ABSTRACT

FUNGI AND ‘FUNGAL-LIKE’ ORGANISMS ASSOCIATED WITH ROOT SYSTEMS OF CUCURBITA MAXIMA AND THE SURROUNDING RHIZOSPHERE

by Jonathan Daniel Hulse

Cucurbita maxima is an incredibly diverse species, and it is suggested to have more cultivated forms than any other crop. It has many medicinal uses including anti-diabetic, anti-oxidant, anticancer, and anti-inflammatory properties. It is also a major food source for wide variety of world cultures, due to its fiber content, carbohydrates, β-carotene, vitamins, alkaloids, minerals, fatty acids, flavonoids, and polysaccharides. Detection of root-associated microbes in C. maxima has not been well documented in the scientific literature. A multi-phase approach was implemented to first verify fungal associations in C. maxima, and then second, to document the colonization of fungi in C. maxima grown in a conventional agricultural system. Cucurbita maxima grown in southwest Ohio showed relationships with members of the Ascomycota, Basidiomycota, Chytridomycota, Glomeromycota, Dark Septate Endophytes, and Plasmodiophorid Slime Molds. This study provides a first report of a relationship between Dark Septate Endophytes, Glomeromycota, Olpidium spp., and Plasmodiophorid Slime Molds in C. maxima in the United States. The images provided in this manuscript are the first photographic documentation of these organisms in C. maxima to date.
FUNGI AND ‘FUNGAL-LIKE’ ORGANISMS ASSOCIATED WITH ROOT SYSTEMS OF *CUCURBITA MAXIMA* AND THE SURROUNDING RHIZOSPHERE

Thesis

Submitted to the

Faculty of Miami University

in partial fulfillment of

the requirements for the degree of

Masters of Science

by

Jonathan Daniel Hulse

Miami University

Oxford, Ohio

2016

Advisor: Michael Vincent

©2016 Jonathan Daniel Hulse
This thesis titled

FUNGI AND ‘ FUNGAL-LIKE’ ORGANISMS ASSOCIATED WITH ROOT SYSTEMS OF CUCURBITA MAXIMA AND THE SURROUNDING RHIZOSPHERE

by

Jonathan Daniel Hulse

has been approved for publication by

College of Arts and Science

and

Department of Biology

____________________________________________________
Michael Vincent

____________________________________________________
James Hickey

____________________________________________________
Nicholas Money
# Table of Contents

Abstract

Title Page

Committee Signatures

Table of Contents

List of Tables

List of Figures

Dedication

Acknowledgments

Introduction

Methods

Results

Discussion

Conclusion

Tables and Figures

Literature Cited
List of Tables

Table 1: Presence of fungi and 'fungal like organisms' associated with different cultivars of *Cucurbita maxima* sampled during Phase I in September 2014, and Phase II in July, August, September 2015 in the eastern United States.

Table 2: *Cucurbita maxima* cv. Burgess Buttercup (BB), Rouge Vif d'Etampes (RD), Mariana de Chioggia (MC), and Golden Hubbard(GH) analyzed for Mean Percent Colonization, Percent Colonization Standard Error, and Percent colonized Standard Deviation.

Table 3: Analysis of Variance (ANOVA) showing significant differences in percent colonization of Arbuscular Mycorrhizal Fungi in *C. maxima* cv. Burgess Buttercup, Rouge Vif d'Etampes, Mariana de Chioggia, and Golden Hubbard at different sampling times.

Table 4: Tukey Post Hoc test analyzing significant differences in percent colonization between *C. maxima* cv. Burgess Buttercup, Rouge Vif d'Etampes, Mariana de Chioggia, and Golden Hubbard at different sampling times.
List of Figures

Figure 1: Research site at Miami University’s Ecology Research Center (ERC) on Somerville Road, north of Oxford, Butler County, Ohio. The field is approximately 1/2 hectare in size and is adjacent to the ERC access road. It is bounded by a gravel access road on one the east, and abandoned fields on the other 3 sides. Across the access road is a secondary/tertiary growth deciduous forest.

Figure 2: Phase II research plot design showing the planting pattern of *Cucurbita maxima* cv. Burgess Buttercup (BB), Rouge Vif d'Etampes (RD), Mariana de Chioggia (MC), and Golden Hubbard (GH). Rows were planted 2.4m apart with 26 individual plants in each row, spaced 2.4m apart.

Figure 3: Histogram of average root colonization levels collected at three sampling times for each cultivar with 0.05% confidence interval error bars. Burgess Buttercup (BB), Rouge Vif d'Etampes (RD), Mariana de Chioggia (MC), and Golden Hubbard (GH). July 2nd (Time 1), August 2nd (Time 2), September 2nd/3rd, 2015 (Time 3).

Figure 4: Ascomycetes found in Phase I. A.) *Chaetomium* spp. on *C. maxima* cv. Rouge Vif d'Etampes at Kensho Farms indicated by arrow B.) DSE microsclerotia and dark staining chlamydospore with two dark staining nuclei and simple septa indicated with arrows C, D.) Blastically produced spores with two dark staining nuclei labeled with arrow on *C. maxima* cv. Rouge Vif d'Etampes collected from Kensho Farms, *C. maxima* cv. Blue Hubbard collected at Five Oaks Farms. E, F.) Dark staining chlamydospore with two dark staining nuclei and simple septa indicated with arrow on *C. maxima* cv. Burgess Buttercup collected at Orr Farms.

Figure 5: Chytridomycota found in Phase I. A, B, C, D. Stellate resting spores produced by the genus *Olpidium* sp. indicated with an arrow, showing the constricted apical morphology typical to members of the Chytridomycota. Mag. bar = 50 µm.
Figure 6: ‘Dark Septate Endophyte’ member of the Ascomycota found in Phase I and II. A. Melanized microsclerotia are shown with arrows in *C. maxima* cv. Kabocha. B. Melanized microsclerotia and melanized hyphae are shown with arrows in *C. maxima* cv. Mariana di Chioggia. C – D. Resting Spores in *C. maxima* cv. Mariana di Chioggia E-H. Melanized hypha and microsclerotia shown with arrows in *C. maxima* cv. Mariana di Chioggia. Mag. bar 50 µm.

Figure 7: *Tetraploa sp.*, a member of the Ascomycota found in Phase I. A. *Tetraploa sp.* observed in *C. maxima* cv. Blue Hubbard, collected from Five Oaks farm, shown with an arrow, having melanized cell wall material and attenuated cells.

Figure 8: ‘Arum’ type arbuscules produced by AMF observed from Phase I and II sampling observed in the following cultivars. A. Developing arbuscule in *C. maxima* cv. Burgess Buttercup collected at Orr Farms. B. Pair of arbuscules imaged in *C. maxima* cv. Golden Hubbard collected at the ERC. C. Imaged with Differential Interference Contrast Microscopy in *C. maxima* cv. Dills Atlantic Giant collected at Mountain Valley Orchard farm. D. *C. maxima* cv. Rouge Vif d'Etampes collected at Kensho Farms. E. *C. maxima* cv. Burgess Buttercup collected at Five Oaks Farms. F. Mariana de Chioggia collected the ERC. G. *C. maxima* cv. Rouge Vif d'Etampes collected at the ERC. H. *C. maxima* cv. Burgess Buttercup collected at Five Oaks Farms.

Figure 9: ‘Hyphal coils’ or ‘Paris’ type arbuscules were observed in *C. maxima* during Phase I and II sampling in the following cultivars. A. *C. maxima* cv. Rouge Vif d'Etampes at Kensho Farms. B. *C. maxima* cv. Mariana de Chioggia collected at Miami University’s ERC. C. *C. maxima* cv. Turks Turban collected from Orr Farms. D. *C. maxima* cv. Golden Hubbard collected at Miami University’ ERC. E. *C. maxima* cv. Turks Turban collected from Orr Farms, imaged with Differential Interference Contrast Microscopy. F. *C. maxima* cv. Golden Hubbard collected at Miami University ERC. Mag. bar is 50 µm.
Figure 10: Vesicles observed with Differential Interference Contrast Microscopy during Phase I and II sampling. A. Micrograph of *C. maxima* cv. Burgess Buttercup from Orr Farms. B. Micrograph of *C. maxima* cv. Turks Turban collected from Orr Farms. C. Differential Interference Contrast Micrograph of *C. maxima* cv. Turks Turban collected from Orr Farms. D – E. Micrographs of *C. maxima* cv. Blue Hubbard collected from Orr Farms. F. Micrograph of *C. maxima* cv. Turks Turban collected from Orr Farms. G. Micrograph of *C. maxima* cv. Turks Turban collected from Orr Farms. H. Micrograph of *C. maxima* cv. Turks Turban collected from Orr Farms. Mag. bar is 50 μm.

Figure 11: Asexual Glomerospores from Glomeromycota were observed during Phase II sampling with bright field light microscopy. A-B. Different glomerospore morphologies, shown with arrows, with 50 μm mag. bars. C-D. Parasitized *Gigaspora* glomerospores were observed amongst detritus with 100 μm mag. bars. E-G. Different glomerospore morphologies, shown with arrows, with 50 μm mag. bars. H. Three parasitized glomerospores shown with 100 μm mag. bars.

Figure 12: Basidiomycota found in Phase II. A-B. Fungal hyphae with clamp connection imaged with Bright Field Light Microscopy, shown with arrow. Mag. bar is 50 μm.

Figure 13: Plasmodiophorid slime molds found during Phase I and II. A. Sporosori of unknown plasmodiophorid species in *C. maxima* cv. Rouge Vif d'Etampes collected from Mountain Valley Orchards. B-H. Sporosori of unknown plasmodiophorid species in *C. maxima* cv. Golden Hubbard collected from Miami University’s ERC. Mag. bar is 50 μm.

Figure 14: Colonization by fungi found Phase I and II sampling in *C. maxima*. A-B. Infection with dense colonization of fungal hyphae in *C. maxima* cv. Turks Turban, collected from Orr Farms, and imaged with Bright Field Light Microscopy. C. Dense hyphal and arbuscule colonization was observed in the bottom hemisphere of *C. maxima* cv. Turks Turban root, from Downing Fruit Farm, imaged with Differential Interference Contrast Microscopy. D. Fungi parasitizing other fungi in *C. maxima* cv. Golden
Hubbard. E. Differential Interference Contrast micrograph of C. maxima cv. Turks Turban root with fungal hyphae and arbuscules, shown with an arrow. Mag. bar is 50 µm.

Figure 15: Plasmodiophorid slime molds sampled during Phase I and II in C. maxima. A-D. Sporosori of unknown plasmodiophorid species in C. maxima cv. Rouge Vif d'Etampes collected from phase I sampling at Mountain Valley Orchards, labeled with arrows, imaged with Laser Scanning Confocal Microscopy.

