al., 2020). This action prevents the fungus from producing the energy necessary for survival and growth by disrupting the fungus's ability to generate ATP by interfering with its normal metabolic processes at a specific site (Sierotzki, 2015).

QoI presents a higher risk of resistance due to single mutations, such as a change from phenylalanine to leucine at codon 129 (F129L) and a change from glycine to arginine at codon 137 (G137R), contributing to partial resistance (Luo et al., 2021; Zhou et al., 2015). Complete resistance occurs when there is a G143A mutation (Gisi et al., 2002). The predominant resistance mechanisms are attributed to target site mutations, efflux transporters, and the use of alternative respiration pathways (Fernández-Ortuño & Torés, 2008).

According to the Fungicide Resistance Action Committee (FRAC), the G143A substitution has been identified in resistant isolates for several species. These include *Mycosphaerella* pathogens, such a*s M. fijensis* (Silva et al., 2023) and *M. graminicola* (Fernández-Ortuño & Torés, 2008; Fraaije et al., 2005; Torriani et al., 2009), as well as powdery mildew and *Alternaria* species (Karaoglanidis et al., 2011; Vega & Dewdney, 2014). Resistance to azoxystrobin using an alternative respiratory pathway has been demonstrated in *M. graminicola* and *Venturia inaequalis* (Miguez et al., 2004; Steinfeld et al., 2001). The use of efflux transporters to confer resistance to QoI has been described in plant pathogenic fungi such as *Phytophthora infestans* (Ziogas et al., 2006), *M. graminicola* ((Roohparvar et al., 2007)), *and Colletotrichum* species (Deising et al., 2008).

Azoxystrobin was labeled for peanut leaf spots in 2000. While information is lacking on the initial efficacy of azoxystrobin against early leaf spot, it was very effective against late leaf spot (Monfort, 2002; Monfort et al., 2004, 2007). By 2016, azoxystrobin still proved effective against late leaf spots but was ineffective against early leaf spots (Elwakil & Dufault, 2016).

There are reports of total control failure against late leaf spot with azoxystrobin in Georgia (Culbreath et al., 2023, 2025) and late leaf spot population in South Carolina (Renfroe-Becton et al., 2024).

DMI: Mode of Action and Mechanisms of Resistance

DMIs are the largest subgroup of fungicides in the Sterol Biosynthesis Inhibitors (SBI) class, which is the most essential fungicide class due to its broad-spectrum mode of action (Mehl et al., 2023). SBIs are divided into four subclasses: (I) DMIs, (II) amines, (III) hydroxyanilides, and (IV) squalene epoxidase inhibitors (Mehl et al., 2023). Ergosterol is the primary sterol found in most fungi and is essential for the normal functioning of fungal cell membranes and overall

cell survival (Alvarez et al., 2007; Choy et al., 2023; Jordá & Puig, 2020). The genes CYP51 and ERG11, which encode sterol C14 demethylase and lanosterol 14α-demethylase, respectively, are the target sites for all DMI (Wieczorek et al., 2015).

The enzyme sterol 14α-demethylase is located within the microsomal membrane and catalyzes eburicol, which is essential in synthesizing sterol at the beginning of the pathway (Wieczorek et al., 2015). DMI fungicides require multiple gene mutations, rather than single point mutations, which leads to the gradual development of resistance in pathogens (Mehl et al., 2023). The factors contributing to resistance are primarily mild, which is why resistance to DMI fungicides developed more slowly (John Innes Centre, UK et al., 2023). The development of resistance mainly arises from mutations in the CYP51 gene, which encodes targets for DMIs (Wieczorek et al., 2015). Other factors have also been involved, such as increased active efflux, which leads to multidrug resistance, and the overexpression of the CYP51 gene ((Pérez-Cantero et al., 2019; Zhang et al., 2019)For example, *M. Graminicola* has been documented to exhibit high resistance levels to many DMI fungicides due to the substitution of valine for isoleucine at position 381 of the CYP51 gene (Delye et al., 1997). A mutation in *Uncinula necator* leading to the substitution of phenylalanine by tyrosine at position 136 of the CYP51 gene confers resistance to triadimenol (De Waard, 1997). Qiu (2010) demonstrated that reduced sensitivity of some *P. arachidicola* isolates to tebuconazole is associated with alterations at codons 453 or 461 in the CYP51 gene.

The DMI fungicide tebuconazole was approved for use on peanuts in 1994 (Culbreath et al., 2005a). In the southeastern U.S., it was usually applied four times after two initial applications of chlorothalonil (Culbreath et al., 2005). However, tebuconazole's effectiveness declined after ten years of use (Chapin & Thomas, 2006; Culbreath et al., 2005b, 2018). Reports

indicate that the resistance prevalence of the leaf spot pathogen populations has significantly increased (Culbreath et al., 2018; Stevenson & Culbreath, 2006).

Assessing Fungicide Resistance

A fungicide resistance assay is a laboratory or greenhouse test used to determine whether fungal pathogens are sensitive or resistant to specific fungicides. These can include *in vitro* assays, which are laboratory tests using fungal cultures; detached leaf assays, involving tests on detached leaves inoculated with fungal pathogens; and whole plant assays.

For fungi that are easily cultivated, *in vitro* assays, such as mycelium growth inhibition, spore germination inhibition, and microtiter plates, are preferred because there is a high degree of control over experimental conditions, allowing for accurate and reproducible results, as well as ease of handling and manipulation of the fungal cultures (Brennan et al., 2003; Cox et al., 2009; da Silva Lehner et al., 2022; Ritchie et al., 2009). However, for biotrophic fungi, which cannot be cultivated, assessments are typically done using tissue assays following fungicide exposure.

Biotrophic fungi require a living host to grow and reproduce, making it challenging to culture them *in vitro*. Tissue assays, such as leaf or plant assays, provide a more suitable environment for assessing fungicide efficacy against these pathogens (Bhattarai et al., 2020; Boydom et al., 2013; Farinas et al., 2019; Patial et al., 2017; Turra et al., 2017).

Various techniques are used to assess *in vitro* fungicide sensitivity, such as spore germination assays (Brenneman & Murphy, 1991; Mushrif et al., 2017). However, spore germination assays are not suitable for large sample sizes because the technique is time-consuming and requires many plates and ample space. They are also ineffective for the DMI fungicide class because fungal spores already contain ergosterol (Ruiter, 2024). Mycelial growth

assays are another traditional method to measure fungicide resistance (Cox et al., 2009). These can be done on solid media (Arias et al., 2025; Cox et al., 2009) or liquid media (Cox et al., 2009; Qiu et al., 2010). Another *in vitro* assay for fungicide resistance is the resazurin assay. The resazurin assay is used to assess the metabolic activity of pathogens (Cox et al., 2009; Riss et al., 2016; Vega et al., 2012).

Resazurin relies on a color change from blue (oxidized form) to pink (reduced form) when viable cells are present (Cox et al., 2009; Riss et al., 2016; Vega et al., 2012). It is stable and non-toxic to cells in a culture medium at low concentrations, allowing for quick *in vitro* measurement of cell growth (Zhang et al., 2004). The metabolic activity of cells in a population can be determined with a microtiter plate reader.

Assessing Fungicide Resistance for the Peanut Leaf Spot Pathogens

While *P. arachidicola* and *N. personata* are considered hemibiotrophs and can be cultivated on media, they do not do so as easily as many other species (Gong, 2016; Hunter et al., 2024). Due to the challenges of culturing these pathogens, some researchers have assessed fungicide resistance using field-collected conidia (Brenneman & Murphy, 1991; Munir et al., 2020; Qiu et al., 2010; Renfroe-Becton et al., 2024) or homogenized stroma as inoculum (Arias et al., 2025; Qiu et al., 2010). Methods used include conidia germination on amended solid media (Brenneman & Murphy, 1991; Mushrif et al., 2017), growth of homogenized stroma in amended liquid media (Qiu et al., 2010), and solid media (Arias et al., 2025) for tebuconazole. It remains to be shown how effectively these assays can screen for fungicide resistance in these pathogens for different fungicide classes.

Purpose

This study aims to (i) develop *in vitro* assays that effectively and efficiently measure fungicide resistance of *P. arachidicola* and *N. personata,* considering the cultivation limitations of these pathogens. (ii) Compares a selection of assays in liquid and solid media with two inoculum types (conidia and homogenized mycelium), each with different physiological phases of the fungi, to see how well each assay detects loss of sensitivity for each of the three classes of fungicides: SDHI, QoI, and DMI. The goal is to understand if one or more assays are more likely to detect reduced sensitivity of isolates.

Chapter II

In Vitro Microtiter Plate Assay Development to Measure Biomass of *Passalora Arachidicola* and *Nothopassalora Personata*

Introduction

Early and late leaf spot diseases are caused by *Passalora arachidicola* (Hori) U. Braun (syn. *Cercospora arachidicola*) and *Nothopassalora personata* (Berk. & M.A. Curtis) U. Braun, C. Nakash., Videira & Crous (syn. *Cercosporidium personatum*), respectively. These two diseases can be devastating, as they can cause complete defoliation (Nutter & Shokes, 1995) and up to 70% if fungicides are not applied (Anco et al., 2020b). Early and late leaf spot disease symptoms include small, circular necrotic lesions with or without a chlorotic halo, premature defoliation, and reduced pod quality and quantity (Jenkins, 1938; McDonald et al., 1985; Nutter & Shokes, 1995; Wilbert, 1938). The two diseases can be diagnosed by lesion color, the position of secondary sporulation within the lesion, and conidial length (McDonald et al., 1985). Sporulation for *P. arachidicola* usually occurs at the upper surface of reddish-brown lesions with a chlorotic halo, with conidia that are 47.6-84 µm long. Sporulation of *N. personata* develops from dark-brown to black lesions on the abaxial side, with conidia measuring 15-45 µm in length (Jenkins, 1938; McDonald et al., 1985).

In vitro research to understand the growth of these pathogens has been limited (Hunter et al., 2024). The limitations are that pathogens grow slowly (Gong, 2016) and do not display typical radial growth (Jenkins, 1938). These pathogens often form uneven colonies that are compact and exhibit convex and irregular shapes in culture, making traditional methods for

measuring mycelium growth difficult (Hunter et al., 2024). Moreover, some isolates fail to sporulate in culture (Abdou & Cooper, 1974), challenging the standardization of propagules for study.

Various techniques are used for assessing *in vitro* fungicide sensitivity, such as spore germination assays (Brenneman & Murphy, 1991; Mushrif et al., 2017), and micro colony growth assay (Qiu et al., 2010). A nondestructive technique has been created to measure biomass for both sporulating and non-sporulating isolates (Hunter et al., 2024), which can be employed in a fungicide resistance test. However, a more rapid, efficient, and automated technique that minimizes the potential for human error for sporulating and non-sporulating isolates is needed.

This study aims to optimize two fungicide screening assays for the leaf spot pathogens, including a liquid media plate reader assay and a resazurin assay, the latter not yet attempted with these pathogens (Cox et al., 2009; Vega et al., 2012). The factors evaluated for optimization were inoculum type, inoculum quantity, and incubation time.

Materials and Methods

Two isolates of *P. arachidicola* and *N. personata* selected from an isolate library at Valdosta State University were evaluated with each assay in this study. The isolate libraries were generated and maintained as follows.

