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| Title | Mitochondrial Visualization in Rice Blast Fungus and its Application to 3D Observation, Quantification, and Distribution Analysis [an abstract of entire text] |
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| Citation | 北海道大学. 博士(農学) 甲第13919号 |
| Issue Date | 2020-03-25 |
| Doc URL | http://hdl.handle.net/2115/77948 |
| Type | theses (doctoral - abstract of entire text) |
| Note | この博士論文全文の閲覧方法については、以下のサイトをご参照ください。 |
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Mitochondrial visualization in rice blast fungus and its application to 3D observation, quantification, and distribution analysis

(イネいもち病菌のミトコンドリアの可視化とその3次元観察, 定量及び分配解析への応用)

Muhammad Akhid Syib'li

1. Introduction

Pyricularia oryzae (teleomorph: *Magnaporthe oryzae*) is a devastating filamentous ascomycetes fungus towards cereal plants yield, massively. This pathogen, well known as a rice blast fungus, is also a deadly plant pathogen for rice plant and a serious threat to our global food security. Every year it is capable of causes economic damage to the rice until about \$66 billion, which is equal to give enough food for 60 million people (Pennisi, 2010).

Recently, resistance to QoI fungicides has occurred in many plant pathogens, including *P. oryzae*. Resistance is caused by mutations in the cytochrome b mtDNA. The mutation prevents QoI from binding in the Qo site. Conidia of *P. oryzae* is an important part of the disease spread. The explanation of the QoI resistance spread in the natural population of *P. oryzae* needs basic information about the dynamics of mitochondria, especially during conidia formation. In this study, visualization of mitochondria by GFP was tried. In addition, this technique was used for three-dimensional observation, quantification, and distribution analysis of conidial mitochondria.

2. Rice blast disease dispersion and conidia formation

Conidia can spread and infect other healthy plants with help of wind and rain. So, the increase of conidia concentration in the air will cause the intense of the lesion because of conidial infection (Pinnschmidt et al. 1993). Rice blast disease usually outbreak mainly when the rainy season or transition to the rainy season comes.

The rice blast fungus attack has a unique pattern of symptoms and is easily recognized. The characteristic symptoms of the vegetative stage are the lesions with spindles and the darker gray lesions color at the center surrounded by orange line boundaries. While attacks on the generative stage show blackish gray with fractures on the panicle axis, fungal attacks in the panicle axis usually are called as rotten neck or neck blast. This infection can be very devastating because they cause the failure of the seed to maturate.

If we look deeper into this symptom, many conidia will be found ready to dispatch. During plant infection, *P. oryzae* also form conidia on the leaf surface, make this disease become contagious. Rao and Jesudhas (1982) observe conidia development among 23 isolates of *Pyricularia*. With the increase of the age, the conidium will become bigger and form septum

until conidium has three cells. Conidia with three cells can be indicated as matured conidia. Therefore, there are three form of conidia, such as conidia without septum (one cell), conidia with one septum (two cells) and conidia with two septa (three cells).

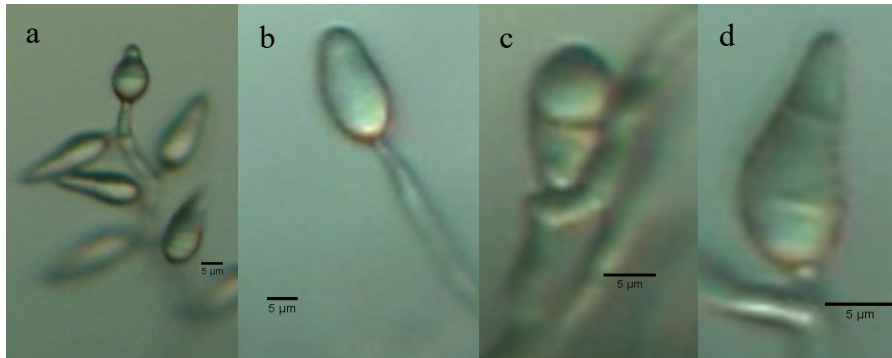


Fig 1. Conidia of *Pyricularia oryzae*. (a) Conidia development with conidiophore, (b) conidium without septa and conidiophore, (c) conidium with one septum and conidiophore, (d) conidium with two septa and conidiophore.

