UNIVERSIDADE FEDERAL DA GRANDE DOURADOS

MICROTUBULE BASED CYTOSKELETON FOR SEPTIN RING ASSEMBLY DURING APPRESSORIUM FORMATION BY THE RICE BLAST FUNGUS

FERNANDA DE PÁDUA DEL CORONA

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Monograph presented to the Universidade Federal da Grande Dourados, as part of the requirements of the Bachelor's degree in Agronomy, to obtain the title of Agronomist Engineer.

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by

Fernanda de Pádua Del Corona

Monograph presented as part of the requirements to obtain the title of
AGRONOMIST ENGINEER

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ABSTRACT: The rice blast fungus, *Magnaporthe oryzae*, causes the major destructive disease on worldwide rice production and understanding its infectious process must be a priority to develop new control strategies. The purpose of this work is to investigate the appressorium formation in germ tube of *M. oryzae* conidia and the involvement of the microtubule based cytoskeleton for septin ring assembly. For that purpose, the experiment was developed at the Department of Plant Pathology at the University of Arkansas and used conidia of the mutant ΔSep5-GFP of *M. oryzae* in seven treatments. The conidia were collected into chamber slides, allocated into seven wells with 2000 conidia/mL concentration. Right after, the fungicide benomyl (5 µg/mL) was applied within a 2 hours difference, each, over a 10-hour period (0, 2, 4, 6, 8 and 10 hours), plus control with 0,5% of dimethyl sulfoxide (DMSO), an organosulfur compound. The chamber slides were kept overnight in an incubator, adjusted to 24ºC and 12:12 light cycles, after the last benomyl application. On the following day, conidia were visualized on the live-cell imaging microcope with magnification of 200x and five frames of each treatment were taken. Data sets were analyzed using linear regression to examine the relationships between fungicide exposure time and apressoria formation. In order to the confirm or not the septin ring assembly in the appressoria, epifluorescence imaging were also taking from the 0 hours, 10 hours and DMSO treatment with magnification of 1000x. The results achieved demonstrated that germination and appressorial formation were lower in conidia at initials exposure times of the fungicide. Furthermore, as benomyl action interrupts mitosis and so depolymerizes microtubules, the septin ring assembly was only visualized in the DMSO treatment.

Keywords: *Magnaporthe oryzae*, appressorium, microtubules, septin ring, benomyl.

CORONA, F.P. **MICROTUBULOS DO CITOESQUELETO PARA A MONTAGEM DO ANEL DE SEPTINA DURANTE A FORMAÇÃO DO APRESSÓRIO PELO PATÓGENO DA BRUSONE.** 2018. Monografia (Graduação em Agronomia) – Universidade Federal da Grande Dourados, Dourados/MS.

RESUMO: O fungo do brusone, *Magnaporthe oryzae*, é o patógeno da principal doença da cultura do arroz e o estudo do seu processo infeccioso deve ser uma prioridade para o desenvolvimento de novas estratégias de controle da doença. O objetivo deste trabalho é investigar a formação de apressórios no tubo germinativo de conídios de *M. oryzae* expostos ao benomil e o envolvimento dos microtubulos do citoesqueleto na montagem do anel de septina, essencial para que o processo infeccioso continue pela formação do peg de penetração. Para isto, o experimento foi desenvolvido no Departamento de Fitopatologia da University of Arkansas e utilizou conídios do mutante ΔSep5-GFP de *M. oryzae* em sete tratamentos. Os conídios foram coletados em *chamber slides*, alocados em sete células com 2000 conídios/mL de concentração. Logo após, o fungicida benomil (5 µg/mL) foi aplicado com uma diferença de 2 horas, entre cada aplicação, ao longo de um período de 10 horas (0, 2, 4, 6, 8 e 10 horas), mais o tratamento controle com 0,5% de dimetilsulfóxido (DMSO), um solvente organossulfurado. As *chamber slides* foram mantidas durante a noite na incubadora, programada a 24ºC e fotoperíodo de 12:12, após a última aplicação. No dia seguinte, os conídios foram visualizados no microscópio de visualização *live-cell*, com resolução de 200 vezes, e cinco fotos de cada tratamento foram tiradas. Os conjuntos de dados foram analisados por meio de regressão linear para examinar as relações entre o tempo de exposição ao fungicida e a formação de apressórios. A fim de confirmar ou não a montagem do anel de septina no apressório, também foram tiradas fotos *epifluorescence* dos tratamentos 0 horas, 10 horas e controle com resolução de 1000 vezes. Os resultados obtidos demonstraram que a formação de apressórios na extremidade dos tubos germinativos foram tanto menores quanto mais cedo ocorreu a exposição dos conídios ao benomil. Além disso, como a ação do benomil interrompe a mitose e depolimeriza os microtúbulos, a montagem do anel de septina foi somente visualizada no tratamento com DMSO.

Palavras-chave: *Magnaporthe oryzae*, apressório, microtubulos, anel de septina, benomil.

1. INTRODUCTION

Rice is the third most staple food produced in the world. The rice paddy production was 741 million tonnes in 2014 (Faostat, 2014) and the Food and Agriculture Organization's forecast for global production in 2017 was stablished at 759.6 million tonnes (FAO, 2018). More than half of the world have rice as staple diet, consequently yield increases are expected by 2050 due to the food demand from population growth (NALLEY et al., 2016).

One of the biggest impediments to those yield increases is the presence of rice blast, caused by *Magnaporthe oryzae* (Nalley et al., 2016). It is the most destructive disease of rice paddies, consequently a menace to global food security. Up to 30% of the rice crop losses are due to blast disease, corresponding enough amount to feed 60 million people (DAGDAS et al., 2012).

According to Filippi et al. (2015), "the blast affects the rice plant from the seedling stage to the grain maturation, and occurs in all federal states of Brazil where the cereal is cultivated". However, the occurence of the disease is "variable, depending on the cropping system, agronomic pratices and climate conditions". Also, Nalley et al. (2016) relates that "yield loss from blast infections depends on varietal susceptibility, the degree of infection, and the timing of fungicide application", and some of those losses have reached 50% or more.

Fungicides have been the simple and important tool to control the blast, yet it can bring inconvenient consequences such as an increase in the costs, resistance development by certain microorganisms and risks to human health and to the environment (Filippi, 2015). Thereby new sustainable strategies to control the disease, besides the use of fungicides, have to be developed. With this in mind, to investigate the morphological and physiological infection structures is therefore a priority to the development of sustainable technologies to control efficiently the blast disease.