Fungal Isolation and Culturing

Single conidia of *P. arachidicola* and *N. personata* were isolated and maintained in culture using arbitrary sampling techniques from early and late leaf spots collected from two fields, Lang Farm and Black Shank Farm, in Tifton, Georgia, USA. One hundred & twelve (112) isolates from both species and populations were collected and cultured in 2023. Additional

isolates from 2012 and 2013 collections from both locations and 2015 isolates from Dawson, Georgia, were added.

In the laboratory, single-spore colonies were initiated using a double-sided tape technique on water agar and then transferred, using a sterile needle, onto potato dextrose agar (PDA) after 24 to 168 hours. All collections were maintained for 2 to 3 months on PDA sealed with Parafilm M (Bemis Company, Neenah, Wisconsin) and incubated at room temperature (20 to 22 °C) under continuous light (AgroSun full spectrum grow lights, 40 watts; Hydro Farm, Shoemakersville, Pennsylvania). The light source was set 48 cm above the media plates.

Tissue increases and conidium production

Entire single spore colonies or sections of colonies, tissues measuring 2 to 3 mm in length and width and 1 to 2 mm in height, were homogenized in 2 mL of sterilized water using a tissue homogenizer (Tissue Miser; Fisher Scientific, Pittsburgh, Pennsylvania). Homogenization was performed in 5 mL round polypropylene bottom tubes (Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ, USA). Homogenates were transferred to fresh PDA plates, 0.25 mL per 100 by 15 mm plate, and spread evenly across the surface using a glass rod. Plates were incubated for 10 to 14 days to induce sporulation, after which they were stored for long-term preservation.

Long-Term Isolate Storage

Isolates were stored in autoclaved 15% glycerol under ultralow temperature as follows. Fungal tissues were cut or scraped with a scalpel and transferred to 1.8 mL cryotubes (Nalge Nunc International, Denmark) containing 1.5 mL of 15% glycerol. Immersed fungal tissues were stored at -800C in an ultracold freezer.

Reactivation of Isolates

Four Isolates, two from each species, were reactivated by spreading the tissues, suspended in 15% glycerol, and stored at -80°C on PDA medium using a sterile loop. Isolates were incubated using the culturing procedure outlined above. They were pat transferred (Hunter et al., 2024) or rinsed with autoclaved 0.05% Tween 20 solution after 7 to 10 days of incubation to replicate and generate more sporulating subculture and biomass.

Plate Assay (Conidial Inoculum)

Conidial suspensions were prepared from 7 to 12-day-old sporulating subcultures by gently and repeatedly rinsing the colonies with 3 to 5 mL of autoclaved 0.05% (v/v) Tween 20 solution. The conidia suspensions were standardized to 5.0 × 104 conidia/mL using a hemocytometer and an Olympus CX binocular Microscope (325 Bustleton Pike, Feasterville, PA 19053). The assay was performed in 96-well, rounded-bottom polystyrene microtiter plates. 150 µL of autoclaved and cooled potato dextrose broth (PDB) (VWR International, West Chester, PA) was pipetted into each well. Inoculations were performed by pipetting 10, 20, 30, 40, and 50 µL of the 5.0 × 104 conidia/mL of each isolate into different wells, resulting in final conidia concentrations of 500, 1000, 1500, 2000, and 2500 conidia/mL. Three replicates were conducted for each concentration.

Additional volumes of Tween 20 solution were added to each well, as needed, to achieve a final volume of 200 µL per well. The plates were sealed with surgical tape to prevent contamination during incubation. Plates were incubated in the dark on a shaker at 150 rpm and 25 °C for 14 days. Absorbance readings were taken on days 0, 2, 4, 6, 8, 10, 12, or 14 at a wavelength of 570 nm. This wavelength was selected because the absorbance spectrum of the wells after 7 days of incubation showed no peaks, suggesting that the biomass filters light

equally; 570 nm was one of the absorbance values of the resazurin assay (See Appendix C). Before reading, plates were manually shaken for 5 seconds to ensure homogeneity.

Plate Assay (Homogenate Inoculum)

Fragments of stroma or mycelium from nonsporulating subcultures were homogenized as previously described. The mycelial suspensions were allowed to settle for 10 minutes, allowing large fragments to settle, prior to inoculation. Inoculations were performed similarly to the conidia inoculum, using volumes of 10, 20, 30, 40, and 50 µL corresponding to inoculum concentrations of 20%, 40%, 60%, 80%, and 100%. Additional sterile water was added to the wells, as needed, to achieve a final volume of 200 µL. The plates were sealed with surgical tape. Incubation and reading times followed the same schedule as above.

Resazurin Assay

The resazurin assay is a technique used to assess the metabolic activity of pathogens (Cox et al., 2009; Riss et al., 2016; Vega et al., 2012). Resazurin relies on a color change from blue (oxidized form) to pink (reduced form) when viable cells are present (Cox et al., 2009; Riss et al., 2016; Vega et al., 2012). It is commonly used in human medicine investigations to evaluate bacterial and fungal cells, as it is stable and non-toxic to cells in a culture medium at low concentrations, allowing for quick in-vitro measurement of cell growth (Zhang et al., 2004). The metabolic activity of cells in a population can be determined with a microtiter plate reader.

The resazurin assay was performed similarly to the plate assay when conidia were the inoculum. 150 µL of autoclaved and cooled PDB was added to each well, 10 to 50 µL of conidial suspensions were added to achieve concentrations of 500, 1000, 1500, 2000, and 2500 conidia/mL, and 20 µL of resazurin was added. The final volume per well was 220 µL. Controls with PDB and resazurin alone, without fungal spores, were included. The plates, sealed with

surgical tape to prevent contamination during incubation, were incubated in the dark on a shaker at 150 rpm at 25 °C and monitored daily for 3 days. Fungal metabolism was assessed by measuring the dye's absorbance at 570 nm (red reflectance, blue light absorbance) and 600 nm (blue reflectance, red light absorbance) using a microplate reader on days 2 and 3. The experiment was repeated twice with three replications.

Similar experiments were performed using homogenate as an inoculum for the resazurin assay, following the protocol of the homogenate microtiter plate assay. The only difference was adding 20 µl of resazurin. The percent reduction of resazurin was calculated using the formula,

Percent Reduction of Resazurin $= \frac{(\mathbf{EOXi600} * \mathbf{A570}) - (\mathbf{EOXi570} * \mathbf{A600})}{(\mathbf{Ered570} * \mathbf{C600}) - (\mathbf{Ered600} * \mathbf{C570})} * \mathbf{100}$, where,

Eoxi 600, the molar extinction coefficient of oxidized Alamar Blue at 600 nm (117216). Eoxi 570, the molar extinction coefficient of oxidized Alamar Blue at 570 nm (80586). Ered 600, the molar extinction coefficient of reduced Alamar Blue at 600 nm (14652).

Ered 570, the molar extinction coefficient of reduced Alamar Blue at 570 nm (155677).

A570 absorbance of the tested well at 570 nm.

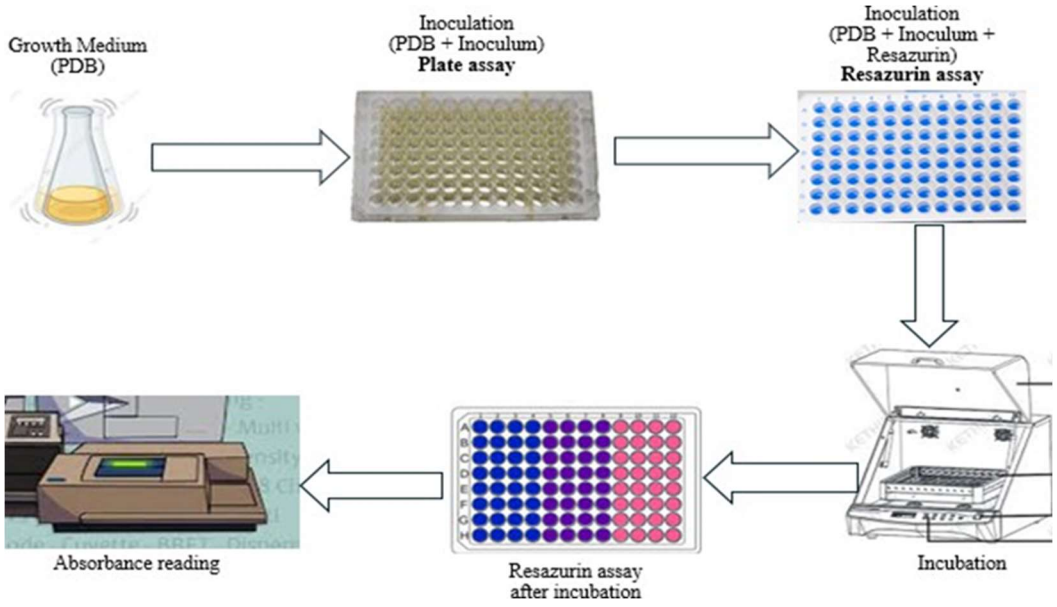
A600 absorbance of the tested well at 600 nm.

C570, the absorbance of the negative control well at 570 nm; and

C600, the absorbance of the negative control well at 600 nm.

Figure 1

Workflow for the plate assay and the resazurin assay



Note. The main differences between the two assays are the addition of the dye to the resazurin assay, the incubation periods, and the wavelengths used for absorbance readings, where absorbance was measured at two different wavelengths for the resazurin assay.

Data Analysis

Univariate analyses of variance (UNIANOVA) using the General Linear Model were performed to examine the interaction between absorbance and inoculum concentrations each day for both standardized and unstandardized absorbance values. The standardized absorbance values were calculated by dividing the absorbance on each assessment day by the initial inoculum concentrations. Analyses were conducted separately for each species, with a significance threshold set at (P = 0.05). Tukey's post hoc tests identified which factor levels differed significantly. For the resazurin study, linear regression was conducted, and Pearson correlation coefficients were determined to describe the relationship between resazurin reduction and inoculum concentration. Tukey's post hoc tests were used to determine whether inoculum

concentration affects resazurin reduction, after calculating the reduction per inoculum unit and log-transforming the data before univariate analysis. All statistical analyses were carried out using SPSS (version 29).

Results

Plate Assay

For the unstandardized conidia and homogenized mycelium datasets, absorbances numerically or statistically increased with increasing inoculum concentrations, and the frequency of significant treatment differences increased with longer incubations (Tables 1-4). In contrast, absorbances per inoculum unit numerically or statistically decreased as concentrations increased (Tables 1-4), and incubation time had a lesser impact on treatment comparisons, with similar significance patterns noted across all assessment periods.

Conidia inoculum

For the unstandardized data, differences in treatments began 2 days after inoculation (dai) for *P. arachidicola*. The treatment of 500 conidia/mL differed from the others until 12 dai, when it started to show similarities with 1000 and 1500 conidia/mL. Both 2000 and 2500 conidia/ml exhibited a similar pattern during incubation, with 2500 having the highest absorbance. The standardized data showed a similar growth rate from day 2 to 14 between 1500, 2000, and 2500 conidia/ml. It was observed that 500 conidia/mL grew at a faster rate compared to other treatments throughout the incubation period (Table 1).