3. Visualizing mitochondria of *Pyricularia oryzae*

Mitochondria are organelles that actively moved, fused, and divided. Within a cell, there might be mitochondria with one or more than one type of mitochondrial DNA (mtDNA). Through random segregation, a fungal cell can have mutated mitochondria that mix with wild-type mitochondria, and it is called heteroplasmy. Another condition is when a fungal cell has completely one type of wild-type or mutated mitochondria, which is called homoplasmy. Both conditions raise the question of what happens with both types of mitochondria when they are mixed.

Fungal cell with mixed mitochondria conditions, which is initially dominated with wild type mitochondria, somehow through segregation might be changed with mutated mitochondria dominance in the new fungal generation. Probably this dominance can cause the fungus to show phenotype of the mutant. This hypothesis might able to explain how pathogenic fungi can gradually turn into resistant towards QoI fungicide and how the mitochondria are passed on to the younger generation. Their dynamics are remained to be explored further, considering the difficulty of observation during the growth and development, especially in the conidia of *P. oryzae*.

Until now, behavior of mitochondria is still unknown in the fungus *P. oryzae*, especially when they are inherited to conidia. Conidia is the part of the fungus that is most responsible for the spread of rice blast disease, so knowing how the mitochondria passed to conidia can answer some of essential questions. This study can reveal basic knowledge to understand how mitochondria behave and are inherited. It is even possible in subsequent studies that the

behavior of mitochondrial DNA can be known, especially when it is inherited and developed in the conidia

Thus, the visualization of mitochondria becomes essential in the observation of mitochondrial behavior in the conidia. Genetic transformation with fluorescent protein genes can be carried out so that the mitochondria will give the expected fluorescence expression under the microscope. Confocal laser-scanning microscopy can be used to visualize and build 3D images of such mitochondria.

Transformation in the fungi can use gene targets in the nucleus that encodes a protein that heading to mitochondria. Most proteins needed by the mitochondria are encoded in the nucleus and synthesized at the cytoplasmic ribosome cytosol, which is then sent to the mitochondrial inner membrane. In this transformation of *P. oryzae* has been carried out by utilizing citrate synthase (CitA)-green fluorescent protein (GFP) fusion. Citrate synthase is a protein encoded from the nucleus of the cell, which then moves to the mitochondria to meet the protein requirements in the tricarboxylic acid (TCA) cycle. By attaching GFP to CitA, this GFP will make mitochondria to express green color under a fluorescence microscope and utilized to reveal mitochondrial distribution patterns through 3-dimensional visualization of mitochondria in the conidia.

Citrate synthase (CitA)-Green Fluorescent Protein (GFP) fusion was utilized to visualize mitochondria in *P. oryzae*, according to the report by Suelmann and Fischer (2000) on *Aspergillus nidulans*. Briefly, DNA sequence encoding N-terminal part of CitA (Accession: MGG_07202) was PCR-amplified with two primers (5'-ATGGCCTCTGCTATGCGC-3' and 5'-CTGGTCAAGAGTGACCTTGTC-3') and cloned into the N-terminal of EGFP gene in pBLASTR-TEF1::eGFP::Mop53BP1 (Ohara et al. 2018) using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs Japan, Tokyo, Japan). The vector was then inverse-PCR amplified with two primers (5'-GCGACGGCCCCGTG-3' and TAGGGGATATCAGCTGGATGG-3') and re-circularized to get rid of Mop53BP1 gene and introduce a stop codon for EGFP. The resulted vector, pBLASTR-TEF1::CitA::eGFP was used for the transformation of *P. oryzae* Ina86-137 strain according to the method by Miki et al. (2009). The transformation was confirmed with PCR and Southern hybridization of *CitA*-EGFP fusion gene.

