CHARACTERIZATION OF *RHIZOPUS* SPP. CAUSING SEED AND PRE-EMERGENCE SEEDLING ROT IN PEANUT (*ARACHIS HYPOGAEA* L.)

by

LUCINDA GAIL MCEACHIN

(Under the Direction of Timothy B. Brenneman)

ABSTRACT

Rhizopus seed and seedling rot is a highly destructive peanut disease, causing rapid seed decay in 36-96 hours after planting and rendering seeds and pre-emerged seedlings indistinguishable from the soil. The seed vigor of thirteen peanut genotypes, molecular identification, pathogenicity, *in vitro* fungicide sensitivity, and temperature response of *Rhizopus* spp. isolated from peanut seeds in Georgia were assessed in this study. Molecular analysis identified three *Rhizopus* spp. (*R. delemar, R. arrhizus,* and *R. stolonifer* with 26, 16, and 3 isolates, respectively) based on the TEF-1α gene. A novel pathogenicity assay for *Rhizopus* spp. on peanut seeds revealed that all tested peanut genotypes were highly susceptible to *Rhizopus* spp. Mycelial growth assays at temperatures of 15-35 °C revealed variability in the thermotolerance across the three species. *Rhizopus delemar* and *R. arrhizus* exhibited rapid mycelial growth and high virulence on peanut seeds at all tested temperatures. The highest germination found at all temperatures was only 12% in inoculated seeds. *Rhizopus stolonifer* exhibited significantly slower mycelial growth rate and no growth at 30 °C and 35 °C, respectively. Due to the reduced growth, some inoculated seeds managed to germinate (40.2% at 30 °C and 72.2% at 35 °C). Seed treatment fungicides fludioxonil, carboxin, and pydiflumetofen

consistently provided the lowest mean fungicide concentrations required to inhibit 50% growth (EC_{50}) values across species (< 0.05 µg/mL), while azoxystrobin, mefenoxam, and ipconazole did not inhibit growth at the highest tested concentration (>10 µg/mL). Overall, these findings enhance the understanding of *Rhizopus* pathogenicity, thermotolerance, and fungicide sensitivity, offering valuable insights for managing this destructive peanut disease.

INDEX WORDS: Peanut *(Arachis hypogaea* L.*),* Rhizopus Seed Rot, Peanut Seed Germination, *Rhizopus* spp., Fungicide Sensitivity

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DEDICATION

I dedicate this thesis to Dr. Annabelle McEachin, my sister, mentor, and lifelong friend. From childhood adventures to late-night phone calls during graduate school, Annabelle has been by my side from the very beginning. Her unwavering support and sage counsel have always just been a phone call away. I will be forever grateful for her friendship, guidance, encouragement, and belief in me.

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CHAPTER 1

INTRODUCTION

1 PEANUT (ARACHIS HYPOGAEA L.)

Peanuts (*Arachis hypogaea* L.) are an annual, but botanically perennial, herbaceous crop and a rich source of plant-based protein, unsaturated fat, minerals, and vitamins in the mammalian diet (Tallury, 2017; Kvien et al., 2022). In addition to their nutritional qualities, peanuts are an affordable source of protein for developing countries and account for a large portion of edible oil and processed food production in developed countries (Variath & Janila, 2017; Pattee et al., 1995). The United States (U.S.) is one of the most high-yielding countries for peanut production. According to the United States Department of Agriculture (USDA), the U.S. is the fourth most productive country for peanuts, following China, India, and Nigeria, respectively (USDA, 2024). A sizable portion of the United States' peanuts are produced in Georgia annually, accounting for 55% of the nation's peanuts (USDA, 2024).

1.1 Origin, Distribution, and Taxonomy of *Arachis*

The genus *Arachis* contains both the domesticated and widely distributed peanut, *A. hypogaea,* and its wild relatives. It is believed that the genus emerged in the southwestern tropical and subtropical highlands of Central Brazil and Paraguay (Gregory et al., 1973). In the sixteenth century, Spanish explorers documented and eventually adopted the practice of peanut cultivation by South American tribes. Subsequently, peanuts were disseminated to Europe, Africa, Asia, and eventually, the southeastern U.S (Hammons, 1973). However, the date of its introduction into the U.S. is unknown (Hammons, 1982). Nowadays, peanuts are cultivated in

tropical and subtropical regions worldwide (Bertioli et al., 2011). In total, the genus contains 69 identified species and is divided into nine sections based on morphological and interspecific characteristics (Krapovickas & Gregory, 2007). Some species are used as forages (Prine et al., 1986; Homem et al., 2024), while their wild relatives are often utilized as sources of resistance to insect pests and diseases (Chen et al., 2023; Bertioli et al., 2021). However, the species of most economic importance is *Arachis hypogaea* (Moretzsohn et al., 2004). *A. hypogaea* is a dicotyledon crop with an extensive taproot system, nitrogen-fixing nodules, tetrafoliate leaves, ovary stalks (or pegs), inflorescences, and pods (Tallury, 2017; Cui et al., 2022; Moss & Rao, 1995). The two subspecies of *A. hypogaea* are *hypogaea* and *fastigiata*. Subspecies *hypogaea* consists of the 'Virginia' and 'Runner' market types and exhibits alternate flowering, indeterminate spreading, and bigger seeds (Kunta et al., 2022; Moss and Rao, 1995). Subspecies *fastigiata*, on the other hand, consists of the 'Spanish' and 'Valencia' with sequential flowering, erect growth, and smaller seeds (Kunta et al., 2022; Moss and Rao, 1995).

1.2 Peanut Crop Development and Establishment

Peanuts express indeterminate growth both vegetatively and reproductively, meaning the plant consistently produces leaves, stems, flowers, and pods throughout the growing season (Song et al., 2022). Although the planting date depends on weather conditions, cultivars, and soil type, most peanuts are planted from mid-April to late May or early June in the southeastern U.S. (Nuti et al., 2013; Woli et al., 2013; Florida Peanut Growers Association, 2024). During the initial stages of seedling development, the plant relies on the end products of photosynthesis and other stored raw materials in the cotyledons. While seed emergence and vigor can be influenced by several factors, such as cultivar selection, soil pH, water availability (Melouk & Backman, 1995; Tarr 1958), mechanical seed damage, temperature, planting depth (Tarr, 1958), and the prevalence of fungi associated with the seed (Shovan et al., 2008; Clinton, 1960), seed

germination can occur five to ten days after planting (National Peanut Board, 2023). The ideal peanut stand consists of four evenly emerging plants per linear foot of row, whether in a single row or combined across two twin rows (Brenneman, 2021). To facilitate successful germination in the field, it is recommended to plant in a sustained, 4-inch soil temperature between 20-32 °C with a seeding rate of six seeds per linear foot (Kvien et al., 2022; Marsalis et al., 2009; Brenneman, 2021; Tillman et al., 2006; Melouk & Backman, 1995). To ensure proper development of the pods, it is crucial to maintain soil moisture levels between 40% and 50% of the total soil volume in the podding zone (Ono et al., 1974). Healthy emerging plants have undamaged cotyledons on a thick hypocotyl tapering above the root, an epicotyl with more than one primary leaf, and a radicle with adventitious roots (Peterson et al., 2018). Although the time of flowering and pod development depends on the cultivar and environmental conditions (Kvein et al., 2022; Cho et al., 2017), most peanut cultivars begin blooming approximately thirty days after planting (Boote, 1982). Self-pollination is common among peanuts due to the proximity of the female and male reproductive structures, called the stigma and anther, respectively, in the keel, or innermost petal (Smith, 1950). Cross-pollination is facilitated by visiting pollinators to flowers. After fertilization, the pegs exhibit positive geotropism, growing downward in response to the force of gravity and penetrating the soil (Yang et al., 2025; Moss & Rao, 1995). As a result, the pegs cease elongation, and pod development will begin approximately ten days after pegging (Lv et al., 2023). Final pod size is documented to occur approximately 20 to 30 days after pegging (Lv et al., 2023). Overall, a peanut crop takes approximately five months from planting to harvesting (National Peanut Board, 2023), and in the southeastern United States, harvest typically occurs in September or October.

1.3 Peanut Seed Anatomy and Function

The peanut seed consists of a thin, papery testa (Dean, 2020), two cotyledons, a plumule, hypocotyl, epicotyl (or primordial leaves), and radicle (Moss and Rao, 1995; Gregory et al., 1973; Brown et al., 1995). The testa, or seed coat, is the maternal tissue derived from integuments that provides varietal recognition and, in some cases, tolerance or resistance to various pathogens such as *Aspergillus* spp. *(*Azaizeh et al., 1990; Mendu et al., 2022; Moss and Rao, 1995). The testa serves as the major physical barrier of the cotyledons against fungal infection and is comprised of five cell layers including the epidermis, hypodermis, sclerenchyma, parenchyma, and chlorenchyma (Commey et al., 2021; Shi et al., 2021). The cotyledons are the major storage tissue for the developing seedling and contain proteins, carbohydrates, and oils (Gregory et al., 1973; Kvien et al., 2022). The plumule develops into the first true leaves (Brown et al., 1995; Kvien et al., 2022). The hypocotyl is located below the epicotyl and aids in the emergence of the radicle. The radicle is the first embryonic part of a dicotyledonous plant to emerge from the seed (Gilbert, 2000; Ma et al., 2017).

1.4 Modern Peanut Cultivars

For many years, breeders have developed different peanut genotypes to meet specific agronomic and consumer needs, such as enhanced sensory characteristics (Sithole et al., 2022), increased oleic acid (Bimro et al., 2020), and disease resistance (Tsai et al., 2024), which has led to the large number of cultivars currently available. While many peanut cultivars are planted in the U.S., ‘Georgia-06G’ accounts for over half of all the nation’s certified peanut acres (Brown, 2023). Released in 2006, ‘Georgia-06G’ is a high-yielding, runner-type cultivar with large seeds and a medium maturity pattern (Monfort et al., 2020). As shown in Table 1, other currently available peanut cultivars include, but are not limited to, the following: ‘Georgia-09B’, ‘Georgia-12Y’, ‘TifNV-High O/L’, ‘Georgia-16HO’, ‘Georgia-18RU’, ‘Georgia-13M’, ‘Georgia-14N’, ‘Tifguard’, ‘TUFRunner™ 511’, and ‘TUFRunner™ 297’ (Monfort et al., 2022). These cultivars

have been characterized for susceptibility to many diseases, but there is no data concerning their relative susceptibility to Rhizopus seed rot.

1.5 Peanut Seed Germination

The main components of seed physiological potential are germination and vigor (Marcos-Filho, 2015). Germination occurs when embryo growth overcomes the constraints of the testa, or seed coat (Bewley & Black, 1994). Although germination involves many processes, it can be described in three general steps: imbibition, reactivation of metabolism by many biochemical processes, and radicle protrusion (Sghaier et al., 2022; Ali & Elozeiri, 2017). During imbibition, water is absorbed by the dormant seed's cell walls and held by the electrostatic forces in hydrogen bonds (Woodstock, 1988). Diffusion and capillary action aid water movement into the seed (Paiva et al., 2006). The seed coat's permeability can influence the rate of water uptake (Woodstock, 1988; Smykal et al., 2014). After imbibition, the second step involves several biochemical processes necessary for germination, including the synthesis of nucleic acids and proteins, hydrolysis, the Krebs cycle, respiration, translation of stored mRNA, and cell elongation (Bewley et al., 2013). Radicle protrusion marks the beginning of seedling emergence (Bewley, 1997), which is the first major event after germination and initiates the plant's production and establishment (Awal & Ikeda, 2002).

1.6 Germination Testing of Peanut Seed Lots

Germination testing and certifications of seed lots assure farmers that seeds meet standards for germination, viability, cultivar purity, and sanitation (Melouk & Backman, 1995). According to the Georgia Seed Law, the minimum germination standard for untreated peanut seeds in Georgia is 70%; however, seeds with germination percentages greater than 60% can be sold with the label "below standard" (Georgia Department of Agriculture, 2012). Seed lots originating

from different environments often vary in pathogen loads, mechanical damage, and quality, leading to differences in germination rates.

1.7 Peanut Seed Vigor

Seed vigor is defined as the seed's potential to quickly emerge uniform stands and maintain quality in storage under a wide range of conditions (Reed et al., 2022). The relative seed and seedling vigor directly affects germination and development under various environmental conditions, reflecting the rate and potential of seeds to establish healthy and uniform plants. Generally, seeds with high vigor more efficiently mobilize reserves from storage tissues and develop healthy seedlings (Marcos-Filho, 2015). However, environmental factors, such as water availability, temperature, and storage conditions, can affect seed vigor and quality (Moreno et al., 2024; Reed et al., 2022; Suriyasak et al., 2020; Woodroof, 1973; Smith et al., 1995). Therefore, selecting high-quality seeds and implementing proper crop management and protection strategies are critical (Melouk & Backman, 1995). The two goals of a seed vigor assay are to assess seed quality and determine differences in physiological potential among commercial cultivars.

2. RHIZOPUS SEED AND PRE-EMERGENCE SEEDLING ROT

2.1 Pathogenicity of Seed- and Soil-Borne Pathogens

Understanding pathogen-host interactions can lead to the development of new strategies for enhancing host resistance, facilitating host tolerance, reducing pathogen loads, and implementing effective crop protection and disease management. Pathogenicity is defined as a microorganism's ability to cause disease in a host, and it is measured in terms of virulence, or the degree of damage caused by the pathogen (Shapiro-Ilan et al., 2005; Veyrier et al., 2011). Disease development is dependent on a favorable environment, a susceptible host, and a virulent pathogen, as demonstrated by the iconic plant disease triangle (Roussin-Léveillée et al., 2024). Environmental conditions such as microbial competition, food availability, soil pH, temperature,

and humidity affect pathogenicity in the field (Bell & Jackson, 1969). Because the nutritionally dense peanut fruit develops in a subterranean environment, the seed is an excellent host for many seed-and-soil-borne pathogens (Hill et al., 1983; Thiessen & Woodward, 2012; Brenneman, 2021). Many abiotic factors such as mechanical and chemical damage (Martinez et al., 2018; Clinton, 1960), poor sanitation and storage practices, susceptible cultivars, and environmental stressors influence plant susceptibility to pathogenic infections.

2.2 *Rhizopus* Taxonomy and Fungal Structures

Rhizopus is a filamentous, cosmopolitan, saprophytic, seed-or-soil-borne pathogen (Bullerman & Caballero, 2003). The most historically documented species are *R. arrhizus, R. oryzae,* and *R. stolonifer* (Schipper and Stalpers, 1984; Middleton and Mayer, 1985; Porter et al., 1982: Thirumalaisamy et al., 2020). As shown in Table 2, each belongs to the phylum Zygomycota, subphylum Mucoromycotina, class Zygomycetes, order Mucorales, and family Mucoraceae (Abe et al., 2010; Bautista-Banos et al., 2014).

The subphylum Mucoromycotina compromises some of earliest fungi to establish a mutualistic relationship with terrestrial plants (Wang et al., 2021; Selosse & Le Tacon, 1998). The class Zygomycetes represents about 1% of known fungal species and contains fungi that reproduce sexually by fusion of morphologically similar gametangia, resulting in zygospores (Kendrick, 2000; Richardson, 2009). The order Mucorales includes all common saprobic zygomycetes, with structures such as sporangiospores, sporangia, branched hyphae, and sporangiophores (Kendrick, 2000). In culture on potato dextrose agar (PDA), the mycelium forms aerial hyphae that start white and later develop spherical black structures called sporangiophores (Bautista-Banos et al., 2014). *Rhizopus* is differentiated from other genera in the order by the presence of well-developed rhizoids and dry sporangiospores with striate walls (Pitt & Hocking, 2009; Baustista-Banos et al., 2014). The family Mucoraceae contains fungi with

distinctive structures, such as a conical-cylindrical columellae from which the sporangium arises, and an outer surface called the peridium (Kendrick, 2000).

Rhizopus species are coenocytic and produce multiple, asexual, darkly pigmented sporangiospores (Ribes et al., 2000; Hawley, 2019). The sporangiospores are released from the sporangium at the end of the sporangiophore, or hyphal stalk, and are typically the most noticeable form on infected plants (Schumann & D'Arcy, 2006). Rootlike rhizoids can be seen underneath the stolon, or the horizontal hyphal stem. The sexual or "resting" spores, called zygospores, are usually dark, large, and produced at the union of compatible hyphae (Schumann & D'Arcy, 2006; Cerdá-Olmedo, 2001).

Rhizopus species can be heterothallic or homothallic. Heterothallism requires the contribution of nuclei from two compatible strains to form a zygospore (Gryganskyi et al., 2010), while homothallism requires only one organism's resources to reproduce sexually (Wilson et al., 2015). However, zygospore germination and progeny development are often rare for several reasons, such as unfavorable moisture and temperature conditions, inadequate nutrients, poor pH, or low selective pressure for dispersal and genetic fitness (Krug et al., 2007; Voigt et al., 2014). Not only do *Rhizopus* species produce sexual zygospores, but they also produce asexual azygospores. Azygospores share the same morphological traits as zygospores, except that development occurs without a sexual partner (Gryganskyi et al., 2010). Overall, the taxonomic classification of *Rhizopus* reflects its distinctive morphological and reproductive structures, distinguishing it from other fungi of the Mucorales order.

2.3 Life Cycle of *Rhizopus* spp.

Rhizopus undergoes various stages in its life cycle, such as dispersal, infection, and reproduction. Under favorable conditions, the pathogen rapidly disseminates from infected seeds to other hosts and locations. Through its ability to be disseminated by seeds, soil, water, vectors,

mechanical means, and air (Pagán, 2022; Cabrera-Rangel et al., 2022; Baggio et al., 2015; Morwood, 1953), *Rhizopus* has a highly effective mechanism of long-range distribution. In a laboratory study, multiple containers of soil placed adjacently in an incubator were contaminated by the spread of *Rhizopus*, spoiling the trial (Moorwood, 1953). In field environments, rapid dissemination also occurs by planting compromised seeds, allowing the pathogen to travel the length of the furrow and infect healthy seeds (Thirumalaisamy et al., 2020; Brenneman, 2021). Due to their small mass, sporangiospores are easily carried by water and air, allowing them to disseminate through streams and air currents. After release from the sporangium, the sporangiospores travel and land on a damp surface or wounded plant tissue and germinate. In the case of pre-emergence seed rot, infection likely occurs through the invasion of exposed cotyledonary tissue (Tarr, 1958). Soon, mycelium produces stolons, which latch onto the surface and produce root-like rhizoids (Agrios, 1988). The rhizoids grow inward and produce sporangiophores. The pathogen macerates the surrounding host tissue by secreting pectinolytic enzymes and subsequently colonizes living host tissues with mycelium (Agrios, 1988; Bautista-Banos et al., 2014). A sour smell often develops due to host cell disintegration. The mycelium will emerge through wounds, often forming thick, gray mats over the host's surface (Kwon et al., 2001: Thirumalaisamy et al., 2020). Soon, the sporangiospores spread to the healthy sections and envelop the entire seed. Infected peanut seeds are reported to completely disintegrate in 36 to 96 hours after planting (Moorwood, 1953). The plumule and cotyledonary laterals of infected seedlings are either partially or entirely destroyed, leading to stunting, wilting, and eventually death (Porter et al., 1982; Moorwood, 1953; Bell, 1967).

2.4 Abundance of *Rhizopus* spp.

Rhizopus is often identified in the rhizosphere, or the area surrounding the roots that is colonized by a vast population of beneficial and pathogenic microorganisms (Hinsinger &

Marschner, 2006). A study in California identified *Rhizopus* to be more common in the rhizosphere of conventional maize systems than in organic soils (Schmidt et al., 2019). *Rhizopus*, among other microorganisms in the soil-fungal community, was identified more abundantly in the rhizosphere of wilted than healthy pepper plants in Mexico (González-Escobedo et al., 2023). An increase in the soil's relative abundance of *Rhizopus*, as well as other pathogenic fungi, was induced by continuous cropping of soybeans (Bai et al., 2015). Due to its small size and mass, its spores are often found in the atmosphere and water systems (Jackson & Bell, 1969; Moustafa et al., 1976; Kiprop et al., 2024; Castro e Silva et al., 2020; Ross et al., 2004; Haleem-Khan & Mohan-Karuppayil, 2012; Ramsey et al., 1938; Bautista-Banos et al., 2014). In 1976-1977, water and bottom sediment samples collected from eight surface irrigation ponds in southern Georgia yielded species of *Rhizopus* (Shokes & McCarter, 1979). Water samples collected from wells in glasshouse cultivation detected at least seven occurrences of *Rhizopus,* which led to plant infections (Bewley & Buddin, 1921). These findings indicate the abundance and diverse distribution of *Rhizopus* in multiple agricultural systems.

2.5 *Rhizopus* Hosts and Geographical Distribution.

Rhizopus is a saprophytic, cosmopolitan pathogen most prevalent in temperate and subtropical regions (Nelson, 2009; Gryganskyi et al., 2010; Pan et al., 2021). It contaminates and inhabits dead or damaged organic matter, in soil, old bread, animal feces, and the mammalian respiratory system (Hawley, 2019; Yang et al., 2022). In years with evenly dispersed rainfall, *Rhizopus* spp., especially *R. stolonifer* and *R. arrhizus,* were most identified in agronomic systems (Melouk & Backman, 1995). Aside from peanuts, *Rhizopus* has been reported to infect over 100 plant species, including tomatoes (Alfaro-Sifuentes et al., 2019), citrus (Kwon & Park, 2002), strawberries (Oliveira et al., 2018) bananas (Kwon et al., 2012), apples (Kwon et al., 2011), pear (Kwon & Lee, 2004), papaya (Singh et al., 2012), grape (Kwon et al., 2007),

jackfruit (Nelson, 2005), sweet potatoes (Jeong et al., 2014), cotton (Simbwa-Bunnya, 1968), squash (Kwon et al., 2000), and sunflowers (Markell et al., 2015).

2.6 Historical Perspective of Rhizopus Seed and Seedling Rot in Peanuts.

Rhizopus was first associated with peanut seed rot in the U.S. in 1943 by Dr. Wingard of the Virginia Agricultural Experiment Station (Taylor & Atkinson, 1944). In the report, random sections of the field were dug, revealing that half of the plant's peanuts were rotten in the soil. It was later revealed in cultures that *Rhizopus*, as well as other soil-borne pathogens, were detected on the rotted seeds. One of the earliest reports identifying *Rhizopus* spp. in Georgia's peanut seeds was published in 1944 (Higgins, 1944). It listed *Rhizopus* spp., among other fungi, as being most attributed to concealed damage of Georgia peanuts. Concealed damage refers to the internal discoloration and decay of peanut seeds that are not visible in unbroken seeds (Garren & Higgins, 1947), which can delay the detection of the pathogen and the implementation of effective management practices. In 1951, *Rhizopus* was identified as one of the major genera associated with the development of peanut seeds in the soil (Arant et al., 1951). In 1953, *Rhizopus*-infected peanut seeds were reported to either rot in the soil before germinating or germinate but rot before breaking the soil line (Moorwood, 1953). From 1955-1958, a study identified high to very high amounts of *Rhizopus spp.* on shelled peanut seeds in Kingaroy, Australia (Purss, 1959). In 1958, seedbed losses in Gezira, Sudan attributed *Rhizopus* as one of the predominant isolates from seedlings before emergence (Tarr, 1958). In 1959, pre-emergence rot of peanut was primarily attributed to *R. arrhizus* in Australia (Purss, 1959). In 1960, peanut seeds inoculated with *R. stolonifer* up to five days after planting showed 90%, 57%, 20%, 11%, and 0% death, respectively (Clinton, 1960). The same study reported that infected seeds were reduced to a rotted pulp within 36 to 96 hours and, by five days, were indistinguishable from the surrounding soil. As a result, the seed and stand quality decreases, reducing plant stands by 60%

and diminishing the crop's value to all divisions of the peanut industry (Clinton, 1960; Jackson, 1964). In 1964 to 1965, two studies conducted by Jackson isolated *Rhizopus spp.* from infected peanut kernels, seeds, and skins in the Coastal Plain region (Jackson, 1964; Jackson, 1965). Because a reduction in *Rhizopus* was observed after subsequent washings of the peanut pod, it was speculated that the fungus was either loosely bound to the pod surface or highly susceptible to the tested washing methods (Jackson, 1965). Between 1965 and 1966, the dominant fungi detected in harvested peanut pods in Cobb, Oklahoma, were *Fusarium* spp., *Penicillium* spp., *Alternaria tenuis*, and *Rhizopus* spp. (Barnes & Young, 1971). In 1970, it was reported that *R. arrhizus* caused maximum seed rot (100%) at 65, 81, 92, 98, and 100% relative humidity and at 19, 25, 31, and 37 °C (Gupta & Chohan, 1970). In the same year, *Rhizopus* spp. were identified in peanut seeds and shells up to nine weeks before harvest in Nigeria (McDonald, 1970). In 1976, a study detected protein decomposition of peanut seeds as soon as two days after infection with *R. oligosporus*, which was later reclassified as a variety of *R. microsporus* (Cherry et al., 1976; Liu et al., 2007). In 1979, a report made by the International Crops Research Institute for Semi-Arid Tropics in India recognized *R. arrhizus* and *R. stolonifer* as causing peanut seed and pre-emergence rots (Chohan, 1979). In 1989, sterilized and non-sterilized peanut seeds from Nadia, West Bengal, were identified as *Rhizopus* at 20% and 56%, respectively. In 1993, *Rhizopus* was identified as one of the most common genera of the 12,744 isolates obtained from peanut shells in two locations near Tifton, Georgia (Baird et al., 1993). These reports across several decades and continents emphasize the severity of Rhizopus seed rot on peanuts and the importance of understanding its pathogenicity to enhance disease management.

2.7 Modern Day Perspective of Rhizopus Seed & Seedling Rot in Peanuts.

Rhizopus remains a major issue in peanut production; however, it is primarily investigated as a storage and fresh market contaminating agent. In a 2004 study, *R. stolonifer* was detected in 80,

90, 85, 82, and 88% in five fresh-produce markets (2000 g of peanuts per sample) in Kenya (Gachomo et al., 2004). In 2013, a study of fungal incidence in peanut cultivars 'Runner IAC Caiapó' and 'Runner IAC 886' isolated *Rhizopus* in over half of the collected pods and kernels during different sampling times after harvest (Zorzete et al., 2013). A study in Brazil assessing five peanut seed lots of cultivars IAC 886 and IAC 503 detected *Rhizopus* in 100% of the lots from the 2010/2011 and 2011/2012 harvests (Santos et al., 2013). Another Brazilian study assessing thirteen peanut seed lots identified *Rhizopus* as one of the predominant fungi associated with unhealthy seedlings and accelerated seed aging (Santos et al., 2016). In 2018, raw peanut samples from Romania and Egypt isolated *Rhizopus* in 65% and 66%, respectively (Popa et al., 2018). In 2014, 1400 isolates collected from peanut seed samples in five Egyptian governorates detected *Rhizopus* at 16% (Embaby et al., 2014). In 2008, a study monitoring the mycoflora of peanut hulls and kernels over twelve months of storage detected *Rhizopus* at an average frequency of 6.7% and 4.8%, respectively (Nakai et al., 2008). In Ghana and Kenya, *Rhizopus* spp. were isolated from peanut samples collected from large regional markets, suggesting the seeds were poorly handled or stored in unfavorable environments (Madilo et al., 2023; Ndung'u et al., 2013). In Ethiopia, *Rhizopus* contamination in stored peanut seeds reached 34% on pods and 59% on seeds (Terefe et al., 2003). In Brazil, *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., and *Rhizopus* spp. were predominately associated with stored peanut seeds, resulting in decreased seed germination and vigor (Santos et al., 2016). In China, peanut seedlings infected with *Rhizopus oryzae* were stunted with chlorotic leaves, reduced growth, and rapid wilting (Xu et al., 2015). In Bangladesh, 24 samples collected from different locations in the country indicated *Rhizopus* at 9.5% frequency of seed-borne fungi with peanut (Khandaker et al., 2019). In recent years, higher incidence of *Rhizopus* spp. were reported in Georgia's commercial seed lots (Brenneman, unpublished). A total of five compromised peanut seed lots

were observed, with two originating from the 2021-2022 harvest (Lot #5002 and #7011) and three from the 2022-2023 harvest (Lot #6832, 677, and 781). Lot #5002 consisted of cultivar 'Georgia-18RU' with 100% incidence of *Rhizopus* spp. Lot #7011 consisted of 'FloRun™ '331' with 10% incidence of *Rhizopus* spp. In 2021 and 2022, varying degrees of seed and seedling disease were observed in plant stands and yield due to Rhizopus pre-emergence seed rot and Aspergillus crown rot. (Brenneman, 2022; Brenneman, 2021). Lots #6832 (Premium Peanut, Douglas, GA) and #677 (Olam Edible Nuts, Sylvester, GA) consisted of 'Georgia-06G' with 54% and 63% incidence of *Rhizopus* spp., respectively (Brenneman, 2023). Although Rhizopus is primarily studied as a peanut seed storage contaminant in most modern studies, these reports emphasize the significant impact of *Rhizopus* on peanut seed quality and the need for understanding its pathogenicity to mitigate its effects on peanut production.