Table 1

Univariate analysis of variance of standardized and unstandardized absorbance of P. arachidicola with conidia as initial inoculum

Unstandardized Absorbance[y]

Inocul[x]	0 d	2 d	4 d	6 d	8 d	10 d	12 d	14 d
500	0.11 a[d]	0.18 a	0.40 a	0.71 a	1.08 a	1.21 a	1.36 a	1.44 a
1000	0.11 a	0. 25 b	0.57 b	0.99 ab	1.33 b	1.46 b	1.60 ab	1.64 ab
1500	0.12 a	0.27 bc	0.62 bc	1.11 bc	1.40 bc	1.51 b	1.68 ab	1.71 ab
2000	0.12 a	0.33 cd	0.69 cd	1.16 bc	1.44 bc	1.59 b	1.75 b	1.76 ab
2500	0.13 a	0.36 d	0.73 d	1.29 c	1.52 c	1.65 b	1.85 b	2.01 b
P-value	0.156	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.004	0.032
Absorbance per inoculum unit [z] ($x10^{-4}$)								
Inocul	0 d	2 d	4 d	6 d	8 d	10 d	12 d	14 d
500	2.17 a	3.56 a	7.92 a	14.2 a	21.6 a	24.2 a	27.2 a	28.8 a
1000	1.12 b	2.45 b	5.73 b	9.86 b	13.3 b	14.6 b	15.9 b	16.4 b
1500	0.76 c	1.78 c	4.13 c	7.38 bc	9.36 b	10.1 bc	11.2 bc	11.4 bc
2000	0.63 d	1.66 c	3.44 cd	5.80 c	7.22 b	7.94 bc	8.76 c	8.80 c
2500	0.48 e	1.45 c	2.90 d	5.16 c	6.09 b	6.61 bc	7.39 c	8.03 c
P-value	0.39	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[x] Inocul = Initial conidia concentrations.

[y] Absorbance = mean absorbance measured at 570nm due to the mycelia concentrations in a potato dextrose broth over time.

[z] Absorbance = means absorbance per inoculum unit calculated by dividing absorbance on each assessment day by the initial conidia concentration.

[d] Different letters represent significant treatment differences based on Tukey's post hoc analysis.

Unlike *P. arachidicola*, *N. personata* exhibited a similar growth pattern across treatments, ranging from 500 to 2000 conidia/ml during incubation, except on day 6. The 2000 and 2500 conidia/ml treatments were comparable, whereas the 2500 treatment differed from the others and recorded the highest absorbance. No treatment differences were observed at 14 dai. The growth rate for *N. personata* treatments was consistent across all treatments from day 2 to day 8, except for the 500 conidia/mL treatment, which showed distinct growth. From day 8 onward, a similar increase was noted between the 500 treatment and the others; however, differences were observed between 500 and 2500 conidia/mL throughout the incubation period (Table 2).

Table 2

Univariate analysis of variance of standardized and unstandardized absorbance of N. personata with conidia as initial inoculum

	Unstandardized Absorbance[y]							
Inocul[x]	0 d	2 d	4 d	6 d	8 d	10 d	12 d	14 d
500	0.11 a[d]	0.14 a	0.23 a	0.39 a	0.54 a	0.66 a	0.81 a	0.96 a
1000	0.12 ab	0.16 a	0.28 a	0.57 ab	0.94 ab	1.06 ab	1.37 ab	1.61 a
1500	0.12 ab	0.17 a	0.34 ab	0.62 ab	1.02 ab	1.16 ab	1.41 ab	1.62 a
2000	0.12 ab	0.20 a	0. 41 ab	0.74 bc	1.20 ab	1.30 ab	1.56 ab	1.73 a
2500	0.13 b	0.21 a	0.52 b	0.87 c	1.34 b	1.46 b	1.74 b	1.88 a
P-value	0.022	0.090	0.008	< 0.001	0.021	0.061	0.038	0.135
	Absorbance per inoculum unit [z] ($x10^{-4}$)							
Inocul	0 d	2 d	4 d	6 d	8 d	10 d	12 d	14 d
500	2.25 a	2.75 a	4.63 a	7.78 a	10.72 a	13.21 a	16.2 a	19. 3 a

1000	1.17 b	1.55 b	2.83 ab	5.68 b	9.41 ab	10.64ab	13.8 ab	16.2 ab
1500	0.82 c	1.12 bc	2.29 b	4.12 bc	6.78 ab	7.74 ab	9.42 ab	10.8 ab
2000	0.62 d	0.97 c	2.09 b	3.70 bc	5.98 ab	6.48 b	7.80 bc	8.67 b
2500	0.50 d	0.84 c	2.72 b	3.47 c	5.35 b	5.84 b	6.95 c	7.52 b
P-value	< 0.001	< 0.001	0.004	< 0.001	0.019	0.014	0.003	0.100

[x] Inocul = Initial conidia inoculum concentrations.

[y] Absorbance = mean absorbance measured at 570nm due to the mycelia concentrations in a potato dextrose broth over time.

[z] Absorbance = means absorbance per inoculum unit calculated by dividing absorbance on each assessment day by the initial conidia concentration.

[d] Different letters represent significant treatment differences based on Tukey's post hoc analysis.

Homogenized mycelium inoculum

No growth difference was observed in *P. arachidicola* treatments from the inoculation day to 2 dai. Treatments 20 and 40 exhibited similar growth patterns from day 2 to day 6 but diverged thereafter. A consistent similarity was observed between treatments 40 and 60 from 2 to 14 dai, and between treatments 60 and 80, except on day 4. From days 2 to 6, growth rates were similar for all treatments with *P. arachidicola*. The exception was treatment 20, which began to grow differently starting on day 8, but was comparable to treatments 40 and 60 for the remainder of the incubation period. Treatment 20 exhibited a higher growth rate than the other treatments (Table 3).

Table 3

Univariate analysis of variance of standardized and unstandardized absorbance of P. arachidicola with homogenized mycelium as initial inoculum

	Absorbance[r]							
Inocul[q]	0 d	2 d	4 d	6 d	8 d	10 d	12 d	14 d
20	0.46 a[t]	0.45 a	0.65 a	0.86 a	1.13 a	1.26 a	1.37a	1.50a
40	0.89 b	.91 ab	1.23 ab	1.45 ab	1.77 b	1.91 b	2.04b	2.23b
60	1.31 c	1.32 bc	1.56 b	1.82 b	2.17 b	2.32 b	2.60bc	2.74bc
80	1.49 c	1.47 c	1.63 c	1.97 b	2.32 bc	2.49 bc	2.70 c	2.93cd
100	1.99 d	1.97 d	2.19 c	2.64 c	2.87 c	3.10 c	3.40 d	3.49d
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Absorbance[s] ($x10^{-3}$)							
Inocul	0 d	2 d	4 d	6 d	8 d	10 d	12 d	14 d
20	22.9 a	22.7 a	32.6 a	43.9 a	56.4 a	63.1 a	68.6 a	75.1 a
40	22.4 ab	22.8 a	28.2 a	36.3 a	44.3 ab	47.9 ab	51.1 ab	55.8 ab
60	21.8 ab	22.2 a	26.1 a	30.3 a	36.2 ab	38.6 ab	43.3 ab	45.6 ab
80	18.6 b	18.5 a	20.3 a	24.7 a	29.0 b	31.1 b	33.4 b	36.7 b
100	19.9 ab	19.8 a	21. 9 a	26.4 a	28.7 b	31.0 b	33.9 b	34.9 b
P-value	0.036	0.134	0.133	0.084	0.023	0.012	0.009	0.007

[q] Inocul = Initial inoculum of homogenized mycelium inoculated in terms of percent concentration.

[r] Absorbance = mean absorbance measured at 570nm due to the mycelia concentrations in a potato dextrose broth over time.

[s] Absorbance = means absorbance per inoculum unit, calculated by dividing absorbance on each assessment day by the initial homogenized mycelium concentration

[t] Different letters represent significant treatment differences based on Tukey's post hoc analysis.

There was a decrease in absorbance on day 2 compared to the day of inoculation for *N. personata.* Treatments 20, 40, and 60 showed similar growth patterns throughout the incubation period, except on day 8, when differences were observed between treatments 20 and 60. No additional differences between treatments were noted after day 10, except on day 12 for treatments 20 and 100 (Table 4).

Treatments 20 and 40 showed similar growth rates until after day 6, when treatment 20 began to exhibit a different growth pattern, a trend that continued until day 12 of *N. personata*. Excluding treatments 20 and 100, there was a similar growth rate between the treatments from day 2 to day 6. The growth rate decreased at higher concentrations of treatment (Table 4).

Table 4

Univariate analysis of variance of standardized and unstandardized absorbance of N. personata with homogenized mycelium as an initial inoculum

	Absorbance[r]							
Inocul[q]	0 d	2 d	4 d	6 d	8 d	10 d	12 d	14 d
20	0.22 a[t]	0.20 a	0.28 a	0.49 a	0.81 a	0.95 a	1.13 a	1.27 a
40	0.36 ab	0.33 ab	0.48 ab	0.77 ab	1.17 ab	1.30 ab	1.46 ab	1.64 a
60	0.43abc	0.39 ab	0.56 bc	0.88 bc	1.21 b	1.35 ab	1.52 ab	1.73 a

80	0.59 bc	0.55 c	0.63 bc	0.89 bc	1.27 b	1.40 ab	1.58 ab	1.77 a
100	0.61d	0.55 c	0.75 c	1.11 c	1. 41 b	1.58 b	1.76 b	1.98 a
P-value	0.001	0.001	< 0.001	0.001	0.002	0.013	0.052	0.88
				Absorbances ($*10^{-3}$)				
Inocul[q]	0 d	2 d	4 d	6 d	8 d	10 d	12 d	14 d
20	11.1 a	9.84 a	13.8 a	24.6 a	40.5 a	47.7 a	56.7 a	63.4 a
40	8.92 ab	8.21 ab	11.9 ab	19.2 ab	29.3 b	32.4 b	36.4 b	41.1 ab
60	7.15 b	6.57 ab	9.37 b	14.4 b	21.1 bc	23.4 bc	26.3 bc	29.5 b
80	7.39 b	6.82 ab	9.42 b	13.9 b	17.7 cd	19.8 c	22.0 bc	24.8 b
100	6.08 b	5.47 b	6.30 c	8.87 c	12.1 d	13.5 c	15.2 c	17.3 b
P-value	0.008	0.013	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[x] Inocul = Initial inoculum of homogenized mycelium inoculated in terms of percent concentration.

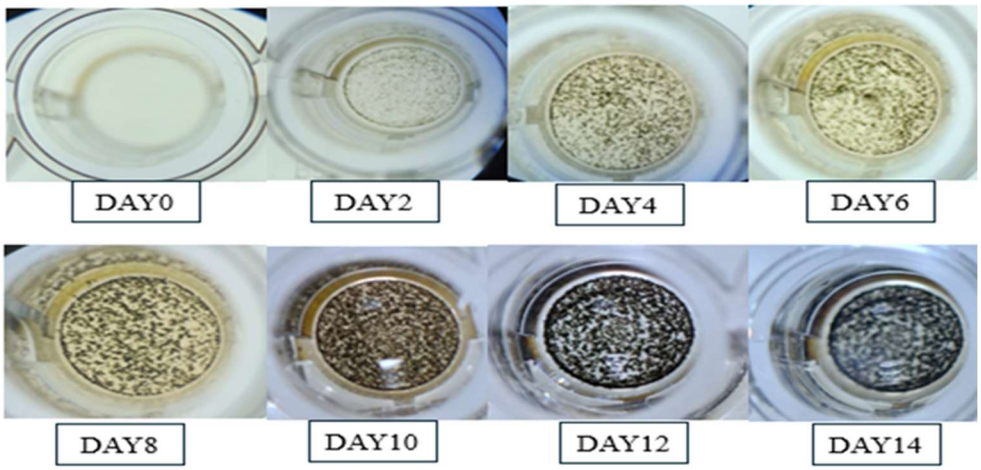
[y] Absorbance = mean absorbance measured at 570nm due to the mycelia concentrations in a potato dextrose broth over time.