One of those morphological structures that has been studied since its discovery in 1971 by Lee Hartwell (Khan, et al., 2015), is the septin, found at the appressorium pore as ring-shaped, required to the infection process in *M. oryzae*. According to Gladfelter and Bridges (2015), "septins are a conserved family of cytoskeletal GTP-binding proteins that function in cytokinesis, cell polarity, and membrane remodeling in many eukaryotic cell types". Also Tabarés and Martín (2010) mentioned the septin involvement in the organization of actin and microtubule cytoskeletons.

In fungi, septin structures have different shapes and functions inside a single cell. The investigation of septins could explain morphogenetic processes such as the capacity of

fungal pathogens to go through morphological transformations in order to penetrate the host (Tabarés and Martín, 2010). A great example of septin mediating host invasion is the rice blast fungus, *Magnaporthe oryzae* (*Pyricularia oryzae*), which has five septins known (Sep1, Sep3, Sep4, Sep5 and Sep6) (Khan et al., 2015) and have an extremely important role in the appressorium as they "provide the cortical rigidity and membrane curvature necessary for protrusion of a rigid penetration peg to breach the leaf surface" (DAGDAS et al, 2012).

To caused disease, *Magnaporthe oryzae*, a filamentous and heterothalic ascomycete fungus, forms a struture called appressorium and it has melanin as a peculiar compound in the cell wall. Early studies of appresorium observed its primary function was believed to be in attachment (Howard and Valent, 1996). However, it has been proved to serves as the place of inicial secretion of fungal effectors, and its growth demands highly coordinated cytoskeleton reorganization and cell cycle regulation (Jenkinson et al, 2016). In addition, Jenkinson and group proves that the appressoria are not only more complex than previously thought, but they also "remain mitotically active during invasive hyphae proliferation and the appressorial nucleus undergoes extreme constriction and elongation through the penetration peg".

Consistent with the importance of rice as a staple food for more than half of the world and the appressorium unquestionable role in pathogenesis, it is clear that the rice blast disease is a dangerous threat and must be a priority currently and in future studies. Therefore, the aim of this work is to investigate the appressorium formation in germ tube of *M. oryzae* conidia exposured to benomyl and the involvement with microtubule based cytoskeleton for septin ring assembly.

2. LITERATURE REVIEW

The plant infection of the rice blast fungus, *M. oryzae*, begins when a asexual three-celled spore (a conidium), lands and germinates on the host cuticle (Figure 1). The conidia adhesion is successful due to spore tip mucilage that is realeased to the hydrophobic leaf surface, maintaining the attachment needed as the conidium germinates (Kershaw et al., 2018). The germination starts within 30 minutes after the contact with a substrate or a liquid (Howard and Valent, 1996). The single polarised germ tube releases from the conidium and after 4 to 6 hours, the tip of the germ tube develops completely the appressorium, depending upon environmental conditions (Kershaw et al., 2018). Within 2-4 hours, while the germ tube grows on the host surface, it starts a "hook" formation at its apex, swelling and differentiating, marking the onset of the specialised infection cell called an appressorium (Howard and Valent, 1996). In the meantime, a single mitosis event generates two daughters nuclei from the only nuclei contained in the spore that migrated into the tube. Right after, one daughter migrates back to the conidium that later undergoes through an autophagic cell death process, while the other daughter migrates into the unripe appressorium and a septum is arranged (Fernandez and Orth, 2018). After maturation, the appressorium remains mitotically active for invasive hyphae primary development and subsequent proliferation (JENKINSON et al., 2016).

The most peculiar feature about this specific infectious process described by Dagdas et al. (2012), is that *M. oryzae* forms the dome-shaped appressorium, who develops enormous cellular turgor of up to 8.0 MPa (equivalent to 40 times that of a car tire) and converts this high pressure into physical force to pierce the host surface. Once formed, the fungi appressorium develops huge cellular tugor due to the accumulation of glycerol against the rapid influx of water into the cell, maintained in the appressorium by a melanin-rich cell wall. The turgor is converted into a physical force which pushes a narrow penetration peg that ruptures the leaf cuticle.

After the appressorium maturation and melanization, the appressorium pore receives a "pore wall overlay", where the penetration peg emerges. The penetration peg is the great responsible for the rupture of the plant cuticle and cell wall, and obvious entrace into the leaf epidermis. The tip-growing cellular protuberance, with around 7 μm in diameter, transfers the contents of the appressorium into the plant tissue cells. Posteriorly, the narrow peg expands and becomes the primary infection hypha that later ramifies and starts the invasive hyphae proliferation within the host (HOWARD AND VALENT, 1996).

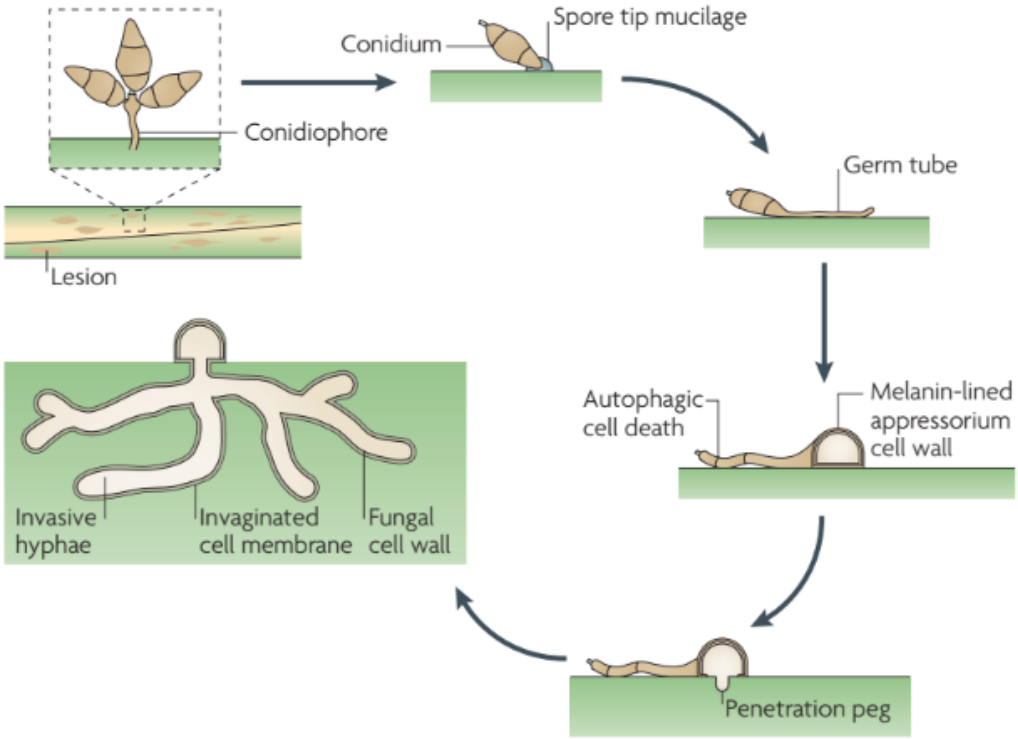

FIGURE 1. Life cycle of the rice blast fungus *Magnaporthe oryzae*. (WILSON, R. A.; TALBOT, N. J., 2009)

