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Research Article

First Report of Dark Septate Endophytes imaged in *Cucurbita maxima* grown in the Eastern United States

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Abstract

Dark Septate Endophytes (DSE) are a type mycorrizal fungi that has mixed ecological rolls in the host plant. The cucurbit, *Cucurbita maxima* is an incredibly diverse species, and suggested to have more cultivated forms than any other crop species. *C. maxima* has many medicinal uses, including anti-diabetic, anti-oxidant, anticancer, and anti-inflammatory properties, and is also a major food source due to its fiber content, carbohydrates, β-carotene, vitamins, alkaloids, minerals, fatty acids, flavonoid, and diverse polysaccharide content. Currently, the scientific literature is missing definitive documentation of DSE associated with *Cucurbita maxima* in the United States of America. This study utilizes light microscopy as supportive evidence to show dark septate endophyte relationship with different varieties of *Cucurbita maxima.*

Keywords: Ascomycota; Ascomycetes; Dark Septate Endophytes; DSE; Fungi; Fungal; Cucurbit; Cucurbita maxima; Cucurbitaceae; Microscopy; Microscope; Tetraploa; Hyphae; Microsclerotia

Introduction

The genus *Cucurbita* includes 13 species, with *Cucurbita maxima* producing the most morphologically distinct fruit of any species in this economically important clad [1]. The Latin binomial name suggests vigorous growth of fruit, which is the most massive fruit on Earth. *C. maxima* is an incredibly diverse species, and it is suggested by Ferriol and colleagues to have more cultivated forms than any other crop species [2]. The polymorphic nature of *C. maxima* can result in a misidentification of the species, and lead to confusion when using this cucurbit for botanical research. *C. maxima* originated in South America and are thought to have been domesticated 4,000 years ago [2]. Various forms of *C. maxima* were disseminated to Europe in the 16th century, and subsequently were taken by European explorers to the Indian sub-content, and Southeast-Asia [2].

Cucurbita maxima has many medicinal uses, including anti-diabetic, anti-oxidant, anticancer, and anti-inflammatory properties [3]. It is also a major food source for a wide variety of world cultures, due to its fiber content, carbohydrates, -carotene, vitamins, alkaloids, minerals, fatty acids, flavonoid, and polysaccharide content [3]. Studies have shown bioactivity of certain polysaccharides extracted from C. maxima and much of this activity is derived from the molecular structure of these compounds, which directly affects the hypoglycemic and anti-oxidant properties of the fruit [3].

Dark Septate Endophytes (DSE) is a group of common endophytic fungi that inhabit the root systems of many plant species (Muthukumar and Tamilselvi 2010). Jumpponen and Trappe originally suggested that DSE are Deuteromycotina, or in common terms, 'Fungi inperfecti', which are conidial ascomycetous fungi with no known sexual stage [4]. Jumpponen and Trappe reviewed literature and the major plant host families, as well as species level recognition of angiosperm and gymnosperm hosts [4]. Jumpponen and Trappe also provide a consortium of DSE species known in the literature, and they provided the GeneBank accession numbers for the corresponding fungi [4]. These fungi are characterized by highly melanized hyphae, and they produce microsclerotia, which are thought to serve as overwintering structures (Muthukumar and Tamilselvi 2010). DSE have been shown to provide protection to the host plant, although the extant of this protection is unknown (Muthukumar and Tamilselvi 2010). Like many other types fungi, DSE's relations with its host can span from beneficial to pathogenic in a spectrum, depending on biological and environmental factors. Menkis and colleagues have shown DSE associations with angiosperms and gymnosperms in Lithuania and Sweden [5]. Mandyam suggests that DSE function in the same ecological niche as other mycorrhizaes, and they are multifunctional in the fact that they acquire nutrients for the host, and they have been shown to protect the host from environmental stresses [6]. Jumpponen suggests that the niche of DSE allows this group of fungi to colonize a variety of hosts, which normally would not form ectomycorrhizal, or ectendomycorrhizal associations [7]. This intracellular and intercellular colonization suggests that DSE are ectendomycorrhizal, and thus should be considered mycorrhizal as an ecological role [7].