Figure 16: Laser Scanning Confocal Micrographs of ‘Dark Septate Endophytes’ observed during Phase I and II sampling from C. maxima. A-C, F. Microsclerotia and hyphae were observed Dark Septate Endophyte fungi were observed in C. maxima cv. Rouge Vif d'Etampes collected at Mountain Valley Orchards, and C. maxima cv. Burgess Buttercup from Orr Farms. D, F. Intercellular conidia observed in C. maxima cv. Rouge Vif d'Etampes collected at Mountain Valley Orchards, and C. maxima cv. Burgess Buttercup from Orr Farms.

Figure 17: Laser Scanning Confocal Micrographs of Glomeromycota sampled during Phase I and II in C. maxima labeled with arrows. A, C, G, H. Vesicles were observed in C. maxima cv. Dills Atlantic Giant from Mountain Valley Farms, C. maxima cv. Turks Turban from Orr Farms, and in C. maxima cv. Rouge Vif d'Etampes collected from Kensho Farms. B, E, F. Arbuscules were observed in C. maxima cv. Burgess Buttercup collected from Orr Farms. D. Glomeromycota hyphae were observed in C. maxima cv. Dills Atlantic Giant from Mountain Valley Orchard.
Dedication
I dedicate this manuscript to my family, for putting up with my leaf collections, fungal collections, insect collections, vinyl records, antique marbles, musical instruments, and everything else that has come with spending almost three decades growing up in an educational environment. Without my family, I would have never made it to where I am now in my career. They have supported me through thick and thin, and as always, been there when I may not have been emotionally available. They have taught me to be strong, by loyal, be kind, nice, supportive, to love unconditionally, and to always help a friend that is in need. I also want to dedicate my thesis to Miss Chante Robertson for teaching me love, RNB, and getting me through one of the toughest times of my life.
Acknowledgements

I want to acknowledge my committee novo for helping me out in a tight situation, and never giving up on me. I want to graciously thank Matt Duley for his training and technical expertise in the Miami University Center for Advanced Microscopy and Imaging. I want to thank Kristi Hutchinson at Five Oaks Organic Farm, John Krowka at Kensho Farms, Scott Downing at Downing Fruit Farm, Leroy Tracey at Mountain Valley Orchard, and Mike and Mark Orr Farm for allowing samples to be collected and processed for imaging. Many thanks to Miami University for providing internal support for the Center for Advanced Microscopy and Imaging and the Willard Sherman Turrell Herbarium during my time at Miami University. I want to send many thanks to Jeremy Fruth for field assistance at the Ecology Research Center during the 2015 field season. I want to thank Jack Keegan for his horticultural expertise. I could not have done it without Kel Arlinghaus, Aaron Anderson, Adam Parlin, Thomas Peterson, Patrick Garrett, Mike Mahon, Ty Hoskins, Michael Stanley, Mark Daniels, and the rest of the Biology Department graduate students and faculty from 2014-2016.
Introduction

Fungi and fungal-like organisms are ubiquitous on every continent, and are thought to have been the first Eukaryotic organisms to colonize terrestrial habitats roughly 460 mya (Bonfante and Genre 2008). These organisms have been documented to fill almost every ecological niche, ranging from antagonists, biotrophs, mutualists and parasites, to opportunistic pathogens, and have notable roles as endophytes, epiphytes, and saprophytes. Fungi and fungal-like organisms have been shown to live in aquatic, marine, terrestrial, and atmospheric habitats, and are often noted as extremophiles, living in a range of harsh and forbidding conditions (Alexopoulos et al. 1996).

The Kingdom Fungi includes the following phyla: Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Cryptomycota, Entomophthoromycota, Entorrhizomycota, Glomeromycota, and Neocallimastigomycota (Alexopoulos et al. 1996, Bauer et al. 2015, Gryganskyi et al. 2013, James et al. 2006, Jones et al. 2011, Liggenstoffer et al. 2010). Ascomycota are characterized by production of spores in an ascus. Basidomycota is a phylum of fungi that is recognized by production of sexual basidiospores, borne on a basidium. Chytridiomycota are identified by the ‘flask/pot’ shaped zoosporangium that holds motile zoospores. Cryptomycota is characterized by not having chitinous cell walls, and was once thought to be a sister clade to Fungi, but is now currently placed in the kingdom Fungi by molecular evidence. Entomophthoromycota is a new phylum that is known for its niche associations with arthropoda. Entorrhizomycota is a new phylum, which was described by Bauer et al. (2015), are characterized by their production of galls on Juncaceae and Cyperaceae and by their dikaryotic meiosporangium, septal pore structure, cell wall, and spindle pole bodies (Bauer et al. 2015). Glomeromycota are identified by the production of arbuscules, and sometimes vesicles in the cortex cells of plant roots. Neocallimastigomycota is a new phylum that is characterized by its niche associations with ruminant mammals. Zygomycota is an old term used to describe a paraphyletic group that is currently not recognized as a distinct phylum, and is being molecularly characterized into distinct phylogenetic divisions.

The diverse Kingdom Fungi can be found in overlapping ecosystems, habitats, niches, and geographic locations, forming diverse communities. ‘Fungal-like organisms’ is a broad term used to characterize microorganisms classified in multiple domains, which contain morphological similarities to ‘true fungi’, but are genetically dissimilar based upon current
molecular characterization tools. Fungi and fungal-like organisms inhabit root systems of many plants, forming many different types of mycorrhizal associations, ranging from endo-, ecto-, ectendo-, and orchidaceous- mycorrhizae. Fungi and fungi-like organisms also live in the rhizosphere that surrounds plant roots, forming complex communities. These organisms may interact with a host plant in a spectrum of ways, from beneficial to pathogenic. The types of organisms that form mycorrhizal associations with herbaceous plants are not entirely known, and could be important links to understanding agricultural ecosystems. The following research on host/microorganism interactions has been described in the literature for fungi and fungal-like organisms.

Dark Septate Endophytes

A common group of endophytic fungi that inhabits the root systems of many plant species is Dark Septate Endophytes (DSE) (Thangavelu and Tamilselvi 2010). Jumpponen and Trappe (1998) suggest that DSE are conidial Ascomycetous fungi with no known sexual stage. Jumpponen and Trappe (1998) reviewed the literature on DSE, and listed angiosperm and gymnosperm hosts, and known species of DSE. Dark Septate fungi are characterized by highly melanized hyphae, and production of microsclerotia, which are thought to serve as overwintering structures (Thangavelu and Tamilselvi 2010). DSE may provide protection to the host plant, although the extent of this protection is unknown (Thangavelu and Tamilselvi 2010). Like that of many other fungi, DSE’s relations with its host can span from beneficial to pathogenic in a spectrum, depending on biological and environmental factors. Menkis et al. (2004) have shown DSE associations with angiosperms and gymnosperms in Lithuania and Sweden. Mandyam and Jumpponen (2005) suggest that DSE function in the same ecological niche as other mycorrhizae, and that they are multifunctional in that they acquire nutrients for the host and have been shown to protect the host from environmental stresses. Jumpponen (2001) suggests that DSE colonizes a variety of hosts, which normally would not form ectomycorrhizae or ectendomycorrhizal associations. The intracellular and intercellular colonization by DSE suggests that DSE are ectendomycorrhizal, and thus should be considered mycorrhizal as an ecological role (Jumpponen 2001). Newsham (2011) conducted a meta-analysis on DSE, and reviewed 56 peer-reviewed articles published over a 40 years period. This literature review indicates that, based upon Internal Transcribed Spacer (ITS) gene sequences, the majority of DSE belong to the order Helotiales, while others belong to the Chaetothyriales, Chaetosphaeriales, Capnodiales,
Pleosporales, and Sordariales (Newsham 2011), while some genera cannot be placed in an order based upon ITS sequences. This diversity of lineages has resulted in coevolution of mycorrhizal associations with a wide range of hosts.

*Arbuscular Mycorrhizal Fungi*

Another common group of root-associated fungi is Arbuscular Mycorrhizal Fungi (AMF), which are categorized in the phylum Glomeromycota, and are known to inhabit the root systems of many herbaceous plant species (Akhtar et al. 2011, Pozo et al. 2002, Pozo and Azcon-Aquilar 2007, Pozo et al. 2009). Akhtar et al. (2011) report that nearly 80% of herbaceous plants form relationships with AMF. The plant receives nutrients, water, and protection from pathogenic invaders, whereas the fungus receives protection, nutrients, and a niche in which to survive (Akhtar et al. 2011; Pozo and Azcon-Aquilar 2007; Pozo et al. 2002, 2009). AMF are identified by the presence of branched haustoria-like structures, which are found in association with the plasma membrane of cortical cells in the host plant, and are termed arbuscules (Akhtar et al. 2011). These modified hyphal structures are the location where nutrient exchange occurs between the host plant and the AMF symbiont (Akhtar et al. 2011). Once the arbuscule is formed, the AMF will grow from the root surface and penetrate the surrounding soil, expanding the zone in which water and nutrients can be acquired (Akhtar et al. 2011). AMF are dichotomized into two distinct groups based upon the morphology of their arbuscules (Dickson et al. 2007). The first group of AMF is the ‘Arum’ type, which has highly branched arbuscules, and the second group is the ‘Paris’ type, which has regions of coiled intracellular hyphae and intercalary arbuscules (Dickson and Kolesik 1999). Additionally, some species of AMF produce vesicles, which are lipid storage structures, and are important for survival during adverse conditions (Helgason and Fitter 2009).

*Plasmodiophorid Slime Molds*

“Fungal-like organisms” include Plasmodiophorid Slime Molds, which are an asexual protozoan clade within the Rhizaria supergroup (Esser et al. 2015). No sexual stage of the plasmodiophorid group has been elucidated, so the biological species concept is very difficult to implement in this small, but economically significant, clade (Esser et al. 2015). Plasmodiophorids were first characterized by a cruciform morphology of the nucleus in 1899.
Since the discovery of this clade, morphological and molecular analyses have revealed 10 genera (*Ligniera* Maire & A. Tison, *Membranosorus* Ostenfeld & H.E. Petersen, *Octomyxa* Couch, J. Leitn. & Whiffen, *Plasmodiophora* Woronin, *Polymyxa* Ledingham, *Sorodiscus* Lagerh. & Winge, *Sorosphaera* Brunch., *Spongospora* Wallr., *Tetramyxa* Goebel) and 35 species (Esser et al. 2015). Classic morphological characterization was determined by the shape and number of associated sporosori, but new characterization and classification are based upon ribosomal 18S sequences (Esser et al. 2015). Ecologically, Plasmodiophorids are obligate parasites of hosts that range from angiosperms to algae and Oomycota (Esser et al. 2015). To date, no Plasmodiophorid has been successfully cultured outside of its host (Esser et al. 2015). Plasmodiophorids can be found in terrestrial, freshwater, and marine habitats, since water is needed for the movement of primary and secondary zoospores. Plasmodiophorids are of economic importance to humans, notably as plant pathogens in agricultural and aquatic ecosystems (Esser et al. 2015). The most economically important terrestrial diseases caused by Plasmodiophorids are club root disease of brassicas, caused by *Plasmodiophora brassicae* Woronin, and powdery scab of potatoes, caused by *Spongospora subterranea* (Wallr) Lagerh., (Esser et al. 2015).