Interestingly, Dagdas et al. in 2012 relates a toroidal ring-shaped strutured at the bottom of the appressorium that is formed by septins, F-actin (filamentous actin) and others cytoskeletal elements. The same structures, according to the aurthors, responsible to "provide the cortical ridity and membrane curvature" needed for the penetration peg projection and break into the host surface. Septins are a conserved family of cytoskeletal – from yeast to humans- of small morphogenetic guanosine triphosphatases (GTPases) binding proteins that form higher-order structures on the cell cortex and link with microtubule and actin cytoskeletal networks (Gladfelter and Bridges, 2015). To understand the cytoskeleton organization in the appressorium during cell invasion, it was generated GFP-tagged versions of four septins that later was observed a formation of a 5.9 µm ring colocalized with the F-actin network. Later, they visualized that in isogenic mutants without any of the four septins, the others septins and F-actin mislocalized and missed to form the ring structure, concluding the necessity of association between septins to organize F-actin network in the appressorium (DAGDAS et al., 2012).

Microtubules, actin filaments and intermediate filaments are the three broad classes of protein that constitute the cytoskeleton. Together, these proteins establish eukaryotic cell and tissue shape by its polymerization and depolymerization, especially actin and microtubules, that

create directed forces that move changes in cell shape and guide the organization of cellular components (Fletcher and Mullins, 2010). However, it is along with associated motor proteins (myosin, kinesin, and dynein) that the cytoskeleton guide a wide range of fundamental cellular functions, involving cell migration, movement of organelles and cell division (Nath, 2003). Yet, Mostowy and Cossart (2012) related in the nature review more then a hundred studies to evince the increasingly recognition of septin as the fourth component of the cytoskeleton.

Actin is a ~40 kDa globular protein and can be known as globulin actin (G-actin) in its monomeric form or filamentous actin (F-actin) in its filamentous helix polymerized chains. They are polar with a plus end and a minus end, where they assemble and disassemble (Mostowy and Cossart, 2012). On the other hand, microtubules contain 13 parallel protofilaments of α-tubulin and β-tubulin heterodimers arranged in a hollow tube (Fletcher and Mullins, 2010). The cylindrical structures are polar and each tubulin monomer of ~50 kDa binds one GTP molecule. Microtubules polymers can reach a diameter of ~25 nm and are known to be the stiffest of all cytoskeletal components. They can assemble and disassemble at the plus end. Intermediate filaments form a tetrameric subunit assemble by individual proteins, that later forms an unit length filament (ULF) until, finally, join end-to-end with other ULFs to form intermediate filaments with a diameter of ~11nm. Unlike the others, intermediate filaments and septins are non-polar (MOSTOWY AND COSSART, 2012).

Septins have been acknowledge as cytoskeleton components for the ability to form filaments as also their link with cellular membrane, actin filaments and microtubules. Recent works, have shown that septins arrange essential cellular processes, like cytokinesis, ciliogenesis and neurogenesis, acting as scaffolds for protein enrollment and as diffusion barriers to compartmentalize cellular areas. These unique and small GTP-binding proteins form heteromeric complexes, then join end-to-end to form higher-order structures such as filaments, rings and cage-like formations. In addition to their interaction with phospholipid membranes for shape control, studies have shown spetins association with actin filaments and colocalization with microtubules in numerous cell types. In other cases, analyses had shown the dependency of microtubules to the septin rings assembly at the plasma membrame (MOSTOWY AND COSSART, 2012).

As it is known, microtubules are interchanged helices of α- and β-tubulin forming a fundamental part of the cytoskeleton and are the main character in spindle formation and the segregation of chromosomes in cell division, especially in mitosis (Hewitt, 1998). To understand the appressorium formation of *M. oryzae* conidia, it was used the benomyl, a fungicide from the chemical group benzimidazoles, for its mode of action well proved to their

effects on tubulin integrity. According to the Fungicide Resistance Action Committee (FRAC) classification on mode of action 2018, the mode of action group of benomyl is “B: cytoskeleton and motors proteins”and its sub-group is “B1: > β-tubulin assembly in mitosis”, with the FRAC code number “#1: Methyl Benzimidazole Carbamates fungicides”. This highly selective and systemic fungicide demonstrates high affinity and attachment with tubulin proteins causing depolymerization and inducing mitosis disruption during cell division in metaphase. The mitotic spindle is twisted out and the daughter nuclei fail to separate, causing the cell death. Consequently, without mitosis the appressorium formation is null and, evidently, its septin ring (HEWITT, 1998; REIS et al., 2001).

3. MATERIAL AND METHODS

The experiment was conducted in the Fungal Cell Biology laboratory at the Department of Plant Pathology in Dale Bumpers College of Agricultural, Food and Life Sciences of the University of Arkansas, at Fayetteville, Arkansas, from May 29th to August 4th, 2017. The study was part of the Adair/Bollenbacher Summer Research Internship Program along with another research study, under the direction of Dr. Martin Egan, who leads the research group of the rice blast infectious process caused by *Magnaporthe oryzae*. The author was granted a fellowship for the program.

To understand the requirement of microtubules for septin ring assembly during infection-related morphogenesis, a experiment was set up using the fungicide Benomyl Pestanal® by Sigma-Aldrich®. Since microtubules are also required for mitotic division and nuclear migration, the fungicide was applied in seven treatments containing conidia of *M. oryzae*, however within a 2 hours difference, each, over a 10-hours period (0, 2, 4, 6, 8 and 10 hours), plus control with dimethyl sulfoxide (DMSO), an organosulfur compound. The experiment was conducted in order to identify the time groups with mitosis inhibited and consequently the lack of appresoria or appressoria with septin ring affected.

It was used on the mutant ΔSep5-GFP of *Magnaporthe oryzae* (which GFP is green fluorescent protein). The conidia were first collected, filtered and centrifuged for 5 minutes at 5000 rpm. Secondly they were poured into seven wells (350 µL each) separately of a chamber slides (Nunc™LabTek™ II Chamber Slide™ System) in a 2000 conidia/mL concentration. The fungicide benomyl was diluted in DMSO, an organosulfur compound, to 5 µg/mL as stock solution (higher concentrations of benomyl crystallize with water). To prove if there is a relationship between microtubules in the cytoskeleton and septin ring formation as well as its dependency from mitotic division, it was necessary to set up different hours of treatments. The control treatment had only 0,5% of DMSO, and the other 6 treatments had, each, 1,75 µL of benomyl stock solution.