2.8 Pathogenicity Assays of Rhizopus on Peanut Seeds and Seedlings.

Most research regarding the pathogenicity of *Rhizopus* as a peanut seed pathogen can be found in historical literature. In 1953, an Australian study surface-sterilized whole nuts by soaking in 1-400 commercial formalin, or a solution of formaldehyde in water, for 30 minutes. After hand-shelling, the seeds were inoculated with a spore suspension of *R. arrhizus*. The seeds were then planted in autoclaved soils and monitored closely for germination and disease symptoms. It was reported that seeds and seedlings infected with *Rhizopus* are reduced to a pulpy, rotten mass 36 to 96 hours after planting when soil moisture and temperature favor disease development (Moorwood, 1953). After five days, the seeds were indistinguishable from the soil. In 1960, a Nigerian study found that seeds and seedlings inoculated with *R. stolonifer* zero, two, three, four, and five days after planting exhibited kill rates of 90%, 57%, 20%, 11%, and 0%, respectively (Clinton, 1960) The same study revealed that the predominant reason why at least 270 seeds never germinated was due to *Rhizopus* infection. The author stated that this

number could be higher, as some of the *Rhizopus*-infected seed were "almost unrecognizable in the black soil" and unable to be recovered. In 1967, Bell inoculated 2 mg of oven-dry 'Early Runner' peanut seedlings with plumules and green, plump cotyledons with 0.9-2.1 mL of *R. oryzae* and *R. stolonifer*. Inoculum was made by blending 14-day-old cultures and sterile water in a Waring Blendor (Bell, 1967). In total, twenty seedlings were inoculated and incubated for seven days under continuous fluorescent light in four temperature chambers: 18.3, 23.9, 29. 4, and 35 °C (Bell, 1967). The results showed that both *Rhizopus* species were pathogenic to peanut seedlings across all temperatures; however, *R. stolonifer* was the only fungus that caused necrosis of more than 50% of the total surface of the plants at 18.3 °C. Additionally, the tests also revealed that the plumules and cotyledonary laterals were more susceptible to fungal invasion than the roots.

V.K. Gupta and J.S. Chohan conducted several pathogenicity assays and other seed-rot studies of *Rhizopus arrhizus*. In 1970, peanut pods surface sterilized in 0.1% mercuric chloride for five minutes, rinsed three times in sterile water, and dried on sterilized paper towels were syringe inoculated with four-day old cultures of *R. arrhizus* (Gupta & Chohan, 1970). By day forty, 21.7% of seeds inoculated with *R. arrhizus* expressed visible and concealed damage. In the same study, it was found that seeds inoculated with a 1:1 mixture of *A. niger* and *R. arrhizus* harbored more *R. arrihzus* in the space between the two cotyledons than on the testa. A second study revealed that the same mixture caused 60% seed rot. In another study, shelled seeds sterilized by the method mentioned earlier (Gupta & Chohan, 1970) were inoculated by rolling the seeds over sporulating cultures of *R. arrhizus* and planted into pasteurized soil. In two weeks, rotted seeds were extracted from the soil. After further examination, it was found that *R. arrhizus* was mainly detected on the surface with only a small portion of seeds showing internal mycelium. A separate study revealed that *R. arrhizus* caused 100% seed rot in relative humidity

of 65, 81, 92, 98, and 100%. In 1970, the pathogenicity of *R. arrhizus* on peanut seeds was examined at five temperatures: 12, 19, 25, 31, and 37 °C. Peanut seeds surface sterilized in 0.1% mercuric chloride for 5 minutes, rinsed three times in sterile water, and dried on sterilized paper towels were placed in Petri dishes with moist blotting paper. At the lowest temperature, *R. arrihzus* caused the most infection of seeds or cotyledons at 28%. 100% seed rot was reported at 19, 25, 31, and 37 °C. The percentage of seed germination from the lowest to the highest temperature was 0, 68, 36, 31, and 0%, respectively. The root length from the lowest to the highest temperature was 0, 1.5, 0.5, 0.4 and 0 mm, respectively. Even though some germination and radicle protrusion occurred at 19, 36, and 32 °C, all seeds eventually rotted. To facilitate successful germination in the field, the minimum soil temperature for planting is 18 °C (Kvien et al., 2022). This study suggests that even within the optimal soil temperature range for planting, *R. arrhizus* has a severe impact on germination.

Despite these reports, modern studies on *Rhizopus* as a pre-emergence peanut pathogen remain largely unaddressed within the seed and seedling disease complex. Much of the foundational research was conducted over fifty years ago and lost momentum after the mid-1970s. This decline in focus may be due to shifting research priorities to other plant pathogens, such as *Aspergillus* spp., or the development of more effective seed treatment fungicides that reduced the impact of the disease. However, given the rising incidence of *Rhizopus* in commercial lots and the lack of updated research, revisiting Rhizopus seed rot is essential for understanding the pathogen and reevaluating seed health management strategies.

3. MOLECULAR IDENTIFICATION OF *RHIZOPUS*

3.1 Deoxyribonucleic acid (DNA)

Deoxyribonucleic acid (DNA) is a double-stranded molecule that contains the genetic code that dictates the growth, development, and identity of a living system (James, 2016). The structure of DNA is described as a double helix, because the two strands of nucleotides twist around each other (Watson, 1968). The coiled strands build a backbone consisting of alternating sugar and phosphate units, with each sugar linked to one of the four nitrogenous bases: adenine (A), cytosine (C), guanine, (G), or thymine (T) (National Human Genome Research Institute, 2025). Base pairs are two complementary bases that pair together and connect the two DNA strands. Adenine (A) bonds with thymine (T), and cytosine (C) bonds with guanine (G) (Shukla & Leszczynski, 2002).

Identifying *Rhizopus* species in agricultural settings, such as seed lots, can lead to a greater understanding of the pathogen's geographical distribution, abundance, and life cycle. Before the advent of molecular identification, *Rhizopus* species were primarily identified by morphological and physiological characteristics, such as size, color, shape, pathogenicity, presence or absence of certain structures, organic acid production, temperature optimums, and growth interactions on different agar media (Ames, 1915; LeCato, 1916; Zycha, 1935; Long et al., 1950; Inui et al., 1965). However, *Rhizopus* species, or any plant pathogen, should not be identified or diagnosed by morphology or physiology alone. Similar morphological features across plant pathogens in the same or different genus, morphological evolution occurring at different rates, the presence of secondary pathogens on infected sites, latent symptoms, and varying virulence and behavior among strains can lead to incorrect identifications and misguided assumptions about *Rhizopus* (Baum, 2008; Nguyen et al., 2023). Additionally, there is no widely accepted standard for how much morphological difference is needed to classify a *Rhizopus* species as a new species or a strain of an existing species to date (Abe et al., 2010). Therefore, other methods, such as microscopy, serological tests, biochemical tests, and polymerase chain reaction (PCR) testing

(Mendonca et al., 2022), reduce the possibility of an incorrect diagnosis and provide standardization in differentiating *Rhizopus* species. In contrast to morphology-based identification, DNA-based identification, like the polymerase chain reaction (PCR), provides high accuracy, sensitivity, specificity, and a more robust understanding of evolutionary relationships between species (Bell, 1989). Public platforms, such as the NCBI Basic Local Alignment Search Tool (BLAST), provide even further accuracy by comparing DNA against large global databases. Additionally, it establishes a more standardized approach to identification beyond morphological features, which can sometimes be an inaccurate reflection of phylogenetic relationships (Abe et al., 2010). A much greater comprehensive analysis of *Rhizopus* species can be achieved by the utilization of both morphological and molecular identification. Despite the clarity molecular identification provides, the number of *Rhizopus* species is debated, with identifications varying among researchers (Abe et al., 2010; Dolatabadi et al., 2013: Liu et al., 2007; Zheng et al., 2007). However, the most documented *Rhizopus* species in collections are *R. stolonifer, R. arrhizus,* and *R. delemar* (Gryganskyi et al., 2018).

3.2 DNA Extraction

DNA extraction of *Rhizopus* spp. involves breaking down the chitinous cell wall to release the DNA and purifying it for advanced testing, such as PCR analysis. Different methods can be employed to extract the DNA of *Rhizopus* species. Many extraction kits, cell disruption methods, lysis buffers, protocols, and user-specific modifications are available. Some extraction protocols require the physical disruption of cells by a probe sonicator, mortar and pestle with liquid nitrogen, or bead-beating technology (van Burik et al., 1998; Zhao et al., 2024). Additionally, various lysis buffers are used for the chemical disruption of cells, such as sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and phenol-chloroform mixtures (Ankola et al., 2020; Liu et al., 2022; Kenjar et al., 2021; Al-Samarrai et al., 2001).

Moreover, several providers have developed their own fungal and yeast extraction kits, such as Qiagen, Norgen Biotek, Zymo Research, and Omega Bio-Tek. In short, there is no single standardized method for fungal DNA extraction (Karakousis et al., 2006), as it can be customized to meet experimental needs and equipment constraints. However, most extraction techniques involve a method of physical and chemical disruption, purification, and quantification (Conlon et al., 2022).

3.3 DNA Quantification

For quantifying *Rhizopus* DNA, spectrometry methods such as the NanoDrop (Thermo Fisher Scientific Inc., Waltham, MA) are standard practice in diagnostic laboratories and interpret the maximal absorbance of light in the ultraviolet (UV) wavelength by DNA at 260 nm (Versmessen et al., 2024). Because nucleic acids and purified proteins absorb light maximally at 260 and 280 nm, respectively (Thermo Fisher Scientific, 2025), the A260/A280 ratio is critical for quantifying DNA. Generally, genomic DNA is considered pure when its A260/A280 absorbance ratio is above 1.80 (Usman et al., 2014). A low ratio may indicate the presence of contaminants, such as peptides, phenols, and reagents used in the extraction protocol (Okamoto & Okabe, 2000).

3.4 Gel Electrophoresis

Gel electrophoresis is a technique that separates segments of DNA based on their size and charge by applying an electric current through a tray that contains a polyacrylamide or agarose gel matrix (Mesapogu et al., 2012). The gel matrix rests upon an aqueous buffer solution. The left side of the tray carries a negative charge, while the right side is positive. A column of sample wells is located on the left or negative side of the tray. DNA has a negative charge due to the phosphate groups attached to DNA strands (Mirzabekov et al., 1979). As a result, the DNA will travel through the gel matrix towards the positive side of the tray. Larger segments of DNA

travel at a slower rate, while smaller segments travel faster (Lee et al., 2012). The number of base pairs (bp) in a DNA molecule can be determined based on the distance traveled through the gel matrix (Tan et al., 2007), which helps identify differences between species in the same genus. Once DNA separation is complete, a band of DNA is revealed under an ultraviolet (UV) light. This biotechnology measures and visualizes the products of gene amplification using the polymerase chain reaction (National Laboratory of Enteric Pathogens, 1991).

3.5 Polymerase Chain Reaction (PCR)

Developed in the 1980s by American biochemist Dr. Kary Mullis (Mullins, 1990; Kaunitz, 2015), the polymerase chain reaction (PCR) is an in vitro technique for amplifying DNA and RNA sequences. It can efficiently and cost-effectively identify pathogens by applying heat to separate the double-stranded DNA, annealing the two separated strands, joining the separated strands with primers, and amplifying the DNA with polymerase (Garibyan & Avashia, 2013). PCR is often described in three main phases: denaturation, annealing, and extension (Rodriguez-Lazaro & Hernandez, 2019; Delidow et al., 1993). During denaturation, the double-stranded DNA (dsDNA) is separated into single-stranded DNA (ssDNA) at high temperatures (93 to 96 °C) to make the DNA template accessible for replication. During the annealing phase, the complementary primers, also called the left or forward primer (5') and the right or reverse primer (3'), flank opposite sides of the targeted ssDNA segment (National Laboratory of Enteric Pathogen, 1991). After the temperature decreases (55 to 65 °C), the two separated ssDNA strands rehybridize into dsDNA. Once the bond is strengthened, DNA polymerase (DNAP) attaches to the DNA and copies nucleic acid molecules to form a new double-stranded DNA (dsDNA). To activate the DNAP, the reaction is reheated (70 °C). Due to its thermostability, or ability to maintain structure when exposed to high temperatures, polymerase 'Taq' is often an ideal DNAP (Ishino et al., 2014; Parasaeimehr et al., 2013). These three phases comprise one

PCR cycle. Most PCR reactions repeat the cycle twenty to forty times to maximize DNA amplification (Wages, 2005). However, PCR has its limitations. The thick, chitin-dense fungal cell walls can impede efficient lysis of organisms and release of DNA, leading to false-negatives or poor PCR results (Khot et al., 2009). Moreover, developing primers to distinguish closely related fungi can be challenging (Hariharan et al., 2021). Inhibitors in some biological samples (e.g. soil or plant parts) can negatively interfere with PCR testing and results (Sharma et al., 2024). Despite these limitations, PCR has consistently proven to have high sensitivity and specificity for amplifying the DNA of *Rhizopus* spp. (Lass-Flörl et al., 2013; Ala-Houhala et al., 2018, Hariharan et al., 2021).

3.6 Ribosomal Genes

Molecular identification utilizes a system called DNA barcoding, which is comprised of DNA sequences from specific gene markers (Hebert & Gregory, 2005). The three common nuclear ribosomal in fungal identification include 18S (small subunit or SSU), 5.8S, and the 28S (large subunit or LSU) (Imoulan et al., 2017). Because the small and large subunit sequences evolved slower than the ITS regions (Walker et al., 2022), it is most effectively used to identify distantly related organisms (White et al., 1990; Hamby and Zimmer, 1992). Located between the small and large subunits, the two internal transcribed spacer regions (ITS-1 and ITS-2) evolved at a faster rate, and as a result, are the best for identifying most species (Tanaka et al., 2003). Depending on the species, ITS alone is insufficient for identification. For example, because the fungal genera *Aspergillus* and *Penicillium* have narrow barcode gaps in its ITS regions, it does not suffice as a barcoding marker (Samson and Pitt, 2000). Therefore, various protein-coding markers, such as the translation elongation factor 1-alpha (TEF-1α) gene and beta-tubulin, can enhance *Rhizopus* species identification and taxa classification (Rehner and Buckley, 2005; Glass and Donaldson, 1995). After sequences are identified, the NCBI Basic Local Alignment

Search Tool (BLAST) can be used to compare and find regions of local similarity between protein sequences in the database.

3.7 Translation Elongation Factor 1-Alpha (TEF-1α) Gene

To express a gene, the multilayered process of protein synthesis relies on two major steps, transcription and translation. During transcription, the gene's DNA serves as a template to synthesize a strand of messenger RNA (mRNA) by the enzyme, RNA polymerase (Bentley, 2014). During translation, ribosomes synthesize proteins using mRNA as a template (Clancy & Brown, 2008). Initiation, elongation, and termination are three sub-stages in transcription and translation (Sasikumar et al., 2012). To accomplish each step, many ribosomes, enzymes, macronutrients, and soluble proteins factors, such as translation elongation factors, are essential. Translation elongation factors are highly abundant in actively growing cells, comprising up to 2% of the cell's total protein composition (Condeelis, 1995). These factors ensure the proper decoding of messenger RNA (mRNA) to produce proteins (Sasikumar et al., 2012). The building blocks of proteins are amino acids, which utilize energy from ATP to bond to transfer RNA (tRNA) molecules matching each amino acid (Kaziro, 1978). The matching or pairing of amino acids to tRNAs for protein synthesis relies on a group of enzymes known as the aminoacyl-tRNA synthetases (AARs) (Živković et al., 2024). The translation elongation factor 1-alpha (TEF-1α) gene is responsible for the transport of amino-acyl tRNAs to ribosome and is one of the most abundant soluble proteins in eukaryotic cells (Slobin, 1980). The gene is highly conserved, meaning it has not evolved much over a long period of time (Merrick, 1992). Genes that remain conserved typically serve an essential function for the organism's survival (Luo et al., 2015). Therefore, its conserved nature makes it a useful identification marker in differentiating multiple *Rhizopus* spp (O'Donnell et al., 2001; Abe et al., 2010).

3.8 Maximum Likelihood Phylogenetic Tree

Phylogenetic trees illustrate the evolution of *Rhizopus* species from common ancestors and the relatedness between species in the order Mucorales (Gryganskyi et al., 2018; Baum, 2008). The base or root of the tree represents the earliest common ancestor of all *Rhizopus* species in the tree, and the branches represent the genetic history of the species in the tree (Gregory, 2008). Closely related branches on a tree form a clade, which represent the common ancestor and its descendants. The branch tips, or terminal nodes, represent the existing *Rhizopus* species. The relatedness between *Rhizopus* species is determined by the closeness to a shared common ancestor, which is signified on the tree by internal nodes (Blomberg & Garland, 2002). The internal nodes also represent speciation events, or where an ancestral species diverged into two or more *Rhizopus* species (Barraclough & Nee, 2001). Located at the internal nodes, bootstrap values indicate the reliability of a given branch location (Dopazo, 1994). The outgroup or basal species on a tree, such as *R. microsporus* (Gryganskyi et al., 2018), represents the most distantly related species of a given sample and the last common ancestor (Gregory, 2008). A maximum likelihood phylogenetic tree uses a statistical method to estimate the most probable relationship of different *Rhizopus* species based on the sequenced data (Zahin et al., 2025). A model of molecular evolution is chosen to dictate how the genetic sequences are estimated to evolve, and the method calculates the probability that an evolutionary tree could produce the sequenced data (Roch, 2006; Steel, 1994). The phylogenetic tree with the highest probability is chosen. Bootstrap values in the maximum likelihood approach are calculated by a statistical analysis called bootstrap resampling (Dixon, 2001). The higher the bootstrap value, the more likely the grouping is reliable. The maximum likely approach provides reliability by utilizing statistics and models to analyze the evolutionary intricacies of different *Rhizopus* species (Guindon et al., 2009).

4 TEMPERATURE RESPONSE OF RHIZOPUS

4.1 Temperature Optima of Fungal Pathogens

Both plants and pathogens alike require certain minimum temperatures to grow, reproduce, and survive. While some fungi can tolerate a temperature range between 5 to 40 °C (Dar, 2020), the optimal soil temperature range for many soil-borne fungi, including *Rhizopus*, is approximately 25 to 30 °C (Pietikäinen et al., 2005; Dix and Webster, 1995; Vujanovic et al., 1999). Optimal environmental conditions for pathogen development may differ between species and strains (Jackson, 1964; Gryganskyi et al., 2010; Muller, 1956). Moreover, the pathogen's previous habitat, age, moisture conditions, and the nutritive qualities of the media may contribute to the pathogen's response to a certain temperature (Muller, 1956).

4.2 Temperature Responses of *Rhizopus* spp. Across the Century (1915-2022)

Previous studies have characterized the temperature optima of *Rhizopus* spp. on a variety of crops, documenting their adaptability to wide temperature and host ranges. In 1915, cultures of *R. nigricans* (reclassified as *R. stolonifer*) on bean agar were grown in temperatures ranging from 1 to 43 °C, and the time at which germination initiated was recorded (Ames, 1915). The results showed that the fastest germination occurred in five and a half hours at 41, 39, 38 °C and six hours at 37.5, 36, and 35 °C. At 15, 20, 25, and 30 °C, spore germination occurred in 13, 8, 8, and 8 hours, respectively. Spore death occurred at 42 and 43 °C, and no germination occurred at 1 °C. *R. nigricans* at 3 to 5 °C produced considerable mycelium although no growth beyond germination occurred in cultures placed immediately at this temperature range. The spores at 1 °C germinated after being brought to a higher temperature. However, germination was slower and sparser than the cultures initially placed under favorable conditions. The cultures from the maximum temperature ranges did not germinate when transferred to an optimum temperature. Of

the six fungi from the study, *R. nigricans* was the only to reach and maintain maximum growth beyond 30 °C and expressed the highest thermal death point at 60 to 60.5 °C. These findings may suggest that *Rhizopus* is more adaptative to higher temperatures than other fungi causing fruit rots. Additionally, the author noted that sporangia production was highly irregular. In one set of cultures, sporangia developed at temperatures as low as 10 to 12 °C. However, when the test was repeated months later, no sporangia were observed at or above 20 °C. No explanation was offered for this irregularity.

In 1935, *R. arrhizus* growing in culture at 37 °C was prolific but its distinctive morphological features, such as numerous hyphal branching and sporangiophores, were most abundant at 26 °C (Zycha, 1935). In 1956, the growth of *R. nigricans* (reclassified as *R. stolonifer*) on nutrient agar was optimum at 25 °C, poor at 30 °C, greatly inhibited at 32 °C, and nonexistent at 35 °C (Muller, 1956). Despite major inhibitions in spore germination at 32 °C, the spores remained viable for five days. When the sporangiospores were transferred from high temperatures to optimum temperatures, mycelia abundantly produced. In the same study, it was reported that *R. nigricans* expanded its mycelial diameter more rapidly than *Aspergillus niger* and *Penicillium italicum* at their respective optimum temperatures.

In 1966, temperatures exceeding 15 °C increased the infection rate of *R. stolonifer* on peaches within a short timeframe, while lower temperatures merely delayed infection (Pierson, 1966). In 1967, disease indices (0 – healthy to 100 = maximum necrosis) for *R. oryzae* on peanut seedlings at four temperatures was 20, 41, 56, and 50, respectively, at 18, 24, 29, and 35 °C (Bell, 1967). Based on these findings, the author speculates that the maximum infective temperature of *R. oryzae* may surpass 35 °C.

In 1970, the pathogenicity of *R. arrhizus* on peanut seeds was examined at five temperatures: 12, 19, 25, 31, and 37 °C (Gupta & Chohan, 1970). Peanut seeds surface sterilized

in 0.1% mercuric chloride for 5 minutes, rinsed three times in sterile water, and dried on sterilized paper towels were placed in Petri dishes with moist blotting paper. At the lowest temperature, *R. arrihzus* caused the most infection of seeds or cotyledons at 28%. 100% seed rot was reported at 19, 25, 31, and 37 °C. The percentage of seed germination from the lowest to the highest temperature was 0, 68, 36, 31, and 0%, respectively. The root length of germinated seeds from the lowest to the highest temperature was 0, 1.5, 0.5, 0.4 and 0 mm, respectively. Even though germination and radicle protrusion were observed, all seeds eventually rotted. In 1980, a study reported a significant decrease in spore viability when strains of *R. sexualis* and *R. stolonifer* were incubated at –1, 0, and 3 °C for three weeks (Dennis and Blijham, 1980). Similar findings were made by Smith when spores of *R. stolonifer* were incubated at -1, 2, 3, and 4 °C for 10 days (Smith, 1965). In a 2007 study, a total of 203 strains in 17 taxa of *Rhizopus* were tested twice for the maximum growth temperature, and the results showed that the optimum temperature ranges of *R. stolonifer, R. arrhizus,* and *R. microsporus* were 26-32, 37-42, and 40 to 51 °C, respectively (Zheng et al., 2007). At 18 °C, the branches, sporangiophores, rhizoids, spores were noticeability less plentiful and off-color. In 2014, maximum Rhizopus rot on fleshy fruits was reached at 27 °C (Bautista-Banos et al., 2014).

In 2022, in vitro studies reported that *R. stolonifer* grew between 5 °C – 30 °C and 35 – 37 °C, with optimal growth at 25 °C; however, colonies generally ceased growth at 37 °C (Sandoval-Contreras et al., 2022). In the same study, *R. stolonifer* grew faster on jackfruit pericarp agar (AJ) at 13 °C and 25 °C than other fungi in the study, occupying the entire area in the Petri dish in a few days (Sandoval-Contreras et al., 2022). However, modern research regarding the effect of temperature on *Rhizopus*-infected peanut seeds is limited and requires further investigation.

5 SEED TREATMENT AND IN-FURROW FUNGICIDES

5.1 Fungicide Action and Translocation

Seed treatment fungicides disinfest the seed's surface and protect it against seed-and-soil-borne pathogens for the first several days after planting. Each fungicide possesses a mode of action (MOA), which is the specific biochemical and physiological mechanism through which an active ingredient (AI) inhibits fungal growth. Fungicides are categorized into two groups based on their MOA: multi-site and site-specific. Multi-site compounds disrupt multiple metabolic processes in fungal cells, whereas site-specific compounds interfere with only one biochemical reactions (Latin, 2011). Fungicides can be applied preventatively or curatively to avoid or treat an infection (Corkley et al., 2021). Phytomobility describes the fungicide's movement or absorption in or on plant tissues, which can be categorized into two general types: contact and systemic (Latin, 2011). Contact fungicides remain on the plant surface, whereas systemic are absorbed into plant tissues and can remain local to the applied area or translocate greater distances in the xylem or phloem (Klittich, 2014; McGrath, 2004). Ten chemical classes are used for seed treatment fungicides, with the predominant type being xylem mobile (Lamichhane et al., 2020).

5.2 Peanut Seed and Seedling Disease Complex

Rapid and uniform seedling emergence is essential to attain a healthy and persistent stand (Berg et al., 2017); however, seed and seedling diseases are a major deterrent for consistent stands. Seed and seedling disease symptoms include pre-emergence rot, post-emergence damping-off, seed decay, low seedling vigor, stunting, lesions, or decomposition of the cotyledons (Gupta & Chohan, 1970). Under favorable conditions, seed and seedling diseases can severely reduce seed germination, survival, and crop establishment. Many can be involved in the downfall of the crop (Tarr, 1958; Al-Amod, 2015), which can vary dramatically depending on

the environmental conditions and seed quality (Brenneman, 2021). *Rhizopus* and other fungal pathogens like *Aspergillus* spp*., Fusarium, Pythium, and Rhizoctonia* contribute to the seed and seedling disease complex (Thirumalaisamy et al., 2020).

Aspergillus species, particularly *A. niger* and *A. flavus*, are seed-or-soil-borne pathogens responsible for several disorders in various plant stages, including pre-harvest, harvest, processing, and handling (Perrone et al., 2007). The pathogen affects many row crops, such as cotton, corn, and peanuts (Ali et al., 2021). *Aspergillus niger* causes the destructive seedling disease, Aspergillus crown rot (Cantonwine et al., 2011). Infected seedlings generally emerge but quickly and begin to rot at or just below the ground level (Moorwood, 1954). Soon, the seedlings will wilt and die 14 to 35 days after planting (Brenneman, 2021; Damicone, 2017). Some *Aspergillus* species, such as *A. flavus* and *A. parasiticus*, produce carcinogenic secondary metabolites called aflatoxins. Aflatoxins are serious contaminants in animal feeds and are reported to induce acute toxicity in many animal species (Wogan, 1966). Under favorable conditions, such as a hot and dry environment and lower-quality seed, the pathogen can proliferate (Schoustra et al., 2019).

In the Oomycetes order, *Pythium* is a soil-borne organism that favors excessive soil moisture (Kofranek, 2012; Agarwal & Sinclair, 1997; Lewis & Filonow, 1990). *Pythium* infects the seed at or before germination, causing damping-off, poor germination, water-soaked lesions on the pods, and blackened pegs with matted mycelia (Syngenta, 2007; Parkunan et al., 2014; Melouk & Backman, 1995).

Rhizoctonia solani is a seed-and-soil-borne pathogen that survives through sclerotia and prefers tropical environments with wet soil moisture (Senapati et al., 2022; Brenneman, 2021). It can infect all plant parts, causing seed decay, root rot, limb rot, and sunken lesions on the hypocotyls of seedlings (Pegg et al., 2014; Thiessen & Woodward, 2012). Early planting in cool,

damp soil favors seed decay and damping-off caused by *Rhizoctonia solani* (Brenneman, 1996). Often, the soil mycoflora are an indicator of soil conditions (Melouk & Backman, 1995). Conducive soil conditions for *Pythium* and *Rhizoctonia* include cool, poorly drained soils, while hot and dry soils are optimal for *Aspergillus* spp.