**Cucurbitaceae: The Squash Family**

Members of the Cucurbitaceae have traditionally been used as model organisms for botanical experiments. Cucurbits such as *Cucumis sativus* Linnaeus, *Cucumis melo* Linnaeus, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, *Cucurbita moschata* Duchesne ex Poir., *Cucurbita maxima* Duchesne ex Poir., *Cucurbita ficifolia* Bouche, and *Cucurbita pepo* Linnaeus are all economically important members of this family (Choi et al. 2007, Guo et al. 2013, Huang et al. 2009, Kim et al. 2010, Yang et al. 2013, Lin et al. 2013, Zhang et al 2006, Jamiolkowska et al. 2011, Sensoy et al. 2013, Zhou et al. 2014). Cucurbits have been used extensively to study fungal/ host relationships. These research topics range host induced resistance to pathogens, to research that reports on cucurbit nutrient acquisition by beneficial mycorrhizas, as well as projects that study the effects of fungal infection in cucurbits on the tolerance of the host plant to adapt to abiotic stresses (Saldajeno et al. 2011, Sensoy et al. 2013). I propose to use a member of the Cucurbitaceae as a model organism for this research project.
Genus Cucurbita

The genus *Cucurbita* contains 13 species, including economically important species such as *Cucurbita ficifolia*, *Cucurbita maxima*, *Cucurbita moschata*, and *Cucurbita pepo* (Lee et al. 2003). Sanjur et al. (2002) state that *Cucurbita* species were some of the earliest crops to be domesticated in the New World, and *Cucurbita spp.* were domesticated during 6 separate events.

Members of the genus *Cucurbita* are used by different human cultures across the globe, and are a notable source for nutrition and medicinal treatments. *Cucurbita maxima* produces the largest number of different fruit shapes of any species in this economically important clad, and misidentification of the species can lead to confusion when using this cucurbit for botanical research (Lee et al. 2003). The species epithet of *Cucurbita maxima* suggests vigorous growth of fruit, which is the most massive of any cucurbit. *Cucurbita maxima* originated in South America, and were domesticated around 4000 years ago (Ferriol et al. 2004). Various forms of *C. maxima* were disseminated to Europe in the 16th century, and subsequently were taken by European explorers to the Indian sub-continent, and Southeast-Asia (Ferriol et al. 2004). *Cucurbita maxima* is used medicinally, and is reported to have anti-diabetic, anti-oxidant, anti-cancer, and anti-inflammatory properties (Saha et al. 2011, Zhou et al. 2014). It is also a major food source for wide variety of world cultures, due to its fiber content, carbohydrates, β-carotene, vitamins, alkaloids, minerals, fatty acids, flavonoids, and polysaccharides (Zhou et al. 2014).

Detection of root-associated fungi in *Cucurbita maxima* has rarely been discussed in the scientific literature. Ragupathy and Mahadevan (1993) published a paper that surveyed the presence or absence of AMF in common vegetables including *C. maxima*, but did not provide images, or information about the variety of *C. maxima* sampled. Srivastava et al. (2012) published a manuscript that identified *Acaulospora mellea* Spain & Schenck, *Gigaspora margarita* Becker & Hall, *Glomus citricola* Tang & Zang, *Gl. macrocarpum* Tul. & C. Tul and *Gl. minutum* Blaszk., Tadych & Madej from root samples of *C. maxima*. Srivastava et al. (2012) stated that they viewed AMF with light microscopy, but they did not include images of AMF in the publication. Pictures of AMF and DSE in *C. maxima* are missing from the literature, and are a crucial step in verifying the presence or absence of infection.
Hypothesis

The purpose of this research is to document whether AMF, DSE, and Plasmodiophorid slime molds are found in association with *Cucurbita maxima* in the United States.

Null Hypothesis: No microorganisms will be found in association with *Cucurbita maxima* in the United States.

Methods

A two phase approach was implemented to first verify an AMF, DSE, and plasmodiophorid slime mold associations in *Cucurbita maxima* and then second, to document the colonization of Arbuscular Mycorrhizal Fungi in *C. maxima* grown in a conventional agricultural system.

Phase I: Survey

The first phase was a survey of *Cucurbita maxima* roots collected from farms in the eastern United States, and the use of traditional staining techniques to discern the presence or absence of fungal and ‘fungal-like’ organism associations in the host. Various microscopic techniques are commonly utilized to image these fungi, and the use of differential stains is a critical step in the imaging process. Differential stains are used to provide contrast between the fungal tissues and the host plant cells. The first phase was conducted in order to establish a justification for further exploration into the colonization mechanisms of fungus and fungal-like organisms in *C. maxima*.

Root samples were collected from 3 farms in southeastern Ohio, 1 farm in West Virginia, and 2 locations in Maryland (Table 1). Farms were selected based upon their different agricultural practices, ranging from certified USDA Organic, uncertified organic, unsprayed, and conventional treatment. Root samples were collected randomly from multiple plants of each cultivar, and then roots were chosen at random for staining and microscopic analysis. According to the growers at each farm, the cultivars included Blue Hubbard, Burgess Buttercup, Dills Atlantic Giant, Rouge Vif d'Etampes, Red Kuri, Sweet Meat, and Turk’s Turban.

Phase II: Colonization Level Comparison

The second phase of this project focused on the documentation of Arbuscular Mycorrhizal Fungi colonization levels among different cultivars of *Cucurbita maxima*, and at
different times during the growing season. This was accomplished by microscopic scoring of colonization levels in root samples of each cultivar of *C. maxima*, followed by statistical analysis of the levels of colonization in several cultivars of *C. maxima* at 3 different times in the growing season.

*Seed Germination*

Seeds of *C. maxima* cv. Burgess Buttercup, Rouge Vif d'Etampes, Mariana de Chioggia, and Golden Hubbard were purchased from Seed Savers Exchange®. Ten seeds of each cultivar were placed in filter paper lined Petri-plates, moistened with de-ionized water. This was replicated 10 times, for a total of 100 seeds of each cultivar. Seeds were incubated at 22°C under 24 hours of fluorescent lights. Seedlings were transferred from filter paper to 3” peat pots, filled with moistened Farfard® 3B potting soil. Plants were grown on light carts or in light boxes under fluorescent lights at 22°C with a regime of 18 hours of light, and 6 hours of dark.

*Field Cultivation*

Research was conducted at Miami University’s Ecology Research Center (ERC) on Somerville Road, north of Oxford, Butler County, Ohio (Figure 1, Figure 2). The field is approximately 1/2 hectare in size and is adjacent to the ERC access road. The field is bounded by a gravel access road on the east, and abandoned fields on the other 3 sides. Across the access road is a secondary/tertiary growth deciduous forest. The bordering fields are comprised of Poaceae, and invasive forbs, such as *Cirsium arvense* (L.) Scop., and *Taraxacum officinale* F.H. Wigg, and other native herbaceous plants, including *Solidago* sp. The field was previously planted with a *Glycine max* (L.) Merr. - *Zea mays* L. rotation on alternating years. The field was left fallow for a year prior to this study.

The field was disked twice, and tilled before planting. No chemical treatments were applied to the field pre- or post-planting. The field was relatively level, except for a slight depression toward the southwest (Figure 1). The field is partially shaded in early morning by the shadows created from the forest on the east side of the field (Figure 1). This shadow rapidly decreases, allowing for more than 12 hours of direct sunlight on the field.

Four-week-old seedlings of *C. maxima* cv. Burgess Buttercup, Rouge Vif d'Etampes, Mariana de Chioggia, and Golden Hubbard were transplanted in a non-randomized pattern that
contained rows of 26 individuals, with 2.4m spacing between the plants, and between the rows (Figure 2). Two rows of the same cultivar were planted adjacent to each other. Plants were irrigated by hand for the first 10 days post-planting.

**Root Sampling**

During each sampling event, 10 plants of each cultivar were randomly destructively sampled, and five roots from each plant were sub-sampled from the total roots collected. Sampling took place during three evenly spaced times during the growing season. The sampling took place on July 2\textsuperscript{nd}, August 2\textsuperscript{nd}, and September 2\textsuperscript{nd} - 3\textsuperscript{rd}, 2015. Roots were stored in plastic bags at 4°C until processing within 48 hours post harvest. Only one, 1cm segment was used from each root, and the rest of the sample was frozen at -80°C for future analysis.

**Soil Sampling**

Soil samples were collected once during the month of July from the field plot at the ERC, and these samples were processed for AMF Glomerospore extraction. A subsample of 50g of soil was processed from each original soil sample. A (20%/60%) sucrose solution was used for gradient separation of Glomerospores, and was centrifuged at 600 x g for 3 minutes. Soil was sieved through a 500 µm and 43 µm stainless mesh sieve, and spores were washed three times with distilled water. Extracted spores were imaged with bright field light microscopy.

**Staining Techniques**

Root staining was a modification of the Brundrett et al. (1984) methods. Samples were heated in 10% (w/v) aqueous potassium hydroxide (KOH) solution for 50 minutes at 95°C. KOH was decanted off of the samples, and 5% hydrochloric acid (HCL) (v/v) was added to neutralize the pH. The samples were kept in 5% HCL for 5 minutes at 20°C. Then, 1% Trypan blue (w/v) was added to the storage tubes. The samples were incubated at 100°C for 5 minutes, and immediately washed with 20°C distilled water. Samples were cut into 1 cm sections and mounted on glass slides with in 50% lactic acid - glycerol (v/v) and stored at 4°C.
**Light Microscopy**

Root segments were examined for distinct morphological features using bright field light microscopy and laser scanning confocal microscopy, and imaged with an Olympus AX-70 light microscope, and a Zeiss 710 laser scanning confocal microscope. Definitive characteristics of AMF such as arbuscules and vesicles were used to distinguish Glomeromycota from other phyla of fungi. Identification of DSE fungi was made by the presence or absence of microsclerotia structures that were found in the host tissues. Presence of Plasmodiophorid Slime Mold infection was determined by the presence of sporosori.

**Root Scoring**

The Abuscular Mycorrhizal Fungi root colonization rates in samples from the ERC were calculated and statistically analyzed in 4 cultivars of *C. maxima* (Burgess Buttercup, Rouge Vif d'Etampes, Mariana de Chioggia, and Golden Hubbard).

Root colonization by AMF was scored subjectively by visually determining the percent colonization in each 1cm segment of *Cucurbita maxima* on a 0-100% scale.

**Statistical Analyses**

The data were first analyzed with Microsoft® Excel for mean percent colonization levels, standard deviation, and standard error for each sampling time, as well as across all sampling times (Table 2).