Each treatment has a different time application of benomyl over a 10 hours period, which means that right after harvesting the spores the first benomyl application stands for 0 hours. The second application, in the other well, occurred 2 hours after the first and the third application, in the third well, corresponds to 4 hours after the 0 hours application. The fourth, fifth and sixth application correspond, respectively, to the 6, 8 and 10 hours after the first application of the fungicide (0 hours). DMSO was used as a control treatment and for observation of the septin ring tagged with GFP on the microscope, and so was applied, in a

separate well, at the same time as the 0 hours treatment. After application of the stock solution over a 10 hours period, the chamber slide was kept in the incubator programmed at 24ºC with 12:12 light cycles.

On the following day, the conidia were visualized on inverted Nikon Ti-E Eclipse Total Internal Reflection Fluoresce microscope equipped with an iXon Ultra 897 electron multiplier CCD Camera (Andor Technology). Therefore, to quantify the appressorium formation, five photos of live-cell imaging were taken with 20X objective lense for each treatment and visually classified.

Different conidia were classified as: non-germinated conidia, conidia with germ tube and damaged appressorium and germinated conidia with appressorial formation. Non-germinated conidia were those conidia that did not form a germ tube and not formed an appressorium as well. As for “conidia with germ tube and damaged appressorium”, the conidia must have grown only the germ tube and some must develop a poorly formed appressorium (without circular shape). The ones classified as “germinated conidia with appressorial formation”, as the phrase already sugests, the conidia must had highly developed a normal appressorium, fully grown, with circular shape.

Subsequently, epifluorescence images were carried out with 100x 1.49 N.A oil immersion Apo Nikon objective, of the 0 hours, 10 hours and control treatments to observe the appearance of septin ring at the appressoria.

Data sets were analyzed using linear regression to examine the relationships between fungicide exposure time and germ tube and apressoria formation.

4. RESULTS AND DISCUSSION

Germination was noticed in all treatments, independent of exposure time. However, the amount of germinated conidia and appressorial formation were notably different in each treatment. The germination and appressorial formation were greater in conidia when the fungicide exposure time was delayed.

The data sets were analyzed using linear regression to examine the relationships between fungicide exposure time and germ tube and apressoria formation. The graphic from the linear regression (Figure 2) shows the results were significantly (0.01). The graphic shows the effect of conidia exposure to the fungicide, which clearly demonstrated that appressorial formation was increased as the time of benomyl application was delayed. This was due to the mode of action of benomyl, which depolymerizes the microtubule cytoskeleton and inhibits the mitotic division (FRAC, 2018; Hewitt, 1998; Reis *et al*, 2001). As a result, there is no growth of germ tubes, and consequently, neither appressorial development and septin ring assembly.

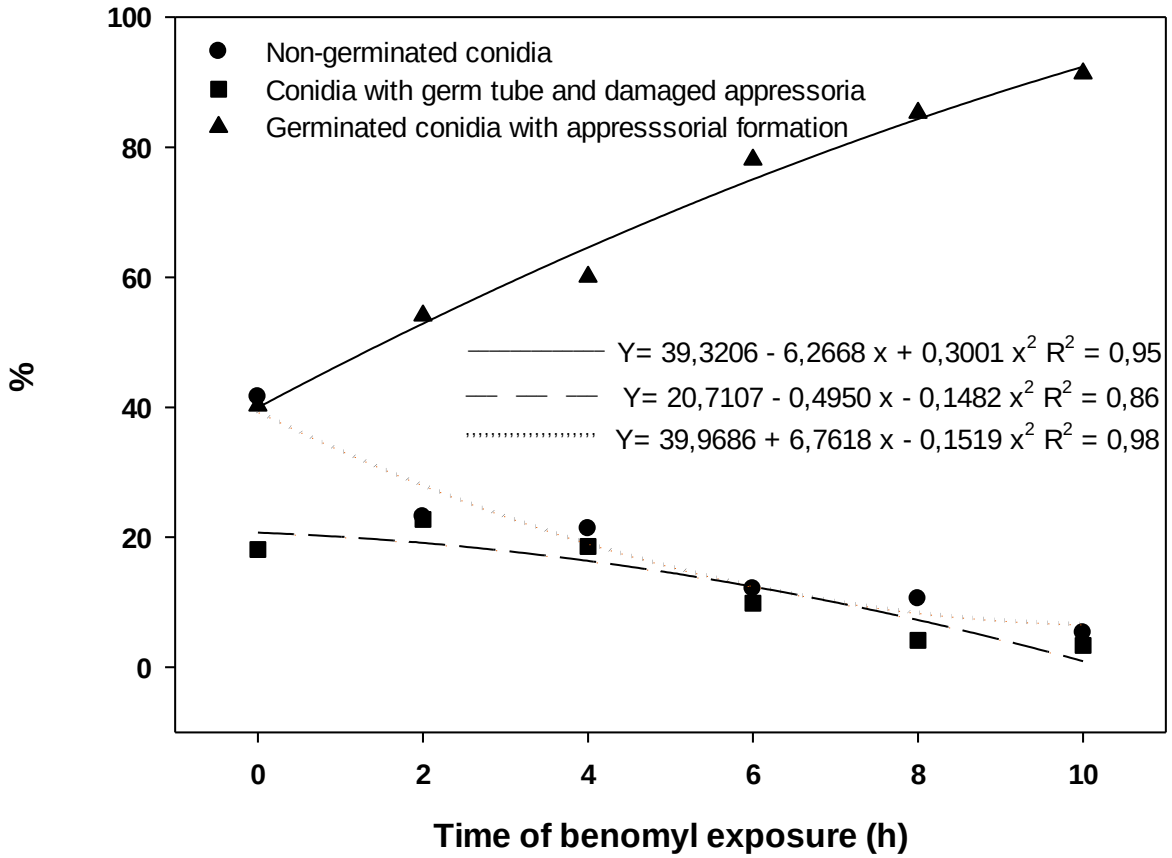

FIGURE 2. Non-germinated, conidia with germ tube and damaged appressoria and germinated *Magnaporthe oryzae* conidia with apressorial formation (%) exposed to benomyl (5 µg/mL) at different times after incubation at 24 ºC, 12:12 light cycles.

At 0 hours exposure, the average of non-germinated conidia (NGC) was 41,6%, for conidia with germ tube and damaged appressoria (CGTDA) was 18,1%, and finally for germinated conidia with appressorial formation (GCAF) was 40,3% (Figure 3A). In the 2 hours exposure, the averages were 23,1% of NGC, 22,7% of CGTDA and 54,1% of GCAF (Figure 3B). Within two hours difference of benomyl application, it was already possible to observe 34,4% increase of appressorial formation. At 4 and 6 hours exposures (Figure 4) the increase of GCAF were, respectively, 60,1% and 78,1%. Meanwhile, NGC were, respectively, 21,3% and 12,1%. At 8 and 10 hours exposures (figure 5), the increase of GCAF were, respectively, 85,3% and 91,4%, while the NGC were, respectively, 10,5% and 5,3%.