Pyricularia oryzae INA 86-137 (Cit-GFP) was cultured on the oatmeal agar with incubation time for two weeks with temperature 25 °C under fluorescence light. After the fungus covered the oatmeal agar surface, the fungal colony was washed by sterilized distilled water (SDW). Then, the fungal cultures were incubated again for 36 hours (H) to produce one,

two, and three cellular conidia. Conidia were harvested with SDW and fixed with formalin 5%. Conidia were set up in the slide to observe mitochondria structure.

Observation of mitochondria was conducted with fluorescence microscopy BX50 (Olympus) for comparing the result from (CitA)-GFP and MitoTracker. 3D images of mitochondria were acquired by Confocal microscopy Leica TCS SP8 STED 3X and Las X software using HC PL APO CS2 100x/1.40 (Oil), equipped with laser Diode 405 and WLL (70%). Conidia were exposed by laser with excitation/emission wavelengths were 488 nm/498–530 nm (CitA-GFP).

The results showed that both green colors from (CitA)-GFP and red color from MitoTracker colocalized inside conidia (Fig. 2). Both colors indicate almost at the same position after merged. MitoTracker has been developed especially for staining mitochondria (Zhang et al. 2015). Based on this result, it suggested that (CitA)-GFP is a reliable method to visualize mitochondria in the *P. oryzae*.

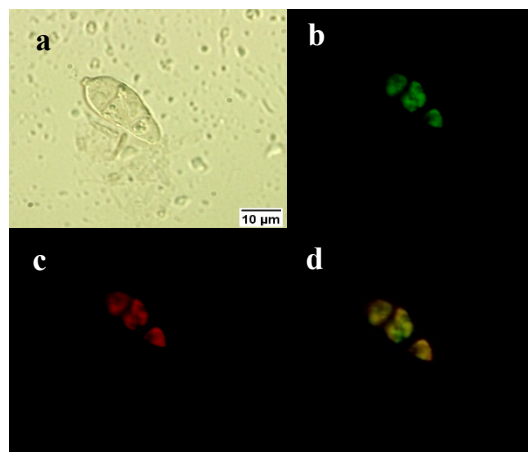


Fig. 2 Conidia with 3 cells and mitochondrial appearance. Mitochondria appearance under fluorescence microscopy with bright field (a), citA-GFP (b), MitoTracker (c) the merged image between the result from citA-GFP (green) and MitoTracker (red) (d). The observation was conducted by using fluorescence microscopy BX 50 and the pictures were adjusted with ImageJ Fiji (<https://imagej.net/Fiji>) for the contrast and brightness.

Through 3D visualization, we would able to observe the display of the mitochondria from many angles without blind spot, so that we could understand the mitochondrial structure much better rather than 2D visualization (Fig. 3). In conidia, mitochondria were observed based on various 3D morphology and the length, mitochondria were categorized into dots, tubules and networks (Table 1).

The relationship between conidial cell number and categorization of conidial mitochondria was analyzed by the enumeration of conidia with each category of mitochondria. 75 conidia of each number of the cell were selected to build 3 dimensional-object of mitochondria. Within each cell number, conidia were grouped into based on mitochondrial morphology appearance, such as dot when mitochondria appearance was only dotted and as tubule or network when we could find tubule or network form in the conidia cell. All types of conidia were dominated by network mitochondria (Fig 4.). There are a few single cellular conidia that could be found without mitochondria and contained with only dot mitochondria.

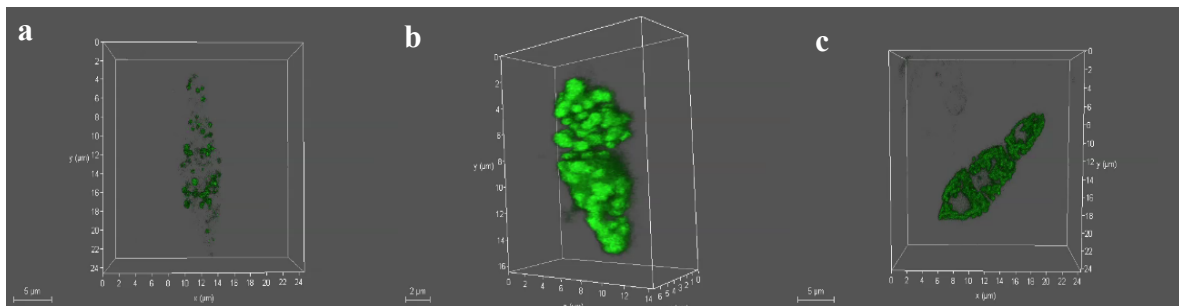


Fig 3. Mitochondria morphology in the conidia and hyphae. 3D image of mitochondria in the single-cellular (a), two-cellular (b) and three-cellular conidia (c).