[s] Absorbance = means absorbance per inoculum unit, calculated by dividing absorbance on each assessment day by the initial homogenized mycelium concentration

[t] Different letters represent significant treatment differences based on Tukey's post hoc analysis.

Figure 2

Images showing the growth of P. arachidicola in potato dextrose broth



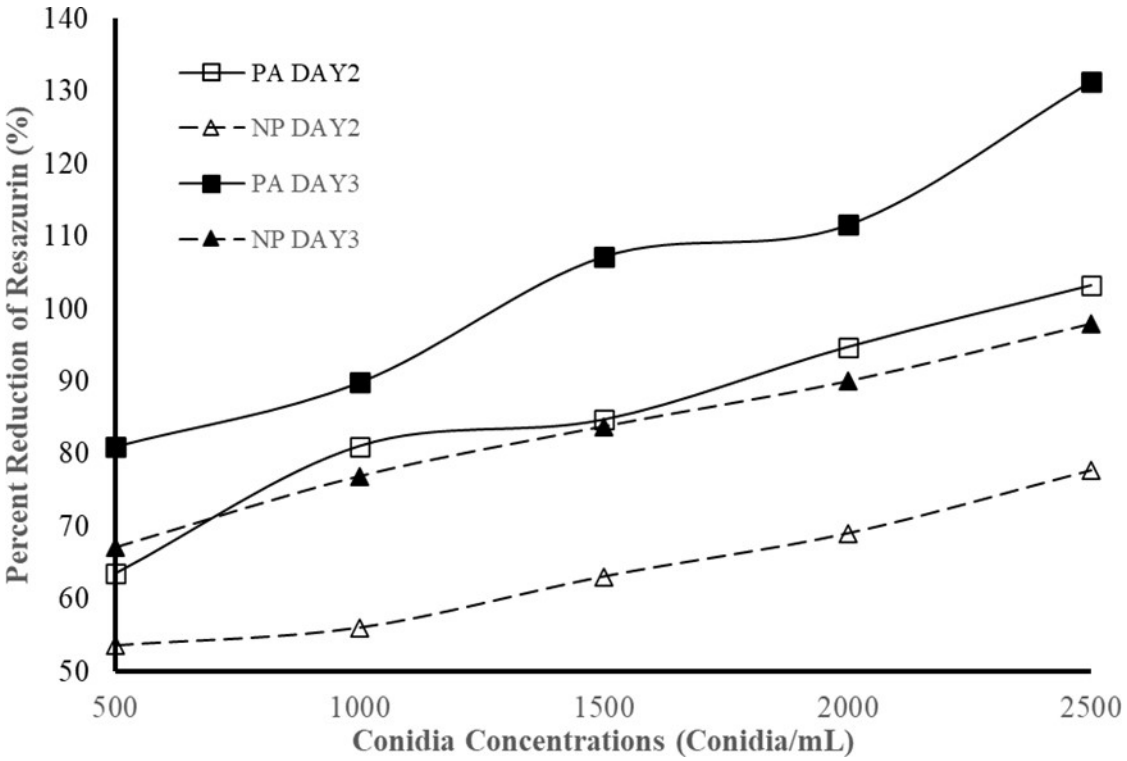
Note: The inoculation was carried out in a microtiter plate at 1500 conidia per milliliter and incubated in the dark on a shaker for 14 days.

Resazurin Assay

There were significant linear relationships ($R2 \geq 0.96$; $P < 0.001$) between the percentage of resazurin reduced and the concentration of conidia for both pathogens at 2 and 3 days of incubation (Figure 3). There was a significant difference in resazurin reduction across conidia concentrations ($P < 0.001$). The groups with 1,500, 2,000, and 2,500 conidia/mL exhibited similar levels of resazurin reduction, whereas the group with 500 conidia/mL showed a significantly lower reduction. Absorbance data were not collected for the experimental trials with the homogenate inoculum because it was qualitatively clear that the higher inoculum concentrations would interfere with absorbance readings.

Figure 3

Reduction of resazurin influenced by conidia concentrations and incubation periods for P. arachidicola (PA) and N. personata (NP)



Discussion

The study examined the growth of two peanut fungal species, *P. arachidicola* and *N. personata*, using absorbance to measure biomass and resazurin to measure metabolism of the pathogens. For the biomass measurement, absorbance levels increased with higher concentrations of the initial inoculum. The species and inoculum type influenced the growth patterns. The conidia inoculum of *P. arachidicola* showed greater differences in absorbance between the inoculation day and 4 dai compared to *N. personata*. This suggests that *P. arachidicola* develops mycelia and increases biomass more rapidly in culture (Hunter et al., 2024).

The most noticeable differences in absorbance were observed between 4 and 8 dai for both fungal species, emphasizing the exponential growth phase (Kung'u, 2019; Peleg & Corradini, 2011). This indicates that the optimal period for assessing treatment effects, such as in a fungicide resistance assay, occurs within this timeframe (Rampersad, 2011), with the initial inoculum concentration range tested. The initial inoculum concentration range provides excellent flexibility in propagules needed for the microtiter plate assay, as some isolates fail to produce enough conidia (Abdou & Cooper, 1974).

The concentration of the inoculum was a crucial factor in this study, as varying concentrations significantly affected the growth rate of the fungi. The growth rate stabilized after day 8, likely due to the depletion of nutrients and limited space. This observation indicates that the fungi had reached the stationary phase, where the rate of cell division is equal to the rate of cell death (Kung'u, 2019; Peleg & Corradini, 2011).

The absorbance readings for the homogenate inoculum differed slightly from those of the conidia on the inoculation day and day two, primarily because there was a drop in absorbance on day two. This drop occurred due to the cloudiness of the well on the inoculation day, which resulted in increased absorbance. After two days of incubation, the mycelium fragments settled, clearing the culture medium and consequently affecting the absorbance levels. Despite the initial drop, the growth patterns of the homogenate inoculum were similar to those of the conidia after day two. The log phase for both species using homogenized mycelium resembled that of the conidia inoculum; however, the homogenates exhibited a higher growth rate in this assay. Specifically, the growth rate when using homogenate as the initial inoculum was approximately three times greater than that of the conidia.

There was some inconsistency in the growth rate of *P. arachidicola* between treatments 80 and 100 from 6 to 14 dai. Treatment 100 recorded a higher growth rate than Treatment 80 during this period, which was contrary to our expectations. This discrepancy may be attributed to challenges in standardizing the inoculum concentration of homogenized mycelium. To effectively compare isolates and species in treatment effects, such as in the fungicide resistance assay, the stroma must be standardized accurately by weighing and using consistent dilutions.

Additionally, it was observed that an inoculum volume between 40% and 60% of the homogenized mycelium aliquot is ideal for inoculation, due to the inconsistencies noted with higher inoculum concentrations. The culturing media darkened as biomass increased over time (See figure 2). The darkening of the media suggests that the fungus produces melanin in culture (Arias et al., 2025), which may have contributed to the increase in absorbance over time (Abdul Manan & Webb, 2018).

Conidia concentrations and incubation period are crucial parameters for achieving optimal results in the resazurin assay (Cox et al., 2009; Vega et al., 2012). At high conidia densities or with longer incubation times, the reduction of resazurin reaches a maximum, where the pink color may fade to colorless (Larson et al., 1997). The resazurin turned colorless for *P. arachidicola* after 3 days of incubation at 2000 and 2500 conidia/mL, despite lower concentrations of 500 and 1000 conidia/mL not reaching a 100% reduction. In this study, the optimal conidia density was 2500 conidia/mL, with incubation periods of 2 days for *P. arachidicola* and 3 days for *N. personata* (See Figure. 3). The resazurin remained blue after 24 hours of incubation across all tested conidia concentrations, prompting the use of extended incubation periods.

Conclusions

The microtiter plate and the resazurin assays are high-throughput method that simultaneously screens multiple isolates (Vega et al., 2012). This feature simplifies comparing different isolates. Additionally, the assays facilitate the optimization of various growth conditions, such as temperature, light, and nutrient levels. It is particularly beneficial when dealing with limited resources, such as time and labor. The process is rapid due to its automation, eliminating the need for microscopy and manual reading of results. It may yield more precise results than traditional assays, such as the spore germination assay. Additionally, homogenized mycelium can replace conidia for the microtiter plate assay when specific isolates do not sporulate in culture, showing the assay's flexibility in terms of the initial inoculum type. However, the inoculum should be standardized, as concentrations mattered in this study for both assays.

Chapter III

Comparing *In Vitro* Assays for Detecting Fungicide Resistance in Early and Late Leaf Spot Pathogens of Peanuts

Introduction

Fungicides play a vital role in modern agriculture by helping growers protect their crops from fungal diseases, which is essential for ensuring food security. However, the repeated application of these chemicals can lead to the development of resistant fungal populations (Brent & Hollomon, 1995; Damicone & Smith, 2009; Massi et al., 2021). This emergence of resistance poses a significant threat to agricultural productivity and sustainability. Fungicide resistance is a complex issue influenced by various factors, including fungicide use patterns, fungal biology, and environmental conditions (Damicone & Smith, 2009; Deising et al., 2008; Lucas et al., 2015; Massi et al., 2021).

Early leaf spot caused by *Passalora arachidicola* (Hori) U. Braun (syn. Cercospora arachidicola) and late leaf spot caused by *Nothopassalora personata* (Berk. & M.A. Curtis) U. Braun, C. Nakash., Videira & Crous (syn. Cercosporidium personatum) are two important foliar diseases of peanuts (*Arachis hypogaea L*) in the southeastern U.S. Both diseases can cause significant damage alone or together, potentially causing 100% defoliation (Nutter & Shokes, 1995) and 70% pod loss without management (Anco et al., 2020b; Mohammed et al., 2018). While integrated disease management strategies, such as planting varieties with partial disease and using crop rotation, are widely practiced, fungicide applications are still essential for successfully managing these diseases in many peanut fields (Culbreath et al., 2019, 2020, 2025).

Most fungicide programs result in 4 to 7 applications throughout a growing season, beginning 30 to 45 days after planting, before primary infections occur, followed by applications at 2- to 3-week intervals (Grichar & Woodward, 2016; Kemerait et al., 2011). This schedule is necessary because the effectiveness of the chemicals diminishes due to extreme weather conditions and the growth of new plant cells. Regular exposure to the same class of fungicide may cause pathogens to develop resistance (Damicone & Smith, 2009; Massi et al., 2021; Yin et al., 2023). The fungicide chemistries most frequently used for peanut leaf spot management include chlorothalonil, a multisite protective chemistry, and single-site chemistries in FRAC groups 3, demethylation inhibitors (DMIs), 7, succinate-dehydration inhibitors (SDHIs), and 11, quinone outside inhibitors (QoIs).

While reduced *P. arachidicola* and *N. personata* sensitivities to a selection of QoI, DMI, and SDHI chemistries have been suspected under field conditions (Culbreath et al., 2018, 2019, 2020, 2023, 2025; Renfroe-Becton et al., 2024; Stevenson & Culbreath, 2006), direct assessments of resistance have been challenging to obtain because these fungi have slow, irregular growth on media (Gong, 2016; Hunter et al., 2024; Jenkins, 1938; Monguillot et al., 2023) and produce relatively few conidia in culture, sometimes lacking the ability to sporulate. Additionally, both species have been reported to form morphological variants in culture (Arias et al., 2025; Hunter et al., 2024). While these hindrances tend to be more limiting for *N. personata* than for *P. arachidicola* (Abdou & Cooper, 1974; Arias et al., 2025; Hunter et al., 2024; Jenkins, 1938), even high sporulating isolates of *P. arachidicola* can fail to produce sufficient inoculum for large-scale studies (Abdou & Cooper, 1974; Smith, 1980; Starkey, 1980).