As observed, the percentage of NGC and CGTDA decreased as the benomyl applications delay. However, the percentage of GCAF had an increase of 126,8% from the 0 hours exposure to the 10 hours. In the case of the control treatment, DMSO only, the averages found were: 8,05% of NGC, 4,4% of CGTDA and 97,5% of GCAF. These results were expected since the conidia from this treatment had no fungicide to interrupt their development.

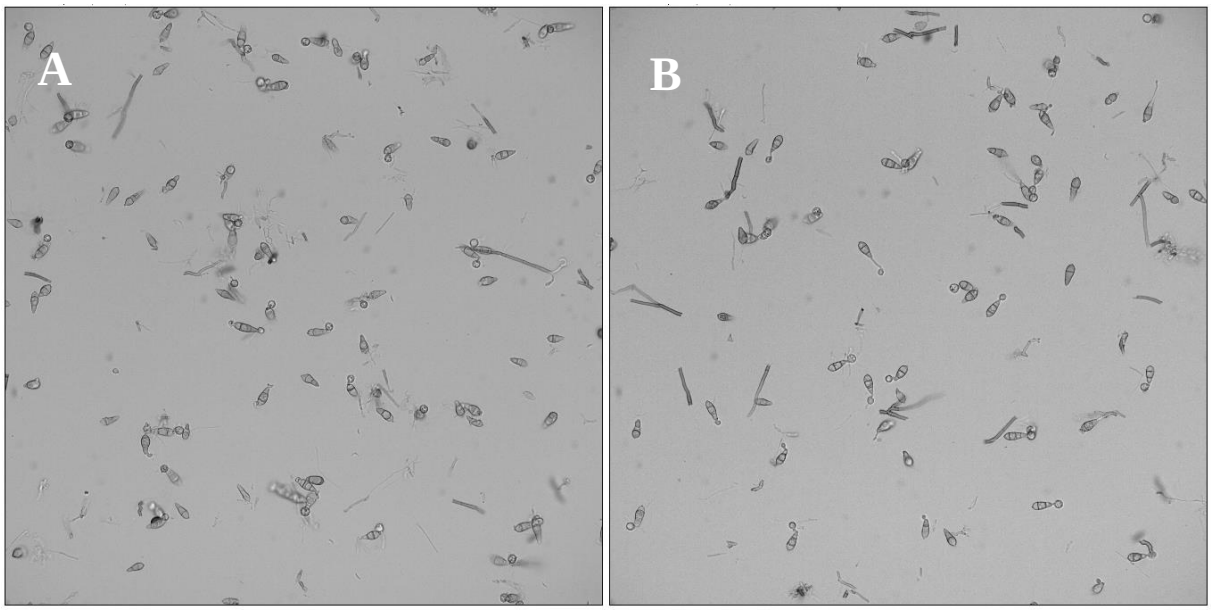

FIGURE 3 . *Magnaporthe oryzae* conidia exposed to benomyl (5 µg/mL) at 0 hours (A) and 2 hours (B), with magnification of 200x.

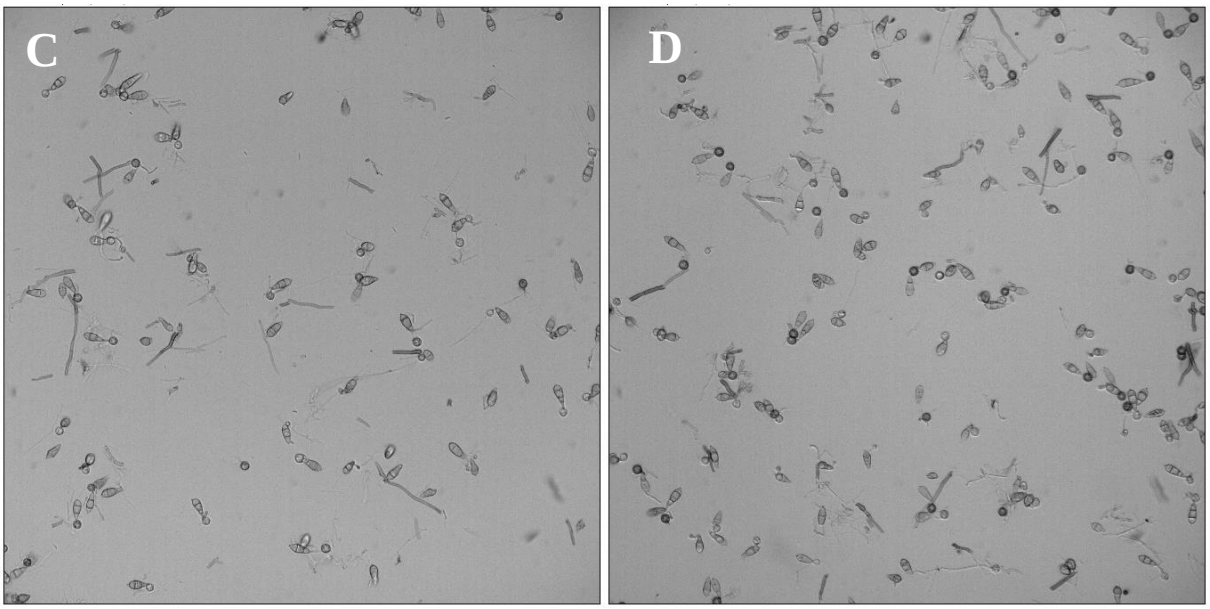

FIGURE 4. *Magnaporthe oryzae* conidia exposed to benomyl (5 µg/mL) at 4 hours (C) and 6 hours (D), with magnification of 200x.