Table 1. Category of Mitochondrial morphology of *P. oryzae*

| No | Category | Description | Length (μm) | Mitochondria image |
|----|----------|------------------------------------------------|-------------|--------------------|
| 1 | Dots | Circle and like ball | 0.23-0.75 | |
| 2 | Tubules | More oval, not branched and formed like a tube | > 1.6 | |
| 3 | Network | Tubules branched and connected | > 1.6 | |

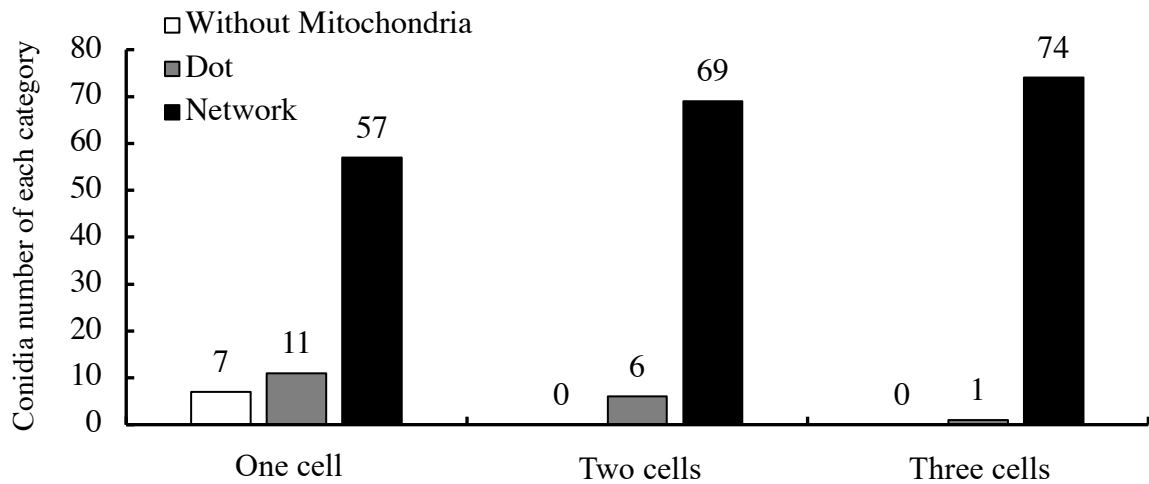


Fig. 4 Mitochondria morphology in each type of conidia. Conidia were categorized based on their cell number. 75 conidia were selected to build 3 dimensional-object of mitochondria. Within categories, conidia were grouped into based on mitochondrial morphology appearance, such as dot when mitochondria appearance was only dotted and as tubule or network when we could find tubule or network form in the conidia cell. For example, a conidia with all types of mitochondria was categorized as network. No conidia was categorized as tubule, which contains only tubule or mixture of dot and tubule.

4. Quantification of Conidial Mitochondrial Volume using MitoGraph software

Mitochondrial quantification is a challenging study, as mitochondria are highly active organelle with diverse shapes from round until becoming interconnected web. Therefore, quantification based on 3D analysis will give more information about the mitochondrial structure and behavior. MitoGraph is an open-source software that can quantify mitochondrial volume in the living cell organism (Viana et al. 2015). This software has been tested using *Saccharomyces cerevisiae* to measure mitochondrial volume (Rafelski et al. 2012). Another research from Harwig et al. (2018) also used MitoGraph software to measure the mitochondrial amount in the mammalian cell. Hence, this study is the first research that uses MitoGraph in the filamentous fungi, *P. oryzae*.