5.3 Significance of Seed Treatment Fungicides in Peanuts

Due to conducive environmental conditions in the southeastern U.S. and the ubiquitous distribution of seed and seedling pathogens, seed treatment fungicides are integral to obtaining healthy and uniform plant stands in conventional peanut production. Applying seed treatment fungicides is typically more cost-effective and results in a more consistent stand compared to increasing the seeding rate (Moorwood, 1953). Untreated seeds, regardless of quality, achieve only 50% stands (Melouk & Backman, 1995), while treated seeds can increase yields by 50-75% (Mahoney et al., 2019). Therefore, the utilization of seed treatment fungicides is common in developed agronomic systems in which seeds are used to establish a crop (Zeun et al., 2012) Seed treatment fungicides are applied to almost all planted corn and peanut seed in the U.S., followed by cotton, potato, wheat, and soybeans (White & Hoppin 2004). For peanut seeds, dust formulations have been preferred, as liquid or polymer-based options can loosen the testa and leave the cotyledonary tissue unprotected from pathogens before emergence (Brenneman, 2021, Tubbs et al., 2013; Melouk & Backman, 1995; Middleton & Mayer, 1985). However, liquids offer many advantages and are rapidly being adopted by the peanut seed industry. Fungicides with varying modes of action (MOA) and chemical classes are used in seed treatments, such as demethylation inhibitors, phenylpyrroles, phenylamides, benzimidazoles quinone outside inhibitors, and succinate dehydrogenase inhibitors.

5.4 Historical Fungicide Use Against Rhizopus Seed and Seedling Rot

The deployment of chemicals as seed treatment fungicides began in the seventeenth and eighteenth centuries with the use of brine, arsenic, lime water and copper sulfate and were often discovered by happenstance (Leukel, 1936; Walker, 1948; Maloy, 1972; Russell 2005). Over time, the development of seed treatment fungicides has since advanced and is now widely accepted as an efficacious practice for disease control. Although *Rhizopus* is documented to cause severe damage to peanut seeds (Clinton, 1960; Moorwood, 1953), few modern research efforts have reevaluated the efficacy of fungicidal seed treatments to Rhizopus seed and seedling rot. Most research regarding the efficacy of peanut seed treatment fungicides to *Rhizopus* can be found in the historical literature. In the mid-1910s to 1920s, the rapid acceptance of dust formulations in the U.S. signified the start of "the era of dust fungicides" (Andrews, 1961). Many of the earliest dust fungicides contained organo-mercury compounds, cited as "outstanding, "superior," "the most consistent in improving field emergence," and "more effective in reducing pre-emergence losses than any other type of seed dressing." (Moorwood, 1953; Jackson, 1963: Purss, 1960; Gibson, 1953). *Rhizopus arrhizus* was often the most sensitive pathogen to mercury-based compounds in these studies (Moorwood, 1953). As a result of its great efficacy, mercury dusts were accepted as a standard practice in the Australian peanut industry in 1937 and applied to "practically all peanut seed in the State" (Moorwood, 1953). The post-World War II rapid advancement and acceptance of chemical control methods in agriculture coincided with the documented success of mercury-based fungicides on peanuts in the U.S (Andrews, 1961). Applying mercurial and nonmercurial mixtures decontaminated hypocotyls of *R. stolonifer* (Bell, 1966), and seed treatments containing phenyl mercury acetate protected mechanically damaged testa from fungal infection (Wood, 1968). Seed treatments containing Agrosan GN (1% mercury) at a rate of 1 gram per pound of seed mitigated the harmful impact of fungal infection through mechanical wounds (Tarr, 1958). When applied at a rate of 1.56 grams

per kilogram of seed, 2% methylmercury dicyandiamide (Panogen 15) resulted in 93% clean seed for the Early Runner variety (Jackson, 1963).

When mercury-based compounds were restricted by the Environmental Protection Agency (EPA) due to the high incidence of fish and mammalian toxicity (Zhao et al., 2022; Rissanen & Miettinen, 1972; World Health Organization, 1993), alternative chemistries were investigated. A mixture of captan (400 g/kg) and quintozene (400 g/mg) applied at 30 g/10kg of seed was accepted as a substitute (Middleton & Mayer, 1985). Dicloran or DCNA (2,6-dichloro-4-nitroaniline) mixed with captan (N-trichloromethylthio-4-cyclohexane-1,2-dicarboximide), thiram (tetramethylthiuram disulfide), or maneb (manganese ethylene-bis-dithiocarbamate) also achieved satisfactory control (Melouk & Backman, 1995; Phipps, 1984). An application of Captan at 1000 ppm provided 100% *in vitro* reduction mycelial radial growth of Rhizopus spp. isolated from cotton bolls (Simbwa-Bunnya, 1968). Captan and thiram became the most used fungicide seed treatments after the restriction of mercury-based compounds to protect against seed-borne and soil-borne diseases (Hairston, 2013).

5.5 Modern Fungicide Use Against Rhizopus Seed and Seedling Rot

Modern fungicide seed treatments, such as Trebuset® (Syngenta Crop Protection, Greensboro, NC) and Rancona VPD and VPL® (UPL Corporation Limited Group Company, King of Prussia, PA), use a combination of active ingredients from various fungicide classes to target a wide range of seed and seedling pathogens, reduce the incidence of single-site fungicide resistance, and extend the longevity of these fungicides. Trebuset® contains azoxystrobin, pydiflumetofen, fludioxonil, and mefenoxam, and Rancona VPL and VPD® contains ipconazole, carboxin, and metalaxyl. Generally, most peanut seed in the southeastern U.S. is treated with mefenoxam or metalaxyl, fluidioxonil, and/or a strobilurin product (i.e. azoxystrobrin).

An active ingredient in Trebuset®, azoxystrobin (methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate) is a broad-spectrum fungicide in

the quinone outside inhibitor (QoI) class (Uppala & Sulley, 2025; Kanetis et al., 2007). Its mode of action inhibits the respiratory electron transport chain of mitochondria by binding to the outer quinol-oxidation site of the cytochrome bc1 enzyme of complex III in several fungal and Oomycete pathogens (Ali et al., 2021; Solorzano & Malvick, 2011). As a result, the respiratory electron transfer chain loses the ability to synthesize adenosine triphosphate (ATP) and halts fungal metabolism (Inoue et al., 2011). When the cytochrome pathway of complex III is inhibited by azoxystrobin, the alternative oxidase (AOX) pathway provides an alternative pathway for the electron transfer chain and aids in the survival of fungi (Song et al., 2022). To combat this compensatory mechanism, salicylhydroxamic acid (SHAM) inhibits the AOX pathway and prevents fungi from avoiding the effects of Qol fungicides. SHAM is commonly used in research to study the *in vitro* chemical sensitivity of fungi to azoxystrobin. Released in 1996, azoxystrobin is primarily applied as a protectant to the seed before planting and in-furrow treatment at planting to inhibit spore germination and mycelial growth (Kanetis et al., 2007; Bartlett et al., 2002). In the past, azoxystrobin was active on many plant pathogens, including *Aspergillus spp, Agroathelia rolfsii*, *Pythium* spp., and *Rhizopus* spp (Bautista-Banos et al., 2014; Northover & Zhou, 2002; Bartlett et al., 2002; Mahoney et al., 2019). Due to its site-specific mode of action, however, serious field resistance is reported in this class of fungicides (Zhang et al., 2009; Inoue et al., 2011; Bartlett et al., 2002). Consequently, seed treatment fungicides containing azoxystrobin have shown reduced efficacy over time (Jordan et al., 2019; Ali et al., 2021; Brenneman, 2021).

Released by Syngenta in 2016, pydiflumetofen (3-(difluoromethyl)-N-methoxy-1-methyl-N-[(RS)-1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-1H-pyrazole-4-carboxamide) is a novel broad-spectrum succinate dehydrogenase inhibitor (SDHI) fungicide that affects the respiratory electron transfer chain by targeting an enzyme known as succinate dehydrogenase

(Shd), thereby blocking the SDH ubiquinone (UQ) binding site of complex II in the respiratory electron transport chain and obstructing cycling of succinate oxidation (Sierotzki and Scalliet, 2013; Liu et al., 2023; Anonymous, 2016; Olaya et al., 2016; Neves & Bradley, 2019). As a result, fungal respiration and ATP synthesis are inhibited (Bian et al., 2021). In fungal cells, succinate dehydrogenase is an integral enzyme for the Krebs cycle, a process that is present in the cells of an aerobic and facultative anaerobic organisms (Alabduladhem & Bordoni, 2022), facilitates ATP synthesis, and produces the carbon skeletons used in amino acid synthesis (de la Peña et al., 2019; Labourdette et al., 2011; Chandel, 2021). Because chemicals such as pydiflumetofen, sedaxane, and fluopyram inhibit succinate dehydrogenase, ATP synthesis and the production of various metabolic compounds used in amino acid synthesis are halted, thereby disrupting cellular respiration. Therefore, SDHI fungicides inhibit all fungal growth stages, including germination to sporulation (Labourdette et al., 2011). On peanuts, pydiflmetofen is often applied in conjunction with other fungicides, such as azoxystrobin and flutolanil, to protect the peanut plant from multiple plant pathogens, such as late leaf spot (*Nothopassalora personata*), southern stem rot *(Agroathelia rolfsii),* and Fusarium root rot (Jordan et al., 2024: da Silva & Langston, 2024; Mao et al., 2024). However, little is known about its efficacy against *Rhizopus* on peanut seeds.

Developed by Syngenta in 2011, sedaxane 2-(1H-pyrazol-1-yl)-4-(trifluoromethyl)-1H-pyrrole-3-carbonitrile is a broad-spectrum, succinate dehydrogenase inhibitor (SHDI) that is reported to provide significant control of *Ustilago nuda, Microdochium nivale, Rhizoctonia solani,* and *Tilletia caries* (Walter et al., 2015; Zeun et al., 2012; Reaves, 2022). In soybean fields throughout the U.S., a combination of sedaxane, mefenoxam, and fludioxonil increased yields by 14% compared to the control group and 7.8% compared to the yield from seeds treated exclusively with mefenoxam and fludioxonil (Munkvold et al., 2014). In greenhouse studies of

corn, sedaxane-treated seeds produced significant increases in seedling shoot and root growth than the control groups infected with *R. solani* (da Silva et al., 2017). In field studies of wheat, sedaxane-treated seeds produced significant increases in yield than the control groups inoculated with *Rhizoctonia* spp (Zeun et al., 2012). In peanuts, sedaxane is registered to control seed decay, seedling blight, and damping-off caused by *Rhizoctonia solani* (Reaves, 2022). However, a lack of peer-reviewed research examining the efficacy of sedaxane as peanut seed treatment is evident, as most studies have focused on its application in other agronomic crops such as soybean, corn, and wheat.

Discovered by Bayer Crop Science in 2001, fluopyram (N-[2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]ethyl]-2-(trifluoromethyl)benzamide) is a broad-spectrum, systemic succinate dehydrogenase inhibitor (SHDI) with activity against certain Ascomycetes and plant-parasitic nematodes, such as *Sclerotinia* spp., *Botrytis* spp., *Monilia* spp., *Erysiphe cichoracearum*, *Rotylenchus reniformis*, *Melodoigyne incognita* (Faske et al., 2022; Labourdette et al., 2011; Hagan et al., 2024; Mandal et al., 2023). To date, fluopyram is registered for use against *Rhizopus* spp. infections in stone fruits and berries (Meredith, 2012). Combinations of fluopyram with chemicals of different fungicide classes, such as trifloxystrobin, are reported to increase its target pathogen range to other plant diseases such as rusts, leaf spots, and fruit rots (Mpina & Mkalanga, 2016; Jaiman et al., 2024; Meredith, 2012). In peanuts, fluopyram is used for the control of root-knot nematodes and suppression of early and late leaf spot (Hagan et al., 2024; Culbreath et al., 2021). However, it is registered and frequently applied as an in-furrow spray on peanuts, but little is known about its efficacy against *Rhizopus* on peanut seeds.

Fludioxonil (4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile), introduced in 1990, hyperactivates the high osmolarity glycerol (HOG) signaling pathway through the sensor protein, histidine kinsase (Kojima et al., 2006). Histidine kinase senses

fluctuations in environmental osmolarity and initiates phosphorylation, thereby activating the HOG pathway and glycerol production (Posas et al., 1996). Glycerol is a protectant compound produced in response to osmotic stress (Duran et al., 2010). The HOG signaling pathway ensures the accumulation of a high intracellular concentration of glycerol to reduce the cellular membrane imbalance of osmotic pressure and prevent water loss (Dihazi et al., 2004). However, the overstimulation in the HOG pathway to produce glycerol by fludioxonil interferes with intracellular osmolarity, causing spore rupture, hyphal swelling, and the inhibition of mycelial growth (Rosslenbroich & Stuebler, 2000; Vetcher et al., 2007; Zhao et al., 2007). Because fludioxonil is fat-soluble, it is partially absorbed into the outer layer of the seeds (Cope & Boobis, 2024). However, most of the applied fludioxonil remains on the seed surface, serving as a protective barrier against soil-borne pathogens (Knauf-Beiter & Zeun, 2012). Therefore, it is classified as a non-systemic, broad-spectrum seed protectant (Cope & Boobis, 2024). Treatment of peanut seeds with Maxim XL® (fludioxonil + metalaxyl) (Syngenta, Greensboro, NC) at the maximum recommended dose (300 mL/100 kg) reduced the incidence of *Rhizopus* and other storage fungi while enhancing seed germination and vigor (Santos et al., 2016). Additionally, field evaluations showed that treating peanut seeds with difenoconazole (1-2 g/kg) and fludioxonil (0.4 g/kg) provided 75% control of *Fusarium solani* and *Aspergillus niger* and increased yield by 5-11% (Lei et al., 2016). Conversely, lab tests from a separate study showed that Maxim XL® (fludioxonil + metalaxyl) (Syngenta, Greensboro, NC) and Maxim Advanced® (fludioxonil + mefenoxam + thiabendazole) (Syngenta, Greensboro, NC) at concentrations of 25 + 10 and 25 + 20 + 150 mL/kg, respectively, controlled *Aspergillus spp.* and *Penicillum* sp. but inhibited peanut seed growth (Barbosa et al., 2013). In field conditions, however, this effect was absent due to water leaching, which reduced chemical concentration (Barbosa et al., 2013).

However, further research is needed to elucidate its efficacy against *Rhizopus* spp. (Bersching & Jacob, 2021).

Phenylamide fungicides, such as mefenoxam (methyl N-(methoxyacetyl)-N-(2,6-xylyl)-D-alaninate), metalaxyl, and benalaxyl, inhibit ribosomal RNA synthesis, specifically RNA polymerization (Gisi & Sierotzki, 2008). As a result, inhibition of mycelia and spore formation occurs (Gomez et al., 2015). In peanut crops, mefenoxam is used as an active ingredient in some seed treatment fungicides to control *Pythium* (Mahoney et al., 2019; Taylor et al., 2002).

An active ingredient in Rancona VPL and VPD® (UPL Corporation Limited Group Company, King of Prussia, PA), ipconazole (2-[(4-chlorophenyl)methyl]-5-(1-methylethyl)-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol) is a systemic, broad-spectrum fungicide that acts as a demethylation inhibitor (DMI) for ergosterol biosynthesis in fungi and manages *Rhizoctonia solani, Fusarium fujikuroi,* and *F. oxysporum* (Villaorduna et al., 2024; Li et al., 2018; Environmental Protection Agency, 2004). Ergosterol is a vital component of fungal cell membranes and facilitates membrane fluidity and permeability (Hata et al., 2010; Rodrigues, 2018). Targeting the C-14 demethylase involved in ergosterol biosynthesis disrupts ergosterol production, excessively increases the membrane's permeability, and results in cell disintegration and death (Lee et al., 2022; Fan et al., 2025). Ipconazole was developed in 1986 and marketed in 1993 as a rice seed disinfectant against many seed pathogens, including *Rhizopus* spp. (Eizuka et al., 1994; Tateishi & Chida, 2000; Li et al., 2018). In a 1998 study, rice seeds treated with wettable powder containing 6% ipconazole showed remarkably reduced mycelial mats of multiple plant pathogens, improved germination rate, and decreased number of diseased seedlings. In the same study, *R. oryzae* isolates extracted from infected rice seeds were particularly sensitive to ipconazole, with EC90 values below 0.5 ug/ml (Tateishi et al., 1998). Applications of ipconazole eventually extended into peanut production. Ipconazole 3.8 FS (0.1

mL) + thiram 75wp (2.5g/kg of seed) was reported to significantly reduced *Aspergillus niger* incidence (6.51%) and sustained abundant pod yield (1864 kg/ha) (Rakholiya et al., 2012).

Launched in 1966, carboxin (5,6-dihydro-2-methyl-1,4-oxathiine-3-carboxanilide) was one of the earliest systemic fungicides inhibiting the respiratory electron transport chain of the mitochondrial succinate dehydrogenase in complex II and primarily controls basidiomycete pathogens, such as rusts, smuts, and *Rhizoctonia* spp (von Schmeling & Kulka, 1966; Newcombe & Thomas, 1990; Sierotzki & Scalliet, 2013; Yanase et al., 2007; Morton & Staub, 2008; White & Georgopoulos, 1992). When combined with thiram, excellent control of-peanut seed and seedling diseases can be achieved (Srinivas et al., 2023; Akgul et al., 2011; Hassuba et al., 2016) In field experiments, applications of equal parts thiram and carboxin (37.5%) (Vitavax 200, Chemtura Corporation 199 Benson Road Middlebury, CT 06749) reduced peanut seed pre-and-post emergence damping off 14 and 42 days after planting (Hassuba et al., 2016). *In vitro* and field experiments in Turkey reported similar results, stating the fungicide significantly decreased disease severity in peanut seeds (Akgul et al., 2011).

Aside from fungicides, the damaging effects of peanut seed and seedling diseases may be reduced with proper handling and cultural practices. Compromised or excessively damaged seed should be discarded and replaced with high-quality seed. Maintaining intact seeds by controlling storage pests and avoiding injury during harvest and shelling decreases fungal infestation of the seed (Jackson & Bell, 1969; Wood, 1968; Moorwood, 1954). To facilitate successful germination in the field, it is recommended to plant in a sustained, 4-inch soil temperature between 20-32 °C with a seeding rate of six seeds per linear foot and soil moisture levels between 40% and 50% of the total soil volume in the podding zone (Kvien et al., 2022; Marsalis et al., 2009; Brenneman, 2021; Tillman et al., 2006; Melouk & Backman, 1995; Ono et al., 1974). Implementing proper handling and cultural practices alongside seed treatment fungicides

can significantly enhance seed health and crop establishment, creating a combined, powerful effect.

In conclusion, this literature review provides insight into peanut physiology and the destructive nature of *Rhizopus* spp. on peanut seeds and seedlings. It connects the historical research with modern agricultural advancements, demonstrating the need for updated studies on *Rhizopus* peanut seed rot to address the decades-long knowledge gaps. The biology, temperature preferences, molecular identification, and chemical sensitivities of *Rhizopus* spp. are discussed in detail. Overall, this review emphasizes the importance of reassessing current seed and seedling disease management strategies against *Rhizopus* spp. in peanut production.

6 OBJECTIVES

The objectives of this study were to: 1) collect a set of *Rhizopus* isolates from infected peanut seeds in Georgia and identify them to species, 2) assess the seed quality and vigor of thirteen modern peanut cultivars to help identify potential physiological advantages related to rapid germination and avoiding seed death by *Rhizopus* infection, 3) develop a novel pathogenicity assay to assess the effect of Rhizopus seed rot on modern peanut cultivars, 4) assess the effect of five temperature conditions (15 °C, 20 °C, 25 °C, 30 °C, and 35 °C) on the radial mycelial growth and virulence of *Rhizopus* spp. on peanut seeds, and 5) evaluate the sensitivity of *Rhizopus* isolates (*R. delemar, R. arrhizus,* and *R. stolonifer*) to eight seed treatment and in-furrow fungicides by determining the effective concentration inhibiting 50% growth (EC_{50}). Altogether, this research contributes to a deeper understanding of peanut seed health and offers insights into the biology, virulence, and management of *Rhizopus* spp.

Tables

Table 1. List of thirteen peanut genotypes assessed for vigor in this study

Commercial Peanut Cultivars		
'TifJumbo'	'TUFRunner™ 297'	'Georgia-09B'
'TifNV-HG'	'Georgia-20VHO'	'Georgia012Y'
'TifNV-High O/L'	'Georgia-19HP'	
'Georgia-06G'	'Florun T61'	
'Georgia-21GR'	'Georgia-22MPR'	

Table 2. Taxonomic Classification of the Genus *Rhizopus*

Taxonomic Levels	**Taxonomic Names**
Kingdom	Fungi
Phylum	Zygomycota
Subphylum	Mucoromycotina
Class	Zygomycetes
Order	Mucorales
Family	Mucoraceae
Genus	*Rhizopus*
Species	*stolonifer, delemar, arrhizus,* etc.

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CHAPTER 2

RELATIVE VIGOR OF PEANUT (*ARACHIS HYPOGAEA* L.) CULTIVARS, MOLECULAR IDENTIFICATION, AND NOVEL PATHOGENICITY ASSAY OF *RHIZOPUS* SPECIES INFECTING PEANUT SEEDS IN GEORGIA[1]

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ABSTRACT

Rhizopus seed and seedling rot is a highly destructive peanut disease, causing rapid seed decay in 36-96 hours and rendering seeds and pre-emerged seedlings indistinguishable from the soil. This study evaluated the seed vigor of thirteen peanut cultivars, as well as the molecular identification and pathogenicity of *Rhizopus* spp. isolated from peanut seeds in Georgia. All tested peanut cultivars exhibited high germination (>95.3%), with some variability in germination rates across cultivars suggesting slight differences in physiological potential across cultivars. In the context of Rhizopus seed rot, faster germination may potentially reduce susceptibility. Analysis of isolates from several commercial seed lots in Georgia identified three *Rhizopus* spp., based on morphology and the TEF-1α gene (*R. delemar, R. arrhizus,* and *R. stolonifer).* A novel pathogenicity assay of *Rhizopus* spp. on peanut seeds revealed that, compared to the non-treated control seeds, all peanut cultivars were highly susceptible, exhibiting a significant reduction in germination and radicle development. However, across both *R. delemar* and *R. stolonifer* inoculations, 'Georgia-12Y' and 'Tufrunner 297' consistently achieved germination values above 22%, suggesting these cultivars may have partial tolerance or an escape mechanism to Rhizopus seed rot. However, future investigations must be conducted to determine this possibility.

Keywords: *Rhizopus* spp., peanut seeds, vigor, identification, pathogenicity

1 INTRODUCTION

Peanuts (*Arachis hypogaea* L.) are an annual, but botanically perennial, herbaceous crop and a rich source of plant-based protein, unsaturated fat, minerals, and vitamins in the mammalian diet (Tallury, 2017; Kvien et al., 2022). The United States (U.S.) is one of the most high-yielding countries and is ranked fourth in the world for total peanut production, proceeding China, India, and Nigeria, respectively (USDA, 2024). A sizeable portion of the United States' peanuts come from Georgia annually which produces approximately 50% of the nation's peanuts (USDA, 2024).

High-quality seed is an important component of any production system. The main components of seed physiological potential are germination and vigor (Marcos-Filho, 2015). Germination occurs when embryo growth overcomes the constraints of the testa, or seed coat (Bewley & Black, 1994). Although germination undergoes many processes, it has been described in three general steps: imbibition, reactivation of metabolism by many biochemical processes, and radicle protrusion (Sghaier et al., 2022; Ali & Elozeiri, 2017). During imbibition, water is absorbed by the dormant seed's cell walls and held by the electrostatic forces in hydrogen bonds (Woodstock, 1988). Diffusion and capillary action aid water movement into the seed (Paiva et al., 2006). The seed coat's permeability can influence the rate of water uptake (Woodstock, 1988; Smykal et al., 2014). After imbibition, the second step undergoes several biochemical processes that are necessary for germination, such as the synthesis of nucleic acids and proteins, hydrolysis, the Krebs cycle, respiration, the translation of stored mRNA, and cell elongation (Bewley et al., 2013). Radicle protrusion marks the beginning of seedling emergence (Bewley, 1997), which is the first major event after germination and initiates the plant's production and establishment (Awal & Ikeda, 2002).

Seed vigor is defined as the seed's potential to maintain quality in storage under a wide range of conditions and quickly emerge to establish uniform plant stands (Reed et al., 2022). The relative seed and seedling vigor directly affects germination and development under various environmental conditions and reflects the rate and potential of seeds to establish healthy and uniform plants. Generally, seeds with high vigor more efficiently mobilize reserves from storage tissues and develop healthy seedlings (Marcos-Filho, 2015). However, environmental factors, such as water availability, temperature, and storage conditions, can affect seed vigor and quality (Moreno et al., 2024; Reed et al., 2022; Suriyasak et al., 2020; Woodroof, 1973; Smith et al., 1995). Therefore, selecting high-quality seeds and implementing proper crop management and protection strategies are critical (Melouk & Backman, 1995). Seed vigor assays are widely used to assess seed performance, with their two goals of assessing seed quality and determining differences in physiological potential among commercial cultivars.

Rhizopus seed and seedling rot is a highly destructive peanut disease, causing rapid seed decay in 36-96 hours and often leaving the seeds and pre-emerged seedlings indistinguishable from the soil (Clinton, 1960). While the historic literature reported its pathogenicity and temperature response on peanut seeds, little is known about the relative susceptibility of modern peanut cultivars to Rhizopus seed rot. Modern peanut cultivars such as 'Georgia-06G', which accounts for over half of the nation's certified peanut acres (Brown, 2023), have not been thoroughly examined for their susceptibility to Rhizopus seed rot. This knowledge gap may be partially due to shifting research priorities to other plant pathogens, such as *Aspergillus* spp., or the development of more effective seed treatment fungicides which have reduced the impact of the disease. However, given its ubiquitous distribution in temperature and subtropical regions and ability to infect over 100 crop species, Rhizopus seed rot remains a threat in peanut production.

Identifying the *Rhizopus* species in peanut seed lots can lead to a greater understanding of the pathogen's geographical distribution, abundancy, and life cycle. Before the advent of molecular identification, *Rhizopus* species were primarily identified by morphological and physiological characteristics. However, *Rhizopus* species, or any plant pathogen, should not be identified or diagnosed by morphology or physiology alone. Similar morphological features across plant pathogens in the same or different genus, morphological evolution occurring at different rates, the presence of secondary pathogens on infected sites, latent symptoms, and varying virulence and behavior among strains can lead to incorrect identifications and misguided assumptions about *Rhizopus* (Baum, 2008; Nguyen et al., 2023). Additionally, there is no widely accepted standard for how much morphological difference is needed to classify a *Rhizopus* species as a new species or a strain of an existing species to date (Abe et al., 2010). Therefore, other methods, such as microscopy, serological tests, biochemical tests, and polymerase chain reaction (PCR) testing (Mendonca et al., 2022), reduce the possibility of an incorrect diagnosis and provide standardization in differentiating *Rhizopus* species. DNA-based identification provides high accuracy, sensitivity, specificity, and a more robust understanding of evolutionary relationships between species (Bell, 1989). Public platforms, such as the NCBI Basic Local Alignment Search Tool (BLAST), provide even further accuracy by comparing DNA against large global databases. This method establishes a more standardized approach to identification beyond morphological features, which can sometimes be an inaccurate reflection of phylogenetic relationships (Abe et al., 2010). A much greater comprehensive analysis of *Rhizopus* species can be achieved by the utilization of both morphological and molecular identification

The translation elongation factor 1-alpha (TEF-1) gene is responsible for the transport of amino-acyl tRNAs to ribosome and is one of the most abundant soluble proteins in eukaryotic cells (Slobin, 1980). The gene is highly conserved, meaning it has not evolved much over a long

period of time (Merrick, 1992). Genes that remain conserved typically serve an essential function for the organism's survival (Luo et al., 2015). Therefore, its conserved nature makes it a useful and standard identification marker in differentiating multiple *Rhizopus* spp (O'Donnell et al., 2001; Abe et al., 2010).

