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Research Article

Three-Dimensional Rendering of an Unidentified Plasmodiophorid Slime Mold in *Cucurbita maxima* roots using Laser Scanning Confocal Microscopy

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Abstract

In 2018, Plasmodiophorid Slime molds were discovered to inhabit roots of *Cucurbita maxima*, an economically important member of the *Cucurbitaceae* family. Plasmodiophorid Slime molds are in the phylum Cercozoa which are protozoan vectors of viruses. They have been shown to be economically important damaging pathogens of members of the Brassicales. Traditionally, Plasmodiophorid Slime Molds are described as the causal pathogen of Club-Root disease and powdery scab diseases, which disrupts vascular transport of photosynthates to vegetative shoots. Light microscopy techniques including laser scanning confocal microscopy has been used to describe the morphologies of these recently discovered slime molds in *Cucurbita maxima*. These studies showed Plasmodiophorid Slime molds with morphologies similar to members of the genera *Ligniera, Polymyxa*, and *Sorosphaera*, but definitive characterization was not completed. This brief publication is the first Three-Dimensional rendering of Plasmodiophorid Slime molds using Laser Scanning Confocal Microscopy and can be a further method to identify them in their host tissues.

Keywords: Plasmodiophorid Sime Mold; Cucurbita maxima; Cucurbitaceae; Laser Scanning Confocal Microscopy; Light Microscopy; Three-Dimensional; Pathology; Pathogen; Ligniera; Polymyxa; Sorosphaera; Z-Stack; Disease; Diseases; Pathogens

Introduction

Plasmodiophorid Slime Molds are asexual protozoans in the Phylum Cercozoa, which share characteristics with fungi and other chitinous microorganisms [1-3]. The sexual stage of the Plasmodiophora group has never been elucidated, so the biological species concept is very difficult to adopt in this small yet economically significant clade [2]. Plasmodiophorids were first characterized by a cruciform morphology of the nucleus in 1899 [2,4,5]. During the twentieth century, the majority of the research on these organisms has been focused on the morphological and biochemical characteristics of these pathogens.

Since this clade was discovered, morphological and molecular research has revealed 10 genera including *Ligniera* Maire and *A. Tison, Membranosorus* Ostenfeld and H.E. Petersen, *Octomyxa* Couch, J. Leitn. and Whiffen, *Plasmodiophora* Woronin, *Polymyxa* Ledingham, *Sorodiscus* Lagerh. and Winge, *Sorosphaera* Brunch., *Spongospora* Wallr., *Tetramyxa* Goebel and contains roughly thirty-five species within these genera [2]. Traditional morphological identification of these slime molds was determined by the number and shape of associated sporosori and crucifer shaped nucleus, but new identification and cladistics are based upon phylogenetic analysis of the ribosomal 18S sequences [6-11].

Ecologically, Plasmodiophorid Slime molds are obligate parasites of angiosperms, algae, and Oomycota [2]. As of the present, no Plasmodiophorid has been successfully cultured outside of its host, but there sporosori has be collected from host tissues and re-inoculated into the host plant, completing Koch's Postulates [2,12]. Plasmodiophorids can be collected in freshwater, marine, and terrestrial habitats, although water is needed for the movement of primary and secondary zoospores [2,13].

Plasmodiphorids are studied due to their economically important to humans as plant pathogens in agricultural and aquatic ecosystems [2,3]. The most economically devastating diseases caused by Plasmodiophorids are the club-root disease of brassicas, which is caused by *Plasmodiophora* brassicae Woronin, and powdery scab of potatoes, caused by *Spongospora subterranea* (Wallr) Lagerh [2,3,14]. Plasmodiophorid Slime Molds are also studied in great detail because they are known vectors of plant viruses which affect economically important plants including cabbage, peanuts, rice, rapeseed, wheat, and numerous other brassicas [15-19]. In the past decade, research on Plasmodiophorid Slime Molds has increased due to the availability of molecular technologies and advances in computer analysis. The genome of economically important Plasmodiophorids have been elucidated in the past decade which is allowing for a revolution of novel molecular studies to be completed [2,20].