Generally, conidia will develop from one cell until becoming three cells; however, after transformation, even though most of them grow until becoming three cells, some conidia remain in the same condition as conidia with one cell or two cells. Nevertheless, quantification towards conidia with one, two, and three cells is essential to reveal basic information about mitochondrial amount distribution and make an analysis based on it. This observation has a purpose to know mitochondrial development patterns through their amount changes.

Conidia suspension of *Pyricularia oryzae* strain INA 86-137 (CitA)-GFP was taken 3 μ l by micropipette and dropped on a slide glass. Conidia suspension on slide glass was given a cover glass. The cover glass was sealed by nail polish with a clear color. This nail polish kept the water under cover glass not to evaporate.

Z stack images were acquired by Confocal microscopy Leica TCS SP8 STED 3X and Las X software using HC PL APO CS2 100x/1.40 (Oil), equipped with laser WLL (70%). Conidia were exposed by laser with excitation/emission wavelengths were 488 nm/498– 530 nm (CitA-GFP). Z stack images were set up with 1024 x 1024 resolution, with z step size 0.2 μ m.

MitoGraph software V3.0 could be downloaded from <http://rafelski.com/susanne/MitoGraph> or <https://github.com/vianamp/MitoGraph>. MitoGraph was operated under mac Operating System. After downloading, there were five files that we could obtain, (1) CropCells.ijm; (2) GenFramesMaxProjs.ijm; (3) LICENSE; (4) MitoGraph; (5) README.md. File (1) and (2) were macros that could be opened in ImageJ software (<https://imagej.net/Fiji>), these two macros had a function to prepare MitoGraph input files. After the input files were ready, MitoGraph Software could be operated from a terminal in the iMac or Macbook. In this study, MitoGraph was operated with iMac (21.5-inch, Late 2013) macOS High Sierra, Processor 3.1GHz Intel Core i7, Memory 16 GB 1600 MHz DDR3, Graphics NVIDIA GeForce GT 750M 1024 MB, Series Number C02M70D3F8J8. MitoGraph output (VTK files) could be opened by Paraview software.

Regression and correlation analysis showed that mitochondrial volume based on voxel (surface) or length (skeleton) was not demonstrated a significant difference against conidial length. This result was different from the result of Rafelski et al. (2012), they showed that mitochondrial volume increased with the increase of yeast size. Based on mitochondrial distribution analysis, there is tendency that mitochondrial volume increased. (Fig 5).

Based on this result, it showed that conidial growth was not synchronized with mitochondrial development, even there is tendency that mitochondrial volume increased in the three cellular mitochondria. Therefore, observation and quantification of mitochondrial volume during conidia development become essential so that we can see the more complete story of mitochondrial dynamic in conidia of *P. oryzae*.

5. Visualization of mitochondrial dynamics during conidia formation using slide culture-confocal microscopy

Until now, some fungal studies including *Pyricularia oryzae*, about mitochondrial dynamics are still limited in the hyphae, because in order to observe conidia, the fungus should be incubated with a particular temperature under fluorescence light treatment, and it is a

difficult effort to achieve. One of the attempts to understand the mitochondrial structure is through collecting many conidia and describe their structure as in the previous chapter. However, of course, some information is still missing.

