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Full Length Article

Half-reserve Mitosis of Sibling Nuclei in *Gymnosporangium asiaticum*

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Abstract

Fluorescent DAPI (4',6-diamidino-2-phenylindole) dyeing showed that *Gymnosporangium asiaticum* (Miyabe ex Yamada) was dikaryotic both in teliospore and basidiospore is different from other rust fungi, and two pathways of nuclei mitosis in basidiospores are found. Directly germinating produces dikaryotic tubes with appressorium at the tip, while indirectly germinating produces secondary basidiospores in 3 pathways. The first, the daughter nucleus disintegrates before the other one takes mitosis in the secondary spore. The second pathway is with one daughter nucleus disintegrated before another one taking mitosis in the mother basidiospore, then the two newborn nuclei moved into the secondary spore. The third pathway is 3 nuclei co-existing with one of daughter nucleus taking mitosis and another one keeping static in the mother basidiospore. Remains of nucleus at the sterigmatal initial can be found by fluorescence microscope, which demonstrates that one of the daughter nucleus disintegrated actually before secondary basidiospore matured. Artificially inoculations on host *chaenomeles* show that secondary basidiospores induce stronger pathogenesis than that of basidiospores. Pycniospores are mono-nucleate. Aeciospores with double cells wall are dikaryotic, and a dikaryotic hypha cell exists between every two bi-nucleus aecium spores. One cofferdam layer is found under the pycnium, and aecium is formed only after hapha has passed through this layer. Somatic cells of mycelium in *Sabina chinensis* are multiple-karyotic, however, in *Chaenomeles lagenaria* are throughout monokaryotic. In the life cycle of *G. asiaticum*, half of two daughter nuclei is valid for new cell born, even haploid basidiospores produce secondary spore abiding by this rule, the daughter nuclei must be dispelled before the secondary spore matured.

Keywords: *Gymnosporangium asiaticum*; *Chaenomeles lagenaria*; DAPI dyeing; Nucleus behavior

Introduction

Biological and genetic evolutions of rust fungi in the mono-karyotic stage are different from the dikaryotic stage (Ono, 2002). For example sexual behavior happens at the monokaryotic state while nucleate flow among isolates occurs at the dikaryotic stage. Both types of nuclear exchange are considered essential and main reasons of pathogenic variation and population division (Kang *et al.*, 1994; Bourasa and Bernie, 1998; Bourasa *et al.,* 2005). Since chromosome exchange and meiosis happen on the stage of basidiospores, which develop sexual spores eventually, hence, pathways of basidiospores yielding, germination of basidiospores and nuclear behavior are all important for population genetic and variation of rust fungi. Many researchers report that the medium of environment affects the germination pathway of basidiospores. Rate change of water in the external environment decides whether the basidiospores germinate tubes or secondary basidiospore. Metzler (1982) reported only secondary basidiospores of *G. fuscum* were found when basidiospores landed on water film. Bauer (1987) reported the same results after his systematic studies with such rust fungi as *Gymnosporangium, Coleosporiun, Cronartiun, Phragmidium, Puccinia and Melampsora*. When the basidiospores were placed on agar substrate of 1.2–3.7% water, they germinated with the un-branched tubes. However, when basidiospores were placed on 3.7–7% water agar, or a glass slide, or on leaves, all fungi except *Coleosporium* and *Cronartium* produced germ tubes, not secondary basidiospores. In water, air or 0–0.7% water agar, all basidospores produced only secondary basidiospores. The same results were found by Freytag for *Uromyces appendiculatus.* Freytag *et al.* (1988). To better understand basidiospore germination and the infection progress, Mims *et al.* (1988; Mims and Richardson, 1989; 1990) studied appressorium and secondary basidiospores of *G. juniperi-virginianae*, and observed all basidiospores were bi-nucleate, so as well in the germtube and secondary basidiospore, while the two nuclei in secondary basidiospores originated from one of the daughter nuclei in the basidiospore.