Recent discoveries of new Plasmodiophorid Slime Molds include Neuhauser's report [21] which provided detailed research on a novel Plasmodiophorid pathogen of *Vitis vinifera* Linnaeus, that was named *Sorosphaera viticola* Neuhauser. This novel pathogen is of world-wide economic importance since grapes are grown on every continent except Antarctica. Also, in 2018, Hulse was the first to document Plasmodophorid Slime in a member of the *Cucurbitaceae*, and more specifically, he was the first to image Plasmodiophorid Slime molds in *Cucurbita maxima* (Hulse 2018). Plasmodiophorid Slime Molds have never been found in a member of the *Cucurbitaceae*, which might indicate that these Plasmodiophorid Slime Molds found in his study could be considered multiple new species (Hulse 2018). Morphologically, the Sporosori found in *C. maxima* most likely resemble members of the genus *Ligniera, Polymyxa* and *Sorosphaera.* Hulse's study did not include molecular identification of these novel slime molds due to financial restrictions at the time of the study (Hulse 2018).

Since Plasmodiophorid Slime Molds are known to vector plant viruses to host organisms, Hulse's report provided justification for further research into the roll of this organism in members of the *Cucurbitaceae* (Hulse 2018). Cucurbits are known to be infected with Cucumber Mosaic Virus, Papaya Ring-Spot Virus, and other viral pathogens. The Plasmodiophorid Slime Molds discovered by Hulse (2018) could be the missing vector of these viruses and these relationships should be studied in more detail.

Hulse's report in 2018 was the first time Plasmodiophorid Slime Molds had been imaged with Laser Scanning Confocal Microscopy (Hulse 2018). This form of microscopy is a useful tool to visualize Sporosori, or the asexual body of the slime mold within the host cortical cells. Since Hulse provided evidence of Plasmodiophorid Slime Molds being detected within a member of the *Cucurbitaceae*, the images Hulse and techniques provided are useful in morphologically characterizing these organisms as well as providing staining methodologies that further researchers can use to conduct similar microscopic studies (Hulse 2018).

This study aims to elucidate the three-dimensional structure of unidentified Plasmodiophorid Slime molds living in C*ucurbita maxima* by using Laser Scanning Confocal Microscopy, in order to pinpoint specific genera for further molecular studies. This manuscript will also provide the first three-dimensional renderings of a Plasmodiophorid Slime mold to date, which can be used by further microbiologists as a reference for morphological and potentially evolutionary development studies.

Methods

Preliminary survey

A survey of *Cucurbita maxima* roots was conducted in the eastern United States, and traditional staining techniques were used to discern the presence or absence of Plasmodiophorid slime mold associations in the host. The first phase was conducted in order to establish a justification for further exploration into the colonization mechanisms of Plasmodiphorid Slime molds in *C. maxima.*

C. maxima roots were collected from three farms in southeastern Ohio, one farm in West Virginia, and two farms in Maryland. The specific farms were selected based upon their different agricultural practices that ranged from certified USDA Organic, uncertified organic, unsprayed, and conventional treatment. Root samples were collected randomly from plants of each cultivar, and then roots were chosen at random for staining and microscopic analysis. According to the farmers, the cultivars in this study include Blue Hubbard, Burgess Buttercup, Dills Atlantic Giant, Rouge Vif d'Etampes, Red Kuri, Sweet Meat, and Turk's Turban.

Seed germination

Seeds of *Cucurbita 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 deionized water. This was replicated ten times, for a total of one hundred seeds of each cultivar. Seeds were incubated at 22°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°C with a regime of 18 hours of light, and 6 hours of dark.

Field cultivation

Field 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. 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. The field is partially shaded in early morning by the shadows created from the forest on the east side of the field. 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. 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 4oC until processing within 48 hours post-harvest. Only one, 1 cm segment was used from each root, and the rest of the sample was frozen at -80°C for future analysis.

Staining techniques

The Brundrett methods of root staining was utilized for this research, except with modifications to concentrations of reagents and times. Root 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 within 50% lactic acid-glycerol (v/v) and stored at 4°C.