2 MATERIALS AND METHODS

2.1. Relative Seed Vigor of Thirteen Commercial Peanut Cultivars

Because peanut seeds grow in the soil and are inherently susceptible to infection by a range of soilborne pathogens, such as *Rhizopus,* colonization can be influenced by many factors, including field conditions, harvest procedures, methods of drying, and others (Fernandez et al., 1997). To accurately compare the susceptibility of peanut cultivars, it is crucial that all cultivars were produced and handled under uniform conditions. Therefore, all seed for this study were grown in a field at the University of Georgia (UGA) Blackshank Farms in Tifton, GA (31.50°N, -83.544°W) in 2023 on Tifton loamy sand with a 2-5% slope. The field was previously planted to peanuts, but prior to planting in 2023, the field was fumigated by injecting 336.25 kg/ha chloropicrin into the soil, and a plastic sheet covered the field for seven days to reduce the presence of harmful soil-borne microorganisms. Soil sample tests revealed a pH of 6.1 and nutrient levels of phosphorus at 12.5 ppm, potassium at 36 ppm, and magnesium at 17.7 ppm. Thirteen peanut cultivars were used in this study, consisting of 'Florun T61', 'Georgia-06G', 'Georgia-09B', 'Georgia-12Y', 'Georgia-18RU', 'Georgia-19HP', 'Georgia-20VHO', 'Georgia-21GR', 'Georgia-22MPR', 'TifJumbo', 'TifNV-HG', 'TifNV-High O/L', and 'TUFRunner™ 297'. Each plot was planted at 29 seeds per meter of row at approximately 5 cm deep into a conventionally tilled seedbed using a 2-row Monosem precision air planter (Monosem, Inc., Edwardsville, KS). Plots consisted of two rows, 4.57 m long and 1.83 m wide, with 0.91 m row

spacing. Standard production practices were followed. Cover sprays of chlorothalonil (1.75 L/ha) were applied using TX-12 tips and 50 mesh ball check screens, with a spray volume of 184.25 L/ha, at a pressure of 2.21 bar, and a ground speed of 6.92 km/h. Cover sprays were applied on 27 June, 12 July, 26 July, 21 August, 5 September, and 19 September. The site was irrigated throughout the growing season to ensure adequate water application. Conventional peanut production practices were used for in-season pest control following UGA Cooperative Extension Service recommendations (Monfort et al., 2022). A randomized complete block design with four replications was used. Peanuts were planted on 19 May, dug on 16 October, and harvested on 20 October. Peanuts were harvested after the taproots sun-dried in the field and threshed using a plot thresher (Kingaroy Engineering Works, Kingaroy, Queensland, AU). To minimize water content and contamination risks, the pods were placed in a wagon at 32 °C until the moisture content reached approximately 8%. All pods were placed in burlap bags and transferred to the Plant Pathology Headhouse at the University of Georgia in Tifton, GA for further processing and analysis. The remaining pods were placed in sealed plastic bags in cold storage (5 °C) for future use.

The Peanut Seed Vigor Test "P50" Method was followed to assess the relative vigor of seeds from all cultivars (ISTA, 2015). For each cultivar, the pods were hand-shelled, and fifty seeds with four replications were placed on a 14 x 2 cm Petri dish (Thermo Scientific, Waltham, MA, USA) with moistened 14 cm blotter paper (Thermo Scientific, Waltham, MA, USA) in a completely randomized design (CRD). The water volume for each Petri dish was calculated by multiplying the weight of the dry blotting paper by two. All Petri dishes were sealed in 63.5 × 46.5 cm plastic bags and stored in an incubator for ten days at 25 °C. To assess the seed germination rate, measurements were taken every six hours for twelve days by documenting radicle protrusion (≥ 2 mm).

The means were compared using the Student T Test at $\alpha < 0.05$ in the software program Germinator package by Wageningen University. The Germinator package is an easy-to-use, cost-effective, and adaptable system for germination scoring and evaluation, providing curve fitting for cumulative germination data, alongside the extraction, summarization, and visualization of key germination parameters (Joosen et al., 2010). The analysis of variance (ANOVA) was conducted on SAS 9.4 (SAS Institute, Cary, NC, USA) to determine differences in germination between cultivars over time where replication and run were treated as random effects. Germination was compared using least significant differences (LSD) with α of 0.05 to determine differences between cultivars at each rating. Germination over time (in hours) of each cultivar was modeled using the Gompertz three-parameter equation in SigmaPlot (Version 16.0; Systat Software, 2024).

[1]

In this equation, *a* is the upper asymptote or maximum germination, *b* is the rate constant reflecting the steepness of the sigmoidal curve, *c* is the mean germination time (T_{50}) or the number of hours required for 50% of the seeds to germinate in each cultivar (Bewley et al., 2013), and *x* is the germination rate. The mean germination time (MGT) represents the time in hours required for the seeds to reach maximum germination (Orchard, 1977). The coefficient of determination (R^2) represents the adjusted goodness-of-fit value. Both tests were combined for analysis due to no significant differences between tests at the 0.05 probability level.

2.2. Assay of Commercial Seed Lots and Identification of Three *Rhizopus spp.*

A total of five compromised peanut seed lots were utilized, with two originating from the 2021-2022 harvest (Lot #5002 and #7011) and three from the 2022-2023 harvest (Lot #6832, #677, and #781). Lot #5002 consisted of cultivar 'Georgia-18RU' with 100% incidence of *Rhizopus* spp. Lot #7011 consisted of 'FloRun™ '331' with 10% incidence of *Rhizopus* spp.

Lots #6832 (Premium Peanut, Douglas, GA) and #677 (Olam Edible Nuts, Sylvester, GA) consisted of 'Georgia-06G' with 54% and 63% incidence of *Rhizopus* spp., respectively. All pods were transferred to the lab, hand shelled, and placed in sealed plastic bags in cold storage (5 °C) for future use.

Rhizopus isolates were sourced from naturally infected peanut seeds in five commercial seed lots in Georgia, two from the 2021-2022 harvests and three from the 2022-2023 harvests. Peanut seeds were surface sterilized with 10% Clorox solution for three minutes, rinsed with sterile water, dried with sterile paper towels, and placed on potato dextrose agar (PDA) medium Petri dishes (100 x 15 mm) at 25 °C in an incubator with continuous fluorescent light for five days. To isolate the pathogen, a single hyphal tip was extracted with a sterile needle from the colony's outermost growth and placed on a sterile Petri dish containing full-strength PDA. Pure cultures were made by extracting a small portion of the colony's outermost growth and streaking it over an agar surface in a Petri dish. All cultures were made on full-strength PDA plates and sealed with Parafilm® M (Bemis Company Inc., Neenah, WI) and incubated at 25 °C with continuous fluorescent light. Microscopic examinations of 7-day-old cultures of each isolate were performed at 40x, 100x, and 400x magnification to observe morphology and assess purity. The spore suspension was made by adding 1 mL sterilized deionized water to 7-day-old cultures in Petri dishes, scraping the agar surface with a sterile inoculation loop to dislodge the spores, and dispensing into sterile 1.5 mL microcentrifuge tubes. For long-term storage, three aliquots of spore suspension in water were made for each isolate, with two stored at -20 °C and one at -80 °C.

Spore suspensions were obtained from naturally infected peanut seeds from five commercial lots in Georgia in 2023 using the aforementioned isolation method. The spore concentrations were determined with a hemocytometer and adjusted to 10^5 spores/mL. An aliquot

of spore suspension (7 µl, 10^5 spores/mL) was cultured on full-strength potato dextrose agar (PDA) media at 25 °C for four days. Approximately 5 mL of 0.9% NaCl solution was added to the plate, and spores were gently scraped with a sterile inoculation loop. 1 mL of washed spores were transferred into a sterile microcentrifuge tube. Thirty and fifteen isolates were processed with the DNeasy Plant Pro Kit (Qiagen, Germantown, MD, US) and the Fungi/Yeast Genomic DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, CA), respectively. The isolates were processed using the DNA extraction method described in the manufacturer's protocol with minor adjustments to the elution step. An additional elution was performed by adding 50 µl of the elution buffer to the column and centrifuging for two minutes at 10,000 revolutions per minute (RPM). The samples were diluted with sterile deionized water to 50 to 70 ng/µL in preparation for DNA quantification. DNA quality control was checked by the Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The samples were stored in a -80 °C freezer.

As described by O'Donnell et al. (2001), the presence of amplifiable DNA in the translation elongation factor 1-alpha gene (TEF-1) was evaluated by PCR using the forward and reverse primers MEF-10 and MEF-4. The T100 Thermal Cycler (Bio-Rad Inc., Hercules CA, USA) was used to perform the polymerase chain reaction (PCR). The cycling condition included an initial denaturation step at 95 °C for 2 minutes, followed by 34 cycles of denaturation at 94 °C for 15 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute, and five minutes of elongation at 72 °C. The cycling procedure was completed in approximately two hours. PCR products were visualized by gel electrophoresis, which required loading 5 ul of PCR products on a 1% agarose gel stained with ethidium bromide (Figure 1). A 100-bp DNA ladder (Biotium, Fremont, CA, U.S.A.) was used to determine the size of the PCR product after fragment separation with electrophoresis at 130 volts (V) for thirty minutes. DNA bands were

observed under the Bio-Rad Molecular Image Gel Doc XR+ with Image Lab Software (Bio-Rad Laboratories, Hercules, CA). Amplified DNA was purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-tec, Inc., Norcross, GA). The samples were premixed with 10 µl DNA and 5 µl the forward primer, MEF-10, and sent for Sanger sequencing by Eurofins Genomics LLC (Louisville, Kentucky, U.S.). Raw reads were then trimmed, edited, and aligned with Molecular Evolutionary Genetics Analysis (MEGA) 7 software using the maximum likelihood method (University of Kent, Canterbury, Kent, United Kingdom) (Tamura et al., 2011; Stecher et al., 2020). *Rhizopus* species were identified from the highest matches based on pairwise identity (≥97%) and query coverage (≥95%) with DNA sequences from known *Rhizopus* species in GenBank from the National Center for Biotechnology Information (Bethesda, MD, U.S.) using the Basic Local Alignment Search Tool (BLAST).

2.3. Pathogenicity of *Rhizopus* spp. to Thirteen Commercial Peanut Cultivars

A novel pathogenicity assay for *Rhizopus* on peanut seeds was developed to accomplish this objective. Plates of full-strength potato dextrose agar (PDA) media were centrally inoculated (7 µl, 10^5 spores/mL concentration) with either *R. stolonifer* or *R. delemar* and incubated for 24 hours at 25 °C. The 24-hour incubation period allowed the culture to grow large enough to adequately fit three surface-sterilized, untreated peanut seeds around the colony's border. After the 24-hour incubation period, three surface-sterilized seeds were placed carefully around the colony's border. Peanut seeds were surface sterilized with 10% Clorox solution for three minutes, rinsed with sterile water, and dried with sterile paper towels. Control plates were inoculated (7 µl) with sterilized water. The plates were placed in the incubator at 25 °C in continuous, fluorescent light and rated for germination for five days. In total eighteen surface-sterilized seeds per cultivar were plated on PDA with three seeds per plate in a nested, randomized complete block design (RCBD) with six replications. Each isolate represented a

whole block, and the seeds nested within the plates served as the experimental units. Final germination and radicle length were measured six days after inoculation (Figure 2). The test was repeated twice for each cultivar and isolate combination, and all thirteen cultivars were tested simultaneously in each test. Although each test was completed within six days, plates were monitored for several additional weeks to observe the longer-term effects of Rhizopus on peanut seeds. During this extended period of monitoring, *Rhizopus* consistently outgrew all seeds, preventing germination in many instances.

To quantify the seed and seedling response of each peanut cultivar to *R. delemar* and *R. stolonifer*, generalized linear mixed model (GLMM) analyses for each *Rhizopus* spp. were conducted using the PROC GLIMMIX procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). The cumulative percentage of germinated seeds, the percentage of seeds with radicle protrusion greater than 10 mm, and the percentage of seeds with radicle protrusion less than 10 mm were analyzed separately for each *Rhizopus* spp., as well as the non-treated control. In the inoculated treatments, peanut cultivar and test, as well as their interaction, were treated as independent variables to evaluate cultivar performance and accommodate for potential variability across test conditions. The replications were treated as random variables nested within each test based on the experimental design. For the non-treated control group, one-way analyses of variance (ANOVA) were conducted using the PROC GLM procedure, with peanut cultivar as the independent variable. Post-hoc pairwise comparisons among peanut cultivars were conducted using the Least Significant Difference (LSD) test at the 95% confidence level ($P \leq 0.05$), and grouping letters were assigned based on significant differences.

3 RESULTS AND DISCUSSION

3.1. Relative Seed Vigor of Thirteen Commercial Peanut Cultivars

Across all peanut cultivars, the adjusted R^2 values ranged from 0.95 to 0.99, demonstrating a strong fit to the Gompertz three-parameter model (Table 3). The maximum germination for all cultivars ranged from 95.3% by 'Georgia-21GR' to 98.8% by 'Florun T61'. All cultivars exhibited high germination, indicating that the seeds were vigorous and high quality. In the Gompertz three-parameter model, parameter *b* represents a rate constant reflecting the steepness of the sigmoidal curve and quantifies the rate of germination during the exponential phase, or the part of the sigmoidal curve where the rate of change is the fastest. Since germination was measured every six hours, *b* is expressed per 6-hour interval. Smaller *b* values indicate a steeper curve and faster germination rate, while larger values indicate a slower, more gradual germination rate. Slope values in the Gompertz three-parameter model significantly varied across peanut cultivars, ranging from 8.1 for 'Georgia-12Y' to 16.8 for 'Georgia-19HP.' 'Georgia-19HP' exhibited a gradual slope and the longest median germination time (80.5 hours), suggesting a delayed and gradual germination progression. Conversely, 'Georgia-12Y,' 'Georgia-06G,' 'Georgia-20VHO,' and 'Georgia-18RU' exhibited *b* values below 10 and median germination time (T_{50}) values at 65.5, 65.8, 63, and 64.8 hours, respectively, indicating a rapid progression in germination. 'Georgia-21GR' was the only peanut cultivar that required more than 148 hours to reach maximum germination. Conversely, 'Georgia-12Y,' 'Georgia-06G,' 'Tufrunner 297,' 'Georgia-20VHO,' 'Georgia-18RU,' 'Florun T61,' and 'Georgia-22MPR' reached maximum germination in under 115 hours, making them statistically distinct from 'Georgia-21GR.' Peanut cultivars with shorter mean germination time (MGT) values may be favorable in avoiding seed infection by Rhizopus seed rot. While all cultivars exhibited high germination, differences in the germination slopes, T_{50} values, and MGT values demonstrate the relative variability of germination characteristics across cultivars.

The two goals of a seed vigor assay are to assess seed quality and determine differences in physiological potential among commercial cultivars. The relative seed and seedling vigor directly affects germination and development under various environmental conditions and reflects the rate and potential of seeds to establish healthy and uniform plants (Basu & Groot, 2023). The seeds of each cultivar tested were high quality, and final germination was equal or greater than 95.3%. Based on the literature, it may be beneficial to select a vigorous cultivar to reduce the chances of seed death by *Rhizopus* infection. Ungerminated seeds are most susceptible to Rhizopus seed rot directly after planting (Melouk & Backman, 1995), and damage often decreases after emergence (Gibson & Clinton, 1953; Clinton, 1960). The seed's susceptibility to preemergence rot increases if germination is delayed or the seed is damaged (Arant et al., 1951; Clinton, 1960). While *Rhizopus* is occasionally recorded to cause aboveground rot symptoms on emerged seedlings (Xu et al., 2015), these findings are not as frequently documented as those described on ungerminated seeds and pre-emerged seedlings (Gibson, 1953; Moorwood, 1953; Arant et al., 1951; Frank, 1969; Gupta & Chohan, 1970). Therefore, selecting a cultivar that quickly and uniformly emerges may reduce the incidence of Rhizopus seed rot.

3.2. Morphological and Molecular Identification of Three *Rhizopus* spp.

In this study, three *Rhizopus* species *(R. delemar, R. arrhizus,* and *R. stolonifer)* were identified from five commercial peanut seed lots in Georgia (Figure 3). DNA was successfully amplified using the primer pairs MEF-10 and MEF-4 targeting the translation elongation factor 1-alpha (TEF-1) gene. Sequences derived from the TEF-1 gene were compared to reference sequences in the NCBI nucleotide database using the BLAST tool and revealed that 26, 16, and 3 isolates shared the highest nucleotide identity with *R. delemar, R. arrhizus,* and *R. stolonifer*, respectively (Figure 4). In total, three main clades were established. The largest group, *R.*

delemar, includes the type strain CBS 120.12. The second largest group, *R. arrhizus*, includes the type strain CBS 112.07. The smallest group, *R. stolonifer*, also includes the type strain NRRL1477 and has the highest bootstrap values, which indicates high confidence in its separation.

According to the selected phylogenetic tree, *R. delemar* and *R. arrhizus* form a monophyletic clade, meaning they share a single common ancestor (Gryganski et al., 2018; Abe et al., 2010). The genetic differences between *R. arrhizus* and *R. delemar* are smaller than their differences from *R. stolonifer.* It is theorized that *R. stolonifer* diverged earlier in time than *R. delemar* and *R. arrhizus,* which may explain its genetic distinction from *R. delemar* and *R. arrhizus.* This phylogenetic analysis is supported by the literature (Gryganski et al., 2018; Abe et al., 2010; Gnanesh et al., 2020; Vebliza et al., 2018; Hartanti et al., 2015; Hartanti et al., 2020). However, given the smaller number of specimens in the *R. stolonifer* clade, this assessment might be modified in further analysis.

Based on the molecular study, the causal organism of pre-emergence seed rot in five commercial seed lots in Georgia was *Rhizopus stolonifer, Rhizopus delemar,* and *Rhizopus arrhizus.* The molecular identification of the three *Rhizopus* spp. was determined by the amplification and sequencing of TEF-1 gene with the paired primers MEF10/MEF4, a method utilized in published studies to precisely distinguish closely related taxa (O'Donnell, 2001, Abe et al., 2010). All tested isolates of *Rhizopus* were highly pathogenic, infecting seeds of thirteen peanut cultivars and significantly reducing germination and radicle development. While this molecular study did not encompass all *Rhizopus* isolates in Georgia, it provided a contribution to the expanding database of mycoflora in Georgia peanut seeds and a precursor for future investigations.

3.3. Pathogenicity of *Rhizopus* spp. to Thirteen Commercial Peanut Cultivars

Table 4 shows the susceptibility of thirteen peanut cultivars to Rhizopus seed rot caused by *R. delemar.* Across all cultivars, the non-treated control seeds exhibited 83.4% to 100% germination. While 'TifJumbo,' 'Georgia-12Y,' 'Tufrunner 297,' 'Georgia-21GR,' 'Georgia-09B,' and 'Georgia-19HP' are statistically similar for maximum germination percentages under inoculated conditions, only 'Tufrunner 297,' 'TifJumbo,' and 'Georgia-12Y,' achieved at least 25% germination. In contrast, 'Florun T61,' 'Georgia-20VHO,' 'Georgia-22MPR,' 'Georgia-22MPR,' 'Georgia-06G,' 'TifNV-HG,' 'TifNV-High O/L,' and 'Georgia-18RU' germinated below 8% under inoculated conditions. Specifically, three cultivars, 'TifNV-HG, 'TifNV-High O/L,' and 'Georgia-18RU,' germinated 3.4%, 0%, and 0%, respectively, indicating severe susceptibility. However, several other cultivars, such as 'Georgia-21GR,' 'Georgia-19HP,' 'Florun T61,' 'Georgia-20VHO,' 'Georgia-22MPR,' and 'Georgia-06G,' shared the same grouping letter and, therefore, were not statistically different from the numerically lowest cultivars. A rapid decline in seed health was observed in radicle development, as most cultivars had no seeds with radicles longer than 10 mm. 'Georgia-22MPR' (4.2%) was the only cultivar to achieve significant radicle length longer than 10 mm. Most germinated seeds under inoculated conditions produced radicles below 10 mm, following similar trends to the maximum germination percentages across cultivars. Compared to the inoculated seeds, the non-treated control seeds performed excellently, with all cultivars achieving over 83% germination, and similar trends observed in radicle lengths above 10 mm.

Table 5 shows the susceptibility of thirteen peanut cultivars to Rhizopus seed rot caused by *R. stolonifer.* For the inoculated seeds, germination rates ranged from 13.8% to 33.4%. 'Georgia-21GR' was the only cultivar to achieve above 33% germination, followed by 'Georgia-12Y' and 'Georgia-22MPR' at 27.8%. In contrast, 'Georgia-09B,' 'Georgia- 19HP,' 'Georgia-20VHO,' 'TifNV-High O/L,' and 'Georgia-18RU,' exhibited the numerically lowest germination

values at 13.8%. Despite these numerical differences, all peanut cultivars were significantly similar in germination at the $P \leq 0.05$ level. These observations in germination suggest a uniform response to inoculation across cultivars, with no cultivar demonstrating higher tolerance or susceptibility based on germination alone. Additionally, *R. stolonifer* may be slightly less virulent on peanut seeds than *R. delemar*, allowing more seeds to germinate and develop radicles.

For the inoculated seeds, radicle development varied among the thirteen peanut cultivars, with values ranging from 0% to 11.2% (Table 3). Despite numerical differences, all cultivars with at least some radicle protrusion above 10 mm were statistically similar. 'Georgia-21GR' was the only cultivar to achieve radicle development above 10 mm at 11.2%, while 'TifJumbo,' 'Georgia-12Y,' 'Georgia-09B,' 'Florun T61,' 'Georgia-06G,' 'TifNV-High O/L' showed radicle development above 10 mm at values below 6%. Six cultivars, including 'Tufrunner 297,' 'Georgia- 19HP,' 'Georgia-20VHO,' 'Georgia-22MPR,' 'TifNV-HG,' 'Georgia-18RU,' exhibited 0% radicle protrusion above 10 mm, indicating severely poor radicle development under inoculated conditions. However, except for 'Georgia-21GR,' the lowest-performing peanut cultivars were statistically similar to those that had radicle protrusion above 10 mm at values below 6%. Compared to the inoculated seeds, the non-treated control seeds performed excellently, with all cultivars achieving over 83% germination, and similar trends observed in radicle lengths above 10 mm.

Across both *R. delemar* and *R. stolonifer* inoculations, 'Georgia-12Y' and 'Tufrunner 297' consistently exhibited germination values above 22%. 'Georgia-12Y' achieved 25% germination under *R. delemar* and 27.8% under *R. stolonifer*, while 'Tufrunner 297' achieved 26.6% and 22.2% germination, respectively. These findings may suggest a possible biochemical or physiological escape mechanism or tolerance in 'Georgia-12Y' and 'Tufrunner 297.' Disease

escape is defined as the plant's ability to avoid or reduce infection through environmental conditions, growth habits, or timing, rather than having inherent resistance (Paveley et al., 2005). The plant or seed "escapes" the disease due to unfavorable conditions for disease development or pathogen and plant interactions. Tolerance is defined as the plant's ability to continue growing without significant reductions in development or yield despite infection (Ney et al., 2012). Biochemical compounds in the testa, such as tannins and flavonoid compounds, have been linked to the inhibition of *A. flavus* (Azaizeh et al., 1990; Mendu et al., 2022). As well as the inhibition of fungal infection by biochemical compounds, it is reported that inhibition can be attributed to the seed's physiological characteristics, such as testa thickness or permeability, the density of palisade cell layers, and the presence of wax layers (Olwari et al., 2013; LaParade et al., 1973; Upadhyaya et al., 2002). However, no research in the existing literature has established a direct association between mechanisms of escape or tolerance in peanut seeds and Rhizopus seed rot. Further investigations must be conducted to determine this possibility.

4 PRACTICAL IMPLICATIONS

Overall, compared to the control seeds, all thirteen peanut cultivars exhibited a severe reduction in germination and subsequent radicle development in response to inoculations of *R. delemar* and *R. stolonifer*. Moreover, the results suggested that *R. delemar* may be more virulent on peanut seeds than *R. stolonifer*. Additionally, the relatively and consistently higher germination values of some peanut cultivars, such as 'Georgia-12Y' and 'Tufrunner 297,' across both *R. delemar* and *R. stolonifer* inoculations suggested that a possible biochemical or physiological escape mechanism or tolerance to Rhizopus seed rot exists. Future investigations must be conducted to determine the possibility of an escape mechanism or tolerance in peanut cultivars to Rhizopus seed rot. The significant negative effects of Rhizopus seed rot on the

thirteen tested cultivars emphasize the necessity of effective management strategies, such as consistent cultural practices and seed treatment fungicides.

Tables

Table 3. Seed Vigor Parameters of Thirteen Peanut Cultivars Based on the Gompertz Three-Parameter Model.[a,b]

	a[c]	*b*[d]	*c*[e]	*MGT*	R^2
Cultivar	%	per 6-hour interval	Hours	hours	
'TifJumbo'	98.4 a	14.1 bc	78.6 f	124.5 ab	0.96
'TifNV-HG'	98.1 a	11.5 ab	73.6 e	126.7 ab	0.98
'TifNV-High O/L'	98.9 a	14.9 bc	73.8 e	123 ab	0.95
'Georgia-06G'	97.5 a	9.6 a	65.8 b	108 a	0.98
'Georgia-21GR'	95.3 b	12.1 b	67.7 c	148.5 b	0.98
'Tufrunner 297'	98.5 a	13.1 b	74.2 e	114 a	0.96
'Georgia-20VHO'	95.6 b	8.4 a	63.0 a	106.5 a	0.97
'Georgia-19HP'	96.8 ab	16.8 c	80.5 f	126.7 ab	0.95
'Georgia-18RU'	97.1 ab	9.7 a	64.8 b	105.7 a	0.96
'Florun T61'	98.8 a	11.2 ab	70.6 d	112.5 a	0.99
'Georgia-22MPR'	98.2 a	15.0 bc	70.3 d	111.7 a	0.96
'Georgia-09B'	98.3 a	11.6 ab	68.4 cd	115.5 ab	0.96
'Georgia-12Y'	97.0 ab	8.1 a	65.5 b	105 a	0.98

[a]The Gompertz three-parameter model was used to generate parameters *a*, *b*, and *c*, where *x* = germination rate. Values within a column followed by the same letter are not significantly different at the $\alpha = 0.05$ probability level according to the 95% confidence intervals.

[b]Abbreviations: NA, not applicable

[c]Maximum germination.

[d]Slope.

[e]T_{50}: Median germination time (hours)

MGT: Mean germination time (hours); values were generated via ANOVA and $\alpha = 0.05$

Table 4. Susceptibility of Thirteen Peanut Cultivar to Rhizopus Seed Rot caused by *R. delemar*[a,b]

	Germination (%)[b]		Radicle Protrusion ≥ 10 mm (%)		Radicle Protrusion ≤ 10 mm (%)		No Germination (%)	
Cultivar	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control
'TifJumbo'	25 a[a]	100 a	0 b	100 a	25 ab	0 b	75 a	0 a
'Georgia-12Y'	25 a	88.8 ab	0 b	88.8 a	25 ab	0 b	75 a	11.2 ab
'Tufrunner 297'	26.6 a	83.4 b	0 b	83.4 ab	26.6 a	0 b	73.4 a	16.6 b
'Georgia-21GR'	16.6 abc	83.4 b	0 b	70.8 b	16.6 abcd	12.6 a	83.4 abc	16.6 b
'Georgia-09B'	20.8 ab	100 a	0 b	100 a	20.8 abc	0 b	79.2 ab	0 a
'Georgia-19HP'	10 abc	90 ab	0 b	90 a	10 bcde	0 b	90 abc	10 ab
'Florun T61'	7.5 bc	93.4 ab	0.7 b	93.4 a	6.8 cde	0 b	92.5 bc	6.6 ab
'Georgia-20VHO'	5.5 bc	100 a	0 b	94.5 a	5.5 cde	5.5 ab	94.5 bc	0 a
'Georgia-22MPR'	4.2 bc	97.2 ab	4.2 a	88.8 a	0 e	8.4 ab	95.8 bc	2.8 ab
'Georgia-06G'	6.6 bc	100 a	0 b	90.5 a	6.6 cde	9.5 ab	93.4 bc	0 a
'TifNV-HG'	3.4 c	88.8 ab	0 b	88.8 a	3.4 de	0 b	96.6 c	11.2 ab
'TifNV-High O/L'	0 c	85.1 b	0 b	85.1 ab	0 e	0 b	100 c	14.9 b
'Georgia-18RU'	0 c	93.4 ab	0 b	93.4 a	0 e	0 b	100 c	6.6 ab

[a]Means in a column followed by a common letter are not significantly different according to the least significant difference (LSD) at $P \leq 0.05$.

[b]Values are means of twelve replications of *R. delemar* for each peanut cultivar

Table 5. Susceptibility of Thirteen Peanut Cultivars to Rhizopus Seed Rot caused by *R. stolonifer*[a,b]

	Germination (%)[b]		Radicle Protrusion ≥ 10 mm (%)		Radicle Protrusion ≤ 10 mm (%)		No Germination (%)	
Cultivar	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control
'TifJumbo'	19.4 a[a]	100 a	5.6 ab	100 a	13.8 a	0 b	80.6 a	0 a
'Georgia-12Y'	27.8 a	88.8 ab	5.6 ab	88.8 a	22.2 a	0 b	72.2 a	11.2 ab
'Tufrunner 297'	22.2 a	83.4 b	0 b	83.4 ab	22.2 a	0 b	77.8 a	16.6 b
'Georgia-21GR'	33.4 a	83.4 b	11.2 a	70.8 b	22.2 a	12.6a	66.6 a	16.6 b
'Georgia-09B'	13.8 a	100 a	2.7 ab	100 a	11.1 a	0 b	86.2 a	0 a
'Georgia- 19HP'	13.8 a	90 ab	0 b	90 a	13.8 a	0 b	86.2 a	10 ab
'Florun T61'	19.4 a	93.4 ab	5.6 ab	93.3 a	13.8 a	0 b	80.6 a	6.6 ab
'Georgia-20VHO'	13.8 a	100 a	0 b	94.4 a	13.8 a	5.6 ab	86.2 a	0 a
'Georgia-22MPR'	27.8 a	97.2 ab	0 b	88.8 a	27.8 a	8.4 ab	72.2 a	2.7 ab
'Georgia-06G'	19.4 a	100 a	2.8 ab	90.5 a	16.6 a	9.5 ab	80.6 a	0 a
'TifNV-HG'	16.6 a	88.8 ab	0 b	88.8 a	16.6 a	0 b	83.4 a	11.2 ab
'TifNV-High O/L'	13.8 a	85.1 b	2.7 ab	85.1 ab	11.1 a	0 b	86.2 a	14.9 b
'Georgia-18RU'	13.8 a	93.4 a	0 b	93.4 a	13.8 a	0 b	86.2 a	6.6 ab

[a]Means in a column followed by a common letter are not significantly different according to the least significant difference (LSD) at $P \leq 0.05$.