G. asiaticum Miyabe ex Yamada (Synonyme: *Gymnosporangium chinense* Long), is the most destructive fungal disease of fruit trees in China, Japan and other Asian countries (Wei 1979; Yang, 1996). It can infect many species of *Chaenomeles, Pyrus, Malus, Cydonia*, and many genera in the *Roseaceae.* It often causes serious yield

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decline. Many studies have reported on the infection cycle, biological control techniques, and ultrastructure of pycniospores and aeciospores (Zhou, 1992; Huang *et al.*, 1994, 1995; Huang and Chen, 1997a, b) of *Gymnosporangium asiaticum*. Following the fluorescent microscope and infecting progress study, the current paper attempts to explore and discuss the following questions: What is the behavior of the nuclei of *Gymnosporangium haraeanum* during basidiospore germination? What role do the secondary basidiospores have in disease epidemiology? Does mitosis happen in the maternal basidiospore or in the secondary basidiospores? What is the role of the disintegrated daughter nucleus in the life cycle? Finally, what initiates or stimulates the daughter nucleus to disintegrate?

Materials and Methods

Germination of Teliospores

Fresh telia were collected after the first spring rain, and placed on the wet filter paper in a watch glass until a telium horn was formed. A cross-sectional cut was made on this telium, and dyed with DAPI (0.5 mg/mL) for 0.5–1.0 h held under microscope. During this period, telium germinated in the drop of DAPI, and observations were made and photographed by a florescent microscope Lecia 4000B. In addition, fresh telium horn in the same sample was simultaneously landed on the glass slide without any water drops and incubated in 20□ chamber at 70% humidity to germinate directly, a glass slide sample was dyed DAPI (0.5 mg/mL) and observed every 30 min. Ten times of each collection are repeated by the same above methods.

Inoculation with Secondary Basidiospores

A fresh telium horn was placed on a wet filter paper for two hours. The filter paper was then placed on the upper surface of Chaenomeles leaves enclosed by a plastic bag to maintain humidity, with fresh telium horn hanging over the *Chaenomeles* leaves inoculated directly as a control. After eight days the bag was removed.

Histology Dyeing of Chaenomeles Leaves

After 7–8 days, pycnium spots began forming on the leaves. A thin section was made and immersed in a solution of DAPI in 70% ethyl alcohol, and held at 4°C for 10–30 min. Pictures of the section were taken using a Lecia 4000B microscope under fluorescent lighting. Histological dyeing was done with acid flushing according to procedure as described by Zheng (1980). Thin sections of the samples were boiled in the acid fuchsine for 5 min, washed with alkaline H_2O_2 (pH 10.6), and re-dyed with acid fuchsine for observation.

For China savin (*Sabina chinensis*), needles with mycocecidium of telia were collected in early spring and fixed in 4% glutaraldehyde ethanol solution, and prepared for transmission electron microscope, referred to Mims *et al.* (1976).Leaves of *Chaenomeles lagenaria* were also prepared in the same way.

Results

Mycocecidium on Needles of *Sabina chinensis*

Mycocecidium on the needles of China savin in the early spring was the early primitive stage of telia of *G. asiaticum*, formed after the first spring rain in most regions in China, and about 2 weeks, the mycocecidium developed as a typical telium horn. However, cellars in the primitive mycocecidium were mono-cellular with multiple nuclei, Fig. 1a, when each two round cells of mycocecidium got longer and merged together, number of nuclear got down and only one nucleus was left in each cell, a septum developed at the equator (Fig. 1b and c), the teliaspore produced and the telium horn then formed.