The data were also analyzed and plotted using R version 3.2.2 using the following packages: ggplot2, graphics, car, Rmisc, stats, and nlme. The ‘ggplot2’ package allows for high definition figures, such as tables and graphs, to be made with ‘R’. The ‘graphics’ package allows for regular definition figures, such as tables and graphs, to be made with ‘R’. The ‘car’ package allows for more selective statistical analyses, including ANOVA and Tukey Post Hoc tests which was used to test for significant differences in colonization levels between cultivar and sampling time. The ‘Rmisc’ package opens miscellaneous software in ‘R’, and is a standard package that is used to write statistical code. The ‘stats’ package allows for basic statistics and random number generation. The ‘nlme’ package allows for non-linear mixed model statistics to be analyzed with the ‘R’ program. Non-linear mixed models include fixed and unfixed effects, but depends more on independent events to show significance of a value.
All of the data were subjected to a Logarithmic (Log) transformed (+0.001) Analysis of Variance (ANOVA), in order to determine the level of significance between the percentage of root colonization, sampling time, and cultivar (Table 4). A Log transformation was performed in order to attempt to normalize the date and residuals, and reduce the effect of large numbers of roots with 0% colonization.

The data were also analyzed with a Log transformed (+0.001) Tukey HSD Post Hoc test to show significant differences in colonization levels between specific C. maxima cultivars and specific sampling time interactions (Table 5). A Tukey HSD Post Hoc test was used in order to make pair wise comparisons between cultivars and percent colonization during specific sampling times. A Tukey HSD Post Hoc test is calculated by subtracting the mean from treatment two (M₂) from the mean of treatment one (M₁), and dividing that difference by the square root of the mean-square-within (MSW) multiplied by the inverse of the number (N) of samples in each treatment  

\[(M₁ – M₂) / \sqrt{(MSW \times (1/N))} \]

A histogram (Figure 3) showing the average percent colonization for each cultivar at every sampling time was produced using the ‘ggplot2’ package. Error bars (in red) represent an alpha value of 0.05, or a 95% confidence interval.

**Results**

*Phase I: Preliminary Sampling Results*

Preliminary Phase I sampling during the fall of 2014 of C. maxima roots show a diversity of fungi and ‘fungal-like’ organisms (Table 1), including fungal members of the Ascomycota (Figure 4 A-D), Chytridiomycota (Figure 5 A-D), ‘Dark Septate Endophytes’ (Figure 6 A-H Figure 14 A-F), *Tetraploa sp.* (Figure 7 A), Glomeromycota (Figure 8 A-F, Figure 9 A-H, Figure 10 A-H, Figure 11 A-H, Figure 12 A-H), and an unknown ‘fungal-like’ organism belonging to the Plasmodiophorid Slime Mold phylum (Figure 13 A-H, Figure 14 A-D). This initial survey provides light micrographs as evidence to show a microbe/host relationship between microbial organisms and *C. maxima.*

The null hypothesis is rejected because fungi and ‘fungal like organisms’ were found in the host’s rhizosphere, and they provide evidence for an association of members of the Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Dark Septate Endophytes, Plasmodiophorid Slime Molds, and ‘Fungi Imperfecti’ and *Cucurbita maxima.* Micrographs of
these groups of organisms provide evidence of a relationship with *C. maxima*, and will help fill in the gaps that might be missing in the literature. Images of these organisms will serve as justification to further study the roles of these organisms in and around *C. maxima*.

After random roots were selected for microscopic observation, characteristic morphological structures of AMF and other fungi were recorded for each cultivar (Table 1). Every sampling location provided plant tissue bearing some mycorrhizal structures, although vesicles and arbuscules were less frequent than normal hyphae and inter-coiled hyphae. ‘Blue Hubbard’ grown under USDA Certified Organic conditions in Ohio, as well as the uncertified organic cultivation from West Virginia showed inter-coiled hyphae, but vesicles were only observed in un-certified organic pumpkins grown in West Virginia. Samples from the cultivar ‘Turk’s Turbans’ were collected from an unsprayed conventional farm in Ohio, and from an uncertified organic farm in West Virginia. The Ohio samples contained hyphae and arbuscules, whereas the West Virginia samples contained hyphae, vesicle, and arbuscules. Samples from ‘Rouge vif d’etampes’ were collected from an uncertified organic farm in West Virginia, as well as from a conventional farm in Maryland. The samples collected from West Virginia had both hyphae, and arbuscules present. The samples from Maryland contained hyphae, and vesicles. The cultivar ‘Burgess Buttercup’ grown under conventional farming techniques in Maryland, USDA Certified Organic conditions in Ohio, and from un-certified organic cultivation from West Virginia showed normal hyphae and inter-coiled hyphae, but vesicle were only observed in un-certified organic pumpkins grown in West Virginia. Arbuscules, vesicles, and hyphae were observed from the conventionally grown ‘Burgess Buttercup’ from Maryland. The cultivar ‘Dill’s Atlantic Giant’ was sampled from a conventional farm in Maryland and showed vesicles, arbuscules, hyphae, and inter-coiled hyphae. ‘Red Curi’ samples were collected from a USDA Certified Organic farm in Ohio. The root samples only contained hyphae, and no other morphological structures were observed. The cultivars ‘Sweet Meat’ was sampled from a USDA Certified Organic Farm in Ohio, and only hyphae were observed. Arbuscular mycorrhizal fungi were found in every cultivar of *C. maxima* sampled.

Members of the Ascomycota were imaged with bright field light microscopy (Figure 4 A-F). *Chaetomium* sp. was observed in *C. maxima* cv. Rouge Vif d'Etampes that was sampled from Kensho Farms (Figure 4 A). DSE microsclerotia and dark staining chlamydospore with two dark staining nuclei and simple septa indicated with arrows (Figure 4 B). Blastically produced spores
with two dark staining nuclei labeled with arrow on *C. maxima* cv. Rouge Vif d'Etampes collected from Kensho Farms, *C. maxima* cv. Blue Hubbard collected at Five Oaks Farms (Figure 4 C, D). Dark staining chlamydospore with two dark staining nuclei and simple septa indicated with arrow on *C. maxima* cv. Burgess Buttercup collected at Orr Farms (Figure 4 E, F). The magnification bar provided on each image represents 50 µm.

Members of the Chytridomycota were observed during Phase I sampling. Stellate resting spores produced by the genus *Olpidium* sp. are indicated with an arrow, showing the constricted apical morphology typical to members of the Chytridomycota (Figure 5 A, B, C, D) (Veranda et al. 2015).

Members of the Dark Septate Endophyte group were observed during Phase I and Phase II sampling. Highly melanized hyphae, microsclerotia, and spores were imaged with bright field light microscopy, and indicated with arrows (Figure 6 A - H). A. Highly melanized hyphae and microsclerotia were observed in *C. maxima* cv. Kabocha and imaged with bright field light microscopy. B-H Highly melanized hyphae and microsclerotia were observed in *C. maxima* cv. Mariana di Chioggia.

*Tetraploa* sp., an asexual member of the Ascomycota, was observed in *C. maxima* cv. Blue Hubbard, collected from Five Oaks farm during phase I sampling (Figure 7 A), and in *C. maxima* cv. Mariana di Chioggia, collected during phase II sampling from Miami University’s ERC (Micrograph not provided due to low resolution of the digital image).

‘Arum’ type arbuscules were observed with bright field light microscopy in *C. maxima* cv. Burgess Buttercup collected at Orr Farms (Figure 8 A), *C. maxima* cv. Mariana de Chioggia collected at Miami University’s ERC (Figure 8 F), *C. maxima* cv. Rouge Vif d'Etampes collected at Miami University’s ERC (Figure 8 G), *C. maxima* cv. Golden Hubbard collected at Miami University ERC (Figure 8 B). An arbuscule, shown with an arrow, was imaged with Differential Interference Contrast Microscopy in *C. maxima* cv. Dills Atlantic Giant collected at Mountain Valley Orchard farm (Figure 8 C), *C. maxima* cv. Rouge Vif d'Etampes collected at Kensho Farms (Figure 8 D), *C. maxima* cv. Burgess Buttercup collected at Five Oaks Farms (Figure 8 E, H).

‘Hyphal coils’ or ‘Paris’ type arbuscules are were observed in samples from *C. maxima* cv. Rouge Vif d'Etampes at Kensho Farms (Figure 9 A), *C. maxima* cv. Mariana de Chioggia collected at Miami University’s ERC (Figure 9 B), *C. maxima* cv. Turks Turban collected from
Orr Farms (Figure 9 C), *C. maxima* cv. Golden Hubbard collected at Miami University’ ERC (Figure 9 D), and imaged with Bright Field Light Microscopy. Hyphal coils’ or ‘Paris’ type arbuscules were observed in *C. maxima* cv. Turks Turban collected from Orr Farms (Figure 9 E), and was imaged with Differential Interference Contrast Microscopy. Hyphal coils’ or ‘Paris’ type arbuscules, were observed with Bright Field Light Microscopy in *C. maxima* cv. Golden Hubbard collected at Miami University ERC (Figure 9 F).

A vesicle was observed with Differential Interference Contrast Microscopy in *C. maxima* cv. Burgess Buttercup from Orr Farms (Figure 10 A), *C. maxima* cv. Turks Turban collected from Orr Farms (Figure 10 C), *C. maxima* cv. Blue Hubbard collected from Orr Farms (Figure 10 D), *C. maxima* cv. Turks Turban collected from Orr Farms (Figure 10 F), *C. maxima* cv. Turks Turban collected from Orr Farms (Figure 10 G), and in *C. maxima* cv. Turks Turban collected from Orr Farms (Figure 10 H). A vesicle and lipid filled hyphae was observed with Differential Interference Contrast Microscopy in *C. maxima* cv. Turks Turban collected from Orr Farms (Figure 10 B).

Plasmodiophorid Slime Molds were observed with Bright Field Light Microscopy after sampling events from Phase I in *C. maxima* cv. Rouge Vif d'Etampes collected from Mountain Valley Orchards (Figure 13 A).

Infection of *C. maxima* observed with along with dense colonization of fungal hyphae in *C. maxima* cv. Turks Turban, which was collected from Orr Farms, and imaged with Bright Field Light Microscopy (Figure 14 A, B). Dense hyphal and arbuscule colonization was observed in the bottom hemisphere of *C. maxima* cv. root, which was imaged with Differential Interference Contrast Microscopy (Figure 14 C). Unknown fungal hyphae were observed in another hyphal strand in *C. maxima* cv. Golden Hubbard (Figure 14 D). Differential Interference Contrast micrograph of *C. maxima* cv. Turks Turban root with fungal hyphae is shown with an arrow (Figure 14 E).

Plasmodiophorid sporosori were observed with Laser Scanning Confocal Microscopy in *C. maxima* cv. Rouge Vif d'Etampes collected from phase I sampling at Mountain Valley Orchards (Figure 15 A-D).