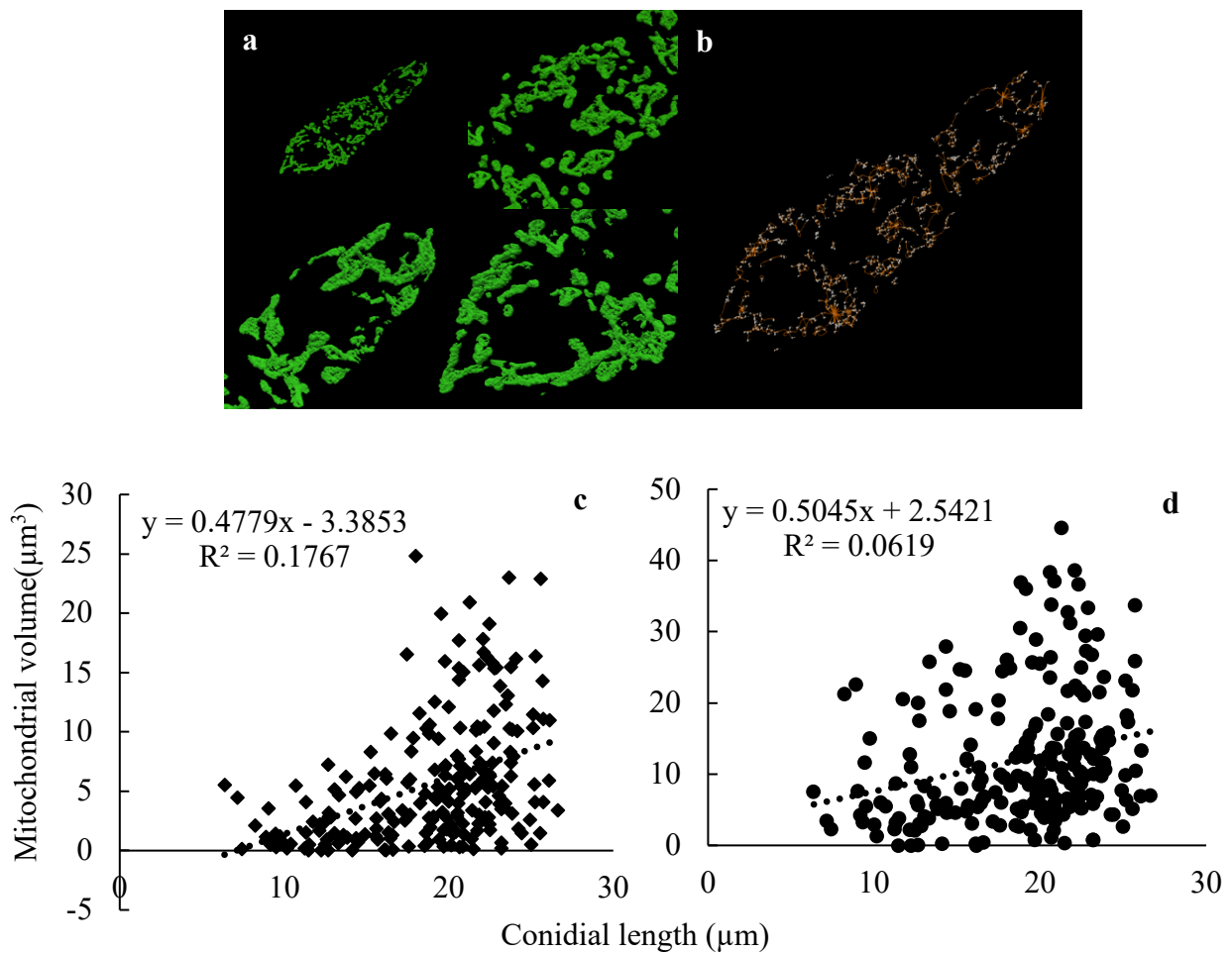


Fig 5. MitoGraph output. 3D conidial mitochondria (a); mitochondrial skeleton (b); the relationship between mitochondrial volume (μm^3) based on voxel (c) and skeleton length (d) towards conidial length (μm) (*continued*).

The development of appropriate methods must be carried out to obtain images of conidia growth from one cell to three cells continuously. One of possible way to do this is to use the slide culture technique. Each researcher has the characteristics of different methods, depending on the microbes used. So many trials and error are required to get the most optimal approach. In this study, in situ observation of conidia development, together with its mitochondria, was carried out using a modified slide culture technique.

Based on the 3D image result, in the complete conidia, mitochondria develop to become a network-shaped (Fig 6). From single-cell conidia, mitochondria have become a network-shaped, but look denser, then fragmented and become a wider network, with the increase of conidia age. During the conidia development, mitochondria are keep changing and some of them disappear.

This result is in accordance with mitochondrial volume analysis since single cell-conidia until there-cellular conidia (Fig 7). The value decreased with the increase of conidia growth and it might be a result of mitophagy events. This is in line with research from Zhong (2016) mitochondrial fission influences the conidiation of *P. oryzae*.