Teliospores Germination

Under a light microscope, teliospores in DIPA drops germinated with 1 or 2 promycleium, but usually only one promycelium was presented. Teliospores are bi-cellular, and each cell contains one nucleus, Fig 1a. When a teliospore germinated, a promycelium developed from one of the cells, and plasma from both cells flowed into the growing promycelium. Promycelium extended and swelled slightly at the tip, and plasma flowed into that tip. When the mature basidium formed, one of the sister nuclei in teliospore took on one meiosis, and divided into 4 cells, Fig. 1b, with each new cell having one nucleus. At the same time, basidia stalk was formed from the mature basidium, and nuclei migrated into the tip, Fig 1c. After a mitosis, a mature basidiospore with two nuclei was formed, Fig. 1d. Another nuclear in teliospore disintegrated before basidium mature.

Basidiospores are broad kidney-shape with an umbilical tip, Fig. 2a. On a dry and hard substrate, such as on a glass side in this paper, the basidiospore germinates only a dikaryotic tube and appressorium Fig. 2b. However, under humid circumstances, basidiospores generally germinated secondary spores instead of tubes. One or two stalk grew from a section of the basidiospore surface. A secondary spore formed at each stalk tapered tip, normally on the left if there are two stalk, Fig. 2a. As cellular material moved into it from the parent basidiospore, the secondary spore enlarged and elongated, and eventually became slightly kidney-shaped. A large vacuole eventually filled the basidiospore and extended into the stalk, then the mother basidiospore disintegrated and a matured secondary spore formed. Throughout the progression three pathways of mitotic division were found in the secondary spore.

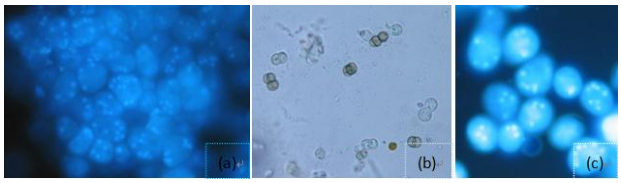

Fig. 1: Mycocedidium cell in the early stage of telia
(a) Mycocedidium cells with multiple nuclei in early stage;(b) Two mycocedidium cells merged together;(c) Mycecedidium cells in the late stage with nuclear number decrease

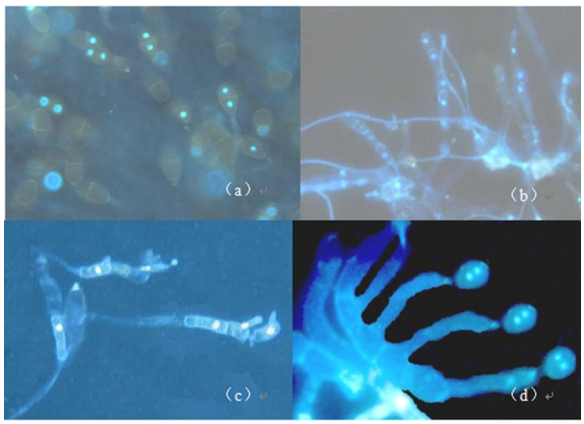

Fig. 2: Germination of Teliospore
(a) Teliospore are bi-nucleate; (b) Basidia with 4 nucleates; (c) Germinated basidium; (d) Basidiospores with 2 nucleates