Dark Septate Endophyte fungi were observed with Laser Scanning Confocal Microscopy in *C. maxima* cv. Rouge Vif d'Etampes collected from phase I sampling at Mountain Valley Orchards, and *C. maxima* cv. Burgess Buttercup from Orr Farms (Figure 16 A-F). Microsclerotia
and hyphae were observed (Figure 16 A, B, C, E) as well as intercellular conidia (Figure 16 D, F) in *C. maxima* cv. Rouge Vif d'Etampes collected from phase I sampling at Mountain Valley Orchards.

Glomerozymycota were observed with Laser Scanning Confocal Microscopy during Phase I sampling events (Figure 17 A-H). Vesicles were observed in *C. maxima* cv. Dills Atlantic Giant from Mountain Valley Farms (Figure 17 A, G), in *C. maxima* cv. Turks Turban from Orr Farms, and in *C. maxima* cv. Rouge Vif d'Etampes collected from Kensho Farms. Arbuscules were observed in *C. maxima* cv. Burgess Buttercup collected from Orr Farms (Figure 17 B, E, F). Glomerozymycota hyphae were observed in *C. maxima* cv. Dills Atlantic Giant from Mountain Valley Orchard.

**Results from Phase II: Field Work 2015**

The raw data collected from three sampling events during the summer of 2015 were analyzed for mean colonization levels, standard error, and standard deviation as regards to cultivar of *C. maxima* and time, with Microsoft® Excel (Table 2).

Microscopic evaluation of roots collected during the second phase of the experiments showed similar microbial associations with *C. maxima*, except for an association with a member of the Basidiomycota (Figure 12 A and B), which was not documented during the preliminary sampling in the fall of 2014. Extra images of unique fungal colonization are provided (Figure 14 A-H). A pair of vesicles was observed with bright field light microscopy in *C. maxima* cv. Golden Hubbard collected from Miami University’s ERC (Figure 10 E). A mixture of different glomerspores morphologies were observed from field samples collected at Miami University’s ERC, and shown with 50 µm magnification bars (Figure 11 A, B, E, F, G). Parasitized *Gigaspora* glomerospores were collected from Miami University’s ERC, imaged with Bright Field Light Microscopy, and shown with 100 µm magnification bars (Figure 11 C, D, H). Sporosori of unknown plasmiodiophorid species in *C. maxima* cv. Golden Hubbard collected from phase II sampling at Miami University’s ERC are shown with an arrow, and imaged with Bright Field Light Microscopy (Figure 13 B, C, D, E, F, G, H). All of the images shown are provided with a 50 µm magnification bars. AMF with lipid filled hyphae and arbuscules are shown with arrows, imaged with Bright Field Light Microscopy in *C. maxima* cv. Mariana di Chioggia (Figure 14 F). AMF hyphae and ‘Arum type’ arbuscules are shown with arrows,
imaged with Bright Field Light Microscopy in *C. maxima* cv. Mariana di Chioggia (Figure 14 G). AMF hyphae with numerous darkly stained nuclei as well as ‘Arum type’ arbuscules shown with arrows, imaged with Bright Field Light Microscopy in *C. maxima* cv. Mariana di Chioggia (Figure 14 H).

The average percent colonization for *C. maxima* cultivars was recorded within 48 hours of sampling on July 2\textsuperscript{nd}, August 2\textsuperscript{nd}, and September 2\textsuperscript{nd}/3\textsuperscript{rd} (Table 2). The average percent colonization for *C. maxima* cv. Mariana de Chioggia during the first sampling event on July 2\textsuperscript{nd} was 3.1\%, 7.72\% on August 2\textsuperscript{nd}, and 13.94\% on September 2\textsuperscript{nd} and 3\textsuperscript{rd}. The overall average percent colonization for *C. maxima* cv. Mariana de Chioggia was made by averaging the mean colonization levels at each sampling time, and was found to be 8.23\% (Table 2).

The average percent colonization for *C. maxima* cv. Golden Hubbard during the first sampling event on July 2\textsuperscript{nd} was 5.86, 8.64\% on August 2\textsuperscript{nd}, and 5.02\% on September 2\textsuperscript{nd} and 3\textsuperscript{rd}. The average percent colonization for *C. maxima* cv. Golden Hubbard was made by averaging the mean colonization levels at each sampling time, and was found to be 6.51\% (Table 2).

The average percent colonization for *C. maxima* cv. Rouge Vif d'Etampes during the first sampling event on July 2\textsuperscript{nd} was 1.94\%, 1.66\% on August 2\textsuperscript{nd}, and 3.72\% on September 2\textsuperscript{nd} and 3\textsuperscript{rd}. The average percent colonization for *C. maxima* cv. Rouge Vif d'Etampes was made by averaging the mean colonization levels at each sampling time, and was found to be 2.44\% (Table 2).

The average percent colonization for *C. maxima* cv. Burgess Buttercup during the first sampling event on July 2\textsuperscript{nd} was 1.94\%, 1.66\% on August 2\textsuperscript{nd}, and 3.72\% on September 2\textsuperscript{nd} and 3\textsuperscript{rd}. The average percent colonization for *C. maxima* cv. Burgess Buttercup was made by averaging the mean colonization levels at each sampling time, and was found to be 10.00\% (Table 2).

Results from the ANOVA performed on the log-transformed data collected in the summer of 2015 show a significant difference in colonization levels by Arbuscular Mycorrhizal fungi among cultivars of *C. maxima* (Table 3). The results also show a significant difference in colonization levels by AMF between cultivars of *C. maxima* at different sampling times during the 2015 growing season (Table 3). P-values under 0.05 are considered significant values, while P-values slightly over 0.05 will be recognized as potentially significant values.

The data suggest that *C. maxima* cv. Mariana de Chioggia has a significant difference in
AMF colonization levels at some point during the growing season than the other varieties (P-value 0.01). The data suggest that C. maxima cv. Rouge Vif d'Etampes has a significant difference in AMF colonization levels at some point during the growing season than other varieties (P-value 0.0001). The data suggest that C. maxima cv. Mariana de Chioggia has a significant difference in AMF colonization levels at time 3, as compared with all other cultivars at all other times (P-value 0.01). All other interactions resulted in P-values that were not significant, suggesting that colonization levels were similar between cultivars and times not previously mentioned (Table 3).

The data were subjected to a nonparametric statistical analysis called a Tukey Post Hoc Test, which is used to make pair-wise comparisons between root colonization levels in varieties of C. maxima at different sampling times (Table 4). The Tukeys Post Hoc Test showed a significant difference between C. maxima cv. Mariana de Chioggia and C. maxima cv. Burgess Buttercup at some sampling time during the growing season (P-value 0.0458). The Tukeys Post Hoc Test showed a significant difference between C. maxima cv. Rouge Vif d'Etampes and C. maxima cv. Golden Hubbard at some sampling time during the growing season (P-value 0.0005). The Tukeys Post Hoc Test showed a significant difference between C. maxima cv. Rouge Vif d'Etampes and C. maxima cv. Mariana de Chioggia at some sampling time during the growing season (P-value 0.003).

The Tukeys Post Hoc Test showed a significant difference in colonization levels of AMF between different C. maxima cultivars during sampling time one and two (P-Value 0.01), and also showed a significant difference in colonization levels of AMF between different C. maxima cultivars during sampling time two and three (P-value of 0.0008).

More specifically, the Tukeys Post Hoc Test showed the highest significant difference in colonization levels of AMF between C. maxima cv. Rouge Vif d'Etampes (August 2\textsuperscript{nd}) and C. maxima cv. Burgess Buttercup (July 2\textsuperscript{nd}) (P-value 0.000003), C. maxima cv. Burgess Buttercup (September 2\textsuperscript{nd} and 3\textsuperscript{rd}) and C. maxima cv. Rouge Vif d'Etampes (August 2\textsuperscript{nd}) (P-value 0.0005), C. maxima cv. Mariana de Chioggia (September 2\textsuperscript{nd} and 3\textsuperscript{rd}) and C. maxima cv. Rouge Vif d'Etampes (August 2\textsuperscript{nd}) (P-value 0.000003).

The Tukeys Post Hoc Test showed a moderate level of significant difference in colonization levels of AMF between: C. maxima cv. Mariana de Chioggia (August 2\textsuperscript{nd}) and C. maxima cv. Burgess Buttercup (July 2\textsuperscript{nd}) (P-value 0.002), C. maxima cv. Rouge Vif d'Etampes
(July 2\textsuperscript{nd}) and \textit{C. maxima} cv. Burgess Buttercup (July 2\textsuperscript{nd}) (P-value 0.009), \textit{C. maxima} cv. Rouge Vif d'Etampes (August 2\textsuperscript{nd}) and \textit{C. maxima} cv. Burgess Buttercup (August 2\textsuperscript{nd}) (P-value 0.0007), The Tukeys Post Hoc Test showed a significant difference in colonization levels of AMF between \textit{C. maxima} cv. Rouge Vif d'Etampes (August 2\textsuperscript{nd}) and \textit{C. maxima} cv. Golden Hubbard (July 2\textsuperscript{nd}) (P-value 0.001), \textit{C. maxima} cv. Mariana de Chioggia (September 2\textsuperscript{nd} and 3\textsuperscript{rd}) and \textit{C. maxima} cv. Rouge Vif d'Etampes (July 2\textsuperscript{nd}) (P-value 0.01), \textit{C. maxima} cv. Mariana de Chioggia (September 2\textsuperscript{nd} and 3\textsuperscript{rd}) and \textit{C. maxima} cv. Mariana de Chioggia (August 2\textsuperscript{nd}) (P-value 0.002).

The Tukeys Post Hoc Test showed a passing level significant difference in colonization levels of AMF between \textit{C. maxima} cv. Rouge Vif d'Etampes (September 2\textsuperscript{nd} and 3\textsuperscript{rd}) and \textit{C. maxima} cv. Burgess Buttercup (July 2\textsuperscript{nd}) (P-value 0.049). The Tukeys Post Hoc Test showed a significant difference in colonization levels of AMF between \textit{C. maxima} cv. Golden Hubbard (September 2\textsuperscript{nd} and 3\textsuperscript{rd}) and \textit{C. maxima} cv. Rouge Vif d'Etampes (August 2\textsuperscript{nd}) (P-value 0.02).

Results from the Phase II summer field trials indicate that \textit{C. maxima} cv. Burgess Buttercup had the highest colonization levels during the July 2\textsuperscript{nd} sampling event of any of the cultivars sampled. The cultivar \textit{C. maxima} cv. Burgess Buttercup also had the highest colonization levels during the August 2\textsuperscript{nd} sampling event. \textit{Cucurbita maxima} cv. Mariana de Chioggia had the highest colonization levels during the September 2\textsuperscript{nd} – 3\textsuperscript{rd} sampling event.