While some have employed creative ways to assess fungicide resistance with these species, there is still no standardized screening assay. Methods include conidia germination on

amended solid media (Brenneman & Murphy, 1991; Mushrif et al., 2017), growth of homogenized stroma in amended liquid media (Qiu et al., 2010), disease development on detached leaves after fungicide exposure (Munir et al., 2020), and, most recently, the growth of homogenized stroma on amended solid media using image analysis (Arias et al., 2025). It remains to be shown how effective these assays are in detecting fungicide resistance in these pathogens and if they do so similarly.

This study aims to compare sensitivity estimates of *P. arachidicola* and *N. personata* isolates with four in vitro assays: (1) inhibition of germination, (2) inhibition of biomass with solid medium and liquid medium, and (3) inhibition of metabolism using resazurin reduction as an indicator. Six fungicide chemistries, an effective and ineffective chemistry from FRAC groups 3, 7, and 11, were investigated. Additionally, the study seeks to determine whether inoculum type (conidia and homogenate) affects the assessment of the pathogens' sensitivity to these fungicides.

Materials and Methods

This section outlines the materials used and experimental procedures followed in this study. Specifically, we describe the preparation of fungicide solutions, inoculum handling, and the setup of the experiment to evaluate fungicide efficacy.

Experimental Design

Four fungicide resistance assays were assessed in this study: germination inhibition (GI), biomass inhibition on solid medium (BI_sm), biomass inhibition in liquid medium (BI_lm), and metabolism inhibition using resazurin as an indicator (RSA) for two *P. arachidicola* isolates (LFPA403B and LFPA403C) and two *N. personata* isolates (BSNP25 and BSNP28). With one exception, the GI assay and DMI fungicides do not suppress germination (Brent & Hollomon, 1995). Each assay was tested with two SDHI, QoI, and DMI chemistries, one with good field

efficacy and one with poor field efficacy. The SDHIs were pydiflumetofen (effective SDHI) (Syngenta Crop Protection, LLC., 410 Suing Road, Greensboro, NC 27409) and penthiopyrad (Ineffective SDHI) (Sigma Aldrich, Merck KGaA, Darmstadt, Germany). The QoIs were pyraclostrobin (Effective QoI) (BASF Cooperation, 26 Davies, Drive, RTP, NC, USA. 27709) and azoxystrobin (Ineffective QoI) (Syngenta Crop Protection, LLC. 410 Suing Road, Greensboro, NC 27409). The DMIs were prothioconazole (Effective DMI) (Bayer Crop Science LP, 800 N. Limdbergh Blvd, Creve Coeur, MO 63167, USA) and tebuconazole (Ineffective DMI) (UPL NA Inc., 15401 Weston Parkway, Suite 170, Cary, NC 27513).

Two types of inoculants were tested for the BI_sm and BI_lm assays: conidia and homogenized stroma. The GI and RSA assays were conducted using conidia only. All assays were conducted simultaneously using the same inoculum and fungicide preparations. Three replications were conducted for each isolate fungicide treatment, and the experiment was performed twice.

Inoculum

Conidial suspensions were prepared from 7- to 12-day-old sporulating subcultures as described in Chapter II. The conidia suspensions were standardized to 5.0 × 104 conidia/ml. The homogenized mycelium inoculum was prepared by homogenizing 100 mg of stroma from nonsporulating subcultures in 3 ml of sterile water as described in Chapter II. The mycelial suspensions were diluted by half and allowed to settle for 10 minutes, allowing large fragments to settle before inoculation.

Fungicide-amended media

For the two assays conducted on solid medium, GI and BI_sm, a stock solution of technical-grade fungicide was prepared by dissolving it in acetone to achieve a stock

concentration of 10,000 µg/mL. This solution was serially diluted in 10-fold increments using acetone to achieve concentrations of 1000, 100, 10, 1, or 0.1 µg/mL. For each of these serial concentrations, 2.8 mL was added to 280 mL of autoclaved PDA medium (55°C) to obtain final concentrations of 10, 1, 0.1, 0.01, or 0.001 µg/mL in the amended medium. The non-amended controls were created by adding 2.8 mL of acetone. Salicylhydroxamic acid (SHAM) was added to the SDHI and QoI- fungicide-amended and non-amended media to block the alternative respiratory pathway. SHAM was dissolved in methanol and added at a concentration of 60 µg/mL. All media contained 0.1% (vol/vol) methanol (Vega et al., 2012). Media were poured into a 100 mm x 15 mm diameter petri dish and allowed to solidify.

For the BI_lm assay, 20 µl of the 1000, 100, 10, 1, or 0.1 µg/mL fungicide concentrations were added to 2 mL of autoclaved potato dextrose broth (PDB) to obtain final concentrations of 10, 1, 0.1, 0.01, and 0.001 µg/mL of amended PDB. Acetone was added to the non-amended controls. SHAM was added to fungicide-amended and non-amended growth media at 55 °C to SDHI and QoI fungicides as described. 150 µL of amended and non-amended PDB were transferred to 96-well, rounded-bottom polystyrene microtiter plates (Corning® 96-well Clear Polystyrene Microplates) before inoculation.

For the RSA assay, 20 µL of 2000, 200, 20, 2, or 0.2 µg/mL fungicide concentrations were added to 2 mL of autoclaved and cooled PDB to obtain final concentrations of 200, 20, 2, 0.2, or 0.02µg/mL of amended PDB. This higher initial concentration was used so the same final concentrations of the test wells, 13.6, 1.36, 0.136, 0.0136, or 0.00136 µg/mL, would be achieved following the addition of the resazurin solution. Acetone was added to the non-amended controls. SHAM was added to fungicide-amended and non-amended media with SDHI and QoI fungicides as described.

Assay Inoculation and Incubation

Solid medium inoculations were performed by pipetting 20 µL of conidia or homogenate onto amended and non-amended PDA. Inoculated plates were undisturbed until the inoculum aliquot was dry (15-30 min) and then sealed with Parafilm (provide parafilm source info). Incubation occurred on a laboratory bench in a randomized complete block design (RCBD) at 22 to 25°C under continuous light (AgroSun full-spectrum grow lights, 40 watts; Hydro Farm, Shoemakersville, Pennsylvania). The light source was positioned 48 cm above the media plates. Plates evaluated for the GI assay were incubated for 48 hours, and those for the BI_sm assay were incubated for 7 days for *P. arachidicola* and 10 days for *N. personata*.

For the liquid media assays (BI_lm and RSA), wells containing 150 µL of fungicide-amended PDB were inoculated with 30 µL of suspended conidia and 20 µL of Tween 20 solution to achieve a spore density of 1.5 × 103 conidia/mL or 50 µL of homogenized mycelium. The final fungicide concentrations in each BI_lm test well were 7.0, 0.75, 0.075, 0.0075, or 0.00075 µg/mL. Plates were incubated in the dark on a shaker at 150 rpm and 25 °C for 6 or 8 days for P. *arachidicola* and *N. personata*, respectively. For the RSA assay, wells containing 150 µL of fungicide-amended PDB were inoculated with 50 µL of suspended conidia to achieve a spore

density of 2.5 × 103 conidia/mL. Immediately after inoculation, 20 µL of resazurin solution (Sigma Aldrich, St. Louis) with a concentration of 440 µM was added. All microtiter plates included internal standards, consisting of PDB and resazurin alone (without fungal spores), at each concentration of the fungicides. The final concentrations of the fungicides in the RSA test wells were 13.6, 1.36, 0.136, 0.0136, or 0.00136 µg/mL.

The plates were then sealed with surgical tape (3M Transpore Medical Tape, St. Louis, MO, USA) to prevent contamination during incubation and covered with foil to restrict light

entry. Plates were incubated in the dark on a shaker at 150 rpm and 25 °C for 2 or 3 days for *P. arachidicola* and *N. personata*, respectively.

Assay Assessments

Percent germination was evaluated at 100X using an Olympus CX binocular microscope (325 Bustleton Pike, Feasterville, PA 19053), following the transfer of media to a slide and staining with 0.01% (v/v) trypan blue in 0.05% (v/v) lactic acid. At least 100 conidia were assessed as germinated or ungerminated. Percent germination was calculated as the number of germinated conidia / total number of conidia × 100.

Biomass on solid media was assessed following 7 and 10 days of incubation for *P. arachidicola* and *N. personata*, respectively, using ImageJ (Hunter et al., 2024) as follows. A 2 cm wide imprint was created around each aliquoted growth area using a core borer, and images were taken with diascopic illumination using the OM system TG-7 digital camera (Olympus Corporation, model No. IM015, Tokyo). In ImageJ, the plugs were outlined to define the assessment area, images were converted to an 8-bit format, and thresholds were adjusted to highlight fungal tissue as described by Hunter et al (2024). Tissue areas (mm*) were then calculated by multiplying the highlighted percentage by the defined area.

A plate reader (Molecular Device SpectraMax, M5, LLC. 3860 N First Street, San Jose, CA 95134) was used to assess the BI_lm and RSA assays. For the BI_lm assay, plates were manually shaken for 5 seconds by the plate reader (prior to measuring absorbances at 570 nm. Absorbance values were treated as the biomass estimate. For the RSA assay, absorbance was measured at 570 nm (red reflectance, blue light absorbance) and 600 nm (blue reflectance, red light absorbance). The reduction of the resazurin dye was determined as the amount of blue, non-fluorescent resazurin reduced to pink, fluorescent resorufin by metabolically active cells. This

change is proportional to the number of living metabolically active cells in the sample. The percentage of resazurin reductions was calculated using the formula described earlier.

Data Analysis

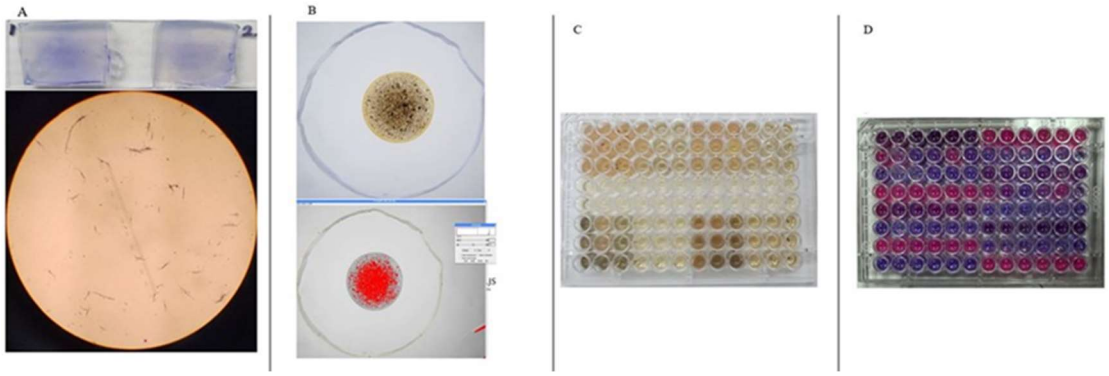
Percent inhibition was calculated as (measurement of the control – measurement of the treated) / measurement of the control x 100. Half-maximal inhibitory concentration (IC50) values were determined by plotting the percentage of inhibition against fungicide concentrations using Excel. When IC50 values could not be calculated, the curves were extended to estimate IC50, capping at 20 µg/mL to avoid overestimations.