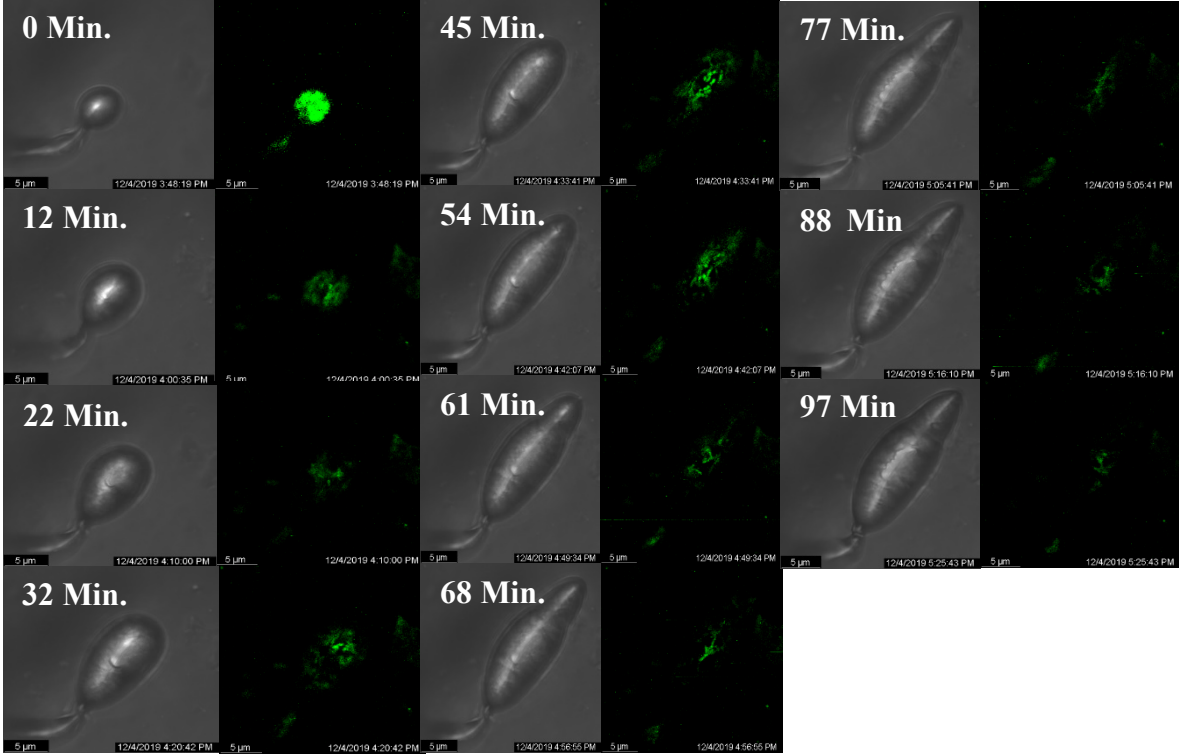


Fig. 6 Mitochondrial dynamic during conidia development

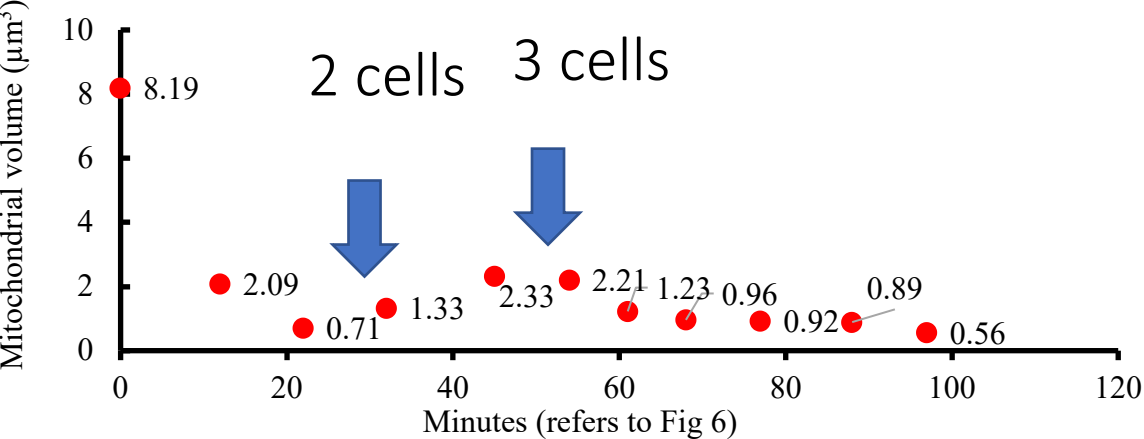


Fig 7. The changes in mitochondrial volume during conidia development

6. Conclusions

Transformation using CitA-GFP were success to visualize mitochondria. Based on 3D observation and categorization, mitochondrial forms were not uniform. Mitochondria might be developed from a dot and become network with the ageing conidia Mitochondrial volume was

not strongly correlated with conidial length. Mitochondrial dynamics during conidia development showed that mitochondrial keep changing during conidia development.

References

1. **Harwig, M. C., Viana, M. P., Egner, J. M., Harwig, J. J., Widlansky, M. E., Rafelski, S. M., & Hill, R. B.** (2018). Methods for imaging mammalian mitochondrial morphology: A prospective on MitoGraph. *Analytical Biochemistry*, 552(March), 81–99. <https://doi.org/10.1016/j.ab.2018.02.022>
2. **Miki S, Matsui K, Kito H, Otsuka K, Ashizawa T, Yasuda N, Fukiya S, Sato J, Hirayae K, Fujita Y, Nakajima T, Tomita F, Sone T** (2009). Molecular cloning and characterization of the AVR-Pia locus from a Japanese field isolate of *Magnaporthe oryzae*. *Mol Plant Pathol* 10(3): 361–374. <http://doi.org/10.1111/j.1364-3703.2009.00534.x>.
3. **Pennisi E** (2010) Armed and dangerous. *Science*. 327:804–805. doi: 10.1126/science.327.5967.804