Overall, \textit{C. maxima} cv. Burgess Buttercup had the highest average colonization levels of 10.00%, while \textit{Cucurbita maxima} cv. Mariana de Chioggia had the second highest colonization of 8.25%, \textit{Cucurbita maxima} cv. Golden Hubbard had the third highest colonization level of 6.51%, and \textit{C. maxima} cv. Rouge Vif d'Etampes had the lowest average colonization levels of 2.44% (Figure 3, Table 1).

\textbf{Discussion}

Cucurbits are cultivated worldwide for nutrition, medical, and cultural uses, and the species \textit{Cucurbita maxima} was used as a model organism for this research project due to a void in information about microbial communities in the scientific literature. \textit{Cucurbita maxima} is an economically important crop and its microbial communities should be studied in more detail because they can either be beneficial to the host plant, or can harbor parasites and pathogens that may potentially kill or weaken the host organism, and reduce agricultural yields.

Members of the Cucurbitaceae are often susceptible to fungal, viral, bacterial, and nematode plant pathogens, including: \textit{Cladosporium cucumerinum} Ellis & Arth., \textit{Colletotrichum},
Monosporascus cannonballus Pollack & Uecker, Rhizoctonia solani J.G. Kühn, members of the Ascomycota, Choanephora cucurbitarum (Berk. & Ravenel) Thaxt., a member of the Zygomycota, Sclerotium rolfsii (Curzi) C.C. Tu & Kimbr., a member of the basidomycota, Erwinia spp., a bacterial pathogen, Pseudoperonospora cubensis Berkeley & Curtis, Pythium spp., a member of the Oomycota, as well as Melodogyn and Pratylenchus spp., which are nematode pathogens (Seebold et al. 2009).

Arbuscular mycorrhizal fungi have been proposed as biological control agents to combat soilborne pathogens of herbaceous plants, and have been show to reduce disease symptoms caused by pathogenic fungi and nematodes (Saldajeno and Hyakumachi 2011). Saldajeno and Hyakumachi (2011) use the term Plant Growth Promoting Fungi (PGPF) to describe beneficial fungi, which includes AMF, which stimulate plant growth, and decrease disease occurrence. Pozo et al. (2009) gave the term Mycorrhiza-Induced Resistance (MIR) for a type of pathogen resistance that arises from the colonization of plant roots by numerous types of mycorrhizal fungus. MIR increases the host’s resistance to a diversity of pathogens, and has been shown to decrease feeding by insect predators (Pozo et al. 2009). In the review by Akhtar’s et al. (2011), AMF, Rhizobium, and fungal pathogen interactions are explored, and a direct correlation was shown between colonization of the host plant by AMF and decreased disease severity. Decreased disease severity in plants was attributed to various AMF species, including species of Glomus Gigaspora, and Sclerocystis (Akhtar et al 2011).

Selecting cultivars of Cucurbita maxima that have the highest levels of AMF colonization would be beneficial for agriculturalists, due to the protective and growth promoting potential by the Glomeromycota. Data from the 2015 field sampling show significant differences in AMF colonization levels between cultivars and sampling times. Further studies should investigate as many cultivars as possible, and select the cultivars with the highest AMF colonization levels, which should be promoted for cultivation in a particular geographic location. Having more than 1000 cultivars of C. maxima grown worldwide allows for agriculturalists to maximize yield by creating customizable field plots, which allow individual landowners to choose more suitable cultivars based upon AMF colonization levels. This in turn can reduce synthetic chemical inputs in the field, generating more profit for landowners at less of an economic expense.

Distribution of fungi and ‘fungal like’ organisms was observed over two seasons, and Glomeromycota was observed in sampling location and in every variety of C. maxima sampled.
Although only four cultivars of *C. maxima* were observed for colonization levels at the local level, varying levels of colonization suggests different levels of disease protection in various cultivars. Molecular studies are needed to study the disease defense pathways turned on by beneficial microbes, such as members of the Glomeromycota. Being able to molecularly identify what species of Glomeromycota is colonizing *C. maxima*, as well as being able to understand the molecular mechanisms that allow for disease resistance, agriculturalists will be able to design integrated pest management strategies ideal to the cultivar or geographic location.

Plasmidiphorids are of economic importance to humans, notably as plant pathogens in agricultural and aquatic ecosystems (Esser et al. 2015). This the first time that Plasmidiphorid Slime Molds have been documented in any member of the Cucurbitaceae, and more narrowly, the first time being documented in *Cucurbita maxima*. Plasmidiphorid Slime Molds are known to vector plant viruses to host organisms, and this report provides justification to further study the roll of this organism in members of the Cucurbitaceae. The host organism as well as the number and distribution of divisions/units of the sporosori were traditionally used to identify Plasmidiphorid Slime molds. Since Plasmidiphorid Slime Molds have never been found in a member of the Cucurbitaceae, the Plasmidiphorid Slime Mold found in this study will be considered a new species, or multiple new species. Morphologically, the sporosori found in *C. maxima* most likely resemble members of the genus *Ligniera* and *Sorosphaera*. Molecular identification should be used to correctly identify this organism. Funding and time constraints did not allow for the molecular identification to be completed during this study, but host root tissues have been stored for further molecular analysis. Molecular identification needs to be conducted in order to identify this unknown species.

This study provides a first report of a relationship between Dark Septate Endophytes, Glomeromycota, *Olpidium sp.*, and Plasmidiphorid Slime Molds in *Cucurbita maxima* in the United States. The images provided in this manuscript are the first photographic documentation of these organisms in *Cucurbita maxima*. Members of the Glomeromycota and Dark Septate Endophyte group were previously documented in *Cucurbita maxima* in India, but images and information regarding the cultivars of *Cucurbita maxima* used for these studies were missing from the literature.

This is a first report of the fungal genus *Olpidium* (A. Braun) J. Schrot. being documented from field sampling in the host *Cucurbita maxima*. Mochizuki et al. (2012) stated...
that they used *C. maxima* as host plants to maintain inoculum in culture, but have never shown *Olpidium sp.* in agriculturally cultivated *C. maxima*. Sekimoto et al. (2011) published a multi-gene phylogeny placing *Olpidium* in the phylum Zygomycota, while Mochizuki et al. (2012) published a manuscript stating that *Olpidium* is in the phylum Chytridiomycota. The publication by Sekimoto et al. (2011) precedes Mochizuki et al. (2012), and the molecular phylogeny should take precedence in this circumstance, placing *Olpidium* sp. into a phylum Zygomycota. Since *Olpidium* has never before been documented from field sampled *C. maxima*, this could be representative of a new species. Molecular identification will need to be conducted in the future, in order to officially name this species.

Based upon the data collected during the 2015 field season, it would be recommended to grow *C. maxima* cv. Burgess Buttercup in the southwest region of Ohio, due to *C. maxima* cv. Burgess Buttercup having the highest AMF colonization levels. It would not be recommended to cultivate *C. maxima* cv. Rouge Vif d'Etampes due to its lower levels of AMF colonization.

Potential limitations of the Phase II portion of this research that should be explored in order to design future research into the microbial communities associated with *Cucurbita maxima*. In the future, it would be beneficial to test the differences in colonization rates between plants grown in a completely randomized plot design, versus a partial randomized plot design. The current study used a partial randomized plot design because the researcher wanted to replicate what had previously been surveyed during the preliminary sampling season before. Cultural practices by farmers did not consistently show a randomized growing field, but rows of individual cultivars. Another potential limitation of the Phase II of this project has is that are no standardized methods that are used to estimate the percentage of host roots tissues that are colonized by AMF (Utobo et al. 2011). Various methods have been proposed, but none of the current methods can completely avoid sampling subjectivity (Utobo et al. 2011). Current methods for quantification of root colonization include the determination of a simple presence or absence of distinct morphological features, creating an average between estimated percent colonization of numerous roots, subjective visual estimation, and gridline intersect method (Utobo et al. 2011). Utobo et al. (2011) suggest that every method has limitations.
Conclusion

*Cucurbita maxima* grown in southwest Ohio showed associations with members of the Ascomycota, Basidiomycota, Chytridomycota/Zygomycota, Glomeromycota, Dark Septate Endophytes, Plasmodiophorid Slime Molds, ‘Fungi Imperfecti’, and Nematoda. This study provides a first report of a relationship between Dark Septate Endophytes, Glomeromycota, *Olpidium spp.*, and Plasmodiophorid Slime Molds in *Cucurbita maxima* in the United States. The images provided in this manuscript are the first photographic documentation of these organisms in *Cucurbita maxima* to date. Based upon the data collected during the 2015 field season, it would be recommended to grow *C. maxima* cv. Burgess Buttercup in the southwest region of Ohio, due to *C. maxima* cv. Burgess Buttercup having the highest AMF colonization levels. Cultivation of *C. maxima* cv. Rouge Vif d'Etampes is not recommended in the southwest region of Ohio due to its lower levels of AMF colonization.

Future research could test the differences in colonization levels between using the subjective visual estimation method and the gridline intersect method for colonization rates in *C. maxima*. Another topic of research that would clarify limitations of this study could test the effects of cultural agricultural practices on colonization levels. This includes tilled versus untilled cultivation, cultivation of plants on berms or on level land, irrigation levels between non-irrigated control plots, certified organic soil verses conventional soil, different geographic locations in the United States, and different sources of the seed stock. Conventional agriculture uses cultural techniques such as the application of synthetic fertilizers, pesticides, monoculture of crops, and tilling, which has been shown to reduce AMF populations by up to 50% (Akhtar et al. 2011). Testing variants of conventional farming practices for *C. maxima* should also be explored, in order to understand the impact of these techniques on microbial communities within agricultural ecosystems.
# Tables and Figures