Because the homogenate inoculum was not evaluated in the GI and RSA assays, the first set of analyses focused on the four assays conducted with conidia as follows. Generalized linear mixed model (GLMM) analyses, with assay defined as a fixed effect and isolate as a random effect, were conducted to test for assay effects on IC50 values. When significant ($P < 0.05$), pairwise contrasts were used to determine assay differences. Similar GLMM analyses were used to test for inoculum and media effects, each set as fixed factors, for the BI_lm and BI_sm assays. When factor effects were significant ($P < 0.05$), pairwise contrasts were used to determine assay differences based on a GLMM with assay as a fixed effect. In the last set of GLMM analyses, the RSA assay was compared to each homogenate assay across species and fungicides or by species when interactions were significant. All analyses were performed in SPSS version 29.

Results

Figure 4

Images showing the various assays



Note: (A). Germination Inhibitory Assay (GI), (B). Biomass Inhibitory Assay on solid medium (BI_sm), (C) Biomass Inhibitory Assay in liquid medium (BI_lm), and
(D). Resazurin Assay (RSA).

Dose-response curves for each assay evaluated, including both inoculum types, were plotted together for each pathogen and each fungicide. The effective chemistry curves are presented in Figure 5, and the ineffective chemistry curves are in Figure 6. For the effective fungicides, IC50 values were calculable for all assays except one, the BIA_lm homogenate assay with pyraclostrobin for *P. arachidicola* (Figure 5). For the ineffective fungicides, IC50s could not be estimated for nearly half of the assays (Figure 6) and were capped at 20 µg/mL. A small selection was estimated to values between the highest concentration evaluated and 20 ug/ml (Refer to Appendix B).

Figure 5

Graph of percent inhibition (%) versus fungicide doses (µg/mL) for the assays estimating IC50 (Half-maximal Inhibitory Concentration) values of P. arachidicola and N. personata for effective SDHI, QoI, and DMI fungicides

P. arachidicola *N. personata*

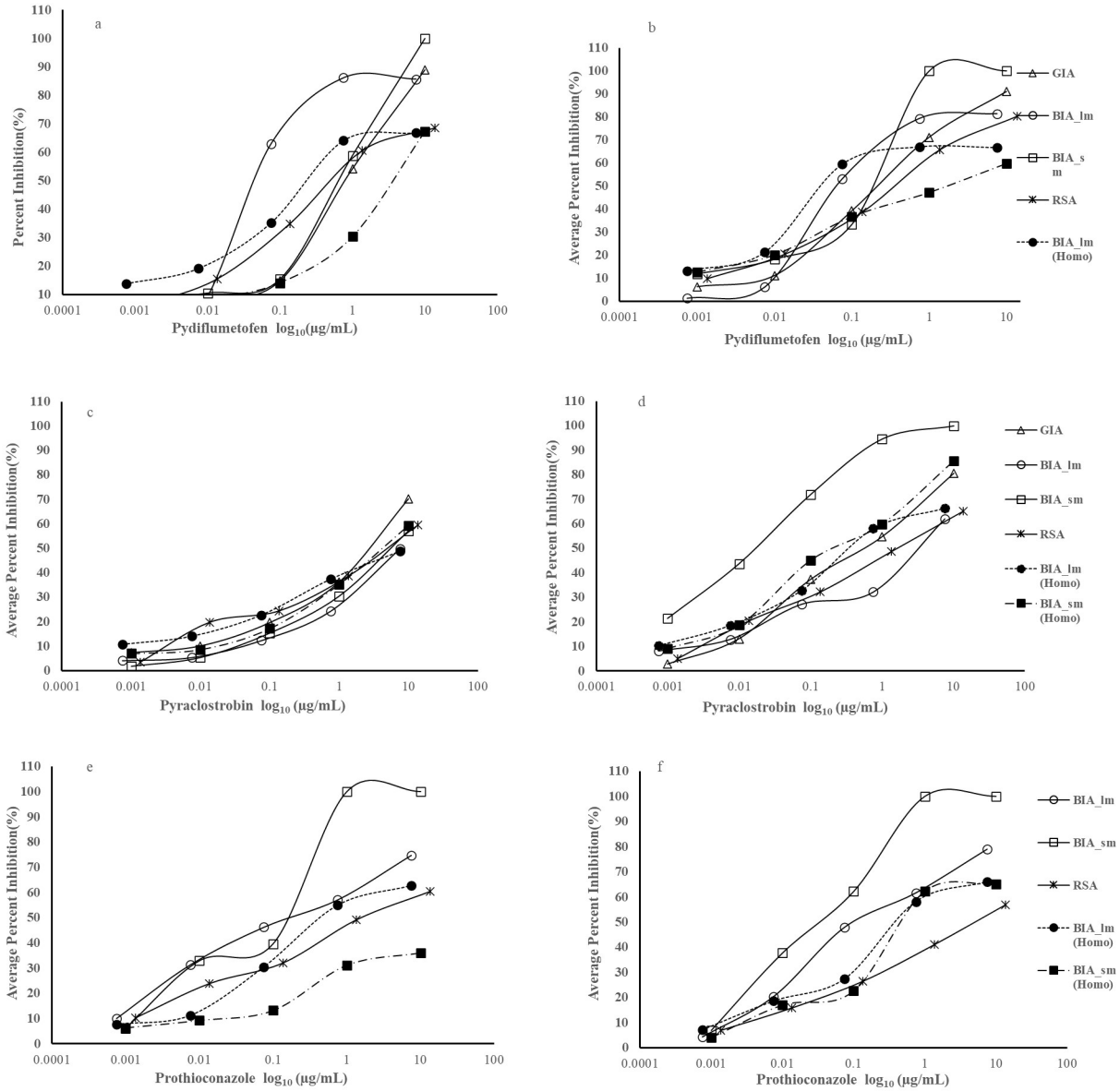
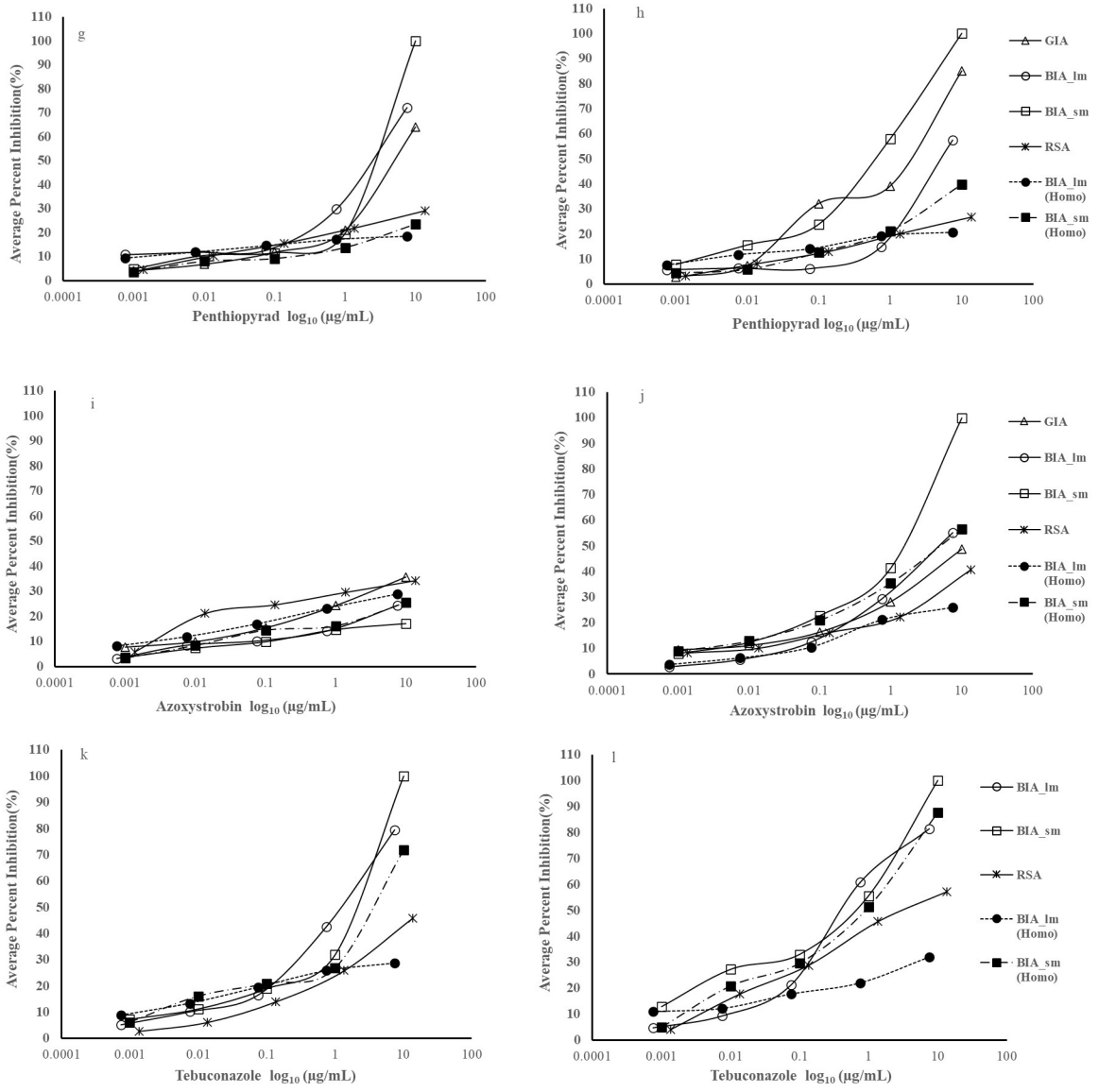


Figure 6

Graph of percent inhibition (%) versus fungicide doses (µg/mL) for the assays estimating IC50 (Half-maximal Inhibitory Concentration) values of P. arachidicola and N. personata for ineffective SDHI, QoI, and DMI fungicides

P. arachidicola *N. personata*



Assay Effects on Conidia Inoculum

Assay type did not significantly affect IC50 values for most of the effective fungicides (Table 5). The only exception was for *N. personata* with pydiflumetofen, where RSA showed a significantly higher IC50 value than the other assays (Table 5). Conversely, there was a significant assay effect on the IC50 values in most of the ineffective fungicides, with the RSA assay having the highest IC50 estimates (Table 6). The only exception to this trend was for *P. arachidicola* with azoxystrobin, where all assays demonstrated resistance at or near the highest estimates of the experiment (Table 6).

Table 5

Comparing IC50 µg/mL values of assays for effective fungicides by species based on pairwise contrast at a 0.05% significance level

Assays	Effective Fungicides					
	Pydiflumetofen		Pyraclostrobin		Prothiconazole	
	IC50[a] (µg/mL)		IC50 (µg/mL)		IC50 (µg/mL)	
	PA	NP	PA	NP	PA	NP
GI	0.80 a[b]	0.18 a	4.63 a	1.10 a	_[c]	-
BI_sm	0.53 a	0.10 a	6.50 a	0.01 a	0.10 a	0.007 a
BI_lm	0.01 a	0.08 a	8.63 a	3.63 a	3.80 a	0.88 b
RSA	0.53 a	0.53 b	5.88 a	2.23 a	2.34 a	6.25 c
P-Value	0.059	0.033	0.737	0.166	0.365	0.098
F-Value	$F_{3,12} = 3.23$	$F_{3,12} = 4.06$	$F_{3,12} = 0.43$	$F_{3,12} = 2.01$	$F_{2,9} = 1.13$	$F_{2,9} = 3.04$

[a] Mean IC50 of three replications and two experiments.