[b]Values are means of twelve replications of *R. stolonifer* for each peanut cultivar

Figures:

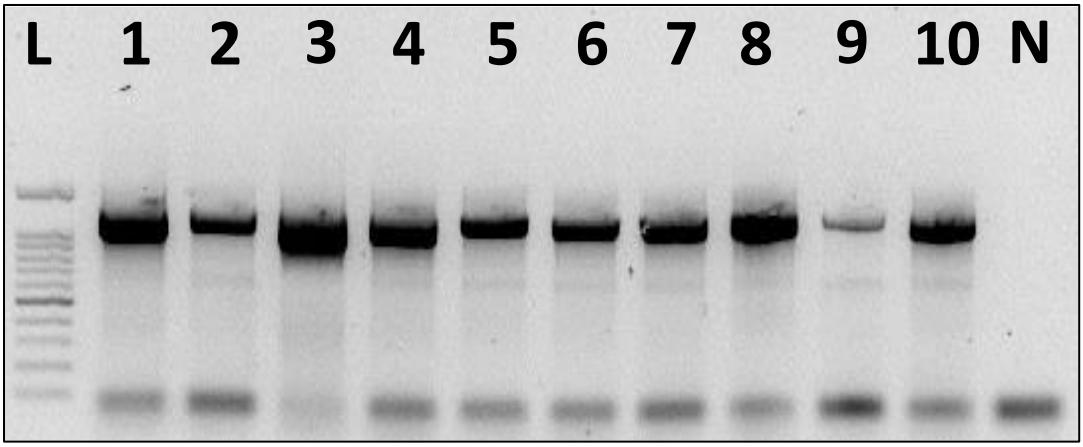

Figure 1. Gel electrophoresis results of PCR assay for the translation elongation factor-1α (TEF-1α) gene target in ten *Rhizopus* isolates. (L) 100-bp DNA step ladder. (1-10) *Rhizopus* isolates. (N) Negative control.

Figure 2. Pathogenicity assay plates of *R. stolonifer* (bottom left), *R. delemar* (bottom right), and control (top) with three surface-sterilized peanut seeds on each plate.

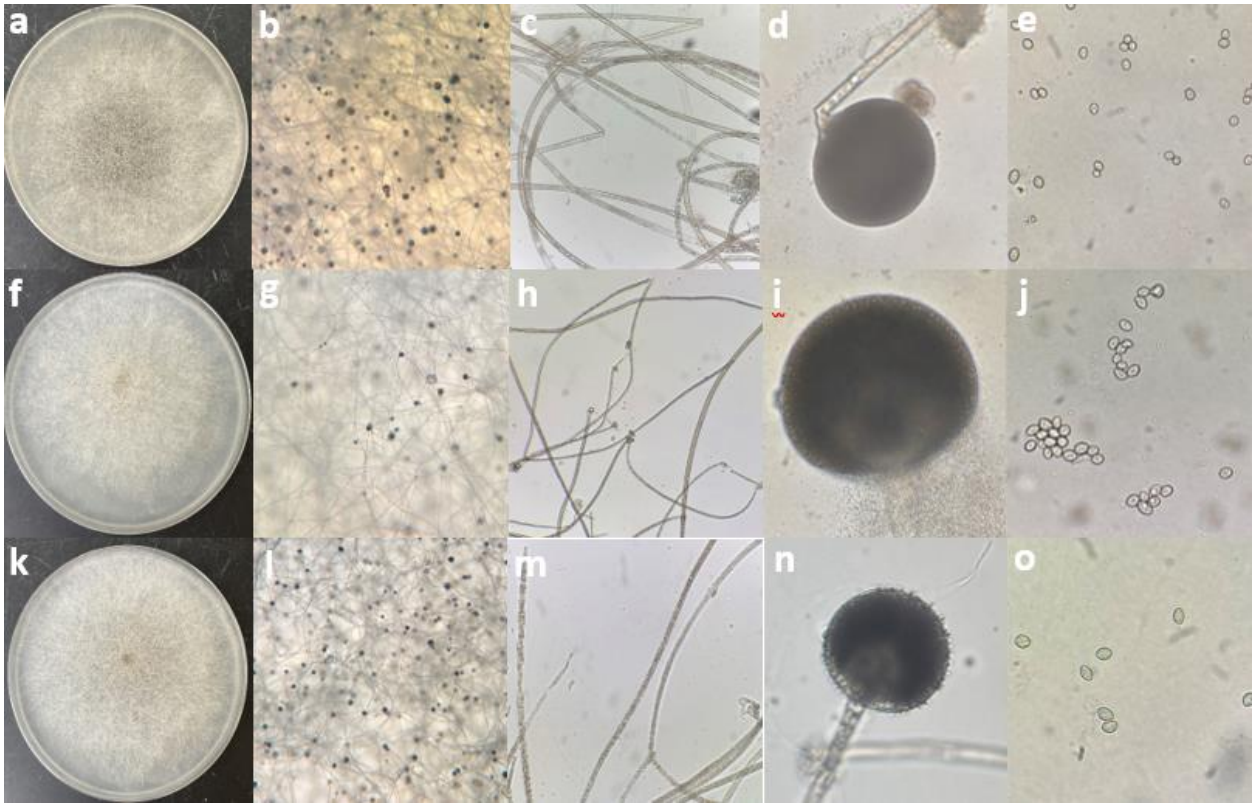

Figure 3. Morphology of three *Rhizopus* spp. (a) *R. arrhizus* colony on PDA after 48 hours of incubation at 25 °C. (b) *R. arrhizus* colony with mycelium, sporangiosphores, and sporangia on PDA under a dissecting microscope. (c) Pigmented hyphae of *R. arrhizus.* (d) *R. arrhizus* sporangium resting upon a swollen columella. (e) Sporangiospores of *R. arrhzius.* (f) *R. delemar* colony on PDA after 48 hours of incubation at 25 °C. (g) *R. delemar* colony with mycelium, sporangiosphores, and sporangia on PDA under a dissecting microscope. (h) Pigmented hyphae of *R. delemar.* (i) *R. delemar* sporangium resting upon a swollen columella. (j) Sporangiospores of *R. delemar.* (k) *R. stolonifer* colony on PDA after 48 hours of incubation at 25 °C. (l) *R. stolonifer* colony with mycelium, sporangiosphores, and sporangia on PDA under a dissecting microscope. (m) Pigmented hyphae of *R. stolonifer.* (n) *R. stolonifer* sporangium resting upon a swollen columella. (o) Sporangiospores of *R. stolonifer.* Total magnification = 500x. Scale bar = 10 µm.

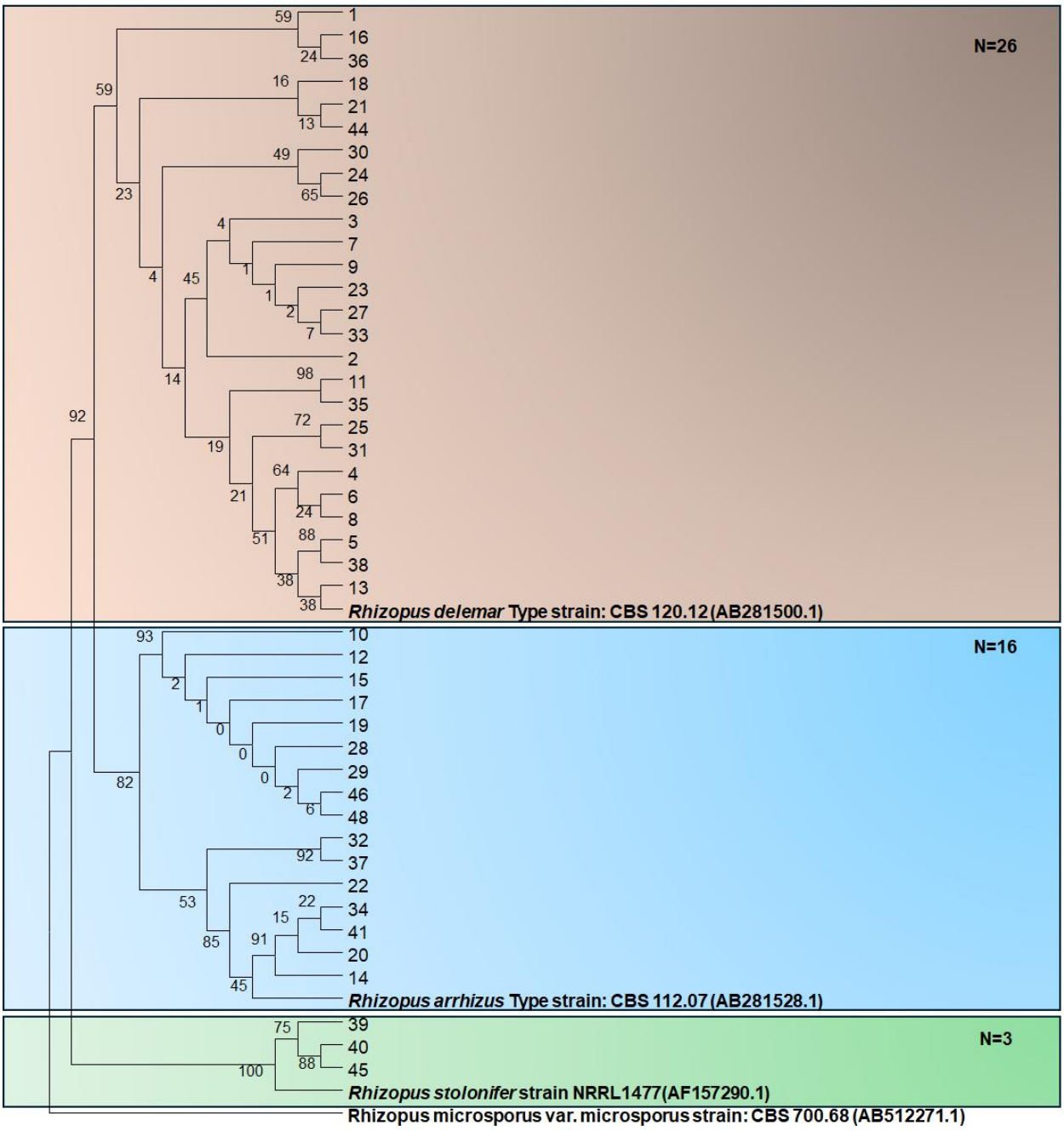

Figure 4. Maximum likelihood phylogenetic tree based on translation elongation factor-1a (TEF-1α) gene in forty-five isolates of *Rhizopus* spp. *R. microsporus* (CBS 700.68) was used as the outgroup. Numbers on the internal nodes indicate bootstrap values.

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CHAPTER 3

IN VITRO FUNGICIDE SENSITIVITY AND TEMPERATURE RESPONSE OF *RHIZOPUS* SPECIES FROM COMMERCIAL PEANUT (*ARACHIS HYPOGAEA* L.) SEEDS IN GEORGIA[1]

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ABSTRACT

Rhizopus seed and seedling rot is a highly destructive peanut disease, causing rapid seed decay in 36-96 hours. Seed treatment fungicides are the first line of defense against this damaging disease. Fifteen isolates of *Rhizopus* spp. (*R. delemar, R. arrhizus,* and *R. solonifer*) collected from Georgia peanut seeds were assessed for sensitivity to eight fungicides. Fludioxonil, carboxin, and pydiflumetofen consistently provided the lowest mean fungicide concentration required to inhibit 50% growth (EC_{50}) values across species (< 0.05 µg/mL). Sedaxane (3.2 – 7.0 µg/mL) and fluopyram (1.0 – 1.9 µg/mL) provided moderate efficacy, while azoxystrobin, mefenoxam, and ipconazole did not inhibit growth at the highest tested concentration (>10 µg/mL). The radial mycelial growth and virulence on peanut seeds of all three species under five temperature conditions (15, 20, 25, 30 and 35 °C) were also evaluated. Temperature assays revealed variability in the thermotolerance across *Rhizopus* spp. *Rhizopus delemar* and *R. arrhizus* exhibited rapid mycelial growth and high virulence on peanut seeds at all tested temperatures, while *R. stolonifer* exhibited significantly slower growth rate and no growth at 30 °C and 35 °C, respectively. Virulence was evaluated with a novel *in vitro* assay. *Rhizopus delemar* and *R. arrhizus* reduced germination to less than 15% at all temperatures. This was also true for *R. stolonifer* at lower temperatures, but due to the reduced growth of 30 °C and 35 °C, some inoculated seeds germinated (40.2% at 30 °C and 72.2% at 35 °C) and developed healthy radicles above 10 mm (37.5% at 30 °C and 63.8% at 35 °C) despite exposure. Overall, these findings verify the high virulence of *Rhizopus* spp. to peanut seed, and show that fludioxonil, carboxin, and pydiflumetofen are excellent candidates for managing *Rhizopus* spp.

Key words: *Rhizopus* spp., temperature, peanut seeds, virulence, fungicides

1 INTRODUCTION

Rhizopus seed and seedling rot is a highly destructive peanut disease, causing rapid seed decay in 36-96 hours and often leaving the seeds and pre-emerged seedlings indistinguishable from the soil (Clinton, 1960). Although the historical literature largely focused on *Rhizopus* spp. affecting ungerminated peanut seeds and pre-emerged seedlings (Gibson, 1953; Moorwood, 1953; Arant et al., 1951; Frank, 1969; Gupta & Chohan, 1970), modern research regarding the temperature responses and chemical sensitivities of *Rhizopus* spp. in peanut production are limited, despite the pathogen's destructive nature and prevalence. Both plants and pathogens alike require certain temperatures to grow, reproduce, and survive. The optimal growth of *Rhizopus* spp. is often reported as 25 °C, with *R. stolonifer* exhibiting growth inhibition at temperature above 30 °C (Muller, 1956; Zheng et al., 2007; Sandoval-Contreras et al., 2022). Even within the optimal planting temperatures for peanuts (18-32 °C) (Kvien et al., 2022), *Rhizopus* spp. can severely impact germination and stand uniformity in the field. This emphasizes the need to revisit the thermal response of *Rhizopus* spp., especially considering changing environmental conditions and production practices.

Due to the warm, humid conditions in the southeastern U.S. favoring most fungal plant pathogens and the ubiquitous distribution of seed and seedling pathogens, seed treatment fungicides are integral to obtaining healthy and uniform plant stands in conventional peanut production. Applying seed treatment fungicides is typically more cost-effective and results in more consistent stands than simply increasing the seeding rate (Moorwood, 1953). Untreated seeds, regardless of quality, achieve only 50% stands (Melouk & Backman, 1995), while treated seeds can increase yields by 50-75% (Mahoney et al., 2019). Therefore, seed treatment fungicides are commonly used in developed agronomic systems where seeds are used to establish a crop (Zeun et al., 2012). Modern seed treatment fungicides, such as Trebuset®

(Syngenta Crop Protection, Greensboro, NC) and Rancona VPD® and VPL® (UPL Corporation Limited Group Company, King of Prussia, PA) use a combination of active ingredients with varying fungicide classes, including azoxystrobin (quinone outside inhibitor, QoI), pydiflumetofen (succinate dehydrogenase inhibitor, SHDI), carboxin (SDHI), fludioxonil, (phenylpyrrole), mefenoxam (phenylamide), and ipconazole (triazoles), to target a wide range of seed and seedling pathogens, reduce the incidence of single-site fungicide resistance, and extend the longevity of these fungicides (Uppala & Sulley, 2025; Sierotzki and Scalliet, 2013; Walter et al., 2015; Gisi & Sierotzki, 2008; Villaorduna et al., 2024; von Schmeling & Kulka, 1966; Kojima et al., 2006). While effectiveness against *Rhizopus* spp. was reported with azoxystrobin (Kortekamp, 2006; Cai et al., 2019), fludioxonil (Sallato et al., 2007; Northover & Zhou, 2002), and ipconazole (Tateishi et al., 1998), limited research has specifically addressed their impact on *Rhizopus* spp. in peanut seeds. A preliminary greenhouse and *in vitro* study revealed that combinations containing ipconazole, carboxin, and metalaxyl suppressed *Rhizopus* growth and improved peanut seedling emergence (Giorando et al., 2024). However, no data were presented on the activity of the individual fungicide components, which limits the ability to assess the individual efficacy of each fungicide. Therefore, more robust *in vitro* chemical sensitivity assays are required to quantify the effectiveness of each active ingredient separately against *Rhizopus* spp. Overall, integrating effective fungicide treatments with proper seed handling techniques and optimal planting conditions remains critical for reducing field stand losses and improving seed health management strategies (Melouk & Backman, 1995; Jackson & Bell, 1969; Wood, 1968; Moorwood, 1953).

2 MATERIALS AND METHODS

2.1. Radial Mycelial Growth of Three *Rhizopus* spp. under Five Temperature Regimes

Forty-five *Rhizopus* spp. isolates were collected from infected peanut seeds from five commercial seed lots in Georgia: two seed lots collected from the 2021-2022 harvest and three from the 2022-2023 harvest. Peanut seeds were surface sterilized with 10% Clorox solution for three minutes, rinsed with sterile water, dried with sterile paper towels, and placed on potato dextrose agar (PDA) medium Petri dishes (100 x 15 mm) at 25 °C in an incubator with continuous fluorescent light for five days. To isolate the pathogen, a single hyphal tip was extracted with a sterile needle from the colony's outermost growth and placed on a sterile Petri dish containing full-strength PDA. Pure cultures were made by extracting a small portion of the colony's outermost hyphal growth and streaking it over an agar surface in a Petri dish. All cultures were made on full-strength PDA plates and sealed with Parafilm® M (Bemis Company Inc., Neenah, WI) and incubated at 25 °C with continuous fluorescent light. Microscopic examinations of 7-day-old cultures of each isolate were performed at 40x, 100x, and 400x magnification to observe morphology and assess purity. The spore suspension was made by adding 1 mL sterilized deionized water to 7-day-old cultures in Petri dishes, scraping the agar surface with a sterile inoculation loop to dislodge the spores, and dispensing them into sterile 1.5 mL microcentrifuge tubes. For long-term storage, three aliquots of spore suspension were made for each isolate, with two stored at -20 °C and one at -80 °C.

Three *Rhizopus* spp. (*R delemar, R. arrhizus,* and *R. stolonifer)* were found in these seed lots as described in the previous chapter. The effect of temperature on the colony growth of these isolates was investigated under laboratory conditions. Six, six, and three isolates of *R delemar, R. arrhizus,* and *R. stolonifer*, respectively, were selected based on branch positions in the phylogenetic tree and used in prior objectives. The spore suspension of the isolates in this experiment were retrieved from -20 °C storage, adjusted to 10^5 spores/mL with a hemocytometer, and used to centrally inoculate plates (7 µl per plate), which were incubated at

one of the five temperatures: 15, 20, 25, 30, and 35 °C. The plates were sealed with Parafilm® M (Bemis Company Inc., Neenah, WI), and the surface radial growth of each colony was measured in 24-hour intervals for eight days. The experiment was repeated twice and followed a randomized complete block design (RCBD), with four replications per isolate at each temperature condition.

Statistical analysis was performed using SAS 9.4 (SAS Institute, Cary, NC, USA). A Proc T-Test was used to compare the results between both tests, and the Proc Mixed procedure was performed to assess the effect of species, temperature, time, as well as their interactions, on radial mycelial growth. Both tests were combined for further analysis due to no significant differences between test and species at the 0.05 probability level. Proc Mixed procedure revealed that the effect of species, temperature, and time were highly significant on radial mycelial growth ($p < 0.0001$). Therefore, the radial mycelial growth varied by species and was significantly influenced by temperature and changes over time. Significant differences across species and temperature were determined according to the least significant difference (LSD) test at $P \leq 0.05$. The mean radial growth over time of each *Rhizopus* spp. was modeled using the Gompertz three-parameter regression in SigmaPlot (Version 16.0; Systat Software, 2024).

$$y = a \times e^{-e^{-b(x-c)}} \quad [2]$$

In this equation, a is the y-coordinate or maximum growth (100%), b is the slope or radial growth rate, c is the number of hours required to reach 50% maximum growth, and x is time. The coefficient of determination (R^2) represents the adjusted goodness-of-fit value. Comparisons among the same parameter estimates for each *Rhizopus* spp. at 15, 20, and 25 °C were evaluated based on non-overlapping 95% confidence intervals. Accurate model fitting and parameter estimates for *R. delemar* and *R. arrhizus* at 30 °C and 35 °C could not be obtained due to rapid

growth between 24-hour intervals. *R. stolonifer* at 35 °C exhibited no growth, making parameter estimates unobtainable.

2.2. Virulence of Three *Rhizopus* spp. on Peanut Seed Germination and Radicle Development under Five Temperature Regimes.

The effect of temperature on the virulence of three *Rhizopus* spp. on peanut seed germination and radicle protrusion was investigated under laboratory conditions. Forty-five *Rhizopus* spp. isolates were sourced, developed in spore suspension, and stored using the previously mentioned method. Fifteen of the forty-five isolates were selected for this study based on use in prior assays and the phylogenetic branches. Plates of PDA media were inoculated centrally with each isolate (7 µl) and incubated at 25 °C for 24 hours. The spore concentrations were determined with a hemocytometer and adjusted to 10^5 spores/mL. The control plates were inoculated with sterilized water (7 µl). The 24-hour incubation period allowed the colony to grow large enough to adequately fit three surface-sterilized peanut seeds around the colony's border. Peanut seeds were surface-sterilized with a 10% Clorox solution for three minutes, rinsed with sterile water, and dried with sterile paper towels. After a 24-hour incubation period, three surface-sterilized 'Georgia-06G' seeds were placed around the colony's border, making a triangular pattern on the plate. 'Georgia-06G' was chosen due to the high frequency in which it is planted in the southeastern U.S. (Brown, 2023). The plates were sealed with Parafilm® M (Bemis Company Inc., Neenah, WI), incubated at a constant temperature of 15, 20, 25, 30, and 35 °C under fluorescent light, and evaluated for germination and radicle protrusion at 24-hour intervals over eight days. Three seeds were nested on each plate, with four replications per treatment. This nested design resulted in twelve seeds per isolate–temperature regime. Final germination and radicle length were measured on day eight, with radicle lengths recorded as either below or above 10 mm. The test was repeated twice and followed a nested, randomized

complete block design (RCBD), with four replications and twelve surface-sterilized seeds per isolate and temperature condition incubated on PDA.

Statistical analysis was performed using SAS 9.4 (SAS Institute, Cary, NC, USA). The Proc T-Test was performed to compare seed performance between both tests, which were then combined for further analysis due to no statistical differences at a 0.05 probability level. The General Linear Model (GLM) procedure in SAS 9.4 (SAS Institute, Cary, NC, USA) was performed to assess differences in the mean cumulative percentage of germination and radicle length on day eight among seeds inoculated with each fungal species at the five temperature conditions. Post hoc comparisons were made using the Least Significant Difference (LSD) test at a significance level of $\alpha = 0.05$. Table 2 displays the comparison made among *Rhizopus* species within the same temperature to assess the effect of species on germination and radicle length. Table 3 displays the comparison made among temperatures within each species to assess the effect of temperature on the pathogenicity of each species. Means sharing the same letter within a column are not significantly different.

2.3. *In vitro* Chemical Sensitivity of Three *Rhizopus* spp. to Eight Seed Treatment and In-Furrow Fungicides

The *in vitro* chemical sensitivity of three *Rhizopus* spp. to the active ingredients of common seed treatment fungicides was evaluated on PDA medium using a mycelial growth inhibition assay. Forty-five *Rhizopus* spp. isolates were sourced, developed into spore suspension, and stored using the previously mentioned method. Fifteen of the forty-five isolates were selected for this study based on use in prior assays and the phylogenetic branches. Six, six, and three isolates of *R delemar, R. arrhizus,* and *R. stolonifer* were selected, respectively. Technical-grade fungicides, including azoxystrobin (99.5% active ingredient; a.i.), pydiflumetofen (99.5% a.i.), and mefenoxam (99.8% a.i.), were obtained from Chem Services

Inc., West Chester, PA 19381). The following formulated fungicides were also evaluated: Rancona® 3.8 FS (40.7% ipconazole; UPL Corporation Limited Group Company, King of Prussia, PA), Vitavax®-34 (34% carboxin; UPL Corporation Limited Group Company, King of Prussia, PA), Velum® (41.5% fluopyram; Bayer Crop Science, St. Louis, MO, USA), Vibrance® (43.7% sedaxane; Syngenta Crop Protection, Greensboro, NC, USA) and Maxim®-4FS (40.3% fludioxonil; Syngenta Crop Protection, Greensboro, NC, USA) were evaluated. For each technical grade fungicide, a stock solution (1 mg/mL) was prepared by dissolving the chemical in 99 mL sterile deionized water and 1 mL ACS-grade acetone. Sterilized media was cooled to 50 °C before the active ingredient was incorporated from stock solutions to achieve final concentrations of 0.0, 0.01, 0.1, 1 and 10 µg/mL. In the azoxystrobin tests, salicylhydroxamic acid (SHAM, 99% a.i.; Thermo Fisher Scientific Inc., Waltham, MA) was dissolved in methanol to prepare a stock solution (20 mg/mL) and added to the cooled media (50 °C) to inhibit the alternative oxidase pathway (Wood & Hollomon, 2003; Grahl et al., 2012). The amended media was dispensed into 100 mm x 15 mm Petri plates (Thermo Scientific, Waltham, MA, USA) and left to solidify. The spore concentrations were determined with a hemocytometer and adjusted to 10^5 spores/mL. A 10 µl-droplet of spore suspension was added centrally to each amended and non-amended PDA plate and incubated for 7 days at 25 °C. Two plates per treatment were inoculated with each isolate, along with two non-amended control plates per treatment. The colony diameters were measured at 36 hours, and each test was repeated twice.

The fungicide concentrations that effectively inhibited mycelial growth by 50% (EC_{50}) were estimated for each species and isolate by calculating the relative growth percentage of each isolate ([Growth at a given concentration/growth in control] x 100) and linearly regressing relative inhibition values (100 – relative growth) on $\log_{10}$-transformed fungicide concentration using PROC REG in SAS 9.4 (SAS Institute, Cary, NC). The log EC_{50} was calculated using the

formula $EC_{50} = 10^{(-\text{intercept/slope})}$, where the negative intercept is divided by the slope, and the EC_{50} was determined by exponentiating this value. Each EC_{50} estimate was summarized using PROC MEANS to calculate the mean log EC_{50} for each fungicide and isolate combination. The PROC UNIVARIATE was used to evaluate the normality of the log EC_{50} values. Two one-way analysis of variance (ANOVA) analyses were performed using PROC GLM to assess significant differences between the $\log_{10}$-transformed values between the three *Rhizopus* spp. and between the fifteen isolates. The least squares mean for each species and isolate was calculated and Tukey's multiple comparison adjustment (P = 0.05) was used to compare groups. *Rhizopus* spp. or isolates sharing the same letter in a column are not significantly different in their mean EC_{50} values.

Isolates with EC_{50} values beyond the tested concentration range (< 0.01 or > 10.0 µg/mL) were excluded from species and isolate-level mean EC_{50} calculations and comparisons to avoid data extrapolation. The fifteen *Rhizopus* spp. isolates tested *in vitro* for sensitivity to azoxystrobin, mefenoxam, and ipconazole exhibited growth exceeding the highest fungicide concentration tested (>10.0 µg/mL), suggesting higher fungicide concentrations may be required to inhibit 50% fungal growth. However, the exact EC_{50} values for these fungicides could not be determined within the tested concentration range. Future assays should broaden the concentration range to determine the EC_{50} values and assess more accurately possible resistance occurrences.