Table 1: Presence of fungi and ‘fungal like organisms’ on different cultivars of *Cucurbita maxima* sampled September 2014, and July, August, September 2015 in the eastern United States.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Features</th>
<th>Novel Associations</th>
<th>Horticultural Practice</th>
<th>State</th>
<th>County</th>
<th>Farm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue Hubbard</td>
<td>H, S</td>
<td>G, A</td>
<td>Organic</td>
<td>OH</td>
<td>Butler</td>
<td>Five Oaks</td>
</tr>
<tr>
<td></td>
<td>H, V, M</td>
<td>G, D</td>
<td>Conventional</td>
<td>MD</td>
<td>Washington</td>
<td>Orr</td>
</tr>
<tr>
<td>Burgess Buttercup</td>
<td>H, Ar</td>
<td>G</td>
<td>Organic-Un</td>
<td>WV</td>
<td>Berkeley</td>
<td>Kenso</td>
</tr>
<tr>
<td></td>
<td>H, V, Ar, Si, M</td>
<td>G, D, A, P</td>
<td>Conventional</td>
<td>MD</td>
<td>Washington</td>
<td>Orr</td>
</tr>
<tr>
<td></td>
<td>H, Ar, S, V</td>
<td>G</td>
<td>Conventional/Unsprayed</td>
<td>OH</td>
<td>Butler</td>
<td>ERC</td>
</tr>
<tr>
<td></td>
<td>H, V</td>
<td>G</td>
<td>Organic-Un</td>
<td>WV</td>
<td>Berkeley</td>
<td>Kenso</td>
</tr>
<tr>
<td>Dills Atlantic Giant</td>
<td>H, Ar, V, S</td>
<td>G, C</td>
<td>Conventional</td>
<td>MD</td>
<td>Washington</td>
<td>Mountain Valley</td>
</tr>
<tr>
<td>Golden Hubbard</td>
<td>H, Ar, S, V, Si</td>
<td>G, P</td>
<td>Conventional/Unsprayed</td>
<td>OH</td>
<td>Butler</td>
<td>ERC</td>
</tr>
<tr>
<td>Kabocha</td>
<td>H, M, S, Ar</td>
<td>G, D</td>
<td>Organic-Un</td>
<td>WV</td>
<td>Berkeley</td>
<td>Kenso</td>
</tr>
<tr>
<td>Mariana di Chioggia</td>
<td>H, Ar, V, S</td>
<td>G, C</td>
<td>Conventional/Unsprayed</td>
<td>OH</td>
<td>Butler</td>
<td>ERC</td>
</tr>
<tr>
<td>Rouge Vif d'Etampes</td>
<td>H, Ar, S</td>
<td>G, A</td>
<td>Organic-Un</td>
<td>WV</td>
<td>Berkeley</td>
<td>Kenso</td>
</tr>
<tr>
<td></td>
<td>H, Ar, V, S</td>
<td>G</td>
<td>Conventional/Unsprayed</td>
<td>OH</td>
<td>Butler</td>
<td>ERC</td>
</tr>
<tr>
<td></td>
<td>H, V, Si, S, Ar</td>
<td>G, C, P</td>
<td>Conventional</td>
<td>MD</td>
<td>Washington</td>
<td>Mountain Valley</td>
</tr>
<tr>
<td>Red Kuri</td>
<td>H</td>
<td>G</td>
<td>Organic</td>
<td>OH</td>
<td>Butler</td>
<td>Five Oaks</td>
</tr>
<tr>
<td>Sweet Meat</td>
<td>H</td>
<td>G</td>
<td>Organic</td>
<td>OH</td>
<td>Butler</td>
<td>Five Oaks</td>
</tr>
<tr>
<td>Turk's Turban</td>
<td>H, Ar</td>
<td>G</td>
<td>Unsprayed</td>
<td>OH</td>
<td>Darke</td>
<td>Downing</td>
</tr>
<tr>
<td></td>
<td>H, V, Ar, Si</td>
<td>G, P</td>
<td>Conventional</td>
<td>MD</td>
<td>Washington</td>
<td>Orr</td>
</tr>
<tr>
<td></td>
<td>H, V, Ar</td>
<td>G</td>
<td>Organic-Un</td>
<td>WV</td>
<td>Berkeley</td>
<td>Kenso</td>
</tr>
</tbody>
</table>

Morphological Features designated by Ar = Arbuscula, H= Hyphae, M= Microsclerotia, S= Spore, Si= Sporosori, V= Vesicle, Novel Associations designated by A= Ascomycota, B=Basidiomycota, C=Chytridiomycota, D=DSE G= Glomeromycota, P= Plasmodiophorid, Horticultural practice designed by Unsprayed= No chemical treatment, Organic= USDA Certified Organic, Organic-UN= Uncertified Organic, Conventional= Grown with synthetic fertilizers and pesticides, States include OH= Ohio, WV= West Virginia, MD= Maryland, Farm name designates 2014 sampling, ERC designates
Table 2: *Cucurbita maxima* cv. Burgess Buttercup (BB), Rouge Vif d'Etampes (RD), Mariana de Chioggia (MC), and Golden Hubbard (GH) analyzed for Mean Percent Colonization, Percent Colonization Standard Error, and Percent colonized Standard Deviation.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Sampling Time</th>
<th>Mean Percent Colonization</th>
<th>Sample Size</th>
<th>Standard Error</th>
<th>Standard Deviation</th>
<th>Mean For Cultivar</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC</td>
<td>1</td>
<td>3.1</td>
<td>50</td>
<td>0.692</td>
<td>4.9</td>
<td>8.25</td>
</tr>
<tr>
<td>MC</td>
<td>2</td>
<td>7.72</td>
<td>50</td>
<td>3.011</td>
<td>21.29</td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>3</td>
<td>13.94</td>
<td>50</td>
<td>3.126</td>
<td>22.109</td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>1</td>
<td>5.86</td>
<td>50</td>
<td>1.203</td>
<td>8.5069</td>
<td>6.51</td>
</tr>
<tr>
<td>GH</td>
<td>2</td>
<td>8.64</td>
<td>50</td>
<td>2.975</td>
<td>21.037</td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>3</td>
<td>5.02</td>
<td>45</td>
<td>1.015</td>
<td>6.8107</td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>1</td>
<td>1.94</td>
<td>50</td>
<td>0.413</td>
<td>2.9235</td>
<td>2.44</td>
</tr>
<tr>
<td>RD</td>
<td>2</td>
<td>1.66</td>
<td>50</td>
<td>0.57</td>
<td>4.0335</td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>3</td>
<td>3.72</td>
<td>50</td>
<td>1.33</td>
<td>9.4069</td>
<td></td>
</tr>
<tr>
<td>BB</td>
<td>1</td>
<td>8.23</td>
<td>47</td>
<td>1.679</td>
<td>11.514</td>
<td>10</td>
</tr>
<tr>
<td>BB</td>
<td>2</td>
<td>11.42</td>
<td>50</td>
<td>2.917</td>
<td>20.631</td>
<td></td>
</tr>
<tr>
<td>BB</td>
<td>3</td>
<td>10.35</td>
<td>45</td>
<td>2.097</td>
<td>14.071</td>
<td></td>
</tr>
</tbody>
</table>

Samples were collected from the Ecology Research Center (ERC) during July 2nd, August 2nd, and September 2nd/3rd, Time 1, Time 2, Time 3.
Table 3: Analysis of Variance (ANOVA) showing significant differences in percent colonization of Arbuscular Mycorrhizal Fungi in *C. maxima* cv. Burgess Buttercup, Rouge Vif d'Etampes, Mariana de Chioggia, and Golden Hubbard at different sampling times.

| Interaction          | Standard Deviation | Standard Error | Pr(>|t|) |
|----------------------|--------------------|----------------|---------|
| (Intercept)          | 0.31862            | 0.59523        | 0.592657|
| varietyGH            | 1.10202            | 0.82907        | 0.184299|
| varietyMC            | 2.09481            | 0.82907        | 0.011781*|
| varietyRD            | 3.131              | 0.82907        | 0.000176***|
| as.factor(time)2     | 1.00105            | 0.82907        | 0.227758|
| as.factor(time)3     | 0.82085            | 0.85109        | 0.335218|
| varietyGH:as.factor(time)2 | 0.01492          | 1.16337        | 0.989773|
| varietyMC:as.factor(time)2 | 0.31888          | 1.16337        | 0.784108|
| varietyRD:as.factor(time)2 | 0.45787           | 1.16337        | 0.694046|
| varietyGH:as.factor(time)3 | 0.2694            | 1.19476        | 0.821683|
| varietyMC:as.factor(time)3 | 2.84686           | 1.17917        | 0.016077*|
| varietyRD:as.factor(time)3 | 1.22831           | 1.17917        | 0.298003|

An ( * ) indicates adjusted P-values under a 0.05% alpha value or 95% confidence interval. Significant Value codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05
Table 4: Tukey Post Hoc test analyzing significant differences in percent colonization between *C. maxima* cv. Burgess Buttercup, Rouge Vif d'Etampes, Mariana de Chioggia, and Golden Hubbard at different sampling times.

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Adjusted P-Value</th>
<th>Sig Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH-BB</td>
<td>0.1548772</td>
<td></td>
</tr>
<tr>
<td>MC-BB</td>
<td>0.0458129 *</td>
<td></td>
</tr>
<tr>
<td>RD-BB</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MC-GH</td>
<td>0.9598524</td>
<td></td>
</tr>
<tr>
<td>RD-GH</td>
<td>0.0005977 *</td>
<td></td>
</tr>
<tr>
<td>RD-MC</td>
<td>0.0034541 *</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Adjusted P-Value</th>
<th>Sig Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD:2-MC:1</td>
<td>0.095378</td>
<td></td>
</tr>
<tr>
<td>BB:3-MC:1</td>
<td>0.9349585</td>
<td></td>
</tr>
<tr>
<td>GH:3-MC:1</td>
<td>0.9999958</td>
<td></td>
</tr>
<tr>
<td>MC:3-MC:1</td>
<td>0.3513291</td>
<td></td>
</tr>
<tr>
<td>RD:3-MC:1</td>
<td>0.9997994</td>
<td></td>
</tr>
<tr>
<td>BB:2-RD:1</td>
<td>0.2765555</td>
<td></td>
</tr>
<tr>
<td>GH:2-RD:1</td>
<td>0.9853868</td>
<td></td>
</tr>
<tr>
<td>MC:2-RD:1</td>
<td>0.9999999</td>
<td></td>
</tr>
<tr>
<td>RD:2-RD:1</td>
<td>0.8244023</td>
<td></td>
</tr>
<tr>
<td>BB:3-RD:1</td>
<td>0.2025468</td>
<td></td>
</tr>
<tr>
<td>GH:3-RD:1</td>
<td>0.8377679</td>
<td></td>
</tr>
<tr>
<td>MC:3-RD:1</td>
<td>0.0104052 *</td>
<td></td>
</tr>
<tr>
<td>RD:3-RD:1</td>
<td>0.9999976</td>
<td></td>
</tr>
<tr>
<td>GH:2-BB:2</td>
<td>0.9689701</td>
<td></td>
</tr>
<tr>
<td>MC:2-BB:2</td>
<td>0.1241834</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sampling time vs cultivar of <em>Cucurbita maxima</em></th>
<th>Adjusted P-Value</th>
<th>Sig Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 by 1</td>
<td>0.010289 *</td>
<td></td>
</tr>
<tr>
<td>3 by 1</td>
<td>0.740529</td>
<td></td>
</tr>
<tr>
<td>3 by 2</td>
<td>0.0008976 *</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cultivar of <em>Cucurbita maxima</em> as a factor of time</th>
<th>Adjusted P-Value</th>
<th>Sig Diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH:1-BB:1</td>
<td>0.9750721</td>
<td></td>
</tr>
<tr>
<td>MC:1-BB:1</td>
<td>0.3253504</td>
<td></td>
</tr>
<tr>
<td>RD:1-BB:1</td>
<td>0.009517 *</td>
<td></td>
</tr>
<tr>
<td>BB:2-BB:1</td>
<td>0.9882996</td>
<td></td>
</tr>
<tr>
<td>GH:2-BB:1</td>
<td>0.3084296</td>
<td></td>
</tr>
<tr>
<td>MC:2-BB:1</td>
<td>0.002547 *</td>
<td></td>
</tr>
<tr>
<td>RD:2-BB:1</td>
<td>0.0000031 *</td>
<td></td>
</tr>
<tr>
<td>BB:3-BB:1</td>
<td>0.9983226</td>
<td></td>
</tr>
<tr>
<td>GH:3-BB:1</td>
<td>0.7316733</td>
<td></td>
</tr>
<tr>
<td>MC:3-BB:1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>RD:3-BB:1</td>
<td>0.0494778 *</td>
<td></td>
</tr>
<tr>
<td>MC:1-GH:1</td>
<td>0.9875733</td>
<td></td>
</tr>
<tr>
<td>RD:1-GH:1</td>
<td>0.3508119</td>
<td></td>
</tr>
<tr>
<td>BB:2-GH:1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GH:2-GH:1</td>
<td>0.9850429</td>
<td></td>
</tr>
<tr>
<td>MC:2-GH:1</td>
<td>0.1687407</td>
<td></td>
</tr>
<tr>
<td>RD:2-GH:1</td>
<td>0.0013449 *</td>
<td></td>
</tr>
<tr>
<td>BB:3-GH:1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GH:3-GH:1</td>
<td>0.9999583</td>
<td></td>
</tr>
<tr>
<td>MC:3-GH:1</td>
<td>0.982907</td>
<td></td>
</tr>
<tr>
<td>RD:3-GH:1</td>
<td>0.7023767</td>
<td></td>
</tr>
<tr>
<td>RD:1-MC:1</td>
<td>0.9825179</td>
<td></td>
</tr>
<tr>
<td>BB:2-MC:1</td>
<td>0.9734776</td>
<td></td>
</tr>
<tr>
<td>GH:2-MC:1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>MC:2-MC:1</td>
<td>0.9023926</td>
<td></td>
</tr>
</tbody>
</table>