[b] Different letters show significant differences according to pairwise contrast at a 0.05% significance level.

[c] Results are unavailable because the assay is incompatible with the fungicide. PA = *Passalora arachidicola*, NP = *Nothopassalora personata*

Table 6

Comparing IC50 µg/mL values of assays for ineffective fungicides by species based on pairwise contrast at a 0.05% significance level

Assays	Ineffective Fungicides					
	Penthiopyrad		Azoxystrobin		Tebuconazole	
	IC50[a] (µg/mL)		IC50 (µg/mL)		IC50 (µg/mL)	
	PA	NP	PA	NP	PA	NP
GI	7.75 b[b]	2.93 ab	17.25 a	11.50 bc	_[c]	-
BI_sm	4.10 a	0.95 a	20.00 a	2.75 a	3.00 a	0.80 a
BI_lm	3.80 a	12.00 b	20.00 a	5.20 ab	2.80 a	0.70 a
RSA	19.50 c	20.00 c	20.00 a	19.50 c	20.00 b	20.00 b
P-Value	<0.001	0.006	0.239	0.003	<0.001	<0.001
F-Value	$F_{3,12}$ = 71.27	$F_{3,12}$ = 6.91	$F_{3,12}$ = 1.61	$F_{3,12}$ = 8.55	$F_{2,9}$ = 167.53	$F_{2,9}$= 488.62

[a] Mean IC50 of three replications and two experiments.

[b] Different letters show significant differences according to pairwise contrast at a 0.05% significance level.

[c] Results are unavailable because the assay is incompatible with the fungicide. PA = *Passalora arachidicola*, NP = *Nothopassalora personata*

Effects of Inoculum and Media

In the analysis examining inoculum and media effects, significant interactions were observed in three of six cases for the effective fungicide and two of five cases for the ineffective fungicide (refer to Tables 7 and 8). When interactions were not significant, inoculum-type did not affect either pathogen's IC50 estimates with pyraclostrobin, and *N. personata's* with prothioconazole or penthiopyrad, but it did affect *P. arachidicola's* IC50 estimates with penthiopyrad and *N. personata's* with azoxystrobin (Table 8). Likewise, media-type did not significantly affect IC50 estimates for *P. arachidicola* with pyraclostrobin or penthiopyrad, and

N. personata with prothioconazole, but it did affect *N. personata's* estimates with pyraclostrobin, penthiopyrad, and azoxystrobin, when interactions were not significant (Tables 7 and 8).

Table 7

Effects and interactions of inoculum and media in assays for effective fungicides by species at a 0.05% significance level

Fungicides	Species	Factors	F-Value	df1	df2	P-Value
Pydiflumetofen	PA	Inoculum	65.74	1	12	< 0.001
		Media	83.19	1	12	< 0.001
		Ino X Med	56.04	1	12	< 0.001
	NP	Inoculum	0.85	1	12	0.374
		Media	5.09	1	12	0.044

		Ino X Med	4.94	1	12	0.046
Pyraclostrobin	PA	Inoculum	0.05	1	12	0.828
		Media	3.04	1	12	0.107
		Ino X Med	0.05	1	12	0.827
	NP	Inoculum	0.31	1	12	0.587
		Media	8.62	1	12	0.012
		Ino X Med	2.98	1	12	0.110
Prothioconazole	PA	Inoculum	1.88	1	12	0.195
		Media	9.19	1	12	0.010
		Ino X Med	46.99	1	12	< 0.001
	NP	Inoculum	0.18	1	12	0.683
		Media	1.74	1	12	0.212
		Ino X Med	0.18	1	12	0.680

PA = *Passalora arachidicola*, NP = *Nothopassalora personata*

Table 8

Effects and interactions of inoculum and media in assays for ineffective fungicides by species at a 0.05% significance level

Fungicides	Species	Factors	F-Value	df1	df2	P-Value
Penthiopyrad	PA	Inoculum	333.62	1	12	< 0.001
		Media	0.21	1	12	0.659
		Ino X Med	0.21	1	12	0.659

	NP	Inoculum	3.89	1	12	0.072
		Media	6.59	1	12	0.025
		Ino X Med	2.63	1	12	0.131
Azoxystrobin	PA	Inoculum	_a	-	-	-
		Media	-	-	-	-
		Ino X Med	-	-	-	-
	NP	Inoculum	6.50	1	12	0.026
		Media	7.54	1	12	0.018
		Ino X Med	1.19	1	12	0.296
Tebuconazole	PA	Inoculum	23.75	1	12	< 0.001
		Media	63.52	1	12	< 0.001
		Ino X Med	67.23	1	12	< 0.001
	NP	Inoculum	97.08	1	12	< 0.001
		Media	456.55	1	12	< 0.001
		Ino X Med	468.57	1	12	< 0.001

[a] Unable to analyze due to insufficient variation.

PA = *Passalora arachidicola*, NP = *Nothopassalora personata*

When the four assays representing both inoculum-types and media-types were considered the fixed effect, there was no case where the conidia inoculum was more resistant to a fungicide than the homogenate inoculum. However, the homogenate inoculum provided significantly higher IC50 estimates than conidia for *P. arachidicola* in the solid media assay for three of the five fungicides analyzed and in the liquid media assay for three of the five (Tables 3.5 and 3.6).

For *N. personata*, a significantly higher IC50 value was observed with homogenate in the tebuconazole liquid media assay, and many of the other fungicides presented numerically higher IC50 estimates (Tables 9 and 10). While no differences in media type were observed with conidia, homogenized *P. arachidicola* had significantly higher IC50 values in the solid media assay than in the liquid media assay for two of the effective fungicides, pydiflumetofen and prothioconazole, and both species demonstrated significantly higher IC50 estimates in liquid media than in solid media with tebuconazole (Tables 9 and 10).

Table 9

Comparison of conidia and homogenate inoculum in assays based on C50 values for effective fungicides at a 0.05% significance level

Assays	Effective Fungicides					
	Pydiflumetofen		Pyraclostrobin		Prothioconazole	
	IC50[a] (µg/mL)		IC50 (µg/mL)		IC50 (µg/mL)	
	PA	NP	PA	NP	PA	NP
BI_sm (conidia)	0.53 a[b]	.10 a	6.50 a	0.001 a	0.10 a	0.001 a
BI_lm (conidia)	0.001 a	0.08 a	8.63 a	3.63 a	3.80 ab	0.88 a
BI_sm (homo)	5.53 b	3.50 a	5.25 a	0.32 a	10.00 b	0.55 a
BI_lm (homo)	0.20 a	0.10 a	8.00 a	1.26 a	0.43 a	2.25 a

P-Value	< 0.001	0.356	0.923	0.353	0.016	0.853
F-Value	$F_{3,12}$ = 18.42	$F_{3,12}$ = 1.20	$F_{3,12}$ = 0.16	$F_{3,12}$ = 1.20	$F_{3,12}$ = 5.20	$F_{3,12}$ = 0.26

[a] Mean IC50 of three replications and two experiments.

[b] Different letters show significant differences according to pairwise contrast at a 0.05% significance level. PA = *Passalora arachidicola*, NP = *Nothopassalora personata*

Table 10

Comparison of conidia and homogenate inoculum in assays based on C50 values for ineffective fungicides at a 0.05% significance level

Assays	Ineffective Fungicides					
	Penthiopyrad		Azoxystrobin		Tebuconazole	
	IC50[a] (µg/mL)		IC50 (µg/mL)		IC50 (µg/mL)	
	PA	NP	PA	NP	PA	NP
BI_sm (conidia)	4.10 a	0.95 a	_c	2.75 a	3.00 a	0.80 a
BI_lm (conidia)	3.80 a	12.00 a	-	5.20 a	2.80 a	0.70 a
BI_sm (homo)	20.00 b	17.50 a	-	12.75 a	5.90 a	1.50 a
BI_lm (homo)	20.00 b	20.00 a	-	20.00 a	20.00 b	20.0 b

P-Value	< 0.001	0.088	-	0.055	< 0.001	< 0.001
F-Value	$F_{3,12}$ = 144.74	$F_{3,12}$ = 2.77	-	$F_{3,12}$ = 3.37	$F_{3,12}$ = 23.63	$F_{3,12}$ = 131.72

[a] Mean IC50 of three replications and two experiments.

[b] Different letters show significant differences according to pairwise contrast at a 0.05% significance level.

[c] Unable to analyze due to insufficient variation.

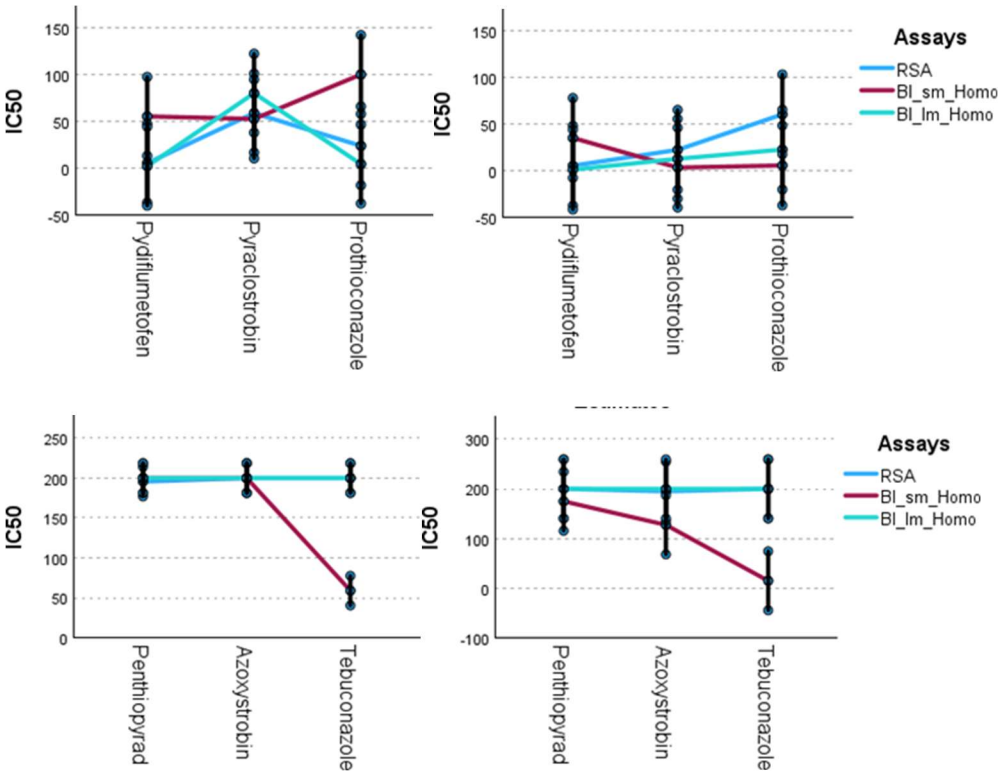
PA = *Passalora arachidicola*, NP = *Nothopassalora personata*

In the pooled analyses comparing the most sensitive conidia assay (RSA) to each homogenate assay, the following results were noted. No differences were detected between the IC50 estimates of the RSA assay and the BI_lm_homo assay across species and fungicides (P = 0.842); this analysis also lacked a significant interaction between assay and fungicide (P = 0.704) and between assay and species (P = 0.242). While there were significant assay x fungicide and assay x species interactions (P < 0.01) when RSA was compared to the BI_sm_homo assay (Figure 7), when species were analyzed separately across all fungicides besides tebuconazole, no difference in the IC50 estimates was detected between the RSA and BI_sm_homo assays for P. arachidicola (P = 0.231) or *N. personata* (P = 0.466). For tebuconazole, the IC50 estimates of both species were significantly less with the BI_sm_homo assay than the RSA or BI_lm_homo assays (P < 0.01) (See Figure 7).