3 RESULTS AND DISCUSSION

3.1. Response of Three *Rhizopus* spp. on Radial Mycelial Growth under Five Temperature Conditions

In Figure 5, the temperature response of *Rhizopus arrhizus* at 15, 20, 25, 30, and 35 °C, as measured by the mean radial growth, is displayed. By 24 hours, the mean mycelial diameters at 15, 20, 25, 30, and 35 °C were significantly different from each other, with measurements of

2, 11.7, 23.2, 43, and 51.1 mm, respectively. By 48 hours, the colony diameters at 30 °C and 35 °C reached maximum growth (85 mm), with 25 °C nearing the maximum at 82.9 mm. The colony diameters at 15 °C and 20 °C, however, grew at a slower rate, with measurements of 15.3 and 58.2 mm, respectively. Between 48 and 72 hours, the colonies at 25 °C reached maximum growth (85 mm), with 20 °C nearing the maximum (79.9 mm). The colonies at 15 °C, however, continued to grow at a slower rate, with measurements of 39 mm. By 96 hours, colonies at 20 °C reached maximum growth (85 mm), while colonies at 15 °C measured 59.4 mm. By 120, 144, and 168 hours, colonies at 15 °C reached 72, 79.1, and 83.7 mm, respectively.

Rhizopus delemar exhibited similar trends in growth rates across the five temperatures as *R. arrhizus* (Figure 6). By 24 hours, the mean mycelial diameters at 15, 20, 25, 30, and 35 °C varied significantly from each other, with measurements at 1.7, 5.5, 23.8, 43, and 53.1 mm, respectively. By 48 hours, the colony diameters at 30 °C and 35 °C reached maximum growth (85 mm), with 25 °C nearing the maximum at 82 mm. The colony diameters at 15 °C and 20 °C, however, grew at a slower rate, with measurements at 13 and 55.2 mm, respectively. Between 48 and 72 hours, colonies at 25 °C reached maximum growth (85 mm), with 20 °C nearing the maximum at 79.4 mm. The colonies at 15 °C, however, continued to grow at a slower rate, with measurements at 35 mm. By 96 hours, colonies at 20 °C reached maximum growth (85 mm), while colonies at 15 °C measured 56.2 mm. By 120, 144, and 168 hours, colonies at 15 °C reached 69.3, 77.5, and 83.7 mm, respectively.

The temperature response of *Rhizopus stolonifer* exhibited an apparent difference in growth from *R. delemar* and *R. arrhizus*, particularly at 30 °C and 35 °C (Figure 7). Because colony growth ceased at 35 °C, the regression parameter estimates were unable to be calculated and displayed within a graph. By 24 hours, the colony diameters at 15, 20, 25, 30, and 35 °C varied, with measurements at 2.1, 13.9, 29, 21.9, and 2 mm, respectively. Unlike *R. delemar* and

R. arrhizus, *R. stolonifer* colonies across the five temperatures did not reach maximum growth (85 mm) by 48 hours. By this time, the colony diameters at 15, 20, 25, 30, and 35 °C were 25, 63.6, 76.7, 64.5, and 2 mm, respectively. By 72 hours, the colony diameters at 15, 20, 25, 30, and 35 °C were 55, 82.1, 84.7, 79.8, and 2 mm, respectively. By 96 hours, colonies at 20, 25, and 30 °C reached maximum growth (85 mm), while colonies at 15 °C and 35 °C were 73.2 and 2 mm respectively. By 120, 144, and 168 hours, colonies at 15 °C reached 80, 82.4, and 83.1 mm, respectively. The colony diameters at 35 °C did not change over time throughout the experiment.

Figure 8 displays the growth response of the three *Rhizopus* spp. at 15 °C, as measured by the mean radial growth. By 24 hours, the colony diameters of *R. arrhizus, R. delemar,* and *R. stolonifer* were similar, with measurements at 2, 1.7, and 2.1 mm, respectively. By 48 hours, however, differences in colony diameters across the species became apparent, with *R. stolonifer* measuring significantly larger at 25 mm and *R. arrhizus* and *R. delemar* at 15 mm and 13 mm, respectively. This trend continues through the 72, 96, and 120-hour points. By 72 hours, the colony diameters of *R. arrhizus, R. delemar,* and *R. stolonifer* were 39, 35, and 55 mm, respectively. By 96 hours, *R. stolonifer* colonies approached maximum growth faster than *R. arrhizus* and *R. delemar*, with measurements at 73.2 mm, 59.4, and 56.2 mm, respectively. By 120 hours, the colony diameters of *R. arrhizus, R. delemar,* and *R. stolonifer* were 72, 69.3, and 80 mm, respectively. By 144 hours, this trend continued but the differences between *R. stolonifer* and *R. arrhizus* narrowed, with colony diameters at 82.4 mm and 79.5 mm, respectively. By 168 hours, the colony diameters of *R. arrhizus, R. delemar,* and *R. stolonifer* were statistically similar at 83.7, 83.7, and 83.1 mm, respectively. Overall, *R. stolonifer* had the steepest slope (21.1 hours) compared to R. arrhizus (31.6 hours) and R. delemar (32.7 hours). These estimates indicate that *R. stolonifer* had the fastest growth rate of the three species at the lowest temperature. The time taken for *R. arrhizus, R. delemar,* and *R. stolonifer,* to reach 50%

maximum growth, represented by the variable *c* in the Gompertz three-parameter equation, was 65.5, 68, and 52.4 hours, respectively (Table 6). These data indicate that *R. stolonifer* required at least 13 hours less to reach 50% growth compared to the other two *Rhizopus* spp. Due to its rapid growth at the lowest temperature, *R. stolonifer* may be more virulent at lower temperatures than *R. delemar* and *R. arrhizus.*

Figure 9 displays the growth response of the three *Rhizopus* spp. at 20°C, as measured by the mean radial growth. By 24 hours, the colony diameters of *R. arrhizus, R. delemar,* and *R. stolonifer* were statistically significant from each other, with measurements at 11.7, 5.5, and 13.9 mm, respectively. This difference in colony diameters continues into the 48-hour point, where the colony diameters of *R. arrhizus, R. delemar,* and *R. stolonifer* were 58.2, 55.2, and 63.6 mm, respectively. The growth differences between species narrowed by 72 hours, with colony diameters of *R. arrhizus, R. delemar,* and *R. stolonifer* at 79.9, 79.4, and 82.1 mm, respectively. This trend continued throughout the rest of the experiment, with each *Rhizopus* spp. reaching maximum growth (85 mm) by 96 hours. The time taken for *R. arrhizus, R. delemar,* and *R. stolonifer* to reach 50% maximum growth varied among species, with values at 34.4, 37.5, and 31.9 hours (Table 6). This data indicates that *R. stolonifer* (31.9 hours) was slightly faster in reaching 50% maximum growth than *R. arrhizus* (34.4 hours) and *R. delemar* (37.5 hours), with *R. delemar* being the slowest. The slope estimates of each species were similar (13.19 – 14.1 hours), indicating that overall growth occurred at similar rates.

The mycelial radial growth response of the three *Rhizopus* spp. at 25 °C is displayed in Figure 10. By 24 hours, the colony diameters of *R. arrhizus* and *R. delemar* were similar, with measurements at 23.2 mm and 23.8 mm. *Rhizopus stolonifer*, however, had slightly larger growth at 29 mm. Despite this initial difference in colony diameter, *R. arrhizus* (82.9 mm) and *R. delemar* (82 mm) surpassed *R. stolonifer* (76.5 mm) in growth by 48 hours. Between 48 and 72

hours, both *R. arrhizus* and *R. delemar* reached maximum growth, with *R. stolonifer* slightly behind at 84.7 mm. However, all species were statistically similar at this point and for the rest of the experiment, as each reached maximum growth (85 mm) after 72 hours. Each species had similar times to reach 50% maximum growth, ranging from 24.7 – 25.4 hours (Table 6). This revealed that each species required approximately 24-hours to reach 50% maximum growth. Overall, the slope estimates of *R. delemar* and *R. arrhizus* were the same (both 6.1 hours), while the slope of *R. stolonifer* was significantly greater (10.7 hours). This data indicates that at 25 °C, *R. delemar* and *R. arrhizus* grew at a faster rate than *R. stolonifer.*

Figure 11 displays the mycelial radial growth response of the three *Rhizopus* spp. at 30 °C. By this temperature, the reduced thermotolerance of *R. stolonifer* compared to the other two species became increasingly apparent. While *R. arrhizus* and *R. delemar* had colony diameters at 43 mm by 24 hours, *R. stolonifer* was nearly twice as small at 21.9 mm. This trend became more pronounced at 48 and 72 hours, where both *R. arrhizus* and *R. delemar* reached maximum growth (85 mm) before 48 hours. Conversely, *R. stolonifer* only reached 64.5 mm and 79.8 mm by 48 and 72 hours, respectively. Finally, *R. stolonifer* reached maximum growth (85 mm) by 96 hours. Although the *b* parameter estimates for *R. delemar* and *R. arrhzius* were unobtainable due to rapid growth between 24-hour intervals, *R. stolonifer* exhibited a large slope value at 15.6 hours and required 28.8 hours to reach 50% maximum growth. At a slower rate, *R. stolonifer* took more than 24 hours to reach maximum growth (85 mm). These data suggest that *R. stolonifer* has reduced thermotolerance at higher temperatures, which may influence its virulence in warmer temperatures.

Figure 12 displays the growth response of the *R. delemar* and *R. arrhizus* at 35 °C. At this temperature, *R. stolonifer* exhibited no growth, while both *R. arrhizus* and *R. delemar* grew so rapidly between 24-hour intervals that regression parameter estimates could not be calculated

and were omitted from Table 6. Additionally, the measurements of *R. stolonifer* could not be meaningfully compared due to no growth at this temperature. By 24 hours, the colony diameters of *R. arrhizus* and *R. delemar* were similar, with measurements at 51.1 mm and 53.1 mm, respectively. Between 24 and 48 hours, both *R.* reached maximum growth (85 mm). All *Rhizopus* spp., except *R. stolonifer* at 35 °C, reached or closely approached maximum growth (85 mm) across temperatures. Overall, these data indicate that as temperature increases, the mycelial growth of *R. delemar* and *R. arrhizus* increased, while R. *stolonifer* expressed reduced thermotolerance at the two highest temperatures.

3.2. Virulence of Three *Rhizopus* spp. on Peanut Seed Germination and Radicle Development under Five Temperature Regimes

Statistical analysis confirmed that the two tests could be combined, and the effect of *Rhizopus* spp. isolates was significant; therefore, each *Rhizopus* spp. was analyzed separately. Seed germination varied with temperature and the specific *Rhizopus* spp. used as inoculum. At 15 °C, the seeds inoculated with *R. delemar, R. stolonifer,* and *R. arrhizus* exhibited significantly low germination percentages of 11.1, 4.1, and 2.7%, respectively (Tables 7 and 8). At 20 °C, seed germination remained consistently low across *R. delemar, R. stolonifer,* and *R. arrhizus* inoculum, with germination percentages of 7.6, 13.8, and 9.0%, respectively. At 25 °C, however, seeds inoculated with *R. stolonifer* experienced a sharp increase in germination to 27.7%, which significantly surpassed the germination percentages of seeds inoculated with *R. delemar* (8.3%) and *R. arrhizus* (7.6%). This trend became more apparent at 30 °C and 35 °C, with seeds inoculated with *R. stolonifer* reaching 40.2% and 72.2%, respectively. Conversely, the seeds inoculated with *R. delemar* and *R. arrhizus* continued to exhibit consistently low germination percentages at 30 °C and 35 °C, with values below 13%.

Similar trends were observed with radicle length. By day eight at 15 °C, germinated seeds inoculated with *R. delemar, R. stolonifer,* and *R. arrhizus* only exhibited radicle lengths below 10 mm, with percentages of 11.1%, 4.1%, and 2.7%, respectively (Tables 7 and 8). In all instances, the seed and radicle were overrun with infection and had become completely soft and rotten. At 20 °C, the radicles of seeds inoculated with *R. delemar, R. stolonifer,* and *R. arrhizus* remained below 10 mm for most germinated seeds, with percentages of 6.9, 13.8, and 6.9%, respectively. A few seeds inoculated with *R. delemar* and *R. arrhizus* developed radicles greater than 10 mm; however, they were completely rotten. At 25 °C, 8.3% of germinated seeds inoculated with *R. stolonifer* maintained radicles greater than 10 mm, which was significantly higher than those inoculated with *R. delemar* (0%) and *R. arrhizus* (0%). This trend continued at the two highest temperatures. At 30 °C, most germinated seeds inoculated with *R. stolonifer* (40.2%) maintained radicle length above 10 mm (37.5%), which far exceeded the radicle lengths of the germinated seeds inoculated with *R. delemar* (0.6%) and *R. arrhizus* (0%). At 35 °C, most germinated seeds inoculated with *R. stolonifer* (72.2%) maintained radicle length above 10 mm (63.8%), while the germinated seeds inoculated with *R. delemar* (1.3%) and *R. arrhizus* (0%).

Despite exposure to *R. stolonifer,* most of the developed hypocotyls of germinated seeds remained green and healthy at 30 °C and 35 °C, indicating that the three *R. stolonifer* isolates tested are less thermotolerant, and therefore, less virulent at the higher temperatures than *R. delemar* and *R. arrhizus*. Similar observations are recorded in the literature. In 1956, the growth of *R. nigricans* (reclassified as *R. stolonifer*) on nutrient agar was optimum at 25 °C, poor at 30 °C, greatly inhibited at 32 °C, and nonexistent at 35 °C (Muller, 1956). Similarly, a 2007 study reported the temperature optima for 203 strains of *R. stolonifer, R. arrhizus,* and *R. microsporus* as 26 – 32 °C, 37 – 42 °C, and 40 – 51 °C, respectively (Zheng et al., 2007). These findings reveal that *R. stolonifer* had the lowest temperature optimum range of the three *Rhizopus* spp.

tested. In 2022, *in vitro* studies reported that *R. stolonifer* grew optimally at 25 °C, with colonies generally ceasing growth at 37 °C (Sandoval-Contreras et al., 2022). The ability to tolerate a greater range of temperatures, or thermotolerance, contributes to enhanced virulence (Kaerger et al., 2015). The literature and temperature assays conducted for this study suggest that the limited thermotolerance of *R. stolonifer* may restrict its virulence, particularly at elevated temperatures, where seed infection by *R. arrhizus* and *R. delemar* was more virulent. Within the scope of this temperature assay, *R. stolonifer* exhibited greater virulence at cooler and moderate temperatures (15–25 °C), while *R. delemar* and *R. arrhizus* consistently showed high virulence across all temperature conditions.

3.3. *In vitro* Chemical Sensitivity of Three *Rhizopus* spp. to Eight Seed Treatment and In-Furrow Fungicides

Fifteen isolates were used for the *in vitro* efficacy tests to determine the effective concentration inhibiting 50% (EC_{50}) growth of *Rhizopus* spp. isolates against fludioxonil, pydiflumetofen, sedaxane, fluopyram, and carboxin. Isolates with EC_{50} values beyond the tested concentration range (< 0.01 or >10.0 µg/mL) were excluded from species and isolate-level mean EC_{50} calculations and comparisons to avoid data extrapolation. The mean EC_{50} values of *R. delemar, R. stolonifer,* and *R. arrhizus* to fludioxonil were 0.031, 0.042, and 0.035 µg/mL, with the minimum and maximum EC_{50} values within species ranging from 0.01 – 0.051, < 0.01 - 0.030, and < 0.01 – 0.072 µg/mL, respectively (Table 9). Two *Rhizopus stolonifer* isolates (40S and 45S) and one *Rhizopus arrhizus* isolate (10A) had EC_{50} estimates below the tested fungicide concentration range (< 0.01 µg/mL), signifying high sensitivity to fludioxonil (Table 10). However, the exact EC_{50} values for isolates 40S, 45S, and 10A could not be determined. Among the most sensitive isolates were one *R. delemar* (3D) isolate and one *R. arrhizus* isolate (10A), with determinable EC_{50} values of 0.010 and 0.012 µg/mL, respectively. The least sensitive

isolates belonged to *R. delemar* (30D and 35D) and *R. arrhzius* (32A and 34A), with EC_{50} values of 0.044, 0.051, 0.069, and 0.072 µg/mL, respectively. The only *R. stolonifer* isolate with a determinable EC_{50} value had an EC_{50} of 0.030 µg/mL, showing moderate sensitivity compared to the other isolates. Similar efficacy against *R. stolonifer* isolates to fludioxonil collected from rotten strawberry fruits and peaches has been reported (Sallato et al., 2007; Northover & Zhou, 2002). Overall, the fifteen isolates exhibited consistently low EC_{50} values to fludioxonil, demonstrating consistently high sensitivity across the three *Rhizopus* species.

The mean EC_{50} values of *R. stolonifer,* and *R. arrhizus* to pydiflumetofen were 0.041 and 0.017µg/mL (Table 9), with the minimum and maximum EC_{50} values within species ranging from < 0.01 – 0.050 and < 0.01 – 0.020 µg/mL, respectively (Table 10). All *R. delemar* isolates, four *R. arrhizus* isolates (17A, 32A, 34A, and 41A), and one *R. stolonifer* isolate (39S) were highly sensitive to pydiflumetofen (< 0.01 µg/mL), with EC_{50} values below the tested concentration range. As a result, calculating the exact EC_{50} values was unobtainable. The variation among the four isolates with determinable EC_{50} values (0.015 – 0.050 µg/mL) was statistically similar, which further indicates the consistently high sensitivity across the three *Rhizopus* spp. to pydiflumetofen. Similar EC_{50} values across different fungal pathogens, such as *Sclerotinia sclerotiorum* (Duan et al., 2019), *Fusarium fujikuroi* (Bai et al., 2021), and *Botrytis cinerea* (He et al., 2020) have been reported for pydiflumetofen. To the best of our knowledge, no previous assays in the literature have investigated the *in vitro* sensitivity of pydiflumetofen against *Rhizopus* spp.

The mean EC_{50} values of *R. delemar, R. stolonifer,* and *R. arrhizus* to sedaxane were 4.3, 7.0, and 3.2 µg/mL (Table 9), with the minimum and maximum EC_{50} values within species ranging from 1.9 – 6.1, 7.0 - >10.0, and 1.9 – >10.0 µg/mL, respectively (Table 10). Within the tested concentration range, sedaxane showed no activity against four *R. arrhizus* (17A, 22A,

32A, and 41A) and two *R. stolonifer* (40S and 45S) isolates, with EC_{50} estimates exceeding the highest concentration range tested (10 µg/mL). As a result, the exact EC_{50} values of these isolates were indeterminable. The most sensitive isolates belonged in *R. arrhizus* (10A) and *R. delemar* (3D), both with EC_{50} values of 1.9 µg/mL. Overall, the sensitivity of the *Rhizopus* spp. isolates varies significantly, with determinable EC_{50} values ranging from 1.9 – 7.0 µg/mL. This range of EC_{50} values reveals significant differences in the efficacy of sedaxane against the tested *Rhizopus* spp isolates. This may be partly due to selective pressure favoring tolerance in some *Rhizopus* spp. isolates than others (Yang et al., 2021), or it may suggest the early stages of quantitative resistance among *Rhizopus* spp., which could eventually lead to more widespread resistance to sedaxane. To the best of my knowledge, no previous assays in the literature have investigated the *in vitro* sensitivity of *Rhizopus* spp. to pydiflumetofen. Therefore, future studies are needed to assess genetic mutations associated with resistance mechanisms, monitor sensitivity changes over time, and determine whether continued exposure to sedaxane may select for resistant individuals in the *Rhizopus* spp. population.

The mean EC_{50} values of *R. delemar, R. stolonifer,* and *R. arrhizus* to fluopyram were 1.04, 1.90, and 1.13 µg/mL (Table 9), with the minimum and maximum EC_{50} values within species ranging from 0.39 – 1.2, 1.1 – 3.4, and 0.38 – 1.70 µg/mL, respectively (Table 10). The most sensitive isolate belonged to *R. arrhizus* (10A), with an EC_{50} value of 0.38 µg/mL. Across the chemical assays, Isolate 10A consistently exhibited some of the lowest EC_{50} values, which may suggest a lack of genetic change or gene expressions favoring resistance, such as fewer efflux pumps removing toxic compounds (Sanchez-Torres, 2021), fewer alternations in complex II of the respiratory electron transport chain in the succinate dehydrogenase enzyme (Amiri et al., 2014), or fewer detoxifying enzymes that degrade fungicides (Naqvi et al., 2025). Alternatively, this may also suggest reduced fitness, which is often characterized by slower growth and

reproduction (Zhan & McDonald, 2013). Isolate 45S, belonging to *R. stolonifer*, exhibited a relatively large EC_{50} value of 3.4 µg/mL, suggesting that not all *Rhizopus spp.* isolates are equally sensitive, and some may be more naturally tolerant to the fungicide. Future research is required to further investigate these possibilities. Similar EC_{50} values have been reported in other plant pathogens, such as *Fusarium virguliforme* and *Fusarium brasiliense* (Sang et al., 2018; Wang et al., 2017).

Excluding the EC_{50} values below the tested concentration range (< 0.01 µg/mL), the mean EC_{50} values of *R. delemar, R. stolonifer,* and *R. arrhizus* to carboxin were 0.021, 0.019, 0.014 µg/mL (Table 9), with the minimum and maximum EC_{50} values within species ranging from 0.015 – 0.031, 0.019, and 0.01 – 0.018 µg/mL, respectively (Table 10). Nine of the tested isolates exhibited EC_{50} estimates below the tested concentration range (< 0.01 µg/mL). Four, three, and two isolates belonged to *R. arrhizus* (10A, 17A, 22A, and 32A), *R. delemar* (3D, 13D, and 35D), and *R. stolonifer* (40S and 45S), respectively. These low EC_{50} estimates signify high sensitivity to carboxin; however, the exact EC_{50} values could not be determined. The least sensitive isolate belonged to *R. delemar* (30D), with an EC_{50} value of 0.031 µg/mL. Overall, the EC_{50} values across *Rhizopus* spp. isolates are consistently low, showing high sensitivity to carboxin.

The four succinate dehydrogenase inhibitor (SDHI) fungicides tested (carboxin, pydiflumetofen, fluopyram, and sedaxane) demonstrated various efficacy against the fifteen tested *Rhizopus* spp. isolates (Figures 13-16, respectively). Carboxin and pydiflumetofen offered the overall highest efficacy against *R. delemar, R. stolonifer,* and *R. arrhizus*, with consistently low EC_{50} values ranging from 0.021, 0.019, and 0.014 for carboxin and < 0.01, 0.041, and 0.017 µg/mL for pydiflumetofen, respectively. Compared to carboxin and pydiflumetofen, fluopyram offered moderate efficacy against *R. delemar, R. stolonifer*, and *R. arrhizus*, with EC_{50} values

ranging from 1.04, 1.90, and 1.13 µg/mL, respectively. Conversely, sedaxane offered significantly variable efficacy against *R. delemar, R. stolonifer*, and *R. arrhizus*, with EC_{50} values ranging from 4.3, 3.2, and 7.0 µg/mL, respectively. This variability in efficacy across the tested SHDI fungicides may suggest that carboxin and pydiflumetofen bind more effectively to the SDH enzyme, uptake more rapidly, or exhibit greater stability in the tested *Rhizopus* spp. isolates than fluopyram and sedaxane. As a result, greater and more consistent mycelial inhibition is offered.

The only phenylpyrrole fungicide tested, fludioxonil, offered consistently effective inhibition of *R. delemar, R. stolonifer,* and *R. arrhizus* at low concentrations, with mean EC_{50} values of 0.031, 0.030, and 0.042 µg/mL, respectively (Figure 17). Based on the results and previous literature, fludioxonil may be a valuable fungicide to incorporate into the fungicide management of *Rhizopus* spp (Sallato et al., 2017; Northover & Zhou, 2002).

The fifteen *Rhizopus* spp. isolates tested for the *in vitro* against azoxystrobin, mefenoxam, and ipconazole exhibited growth exceeding the highest fungicide concentration tested (>10.0 µg/mL), suggesting higher fungicide concentrations may be required to inhibit 50% fungal growth (Figure 18). Few studies have investigated these fungicides against *Rhizopus* spp., and contradictory results are evident in the literature over the past 25 years. In a 1998 study, rice seeds treated with wettable powder containing 6% ipconazole showed remarkably reduced mycelial mats of *Rhizopus* oryzae, improved germination rate, and decreased number of diseased seedlings. In the same study, *R. oryzae* isolates extracted from infected rice seeds were particularly sensitive to ipconazole, with EC_{90} values below 0.5 µg/mL (Tateishi et al., 1998). In a 2024 study, the *in vitro* chemical sensitivity of one *Rhizopus* sp. to combinations of ipconazole, azoxystrobin, and metalaxyl revealed the plates treated with azoxystrobin + carboxin + thiophanate-methyl + metalaxyl reached maximum growth (100%) after 96 hours, and the plates

treated with metalaxyl + carboxin + ipconazole and ipconazole + metalaxyl + carboxin + thiram showed no fungal growth. However, crucial information was omitted from the report, such as the final fungicide concentrations, EC_{50} values, and the evaluations of each active ingredient separately, which limits the ability to assess the individual efficacy of each fungicide. The 25-year gap between these two tests with contradictory results opens the possibility that shifts in sensitivity or resistance development may have occurred in *Rhizopus* spp. Furthermore, these three fungicides are commonly used in commercial seed treatment fungicides, such as Trebuset® (Syngenta Crop Protection, Greensboro, NC) and Rancona VPD and VPL® (UPL Corporation Limited Group Company, King of Prussia, PA). The heavy reliance on these seed treatments fungicides may have selected populations of resistant fungal pathogens, possibly including *Rhizopus* spp. This occurrence in other fungal pathogens, such as *Aspergillus* spp., is supported by the literature, with serious field resistance reported to azoxystrobin (Zhang et al., 2009; Inoue et al., 2011; Bartlett et al., 2002). Consequently, seed treatment fungicides containing azoxystrobin have shown reduced efficacy over time (Jordan et al., 2019; Ali et al., 2021; Brenneman, 2021).

Overall, the results provide valuable insights into the efficacy of fungicides managing *Rhizopus* spp., addressing some of the knowledge gaps within the literature. Fludioxonil, carboxin, and pydiflumetofen provided consistently high efficacy against *Rhizopus* spp., making these fungicides excellent candidates for incorporating into the management of *Rhizopus* spp. in peanut seeds. Fluopyram showed moderate efficacy, while sedaxane exhibited significantly variable sensitivity among the tested *Rhizopus* spp. isolates. This suggests that sedaxane may be used cautiously, as it may not provide consistent efficacy against *Rhizopus* spp. The unanimous lack of inhibition by azoxystrobin, mefenoxam, and ipconazole within the tested concentration range emphasizes the need to broaden the range for future assays and carefully monitor for

resistance development. Future research should also monitor and investigate the potential for genetic resistance to these fungicides.

PRACTICAL IMPLICATIONS

In summary, these studies revealed fludioxonil, carboxin, and pydiflumetofen provided the most effective inhibition of *Rhizopus* spp. The lack of inhibition by azoxystrobin, mefenoxam, and ipconazole at the highest tested fungicide underscores the need for future research to broaden the tested concentration range and monitor for resistance development. Additionally, both temperature assays revealed that *R. stolonifer* was less thermotolerant and virulent at higher temperatures than *R. delemar* and *R. arrhizus,* which achieved maximum growth between 24 to 48 hours and high virulence on peanut seeds across all temperature conditions.

Tables

Table 6. Mean Diameter Growth Parameters of Three *Rhizopus* spp. at 15 °C, 20 °C, and 25 °C, and *R. stolonifer* at 30 °C, Based on the Gompertz Three-Parameter Model.[a,b]

	a[c]	*b*[d]	*c*[e]	R^2
Temperature (15 °C)	%	slope	Hours	
Species				
R. arrhizus	98.7 a	31.6 a	65.6 g	0.98
R. delemar	98.8 a	32.7 a	69 g	0.96
R. stolonifer	100 a	22.1 b	52.4 f	0.87
Temperature (20 °C)				
R. arrhizus	100 a	13.19 a	34.4 d	0.98
R. delemar	100 a	13.18 a	37.5 e	0.98
R. stolonifer	100 a	14.1 a	31.9 c	0.95
Temperature (25 °C)				
R. arrhizus	100 a	6.1 e	25.4 a	0.98
R. delemar	100 a	6.1 e	25.3 a	0.96
R. stolonifer	100 a	10.7 d	24.7 a	0.92
Temperature (30 °C)				
R. stolonifer	100 a	15.6 c	28.8 b	0.87

[a]The Gompertz three-parameter model $y = a \times e^{-e^{-b(x-c)}}$ was used to generate parameters *a*, *b*, and *c*, where *x* = radial growth rate. Values within a column followed by the same letter are not significantly different at the α = 0.05 probability level according to the 95% confidence intervals.

[b]Accurate model fitting and parameter estimates for *R. delemar* and *R. arrhizus* at 30 °C and 35 °C could not be obtained due to rapid growth between 24-hour intervals. *R. stolonifer* at 35 °C exhibited no growth, making parameter estimates unobtainable.