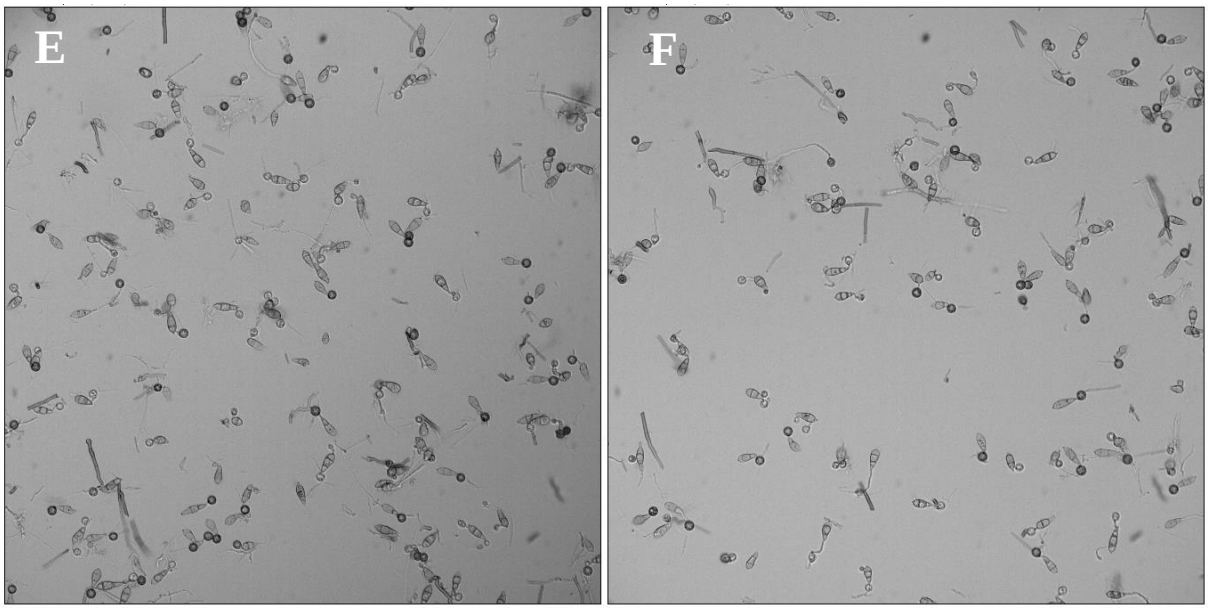

FIGURE 5. *Magnaporthe oryzae* conidia exposed to benomyl (5 µg/mL) at 8 hours (E) and 10 hours (F), with magnification of 200x.

A possible reason for the results obtained in this experiment is well documented in some published research, as Howard and Valent (1996) reported *M. oryzae* conidia germination at around 30 minutes after contact with some substrate and within 2 to 4 hours the germ tube starts the differentiating process (and one mitosis) to form the appressorium. With that in mind, the 0 hours treatment proved the mode of action of benomyl is the interruption of microtubules assembly during mitosis, consequently, inhibition of germ tube and appressorium. However, 40,3% evaluated at the 0 hours treatment was classified as GCAF. This happened probably due

to the time for preparation to start the exposure, since the harvest of conidia to the fungicide application it took 20 to 30 minutes. By the time the exposure started some conidia were already in the germination process, as they were also in an appropriate environmental conditions.

Moreover, Kershaw et al. (2018) related the appressorium formation is completely within 4 to 6 hours. Therefore, this explain the progressive increase of the percentage of GCAF as the benomyl exposure delays. In this case, starting at 4 hours it was already expected to visualize almost all conidia with appressorial formation. And yet, the observed was that from the 2 hours treatment, the variable GCAF had already the majority percentage in comparison to NGC and CGTDA.

Despite the increase of appressorial formation along the time exposure in the treatments, the epifluorescence imaging in 1000x of resolution of those appressoria showed unformed septin rings (not yet with the toroidal-shaped) as observed in the 0 hours (Figure 6) and 10 hours treatments (Figure 7). This obviously happened in the 0 hours treatment, as the microtubules are also required for mitotic division and septin ring assembly. Interestingly, some small circles and filaments were seen in the 10 hours treatment, but not yet the septin ring with toroidal-shaped at the appressorium pore. This showed a potential role of microtubules in the septin ring assembly, as the fungicide acted on β-tubulin assembly and so failed to build the septin ring.

As expected, the DMSO control not only served as the check treatment to the experiment, but also to visualize its toroidal-shaped septin ring at the appressorium pore through the mutant ΔSep5-GFP (Figure 8). With the same treatment, 3-D epifluorescence imaging were taken to visualize the septin ring (Figure 9 and 10). Once used for comparison, the DMSO demonstrated that appressorial development is dependent on cytoskeleton components such as microtubules, these in turn are also necessary for septin ring assembly.

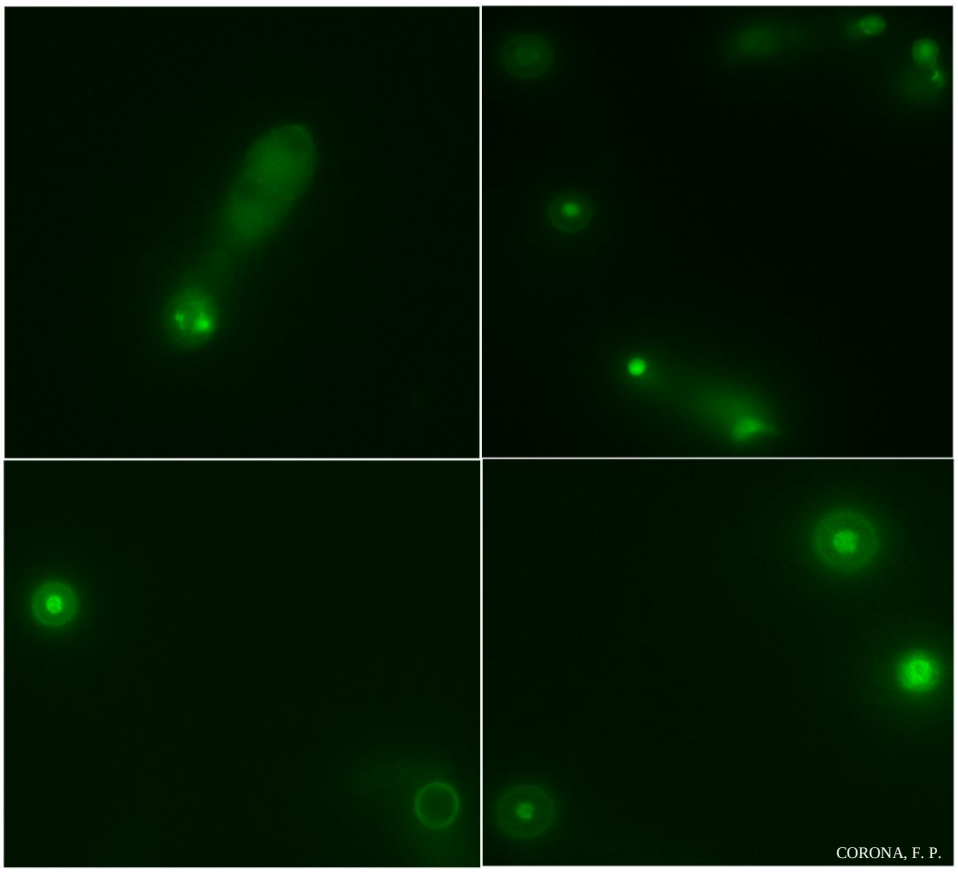

FIGURE 6 . Epifluorescence imaging of ΔSep5-GFP of *M. oryzae* exposed to benomyl (5 µg/mL) at 0 hours, with magnification of 1000x. The mutant did not form the toroidal-shaped septin ring at the appressoria pores.