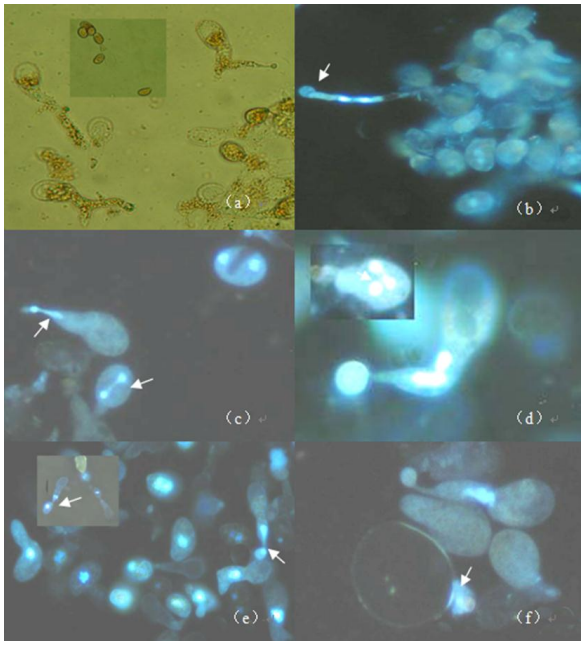

Fig. 3: Germination of basidiospore
(a) Germinated basidiospores and stalk; (b) Basidiospores germinated directly with a tube appressorium (arrow); (c) Mitotic nucleate after another one disintegrated flowing into the initial (arrow); (d) Coexisting daughter nucleates with one undergoing mitosis (arrow); (e) One of the daughter nuclei disintegrated, the moving nucleus (arrow), the un-disintegrated nucleus moving into the stalk (arrow); (f) The remains of the nucleus deposit at the septum (arrow)

1) A single mitotic division happened after one of the daughter nuclei disintegrated, then the newborn nuclei moved into the secondary spore and rested at the two poles of the cell, Fig. 2c.

2) Three nuclei co-existed in the mother basidiospore, one of them is the undivided daughter nucleus. Before the 2 newborn nuclei moved into the initial of secondary spores, the undivided daughter nucleus disappeared, Fig. 2d.

3). After one nuclei disappeared, the other one flowed into the secondary spore initial without mitotic division, so the young secondary spore has only 1 nucleate. A mitotic division when the secondary spore get matured, Fig. 2e.

These findings show that a single mitotic division can occur both in the mother basidiospore and the secondary spores, but it is not clear how the daughter nucleus disintegrated and what the role of the disintegrated nucleus is. The stalk fluoresced under DAPI dyeing and showed that one of daughter nuclei dissolved and the remains deposited at the stalk before the secondary spores matured, Fig. 2f.

Inoculation with Secondary Spores

Mycocecidium will be flowered after spring rain, Fig. 1a, and at one hour later on wet paper, telia began to produce basidopores. Two hours later, most basidiospores developed secondary basidiospores. When the basidiospore-infused filter paper was attached to the upper surface of the host leaves for 7–8 days, like in Fig. 3b, a yellow pycnium was formed, Fig. 3c. Seven days later, aecia were formed on the abaxial leaf surface, Fig. 3d. However, pycnia were formed after 15 days by directly inoculating leaves with a teliospore horn, 7 days later than the inoculation with secondary spores. The experiment was also repeated in controlled chamber and nearly the same results were obtained. Thus, the secondary basidiospores have stronger infective capability than the basidiospores, which implies rainfall and humidity may accelerate the infection rate and disease progression of this rust disease if enough secondary basidiospores are produced in the field.

Dyeing of Pycnium and Aecium

Spermogonia are bottle-shaped. A spermatium is delimited by a centripetally developing septum, and then pycniospores were pushed into the spermogonial cavity by the next spermatium initial. Aecium are cup-shaped, blown out at the under skin of the leave, opposed to the spermogonia, Fig. 4a. Between the spermogonia and aceium, a cofferdam was found. Only after the intercellular hypha grew through this cofferdam, can aceum be formable. Spermatia are ellipsoid with tapered end, and enclosed by a thin wall. Each spermatia contained a single nucleus, which took on more than half the bulk of the spermatia, Fig. 4c. Sexual progress is completed by spermatia adhering on the

Fig. 4: Inoculation with secondary spores
(a) Telia mycocecidium on *Sabina chenesis*; (b) Secondary spore inoculation of *Chaenomeles lagenaria* enclosed by a plastic bag; (c) Pycnium spots formed on the adaxial after inoculating for 8 days; (d) Aecia horn appeared on the abaxial leaf surface