Figure 7

IC50 estimates by species and fungicide for the three most sensitive assays

P. arachidicola *N. Personata*



Discussion

When conidia suspensions were used as the inoculum, and the tested fungicides were effective, the four assays evaluated —GI, BI_sm, BI_lm, and RSA —provided the same fungicide sensitivity estimates for the *P. arachidicola* and *N. personata* isolates in all cases except one. This exception will be discussed in the next paragraph. However, as demonstrated with the ineffective treatments, the assays were not always comparable when fungicide sensitivity was reduced. In these cases, the RSA assay consistently estimated higher IC50 values than the others. This suggests that the RSA assay is a more sensitive method for detecting reduced fungicide sensitivity than germination or biomass inhibition, at least when conidia are used as the inoculum. That said, when resistance is overwhelming, as was the case with azoxystrobin and *P. arachidicola*, all assays detected high levels of resistance.

Considering this trend, the finding that the effective fungicide pydiflumetofen and *N. personata*, where the IC50 for RSA (5.25 µg/mL) was significantly higher than that of the other assays, may indicate an early shift towards resistance by this pathogen against pydiflumetofen. While this is supported by field assessments, where pydiflumetofen has been less effective at controlling late leaf spot in recent years (Culbreath, personal communication), assessments with more isolates are needed to support this possibility.

While the sensitivity estimations were similar for the four assays evaluated in this study when tested with the effective fungicides, the IC50 estimations for the ineffective fungicides suggest that assays differ in their ability to detect sensitivity shifts towards resistance. The only case where the assays produced similar results was with azoxystrobin for *P. arachidicola*, where the IC50 values were mostly capped at 20 µg/mL. The differences in IC50 values for the effective

SDHI (pydiflumetofen) with RSA indicating less fungicide sensitivity, as well as the various resistance levels seen with ineffective fungicides, could be explained by the assays measuring fungicidal effects differently: GI measures conidia germination (Brenneman & Murphy, 1991; Mushrif et al., 2017), BI_sm measures tissue area (Arias et al., 2025; Hunter et al., 2024), BI_lm measures media turbidity caused by fungal growth (Abdul Manan & Webb, 2018), and RSA evaluates fungal metabolism through the color change of resazurin (Cox et al., 2009; Vega et al., 2012). The study would have been improved had we included a 100 µg/ml treatment; we did not expect resistance to exceed 10 µg/mL.

Inoculum type was a significant factor affecting the assays, with the homogenized stroma demonstrating less sensitivity (greater resistance) to most fungicides than the corresponding conidia inoculum in the solid and liquid media biomass inhibition assays. For most fungicides,

the assays using the homogenate inoculant were statistically equivalent to the RSA assay. The one exception was with tebuconazole, where both species appeared to be more sensitive to the fungicide with the BI_sm_homo assay than with the RSA assay.

Inoculum from homogenates may lack uniformity due to the difficulty in standardizing it. Spores are easier to quantify and standardize for experiments. This simplifies the creation of consistent inoculum densities for accurate and reproducible resistance tests (Hu et al., 2007; Vega et al., 2012). Since spores are often the primary means by which fungal pathogens infect plants and spread diseases, testing resistance directly on spores assesses the fungicide's ability to prevent infection at this critical initial stage, reflecting actual field conditions (Secor et al., 2010). However, this study demonstrates that homogenate can be used to evaluate resistance in *P. arachidicola* and *N. personata*. The results showed that the homogenate was less sensitive to fungicides than conidia in some cases suggest that it might be more informative than conidia with the BI_sm and BI_lm assays for detecting sensitivity shifting.

While we do not have a good explanation for why the BI_sm_homo assay differed from the RSA and BI_lm_homo assays for tebuconazole but not the other fungicides, one possibility is that these species are less resistant to tebuconazole than penthiopyrad and azoxystrobin. The BI_sm_homo assay was less sensitive than the RSA and BI_lm_homo assays. It is also possible that our image analysis method may have overestimated sensitivity (underestimated resistance). While we believe this is unlikely, a methodological error occurred during biomass photography that may have had an impact. In our study, images were taken using both diascopic and episcopic illumination. However, Hunter et al. (2024) recommended using only diascopic illumination to maximize contrast.

While this potential error was assumed to be minimal in terms of effective dose treatments, when it did occur, as biomass can appear transparent and be undetectable when contrast is not optimal, it is possible that the tebuconazole treatment altered the biomass's morphology in some way that affected its imaging. *N. personata* can change morphology in response to fungicide exposure in culture (Arias et al., 2025). Regardless, if the imaging method affected biomass estimates with the BI_sm_homo assay, this error may also apply to the BI_sm assay with conidia.

The germination inhibition assay has long been the standard in vitro method for detecting SDHI and QoI resistance in closely related pathogens (Secor et al., 2010). These results suggest that it may fail to detect early shifts in sensitivity. While it is pretty simple to perform, requiring minimal instrumentation, the method is cumbersome and time-intensive, making it less than ideal for testing large sample sizes. The microtiter method is simple to perform and features an automated assessment mode that provides consistent results. It also requires less time than GI and BI_sm, allowing for the simultaneous testing of multiple samples. This supports high-throughput screening, reduces the amount of growth media needed, and decreases the number of plates required, leading to cost savings (Vega et al., 2012). The technique needs careful pipetting of media and inoculum to ensure equal volumes and concentrations in each well, as uneven volumes could affect the results.

The results of this study indicate that the RSA could be a valuable method for monitoring shifts in sensitivity or resistance in pathogens. Like the BI_lm, the RSA allows high-throughput fungicide resistance screening but has shorter incubation times of 2 and 3 days, compared to 6 and 8 days for *P. arachidicola* and *N. personata*, respectively. It is a highly rapid test that requires fewer steps and faces less interference (Petiti et al., 2024; Riss et al., 2016).

Although GI has a similar incubation period to RSA, differences in assessment methods, evaluation timing, and resource needs make RSA less labor-intensive. RSA offers both qualitative and quantitative data that are not available from other assays. Qualitative data, as indicated by the color change of the dye, provide an early indication of fungicide effectiveness before quantitative analysis (see Appendix A). RSA requires pure culture isolates to prevent contamination from yeast, bacteria, and other fungi (Petiti et al., 2024), which limits its use with direct field inoculum. Additionally, RSA is restricted to isolates that can sporulate in culture, making it unsuitable for sensitivity testing of non-sporulating isolates. Optimizing assay parameters, such as inoculation cell density and incubation time, is crucial for obtaining accurate results with RSA.

Conclusions

In vitro assays are essential tools for detecting changes in fungal sensitivity to fungicides. They facilitate the early identification of resistance and assist in resistance management. This study demonstrated that all the assay methods tested are useful for measuring fungicide sensitivity in these pathogens. However, the choice of assay method can significantly impact the accuracy of assessing a fungicide's effectiveness. Our findings revealed that the RSA, BI_lm (Homo), and BI_sm (Homo) were more effective in detecting reduced fungicide sensitivity compared to the other assays. The RSA offers advantages such as high-throughput screening, rapid results, and qualitative data. It's important to note that the type of inoculum and media used can influence the assay outcomes. Specifically, homogenized stroma inoculum exhibited lower sensitivity to fungicides than conidia and showed results comparable to those from the RSA.

References

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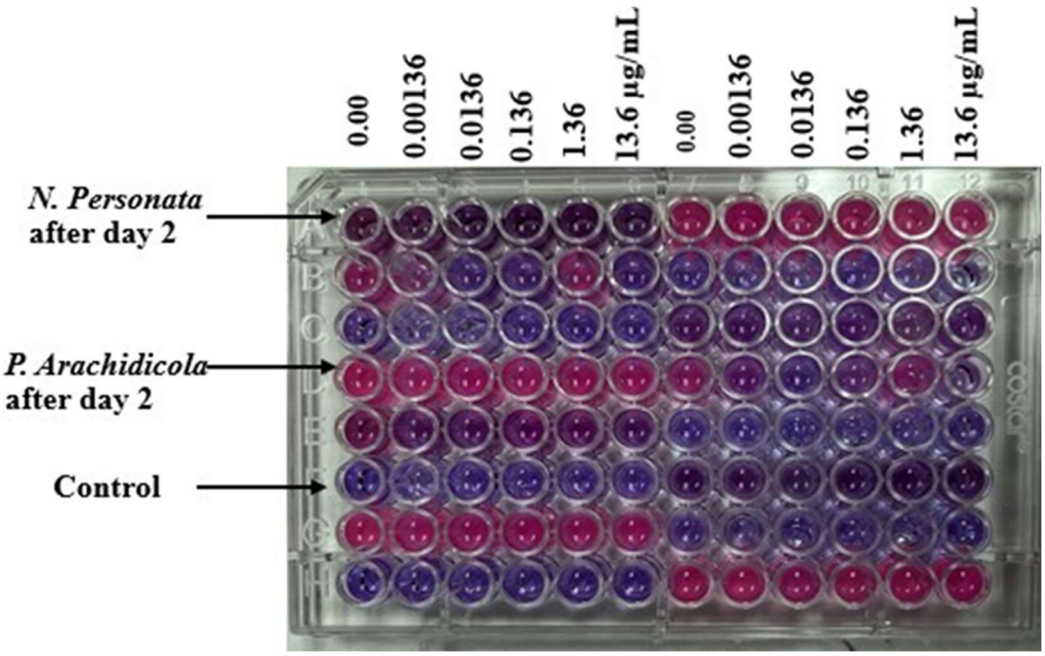
Appendix

Appendix A

Dose-response of P. arachidicola and N. personata to penthiopyrad, with resazurin as an indicator of fungal metabolism



Note: The pink color shows resistance of *P. arachidicola* isolates to penthiopyrad, and the purple color indicates *N. personata*, which was not ready for assessment after 48 hours of incubation.

Appendix B

Percentage of estimated and capped IC50 values for different fungicides across species

Assays	Fungicides											
	Effective						Ineffective					
	Pydiflu		Pyraclo		Prothio		Penthio		Azoxy		Tebu	
	E[a]	C[b]	E	C	E	C	E	C	E	C	E	C
GI	0	0	0	0	-	-	0	0	25	37.5	-	-

BI_sm	0	0	12.5	0	0	0	0	0	0	50	0	0
BI_lm	0	0	25	0	0	0	25	0	12.5	50	0	0
RSA	0	0	0	0	0	0	12.5	87.5	25	75	0	100
BI_sm (Homo)	0	0	25	0	0	0	12.5	87.5	0	75	0	0
BI_lm (Homo)	0	0	25	0	0	0	0	100	0	100	0	100

Note: The percentages were calculated from eight cases.

[a] Percentage of estimated IC50 values

[b] Percentage of capped IC50 values

Appendix C

Graph of absorbance versus wavelength after seven days of incubation