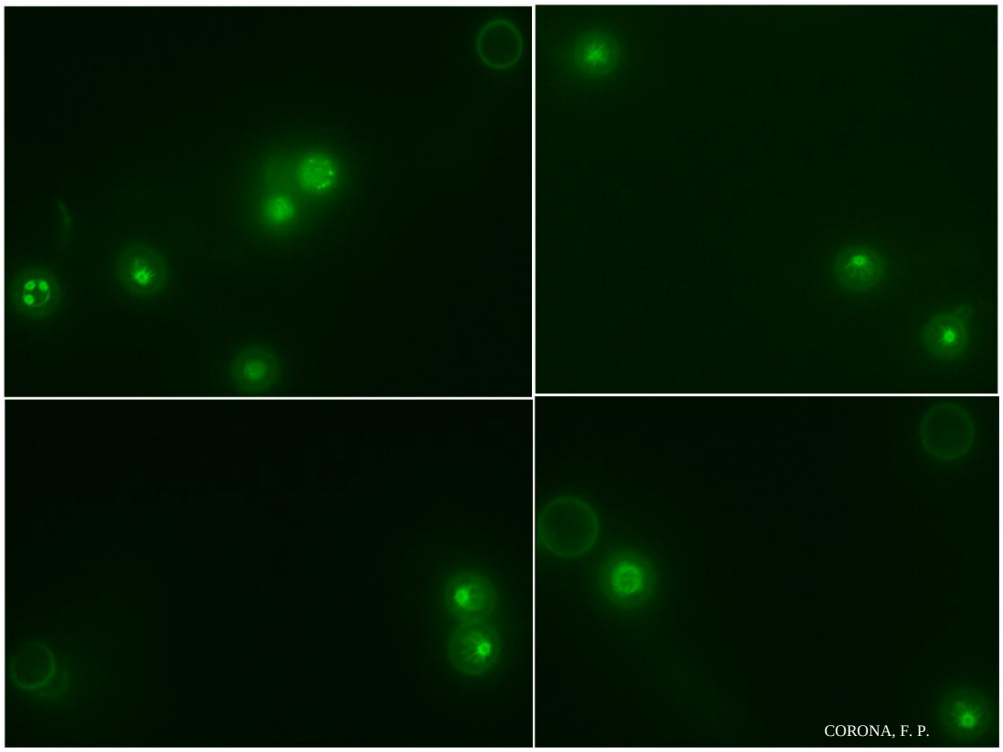

FIGURE 7. Epifluorescence imaging of ΔSep5-GFP of *M. oryzae* exposed to benomyl (5 µg/mL) at 10 hours, with magnification of 1000x. The mutant did not form the toroidal-shaped septin ring at the appressoria pores.

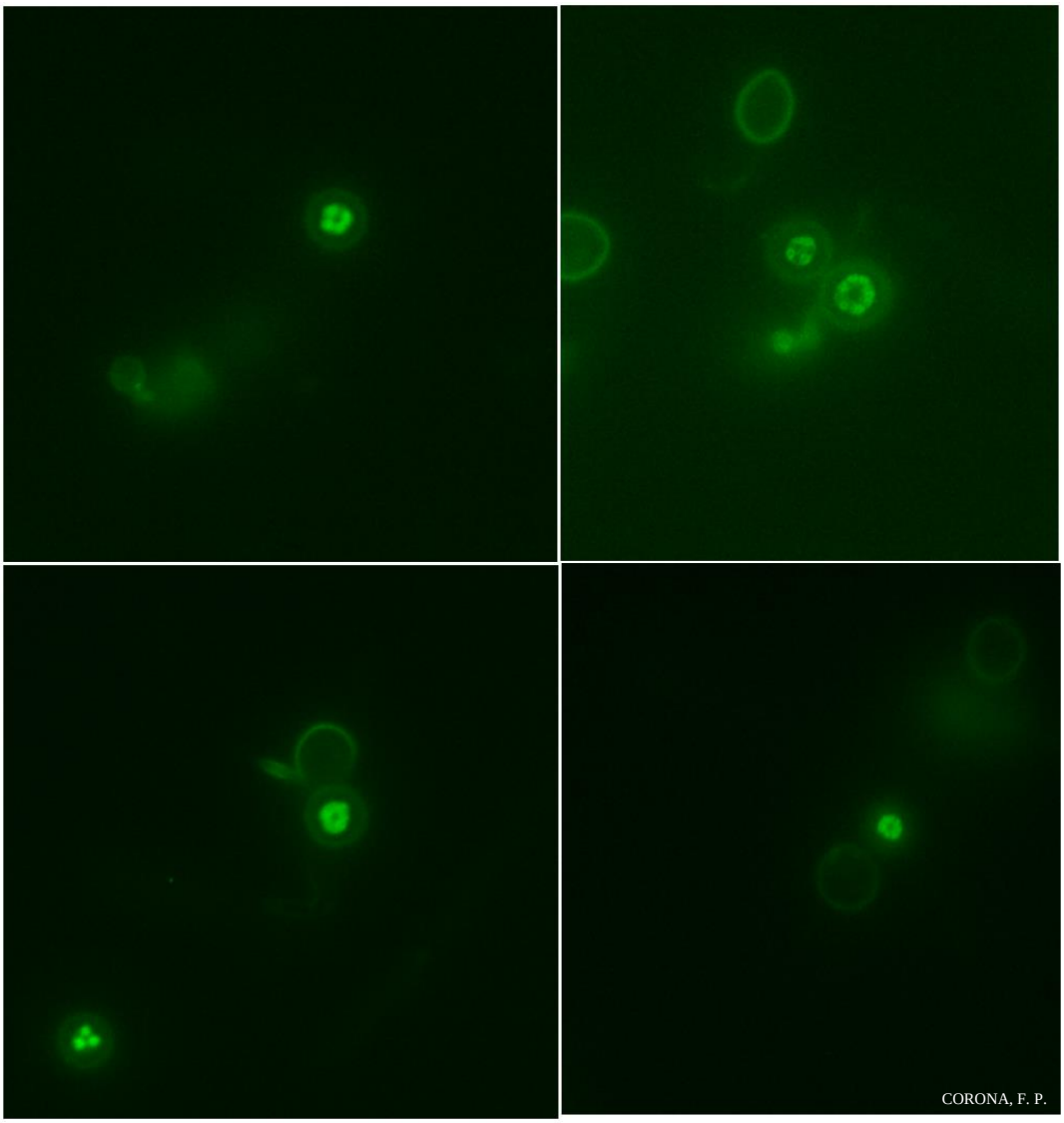

FIGURE 8. Epifluorescence imaging of ΔSep5-GFP of *M. oryzae* control treatment DMSO (0,5%), with magnification of 1000x. The mutant form the toroidal-shaped septin ring at the appressoria pores.