Laser scanning confocal microscopy

Presence of Plasmodiophorid Slime Mold infection was determined by the presence of Sporosori. Root segments were examined three-dimensionally for Plasmodiophorid Slime molds using laser scanning confocal microscopy and imaged with a Zeiss 710 laser scanning confocal microscope. A Z-stack was performed with a Zeiss 710 Laser Scanning Confocal Microscope and image from this method is included in the results section of this manuscript.

Results

Plasmodiophorid Sporosori were three-dimensionally rendered with Laser Scanning Confocal Microscopy in C. maxima cv. Rouge Vif d'Etampes collected from initial and secondary sampling at Mountain Valley Orchards (Figure 1). This 'Z-Stack' was created using Zeiss software that accompanies the Zeiss 710 microscope. Regions for the 'Z-Stack' were selected that encompasses the entire root diameter and shows multiple infection sites in the top left region of Figure 1.

Figure 1: Three-Dimensional rendering of Unidentified Plasmodiophorid Slime Mold Sporosori in *Cucurbita maxima* roots.

On average, the Sporosori of the unknown Plasmodiophorid Slime Molds are approximately 50 µm and have roughly 20-40 individual units that are 5 µm in diameter. Multiple infections sites on the roots of *C. maxima* suggests that Plasmodiophorid Slime Molds maybe transferred between cells of the host plant.

Discussion

Cucurbits are grown worldwide for cultural uses such as materials for musical instruments, water storage devices, food and nutritional resources, and in some regions of the world, medical

purposes. The species *Cucurbita maxima* has been used as a model organism for this research project due to a lac of information about viral vectors in *C. maxima*, which have been missing in the scientific literature. *C. maxima* is an economically important species and its microbial communities should be studied in more detail because they can harbor viral vectors that can potentially kill or weaken the host organism and reduce agricultural yields. This plant belongs to a large family that contains 800 species, and many of them are of significant economic importance to farmers of Africa, Asian, Europe, and North and South America.

Plasmodiphorid Slime Molds can be economically destructive pathogens in agricultural and aquatic ecosystems by causing clubroot diseases, powdery scabs, and vector viruses to host plants and fungi. This the first time that the three-dimensional structure of Plasmodophorid Slime Molds has been rendered using Laser Scanning Confocal Microscopy in *Cucurbita maxima*. Morphological characteristics suggest these are members of the genera *Ligniera, Polymyxa, Sorosphaera*, but external morphology alone cannot be used to determine the species. There are potentially one to three novel species of Plasmodiophorid Slime Molds imaged during this and previous studies. Since Plasmodiophorid Slime molds have never been described in *Cucurbitaceae*, these could potentially belong to a novel genus as well. The morphologies observed during this study and previous studies are not definitive. Further molecular identification of these organisms is required to correctly place these organisms within a clade. 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.

Members of the *Cucurbitaceae* are known to harbor many viruses, but the vectors of these viruses have only partially been elucidated. Further research needs to be conducted in order to see if these recently discovered Plasmodiophorid Slime Molds are infected by viruses, and if so, what viruses they might be transmitting to cucurbits. It is plausible that the slime molds discovered in this and previous studies could also vector hypoviruses which could provide protection to host plants from pathogens. With so little being understood about this novel interaction, it is recommended that more research be conducted within the *Cucurbitaceae* family to find presence of Plasmodiophorid infections and how these interactions effect the host plants [22-32].

Conclusion

Plasmodiphorid Slime Molds are of significant economic importance to humans, notably as plant pathogens and vectors of viruses. This the first time that the three-dimensional structure of Plasmodophorid Slime Molds has been documented in any member of the *Cucurbitaceae*, and more narrowly, the first time being documented in *Cucurbita maxima*. Since these Plasmodophorid slime molds have just recently been discovered in this family of plants, they very well could be new species. Plasmodiophorid Slime Molds are known to vector plant viruses to brassicas, peanuts, wheat, and many other host organisms. This report provides the first three-dimensional structure of Plasmodiophorid Slime molds and provides a justification to further study the ecology of this organism in other members of the *Cucurbitaceae*.

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