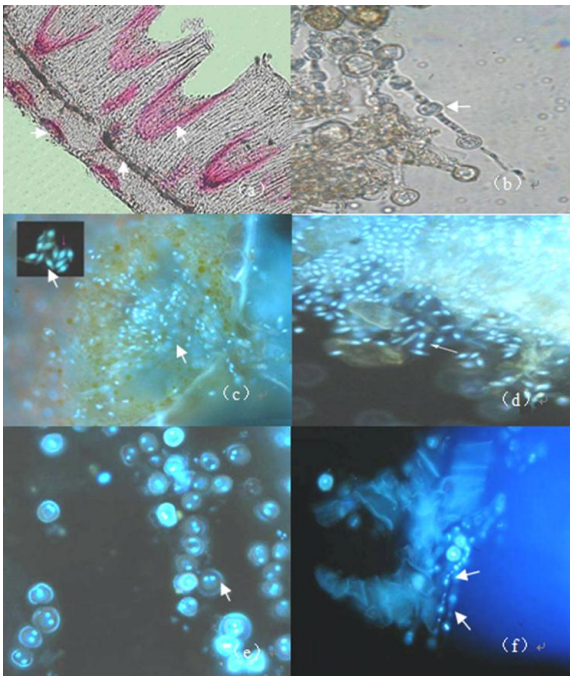

Fig. 5: Nucleus of G. asiaticum on *Chaenomeles lagenaria*
(a) Histology of *Chaenomeles*, showing a pycnium, a cofferdam, and an aecium (arrows); (b) Tandem aeciospore structures (arrow); (c) Pycnium and mono-pycniospores (arrows); (d) Spermatiophore and an adhered spermatium (arrow); (e) Binucleate aeciospores with double walls (arrow); (f) Binucleate hapha between aeciospores (arrows)

spermatiophores, Fig. 4d, cytoplasma and nuclear flow into spermatiophores, and meiosis takes place here, and aecium spore, double-walled and dikaryotic, are produced, matured from top to the bottom cell, Fig. 4b. However, there is a bi-nucleus hypha cell between every two aecium spores, Fig 4e, f.

Mycelium in alternative host, *Sabina chinensis,* is multiple-nuclei, as well as in haustorium, Fig. 6b and c, the tumor cells developed beside infection mycelium are multiple, too, Fig. 6a. However, infected mycelium, pycnium cells and young spermatia in host Chaenomeles are all monokaryotic, Fig. 6c, d and e.

Discussion

One or two promycelia formed from each teliospore. When the promycelia elongated, a single meiosis occurred in the mature promycelia. One of the daughter nuclei in the teliospore bore four nucleates, with another one expelled. The cellular matrix moved into the tip, concentrated, and divided into four basidia each containing one nucleus. When a basidium germinated, a basidiospore stalk grew and a basidiospore formed at the tip. A single mitosis occurred before basidiospore maturation, resulting in each basidiospore containing two nuclei.

From our observations, four basidiospore germination pathways exist. In saturated humid environments, such as in a water–film substrate, mass basidiospores germinate secondary spores, usually using three pathways. In comparison, in dry or low humidity conditions, basidiospores usually germinate tube appressoria with two nuclei. This latter method is similar to the reports of Mims and Richardson (1989). DAPI fluorescent dyeing showed that one of the daughter nuclei in the basidiospore underwent a single mitotic division before the secondary spore formed, whereas another nucleus disintegrated before or after this mitotic event. The remaining material moved and concentrated at the tip of the stalk and formed a septum, and the secondary spore matured and broke away from the tip. Mims *et al.* (1976; Mims and Richardson, 1989, 1990) thought that mitosis can only happen in the secondary spores; even Sundberg (1978) assumed that a similar mitotic division of secondary spores existed in all the *hymenomycetes* fungi. In the current study, a single nucleus in the young secondary spores was also found, but this most eventually matured as dikaryotic secondary spores. Three nuclei basidiospores and the dividing nucleus are found in the mother basidiospores. These results indicate that mitotic conditions in basidiospore germination are complex, and mitosis can occur both in the mother basidiospores and in the mature secondary spores. Whichever part of the cell it is in, half of two daughter nucleus is valid for new cell born, even haploid basidiospores produce secondary spore abiding by this rule, the daughter nuclei must be dispelled before the secondary spore matured, and the remains move to the tip of stalk before forming septum. The mycocecidium cells on China savin get monokaryotic after the other nuclei dispelled, so that ensure to develop the merged telia are dikaryotic, however, mycelium and hausterium in savin are throughout multiple nuclei, and how mycocecidium cells developed into telium cells is still under discovery.