[c]Maximum radial growth.

[d]Slope.

Table 7. Germination and Radicle Length of 'Georgia-06G' Peanut Seeds Inoculated with *Rhizopus* spp. at Day Eight Across Temperatures with Comparisons Made by *Rhizopus* Species[a,b]

Seed Germination (%)	Temperature (°C)				
Treatment	15 °C	20 °C	25 °C	30 °C	35 °C
Control	77.7 a	93.0 a	100 a	95.8 a	97.2 a
R. delemar	11.1 b	7.6 b	8.3 c	9.0 c	11.1 c
R. stolonifer	4.1 bc	13.8 b	27.7 b	40.2 b	72.2 b
R. arrhzius	2.7 c	9.0 b	7.6 c	12.4 c	6.2 c
Radicle Protrusion ≥ 10 mm (%)					
Control	75 a	91.6 a	100 a	93.0 a	97.2 a
R. delemar	0 b	0.6 b	0 c	0.6 c	1.3 c
R. stolonifer	0 b	0 b	8.3 b	37.5 b	63.8 b
R. arrhzius	0 b	2.0 b	0 c	0 c	0 c
Radicle Protrusion ≤ 10 mm (%)					
Control	2.7 b	1.3 b	0 c	0 c	0 b
R. delemar	11.1 a	6.9 ab	8.3 b	8.3 ab	9.7 a
R. stolonifer	4.1 b	13.8 a	9.4 a	2.7 bc	8.3 a
R. arrhzius	2.7 b	6.9 ab	7.6 bc	12.5 a	6.2 ab

[a]Means in a temperature condition followed by a common letter are not significantly different according to the least significant difference (LSD) at $P \leq 0.05$.

[b]Values are means of four replications, each consisting of 12 seeds per treatment at each temperature.

Table 8. Germination and Radicle Length of 'Georgia-06G' Peanut Seeds Inoculated with *Rhizopus* spp. at Day Eight Across Temperatures with Comparisons Made by Temperature[a, b]

Seed Germination (%)	*Rhizopus* spp.			
Temperature	*R. delemar*	*R. arrhizus*	*R. stolonifer*	Control
15 °C	11.1 a	2.9 b	4.2 c	77.8 b
20 °C	7.7 a	9.0 ab	13.8 cd	93.0 a
25 °C	8.4 a	7.7 ab	27.8 bc	100 a
30 °C	9.0 a	12.5 a	40.3 b	93.0 a
35 °C	11.1 a	6.3 ab	72.2 a	97.8 a
Radicle Protrusion ≥ 10 mm (%)				
15 °C	0 a	0 b	0 c	75 b
20 °C	0.8 a	2.0 a	0 c	91.6 a
25 °C	0 a	0 b	8.4 c	100 a
30 °C	0.6 a	0 b	37.5 b	93.0 a
35 °C	1.4 a	0 b	63.8 a	97.8 a
Radicle Protrusion ≤ 10 mm (%)				
15 °C	11.1 a	2.9 b	4.2 bc	2.8 a
20 °C	6.9 a	7.0 ab	13.8 ab	1.4 a
25 °C	8.4 a	7.7 ab	19.4 a	0 a
30 °C	8.4 a	12.5 a	2.8 c	0 a
35 °C	9.7 a	6.3 ab	8.4 bc	0 a

[a]Means in a species or non-treated group followed by a common letter are not significantly different according to the least significant difference (LSD) at $P \leq 0.05$.

[b]Values are means of four replications, each consisting of 12 seeds per treatment at each temperature.

Table 9. Mean EC_{50} Values (µg/mL) of Five Seed Treatment Fungicides Against Three *Rhizopus* spp.[a]

	Mean EC_{50} (µg/mL)				
Species	Fludioxonil[b]	Pydiflumetofen	Sedaxane	Fluopyram	Carboxin
R. delemar	0.031 a[c]	< 0.01[d]	4.3 a[e]	1.04 a[f]	0.021 a[g]
R. stolonifer	0.030 a	0.041 b	7.0 b	1.90 a	0.019 a
R. arrhzius	0.042 a	0.017 a	3.2 a	1.13 a	0.014 a

[a]Least squares mean for each species was calculated and Tukey's multiple comparison adjustment (P = 0.05) was used to compare groups. To avoid extrapolation, isolates with EC_{50} values beyond the tested concentration range were excluded from mean comparisons.

[b]*Rhizopus* spp. sharing the same letter in a column are not significantly different in their EC_{50} values.

[c]Means represent the averages of six, one, and five *R. delemar, R. stolonifer,* and *R. arrhizus* isolates, respectively.

[d]Means represent the averages of two isolates each of *R. stolonifer* and *R. arrhizus*. Mean EC_{50} values for *R. delemar* were not statistically compared because all observed values were below the lowest tested fungicide concentration (< 0.01 µg/mL).

[e]Means represent the averages of six, two, and one *R. delemar, R. stolonifer,* and *R. arrhizus* isolates, respectively.

[f]Means represent the averages of six, three, and six *R. delemar, R. stolonifer,* and *R. arrhizus* isolates, respectively.

[g]Means represent the averages of three, one, and two *R. delemar, R. stolonifer,* and *R. arrhizus* isolates, respectively.

Table 10. Mean EC_{50} Values (µg/mL) of Five Seed Treatment Fungicides Against Fifteen *Rhizopus* spp. Isolates[g,h]

		Mean EC_{50} (µg/mL)				
Isolate[a]	Species	Flud.[b]	Pyd.[c]	Sed.[d]	Fluo.[e]	Car.[f]
10A	*R. arrhizus*	< 0.01	0.015 a	1.9 a	0.38 a	< 0.01
17A	*R. arrhizus*	0.032 bcd	< 0.01	>10.0	1.5 bc	< 0.01
22A	*R. arrhizus*	0.012 a	0.020 a	>10.0	1.6 c	< 0.01
32A	*R. arrhizus*	0.069 e	< 0.01	>10.0	1.7 c	< 0.01
34A	*R. arrhizus*	0.072 e	< 0.01	4.5 b	1.1 bc	0.01 a
41A	*R. arrhizus*	0.025 bc	< 0.01	>10.0	0.90 bc	0.018 b
3D	*R. delemar*	0.01 a	< 0.01	1.9 a	0.39 b	< 0.01
4D	*R. delemar*	0.018 ab	< 0.01	4.5 b	0.83 bc	0.017 ab
13D	*R. delemar*	0.032 bcd	< 0.01	5.2 b	1.8 c	< 0.01
18D	*R. delemar*	0.029 bcd	< 0.01	5.6 b	0.93 bc	0.015 ab
30D	*R. delemar*	0.044 cde	< 0.01	6.1 b	0.90 bc	0.031 c
35D	*R. delemar*	0.051 de	< 0.01	3.0 ab	1.2 bc	< 0.01
39S	*R. stolonifer*	0.030 bcd	< 0.01	7.0 b	0.94 bc	0.019 bc
40S	*R. stolonifer*	< 0.01	0.032 a	>10.0	1.2 bc	< 0.01
45S	*R. stolonifer*	< 0.01	0.050 a	>10.0	3.4 c	< 0.01

[a]Letters A, D, and S represent *R. delemar, R. arrhizus,* and *R. stolonifer,* respectively.

[b]Fludioxonil

[c]Pydiflumetofen

[d]Sedaxane

[e]Fluopyram

[f]Carboxin

[g]Least squares mean for each isolate was calculated and Tukey's multiple comparison adjustment (P = 0.05) was used to compare groups.

[h]*Rhizopus* spp. isolates sharing the same letter in a column are not significantly different in their EC_{50} values.

Figures:

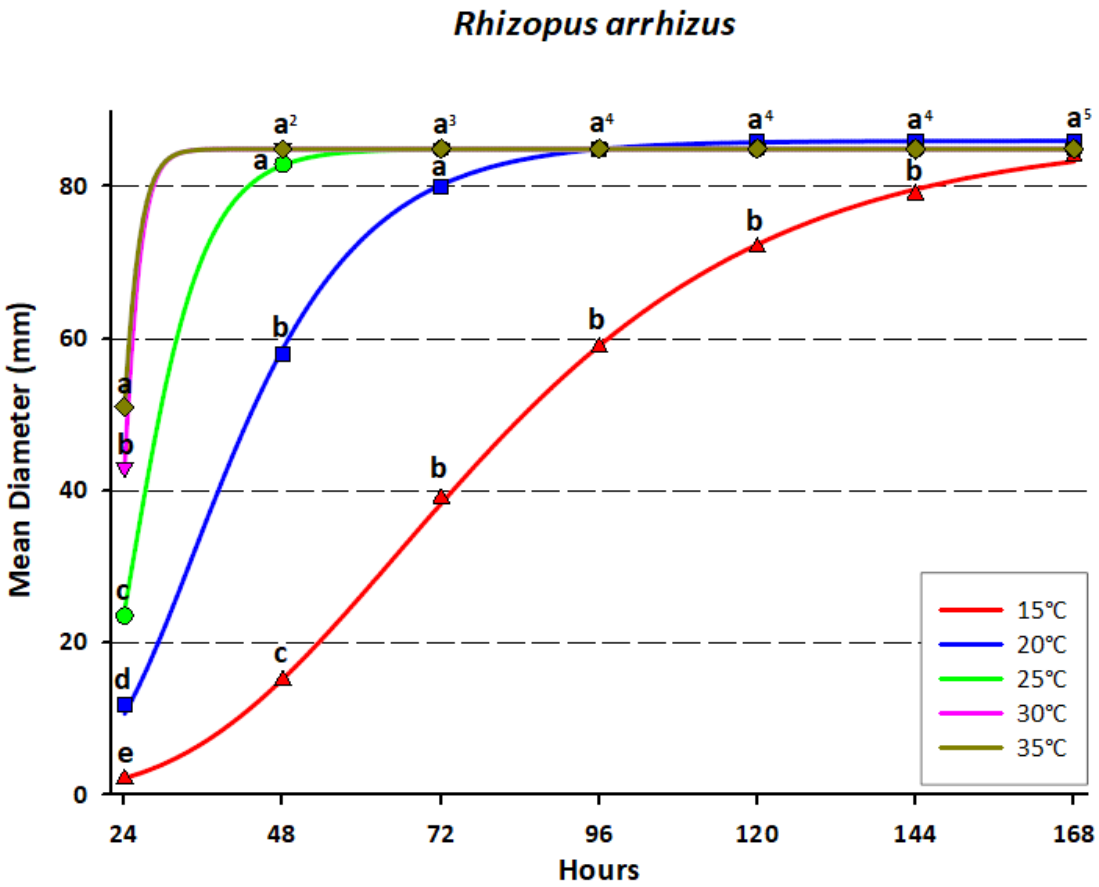

Figure 5. Effect of Temperature on the Mycelial Radial Growth of *Rhizopus arrhizus*[a,b].

[a]The significance of each 24-hour interval across temperature was assessed using the Least Significant Difference (LSD) test $P \leq 0.05$. Mean values sharing a common letter within the same 24-hour interval are not significantly different.

[b]Superscripts with a common letter (e.g., a^2, a^3, a^4, and a^5) represent a cluster of points at the same 24-hour interval with similar significance to one another.

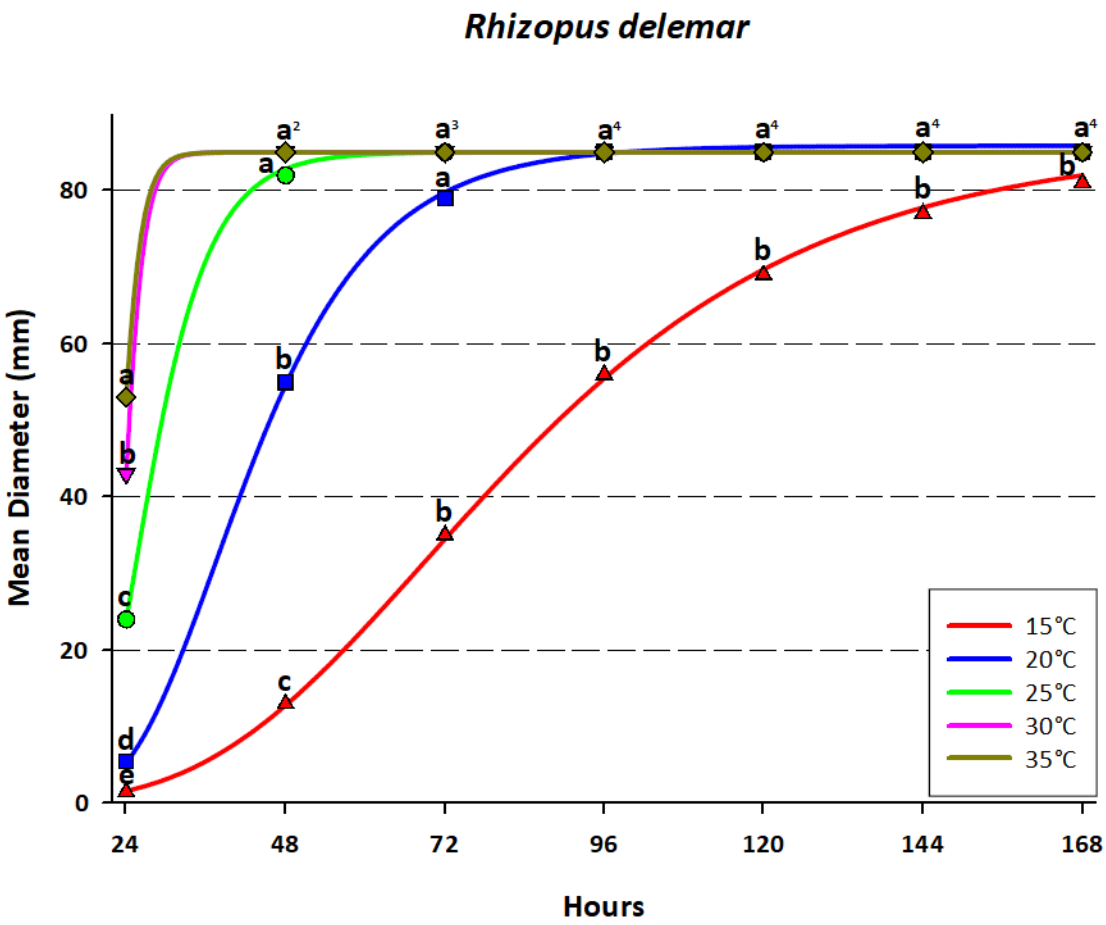

Figure 6. Effect of Temperature on the Mycelial Radial Growth of *Rhizopus delemar*[a,b].

[a]The significance of each 24-hour interval across temperature was assessed using the Least Significant Difference (LSD) test $P \leq 0.05$. Mean values sharing a common letter within the same 24-hour interval are not significantly different.

[b]Superscripts with a common letter (e.g., a^2, a^3, and a^4) represent a cluster of points at the same 24-hour interval with similar significance to one another.

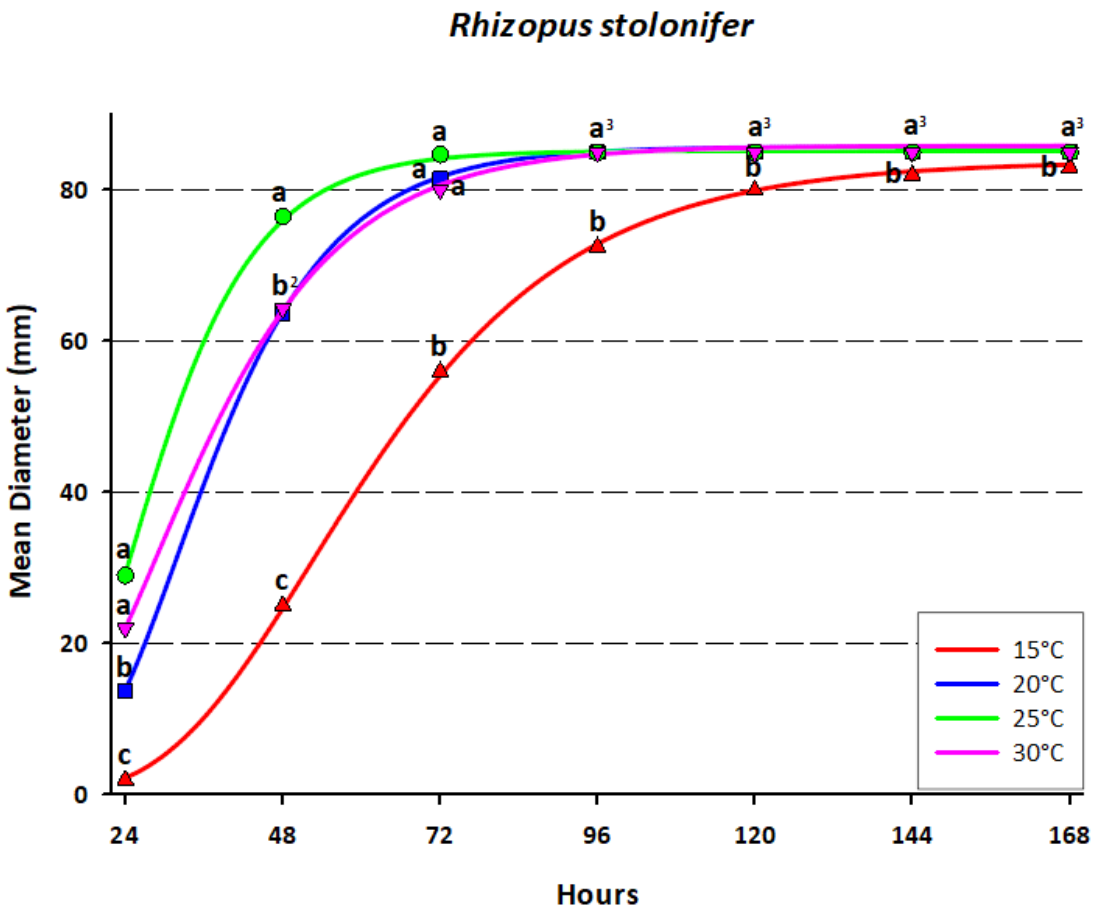

Figure 7. Effect of Temperature on the Mycelial Radial Growth of *Rhizopus stolonifer* [a.b].

[a]The significance of each 24-hour interval across temperature was assessed using the Least Significant Difference (LSD) test $P \leq 0.05$. Mean values sharing a common letter within the same 24-hour interval are not significantly different.

[b]Superscripts with a common letter (e.g., b^2 and a^3) represent a cluster of points at the same 24-hour interval with similar significance to one another.

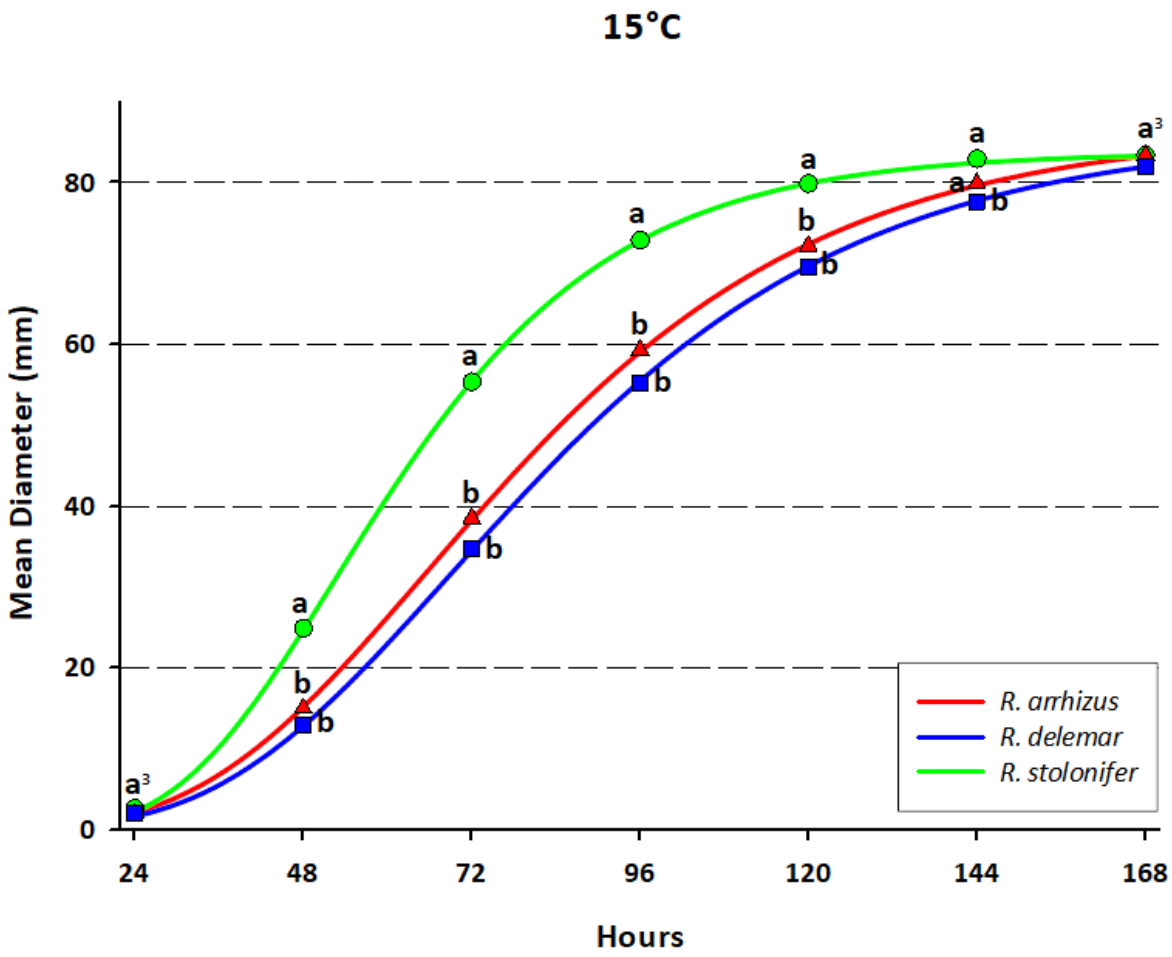

Figure 8. Mycelial Radial Growth Response of Three *Rhizopus* Spp. at 15 °C [a.b].

[a]The significance of each 24-hour interval across species was assessed using the Least Significant Difference (LSD) test $P \leq 0.05$. Mean values sharing a common letter within the same 24-hour interval are not significantly different.

[b]Superscripts with a common letter (e.g., a^3) represent a cluster of points at the same 24-hour interval with similar significance to one another.

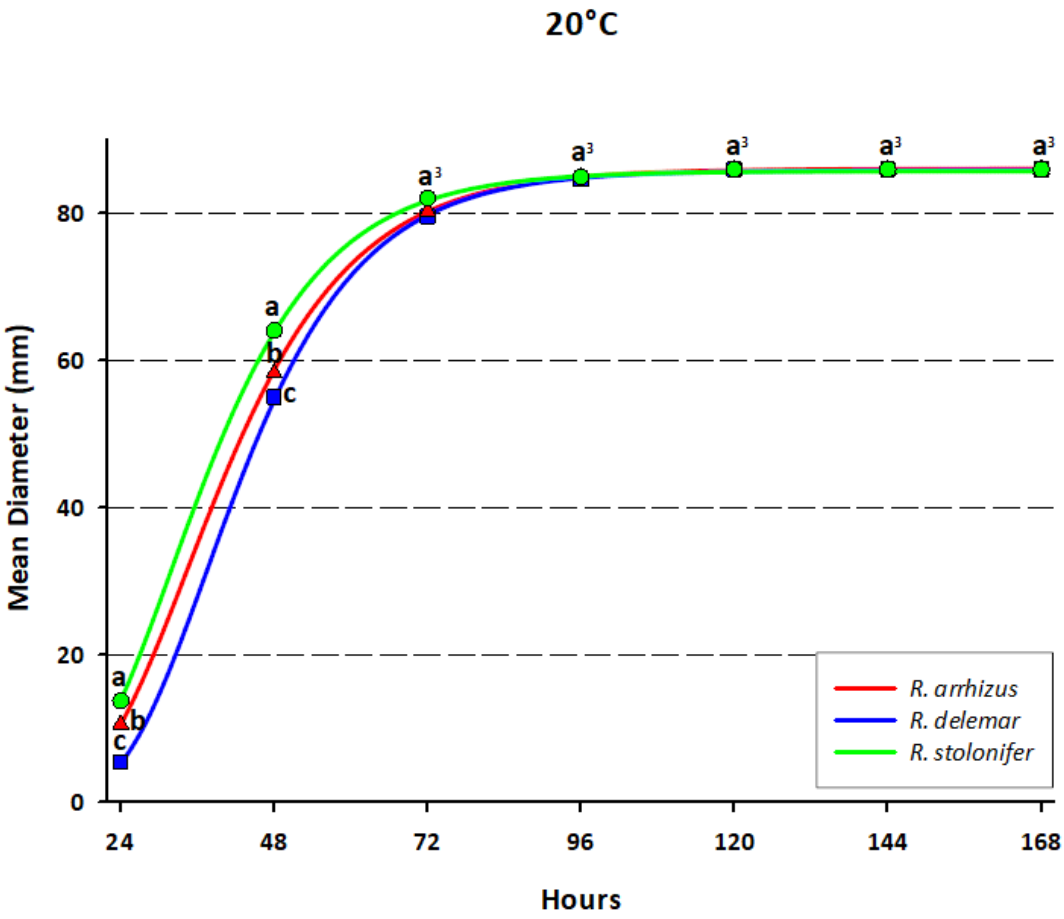

Figure 9. Mycelial Radial Growth Response of Three *Rhizopus* Spp. at 20 °C [a.b].

[a]The significance of each 24-hour interval across species was assessed using the Least Significant Difference (LSD) test $P \leq 0.05$. Mean values sharing a common letter within the same 24-hour interval are not significantly different.

[b]Superscripts with a common letter (e.g., a^3) represent a cluster of points at the same 24-hour interval with similar significance to one another.

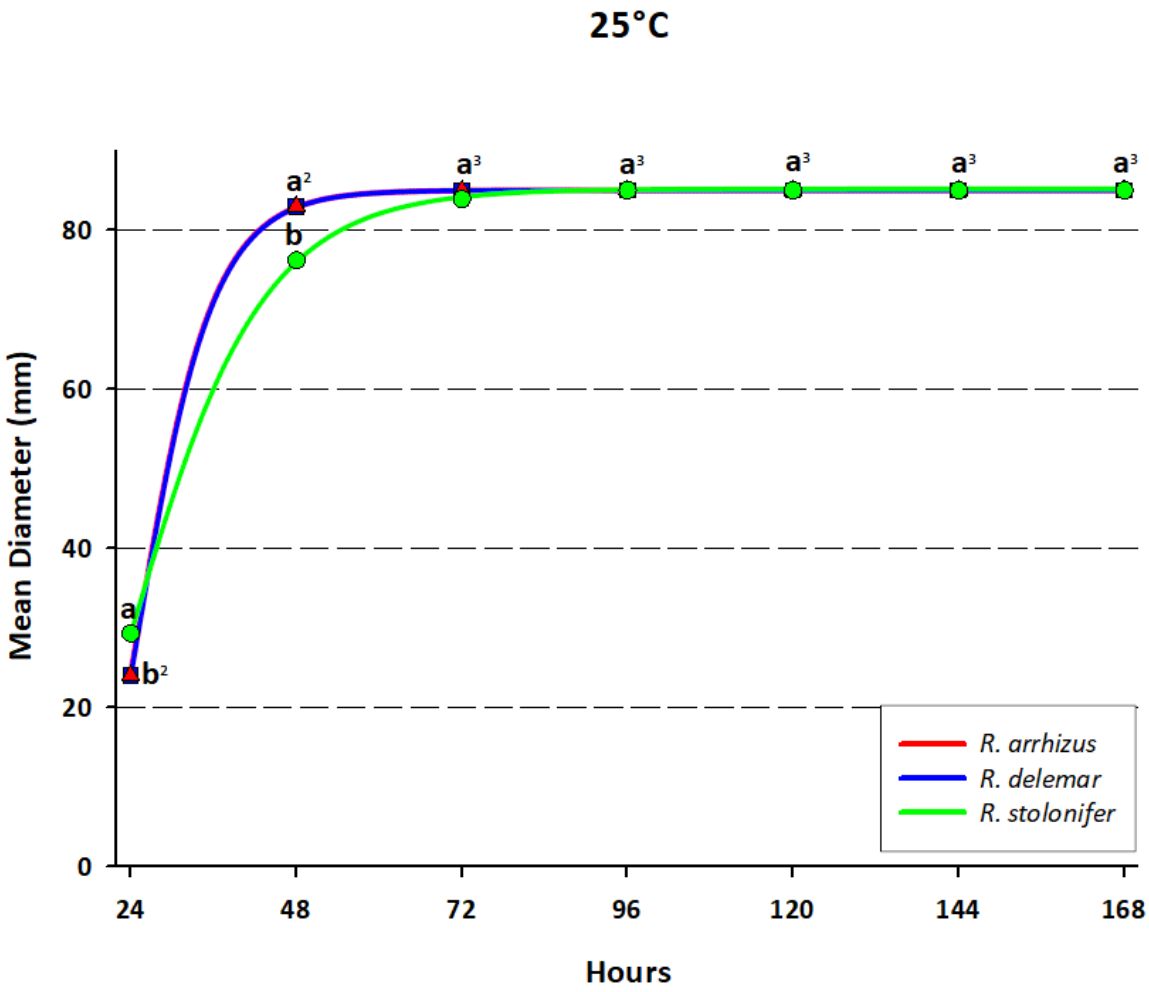

Figure 10. Mycelial Radial Growth Response of Three *Rhizopus* Spp. at 25 °C [a.b].