An ( * ) indicates adjusted P-values under at a 0.05% alpha value or 95% confidence interval.
Figure 1: Research was conducted at Miami University’s Ecology Research Center (ERC) on Somerville Road, north of Oxford, Butler County, Ohio. The field is approximately 1/2 hectare in size and is adjacent to the ERC access road. The field is bounded by a gravel access road on the east, and abandoned fields on the other 3 sides. Across the access road is a secondary/tertiary growth deciduous forest.
Figure 2: Phase II research plot design showing the planting pattern of *Cucurbita maxima* cv. Burgess Buttercup (BB), Rouge Vif d'Etampes (RD), Mariana de Chioggia (MC), and Golden Hubbard (GH). Rows were planted 2.4m apart with 26 individual plants cultivated in each row.
**Figure 3:** Histogram of data collected during Phase II showing average root colonization levels collected at three sampling times for each cultivar of *Cucurbita maxima* with 95% confidence interval error bars. Cultivars designated by Burgess Buttercup (BB), Rouge Vif d'Etampes (RD), Mariana de Chioggia (MC), and Golden Hubbard (GH). Sampling times designated by July 2<sup>nd</sup> (Time 1), August 2<sup>nd</sup> (Time 2), September 2<sup>nd</sup>/3<sup>rd</sup> (Time 3).
Figure 4: Ascomycota found in Phase I. A. *Chaetomium spp.* on *C. maxima* cv. Rouge Vif d'Etampes at Kensho Farms indicated by arrow B. DSE microsclerotia and dark staining chlamydospore with two dark staining nuclei and simple septa indicated with arrows C, D. Blastically produced spores with two dark staining nuclei labeled with arrow on *C. maxima* cv. Rouge Vif d'Etampes collected from Kensho Farms, *C. maxima* cv. Blue Hubbard collected at Five Oaks Farms. E, F. Dark staining chlamydospore with two dark staining nuclei and simple septa indicated with arrow on *C. maxima* cv. Burgess Buttercup collected at Orr Farms. Mag. bar = 50 µm.
**Figure 5:** Chytridomycota found in Phase I. A - D. Stellate resting spores produced by the genus *Olpidium* sp. indicated with an arrow, showing the constricted apical morphology typical to members of the Chytridomycota. Mag. bar = 50 µm.
Figure 6: ‘Dark Septate Endophyte’ found in Phase I and II. A. Melanized microsclerotia are shown with arrows in *C. maxima* cv. Kabocha. B. Melanized microsclerotia and melanized hyphae are shown with arrows in *C. maxima* cv. Mariana di Chioggia. C – D. Resting Spores in *C. maxima* cv. Mariana di Chioggia E-H. Melanized hyphe and microsclerotia shown with arrows in *C. maxima* cv. Mariana di Chioggia. Mag. bar 50 µm.
Figure 7: *Tetraploa sp.*, a member of the Ascomycota found in Phase I. A. *Tetraploa sp.* observed in *C. maxima* cv. Blue Hubbard, collected from Five Oaks farm, shown with an arrow, having melanized cell wall material and attenuated cells.
Figure 8: ‘Arum’ type arbuscules produced by AMF observed from Phase I and II sampling observed in the following cultivars. A. Developing arbuscule in *C. maxima* cv. Burgess Buttercup collected at Orr Farms. B. Pair of arbuscules imaged in *C. maxima* cv. Golden Hubbard collected at the ERC. C. Imaged with Differential Interference Contrast Microscopy in *C. maxima* cv. Dills Atlantic Giant collected at Mountain Valley Orchard farm. D. *C. maxima* cv. Rouge Vif d'Etampes collected at Kensho Farms. E. *C. maxima* cv. Burgess Buttercup collected at Five Oaks Farms. F. Mariana de Chioggia collected the ERC. G. *C. maxima* cv. Rouge Vif d'Etampes collected at the ERC. C. *maxima* cv. Burgess Buttercup collected at Five Oaks Farms.
Figure 9: ‘Hyphal coils’ or ‘Paris’ type arbuscules were observed in *C. maxima* during Phase I and II sampling in the following cultivars. A. *C. maxima* cv. Rouge Vif d'Etampes at Kensho Farms. B. *C. maxima* cv. Mariana de Chioggia collected at Miami University’s ERC. C. *C. maxima* cv. Turks Turban collected from Orr Farms. D. *C. maxima* cv. Golden Hubbard collected at Miami University’s ERC. E. *C. maxima* cv. Turks Turban collected from Orr Farms, imaged with Differential Interference Contrast Microscopy. F. *C. maxima* cv. Golden Hubbard collected at Miami University ERC. Mag. bar is 50 µm.
Figure 10: Vesicles observed with Differential Interference Contrast Microscopy during Phase I and II sampling. A. Micrograph of *C. maxima* cv. Burgess Buttercup from Orr Farms. B. Micrograph of *C. maxima* cv. Turks Turban collected from Orr Farms. C. Differential Interference Contrast Micrograph of *C. maxima* cv. Turks Turban collected from Orr Farms. D – E. Micrographs of *C. maxima* cv. Blue Hubbard collected from Orr Farms. F. Micrograph of *C. maxima* cv. Turks Turban collected from Orr Farms. G. Micrograph of *C. maxima* cv. Turks Turban collected from Orr Farms. H. Micrograph of *C. maxima* cv. Turks Turban collected from Orr Farms. Mag. bar is 50 µm.
**Figure 11:** Asexual Glomerospores from Glomeromycota were observed during Phase II soil sampling with bright field light microscopy. A-B. Different glomerospore morphologies, shown with arrows, with 50 μm mag. bars. C-D. Parasitized *Gigaspora* glomerospores were observed amongst detritus with 100 μm mag. bars, E-G. Different glomerospore morphologies, shown with arrows, with 50 μm mag. bars. H. Three parasitized glomerospores shown with 100 μm mag. bars.
**Figure 12:** Basidiomycota found in Phase II. A-B. Fungal hyphae with clamp connection imaged with Bright Field Light Microscopy, shown with arrow. Mag. bar is 50 µm.
**Figure 13:** Plasmodiophorid slime molds found during Phase I and II. A. Sporosori of unknown plasmodiophorid species in *C. maxima cv.* Rouge Vif d'Etampes collected from Mountain Valley Orchards. B-H. Sporosori of unknown plasmodiophorid species in *C. maxima cv.* Golden Hubbard collected from Miami University’s ERC. Mag. bar is 50 µm.
Figure 14: Colonization by fungi found Phase I and II sampling in C. maxima. A-B. Infection with dense colonization of fungal hyphae in C. maxima cv. Turks Turban, collected from Orr Farms, and imaged with Bright Field Light Microscopy. C. Dense hyphal and arbuscule colonization was observed in the bottom hemisphere of C. maxima cv. Turks Turban root, from Downing Fruit Farm, imaged with Differential Interference Contrast Microscopy. D. Fungi parasitizing other fungi in C. maxima cv. Golden Hubbard. E. Differential Interference Contrast micrograph of C. maxima cv. Turks Turban root with fungal hyphae and arbuscule, shown with an arrow. Mag. bar is 50 µm.
Figure 15: Plasmodiophorid slime molds sampled during Phase I and II in *C. maxima*. A-D. Sporpsori of unknown plasmodiophorid species in *C. maxima* cv. Rouge Vif d'Etampes collected from phase I sampling at Mountain Valley Orchards, labeled with arrows, imaged with Laser Scanning Confocal Microscopy.
Figure 16: Laser Scanning Confocal Micrographs of ‘Dark Septate Endophytes’ observed during Phase I and II sampling from *C. maxima*. A-C, F. Microsclerotia and hyphae were observed. Dark Septate Endophyte fungi were observed in *C. maxima cv. Rouge Vif d’Etampes* collected at Mountain Valley Orchards, and *C. maxima cv. Burgess Buttercup* from Orr Farms. D, F. Intercellular conidia observed in *C. maxima cv. Rouge Vif d’Etampes* collected at Mountain Valley Orchards, and *C. maxima cv. Burgess Buttercup* from Orr Farms.
Figure 17: Laser Scanning Confocal Micrographs of Glomeromycota sampled during Phase I and II in *C. maxima* labeled with arrows. A, C, G, H. Vesicles were observed in *C. maxima* cv. Dills Atlantic Giant from Mountain Valley Farms, *C. maxima* cv. Turks Turban from Orr Farms, and in *C. maxima* cv. Rouge Vif d'Etampes collected from Kensho Farms. B, E, F. Arbuscules were observed in *C. maxima* cv. Burgess Buttercup collected from Orr Farms. D. Glomeromycota hyphae were observed in *C. maxima* cv. Dills Atlantic Giant from Mountain Valley Orchard.
Literature Cited


45