Detection of dark septate endophytic (DSE) fungi in *C. maxima* has not been documented well in the scientific literature. Images of DSE in *C. maxima* from the United States of America is missing from the literature and is a crucial step in verifying the presence or absence of a fungal endosymbiosis in North America.

The purpose of this study is to provide a detailed first report of a DSE association with *C. maxima* in the United States of America. Understanding root colonization DSE in different varieties of *C. maxima* will allow researchers to make recommendations to agriculturalists that are interested in organic and sustainable production of gourds. Varieties that have more colonization by DSE could potentially have less disease prevalence and might be more adapted to specific geographical areas.

Methods

Preliminary Sampling

The first phase was a survey of *Cucurbita maxima* roots collected from farms in the eastern United States, and the use of traditional light microscopy techniques to discern the presence or absence of DSE in the host. Various microscopic techniques are commonly utilized to image these fungi.

Root samples were collected from 3 farms in southeastern Ohio, 1 farm in West Virginia, and 2 locations in Maryland. 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 microscopic analysis. The cultivars included Blue Hubbard, Burgess Buttercup, Dills Atlantic Giant, Rouge Vif d'Etampes, Red Kuri, Sweet Meat, and Turk's Turban.

Experimental Design

Seedlings were planted on June 2nd, 2015 at Miami University's Ecology Research Center. Roots from *C. maxima* were sampled monthly, on July 2nd, August 2nd, September 2nd and 3rd. During each sampling event, 10 plants of each variety were randomly destructively-sampled, and five roots from each plant were sub-sampled from the total roots collected. The individual roots were randomly cut into 1 cm segments and were selected for analysis.

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^{O}C under 24 hours of florescent lights. Seedlings were transferred from filer 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^{O}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. The field is approximately 1/2 hectare in size and is adjacent to the ERC access road. The field was disked twice and tilled before planting. No chemical treatments were applied to the field pre- or post-planting.

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. 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 2nd, August 2nd, and September 2nd - 3rd, 2015. Roots were stored in plastic bags at 4^{O}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^{O}C for future analysis.

Light Microscopy

Root segments were examined for distinct morphological features using bright field light microscopy and imaged with an Olympus AX-70 light microscope. Identification of DSE fungi was made by the presence or absence of microsclerotia and melanized hyphae structures that were found in the host tissues.

Results

Phase I: Preliminary Sampling Results

Preliminary Phase I sampling during the fall of 2014 of *C. maxima* roots show a diversity of fungi that included 'Dark Septate Endophytes' (Figure 1 A-H) including *Tetraploa* sp. (Figure 2A). 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 rolls of these organisms in and around *C. maxima.*

Figure 1: '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 2: *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.

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 1 A - H). A. Highly melonized 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 and in *C. maxima* cv. Mariana di Chioggia, collected during phase II sampling from Miami Universty's ERC (Micrograph not provided due to low resolution of the digital image).

Discussion

Cucurbits are cultivated worldwide for nutrition, medical, and cultural uses, and the species 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. 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.

Distribution of DSE fungi was observed over two seasons, and DSE was observed in almost every location and in every variety of *C. maxima* sampled, although images were only provided from select varieties due to the quality of the image. This study provides a first report of a relationship between Dark Septate Endophytes in *Cucurbita maxima* in the United States. The images provided in this manuscript are the first photographic documentation of these organisms in *Cucurbita maxima*. Presence or absence of this mycorrhizal relationship could have potential to allow the host to be more adapted to environmental stresses and could aid in the uptake of nutrients that would be unavailable to the host. Molecular identification of these DSE fungi should be carried out in the future to determine the correct genus and species of endophytes living in the host [8-20].

Conclusion

This study provides a first report of a relationship between Dark Septate Endophytes in *Cucurbita maxima* in the United States. The images provided in this manuscript are the first photographic documentation of these organisms in *Cucurbita maxima*. Further research into how these organisms are affecting the hosts adaptability to its environment is needed. Future research should attempt to molecularly characterize the species of fungi living in the host tissues. This first report provides evidence of a relationship between Dark Septate Endophytes in *Cucurbita maxima* in the United States and will lead to more research on this new relationship.

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