[a]The significance of each 24-hour interval across species was assessed using the Least Significant Difference (LSD) test $P \leq 0.05$. Mean values sharing a common letter within the same 24-hour interval are not significantly different.

[b]Superscripts with a common letter (e.g., a^3, a^2, and b^2) represent a cluster of points at the same 24-hour interval with similar significance to one another.

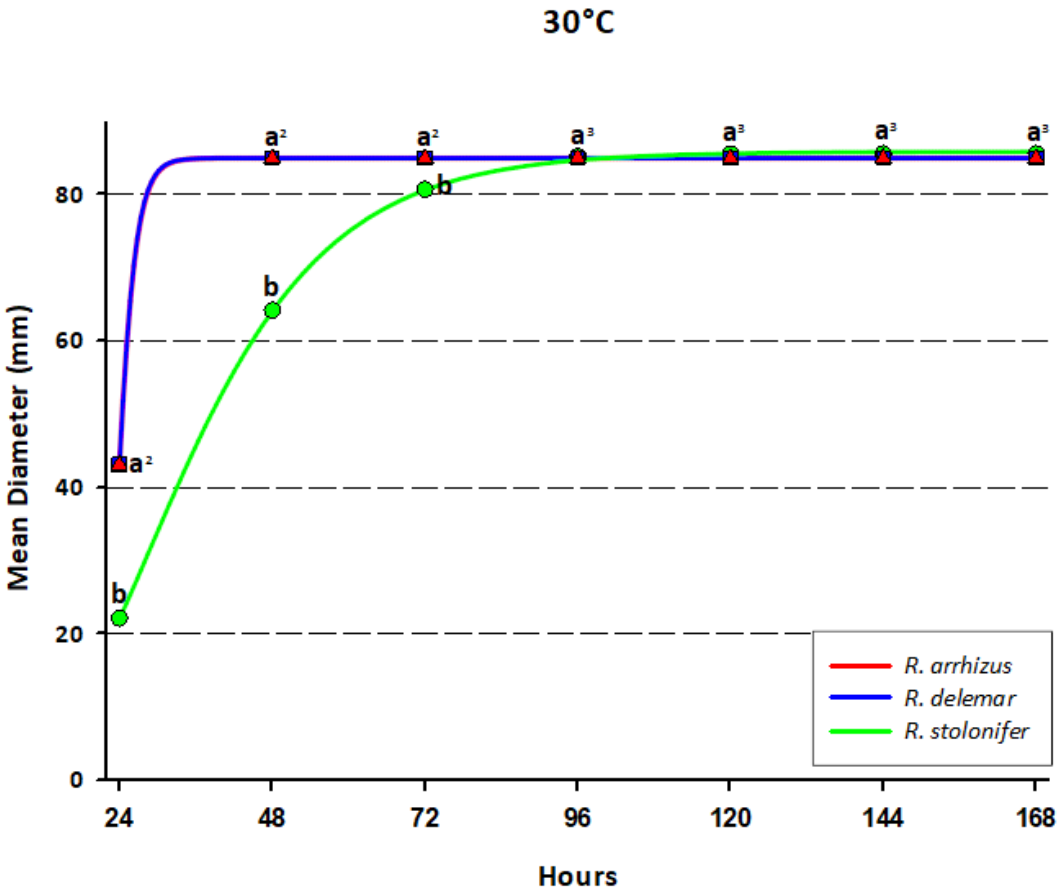

Figure 11. Mycelial Radial Growth Response of Three *Rhizopus* Spp. at 30 °C [a.b].

[a]The significance of each 24-hour interval across species was assessed using the Least Significant Difference (LSD) test $P \leq 0.05$. Mean values sharing a common letter within the same 24-hour interval are not significantly different.

[b]Superscripts with a common letter (e.g. a^3 and a^2) represent a cluster of points at the same 24-hour interval with similar significance to one another.

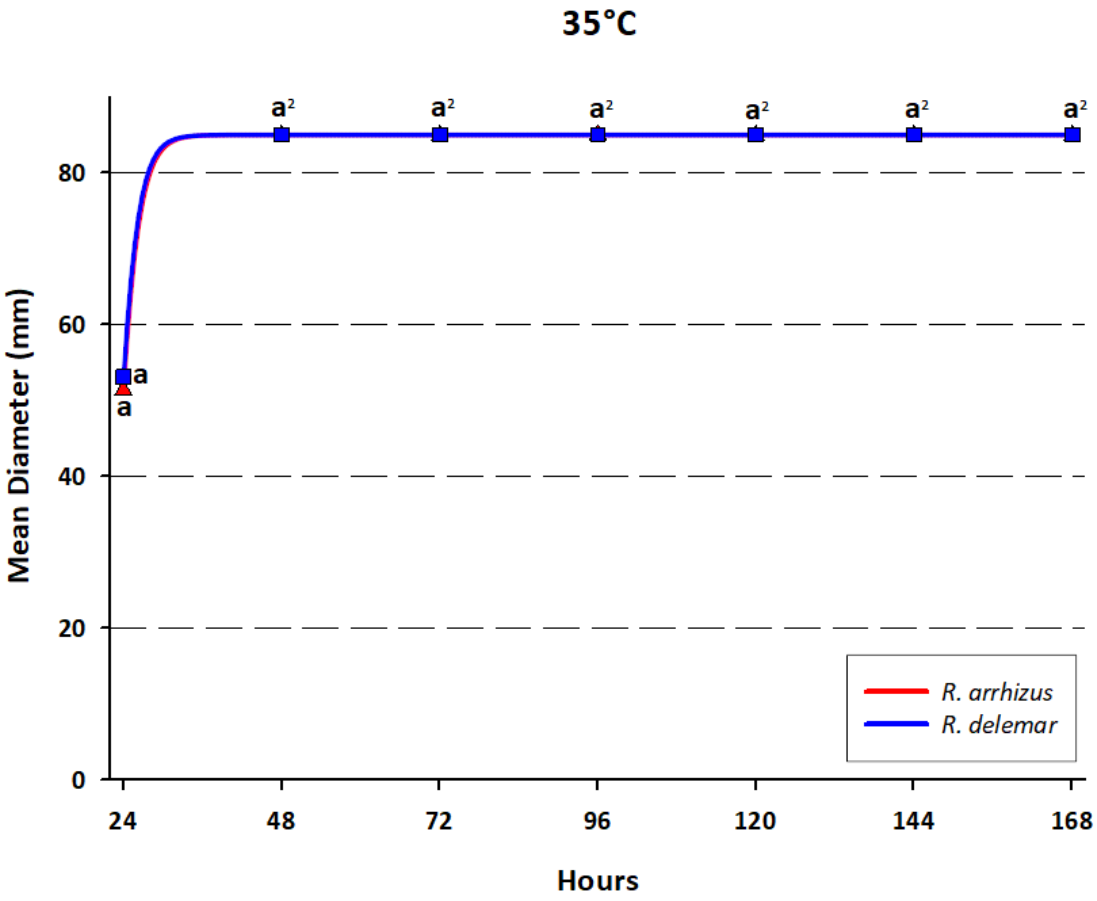

Figure 12. Mycelial Radial Growth Response of *R. delemar* and *R. arrhizus* at 35 °C [a.b]

[a]The significance of each 24-hour interval across species was assessed using the Least Significant Difference (LSD) test $P \leq 0.05$. Mean values sharing a common letter within the same 24-hour interval are not significantly different.

[b]Superscripts with a common letter (e.g., a^2) represent a cluster of points at the same 24-hour interval with similar significance to one another.

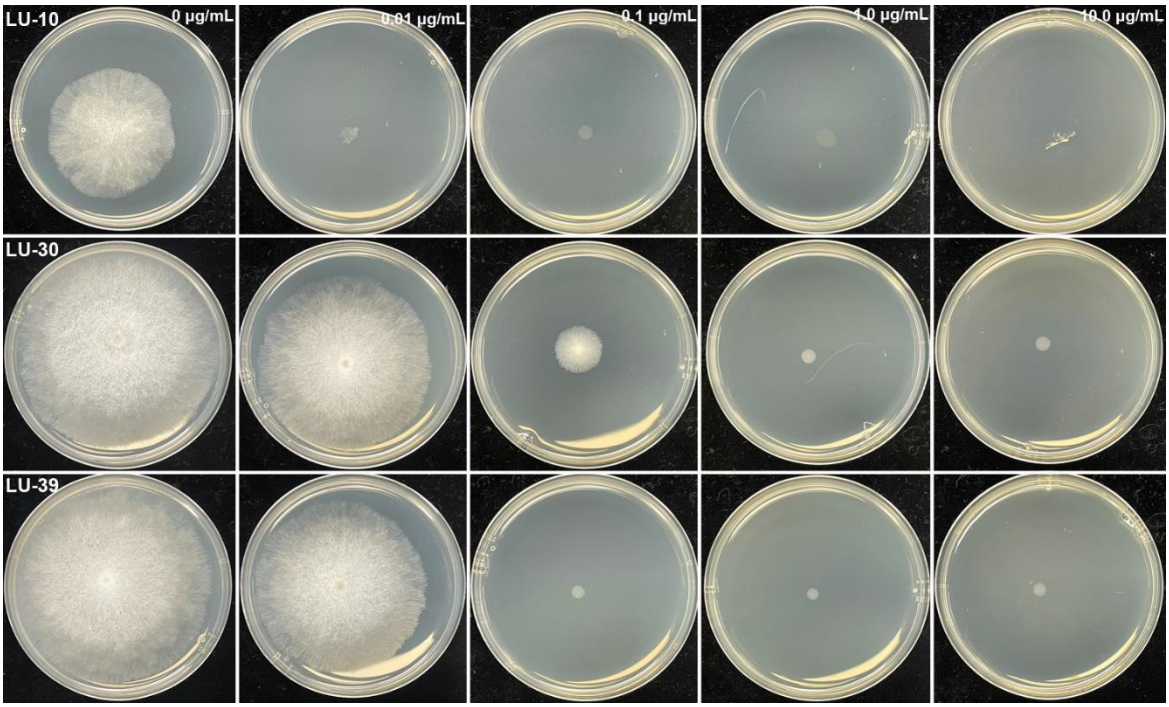

Figure 13. Mycelial growth inhibition of three *Rhizopus* spp. isolated from peanut seeds, exposed to concentrations of carboxin (0, 0.01, 0.1, 1, and 10 µg/mL). Concentrations increase from left (0 ug/mL) to right (10 ug/mL). The top row (LU-10, Isolate 10A) belongs to *R. arrhizus*. The middle row (LU-30, Isolate 30D) belongs to *R. delemar.* The bottom row (LU-39, Isolate 39S belongs to *R. stolonifer*.

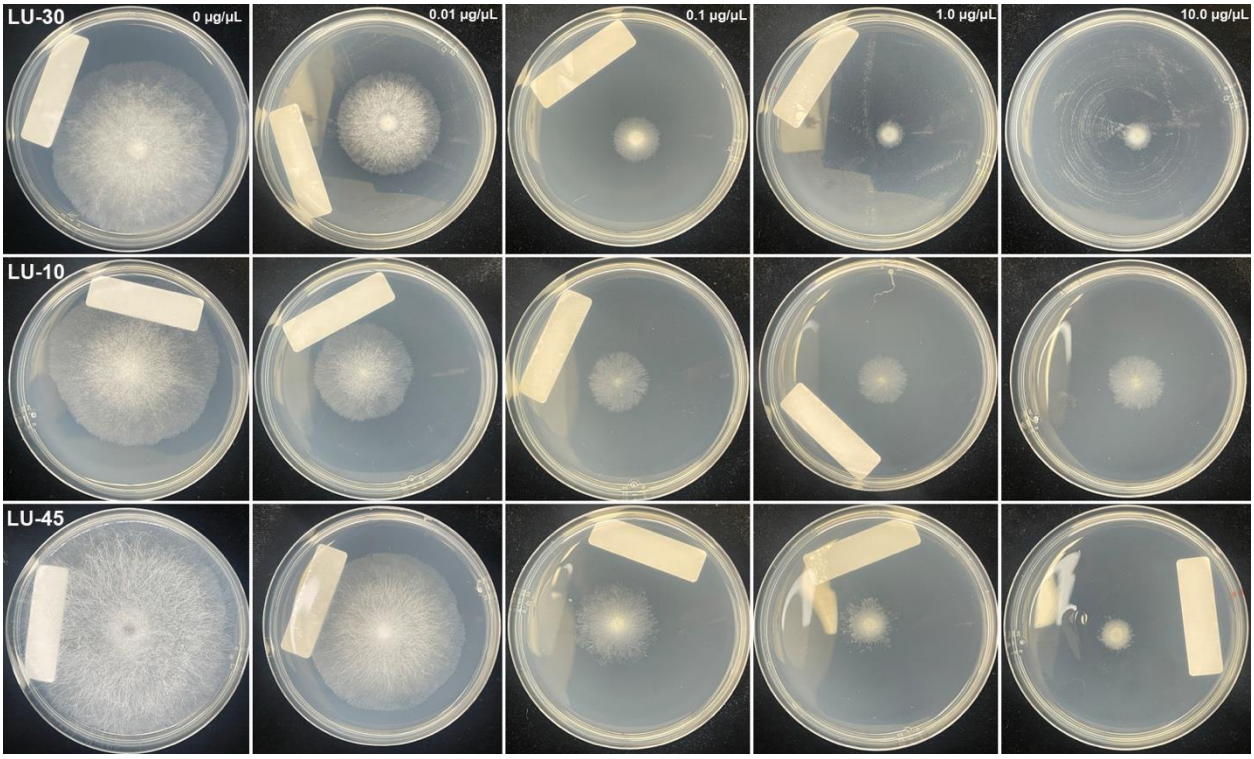

Figure 14. Mycelial growth inhibition of three *Rhizopus* spp. isolated from peanut seeds, exposed to concentrations of pydiflumetofen (0, 0.01, 0.1, 1, and 10 µg/mL). Concentrations increase from left (0 ug/mL) to right (10 ug/mL). The top row (LU-10, Isolate 10A) belongs to *R. arrhizus*. The middle row (LU-30, Isolate 30D) belongs to *R. delemar.* The bottom row (LU-39, Isolate 39S belongs to *R. stolonifer*.

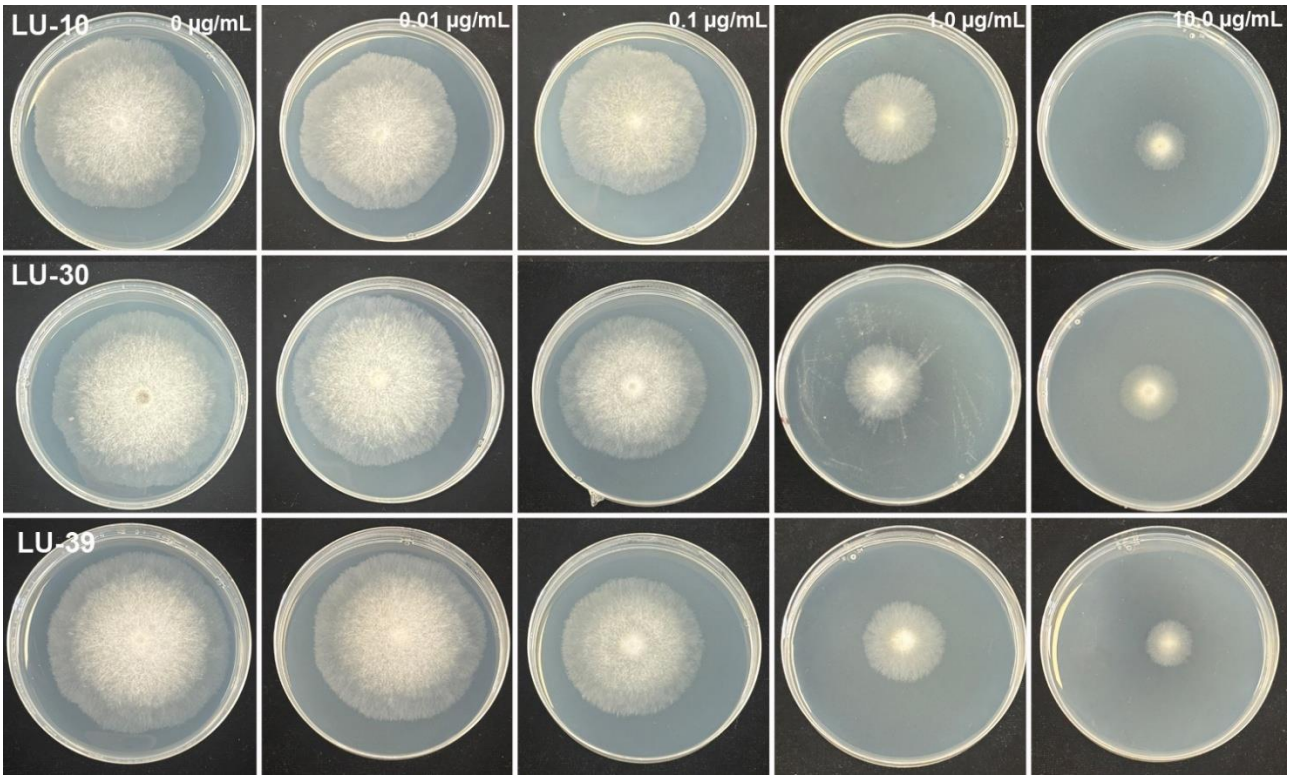

Figure 15. Mycelial growth inhibition of three *Rhizopus* spp. isolated from peanut seeds, exposed to concentrations of fluopyram (0, 0.01, 0.1, 1, and 10 µg/mL). Concentrations increase from left (0 ug/mL) to right (10 ug/mL). The top row (LU-10, Isolate 10A) belongs to *R. arrhizus*. The middle row (LU-30, Isolate 30D) belongs to *R. delemar*. The bottom row (LU-39, Isolate 39S belongs to *R. stolonifer*.

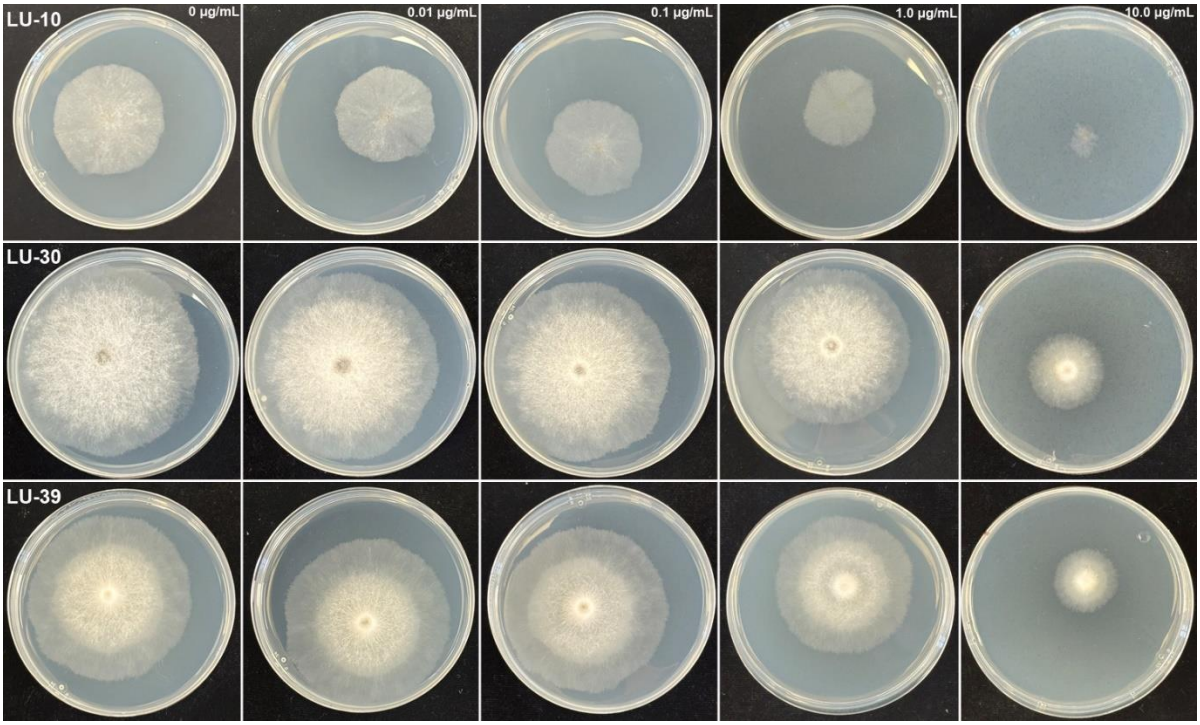

Figure 16. Mycelial growth inhibition of three *Rhizopus* spp. isolated from peanut seeds, exposed to concentrations of sedaxane (0, 0.01, 0.1, 1, and 10 µg/mL). Concentrations increase from left (0 ug/mL) to right (10 ug/mL). The top row (LU-10, Isolate 10A) belongs to *R. arrhizus*. The middle row (LU-30, Isolate 30D) belongs to *R. delemar*. The bottom row (LU-39, Isolate 39S belongs to *R. stolonifer*.

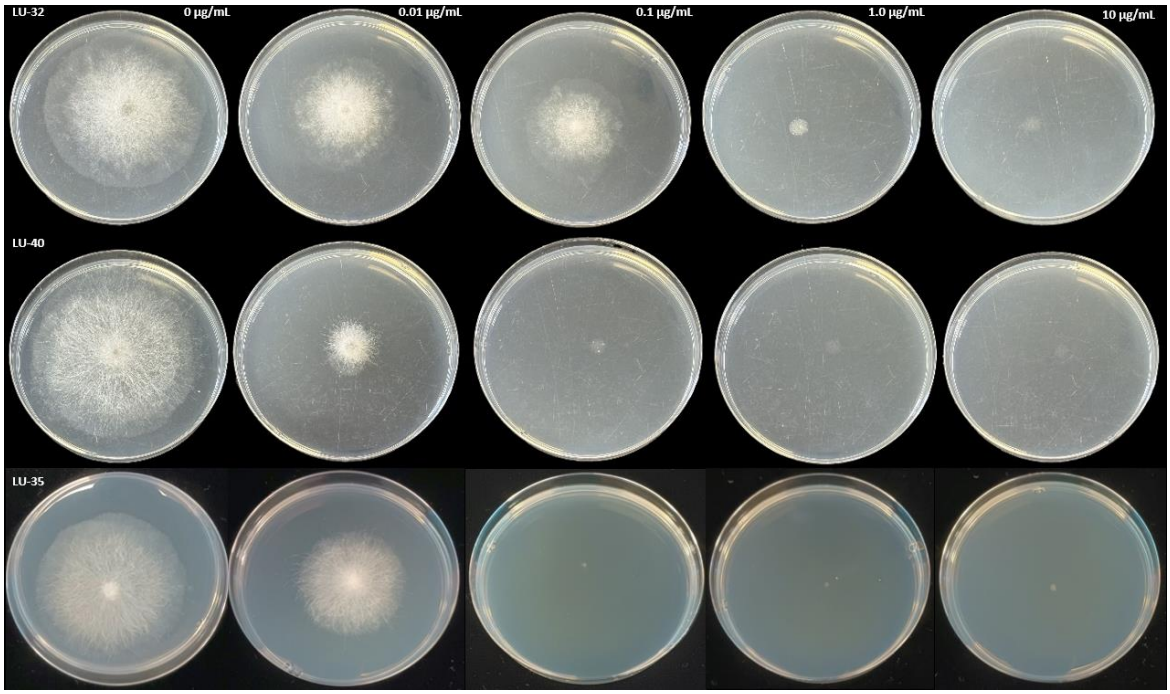

Figure 17. Mycelial growth inhibition of three *Rhizopus* spp. isolated from peanut seeds, exposed to concentrations of fludioxonil (0, 0.01, 0.1, 1, and 10 µg/mL). Concentrations increase from left (0 ug/mL) to right (10 ug/mL). The top row (LU-10, Isolate 10A) belongs to *R. arrhizus*. The middle row (LU-30, Isolate 30D) belongs to *R. delemar.* The bottom row (LU-39, Isolate 39S belongs to *R. stolonifer*.

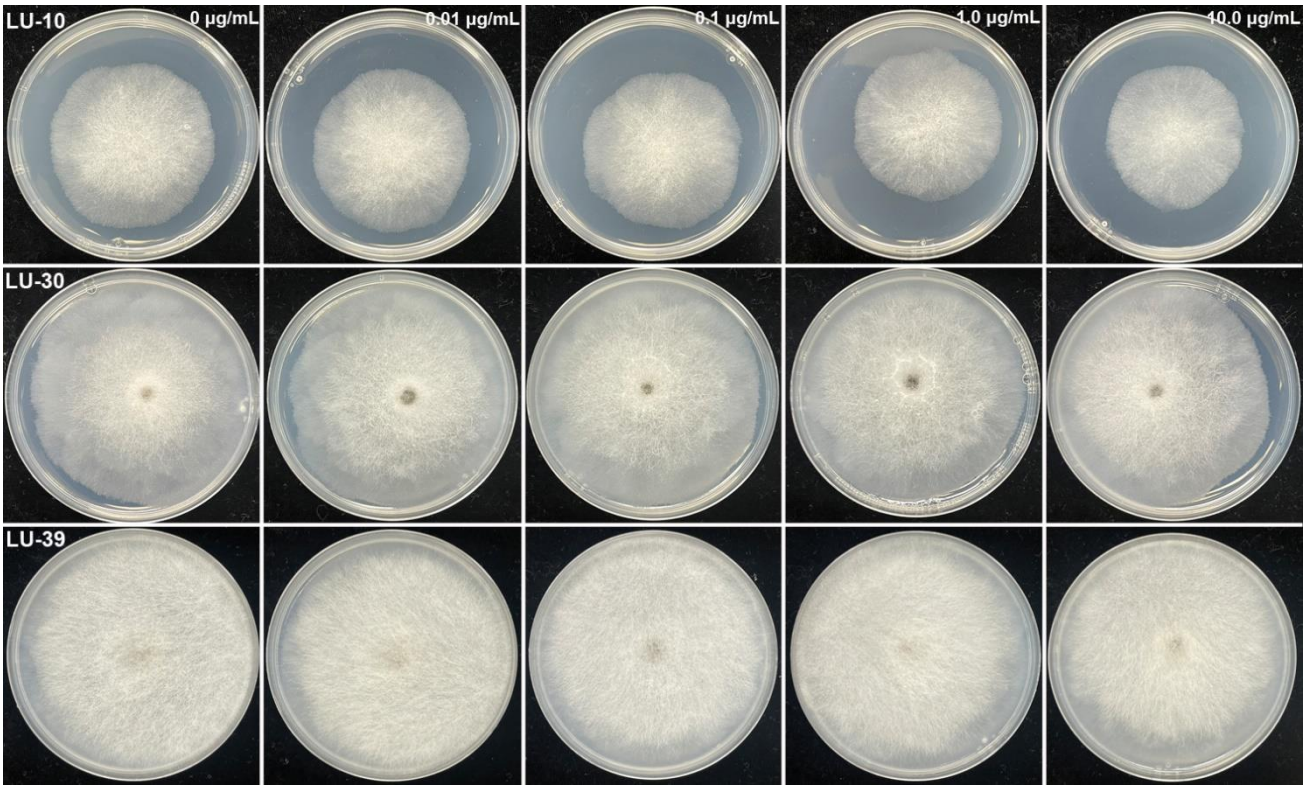

Figure 18. Mycelial growth inhibition of three *Rhizopus* spp. isolated from peanut seeds, exposed to concentrations of ipconazole (0, 0.01, 0.1, 1, and 10 µg/mL). Concentrations increase from left (0 ug/mL) to right (10 ug/mL). The top row (LU-10, Isolate 10A) belongs to *R. arrhizus*. The middle row (LU-30, Isolate 30D) belongs to *R. delemar.* The bottom row (LU-39, Isolate 39S belongs to *R. stolonifer*.

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