4. **Pinnschmidt HO, Klein-Gebbinck HW, Bonman JM, Kranz J** (1993) Comparison of aerial concentration, deposition, and infectiousness of conidia of *Pyricularia grisea* by spore sampling technique. *Phytopathology* 83:1182-1189
5. **Rao KM, Jesudhas ML** (1984) Conidium ontogeny in pyricularia. *Phytopath.* 110 (3): 235—244.
6. **Suelmann R, Fischer R.** (2000). Mitochondrial Movement and Morphology Depend on an Intact Actin Cytoskeleton in *Aspergillus nidulans*. *Cell Motil Cytoskel* 45:42–50. doi:10.1002/(SICI)1097-0169(200001)45:1<42::AID-CM4>3.0.CO;2-C
7. **Zhang Y, Shi H, Liang S, Ning G, Xu N, Lu J, Liu X, Lin F** (2015) MoARG1, MoARG5,6 and MoARG7 involved in arginine biosynthesis are essential for growth, conidiogenesis, sexual reproduction, and pathogenicity in *Magnaporthe oryzae*. *Microbiological Research* 180 : 11-12. doi: 10.1016/j.micres.2015.07.002.
8. **Rafelski, S. M., Viana, M. P., Zhang, Y., Chan, Y.-H. M., Thorn, K. S., Yam, P., et al.** (2012). Mitochondrial network size scaling in budding yeast. *Science* 338 (6108): 822-824. <http://dx.doi.org/10.1126/science.1225720>.
9. **Viana, MP, Lim S, Rafelski, SM.** (2015) Quantifying Mitochondrial Content in Living Cells. In E. Paluch (Ed.), *Biophysical Methods in Cell Biology* **125**: 77-93.
10. **Zhong K, Li X, Le X, Kong X, Zhang H, Zheng X, Zheng X, Wang P, Zhang Z** (2016) MoDnm1 dynamin mediating peroximal and mitochondrial fission in complex with MoFis1 and MoMdv1 is important for development of functional appressorium in *Magnaporthe oryzae*. *Plos Pathogen* 12 (8): 1-34. doi:10.1371/journal.ppat.1005823