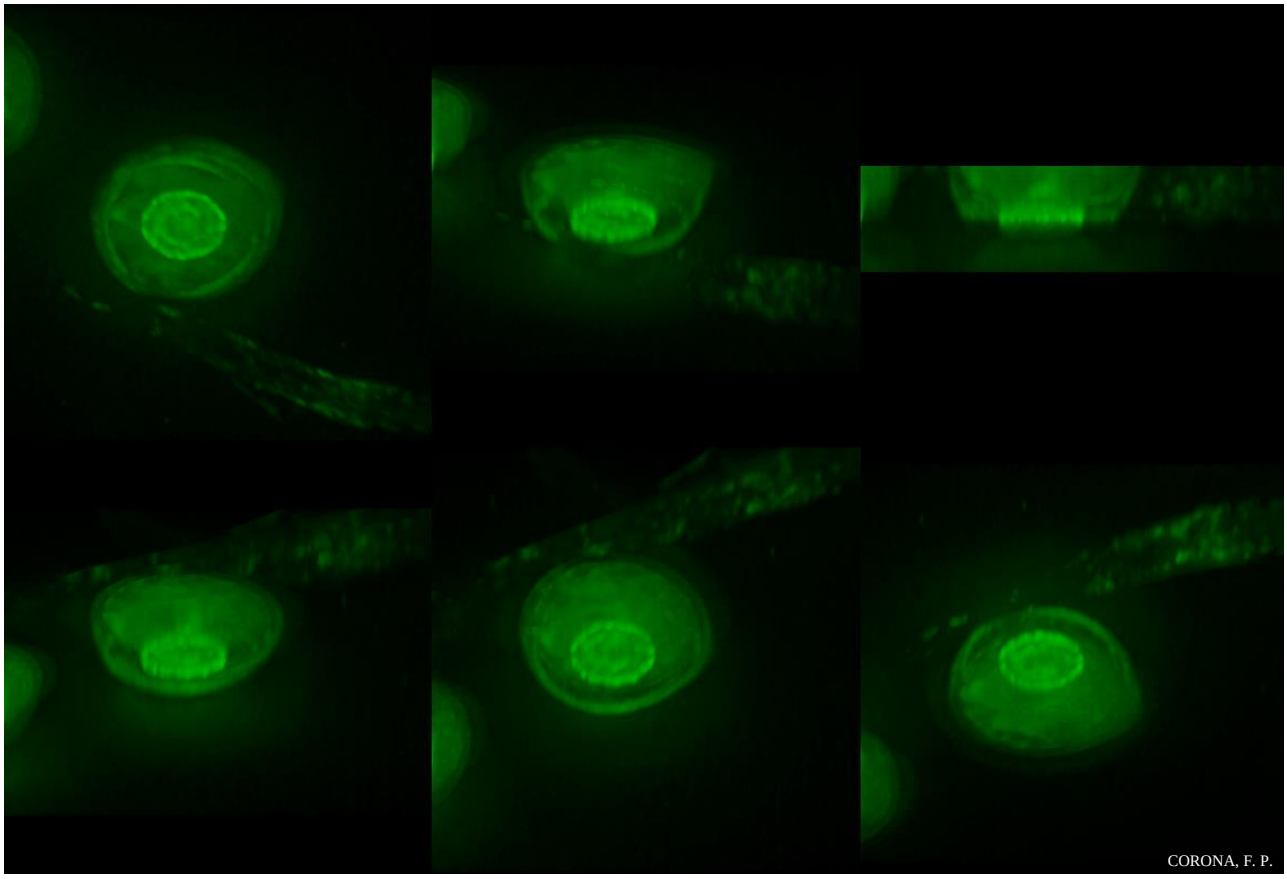

FIGURE 9. 3-D sequences of epifluorescence imaging of ΔSep5-GFP of *M. oryzae* control treatment DMSO (0,5%), with magnification of 1000x. The mutant form the toroidal-shaped septin ring at the appressorium pore.

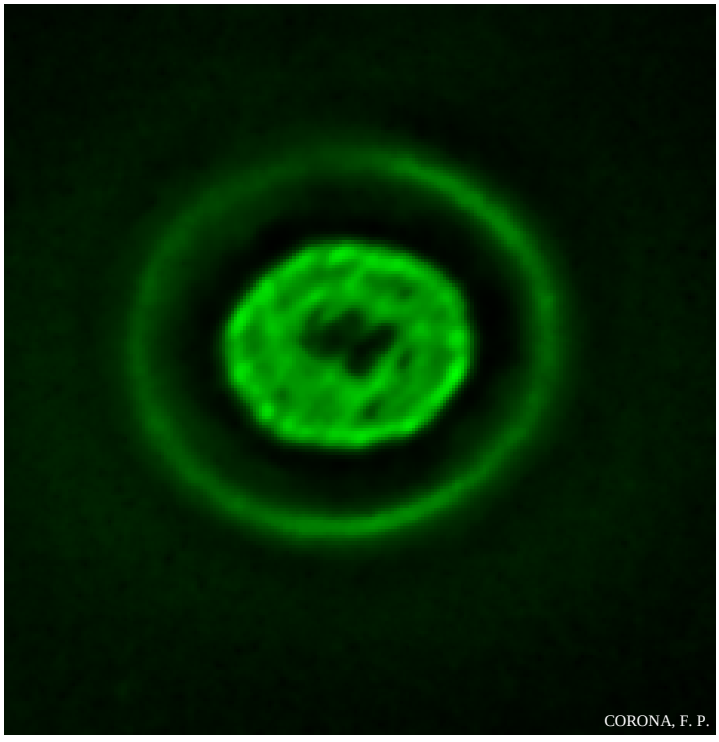

FIGURE 10. 3-D epifluorescence imaging of ΔSep5-GFP of *M. oryzae* control treatment DMSO (0,5%), with magnification of 1000x. The mutant form the toroidal-shaped septin ring at the appressorium pore.

5. CONCLUSION

This study demonstrated a potential role of microtubules in appressorial formation and septin ring assembly.

Appressorial development is dependent on microtubules based cytoskeleton, as they are required to mitotic division in the germ tube. And microtubules are also required to the assembly of septin ring at the appressorium pore.

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