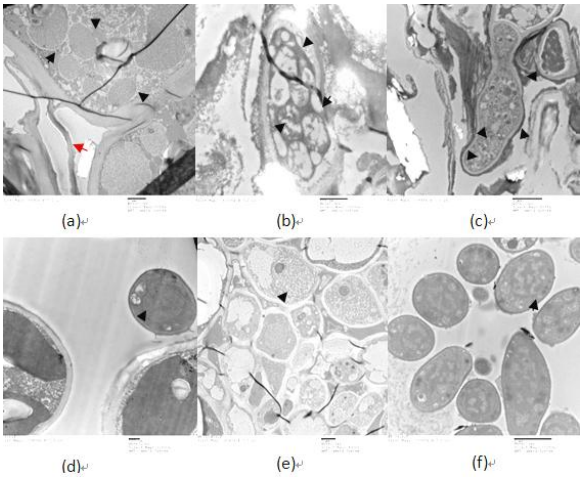

Fig. 6: Nuclear of mycelium and young spermatia in host tissues

(a) Infected mycelium (red arrow) in China savin with multiple nuclei (black arrow); (b) Mycelium cells with multiple nuclei (arrows); (c) Haustorium in savin with multiple nuclei (arrows); (d) Monokaryotic mycelium in Chaenomeles (arrows); (e) Pycinium cells with one nucleus (arrow); (f) Spermatia with one nuclues (arrow)

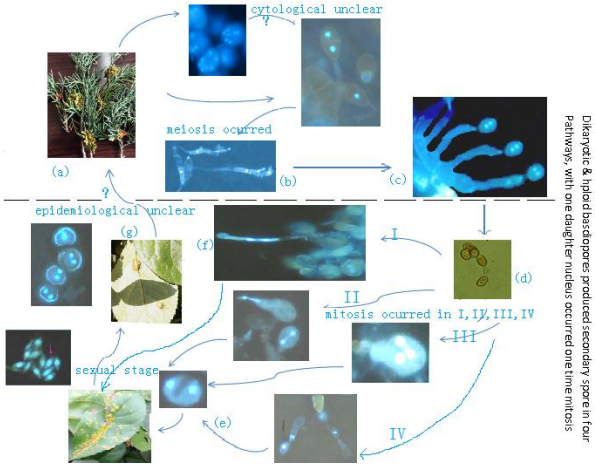

Fig. 7: Nuclei cycle of *Gymnosporangium asiaticum* Miyabe ex Yamada

(a) Telium on *Sabina chinesis;* (b)Germinated pro-basidium hypha with 4 nuclei; (c) Basidia germinated basidiospores with 2 nuclei; (d) Mature basidiospores with 2 nuclei I: Basidiospores germinated tubes directly with appressorium at the end; II: Basidiospores germinated with one of daughter nucleate mitosis after another one disintegrated; III:Basidiospores germinated with one of daughter nucleate mitosis before another one disintegrated; IV: Basidiospores germinated with one of daughter nucleate flew into the secondary basidiospore initial after another one disintegrated; (e)Mature secondary basidiospores; (f) Germ tube and appressorium; (g) *Chaenomeles* leaves with pycium and aeceium, aeciospores with 2 nuclei and pycniospore with 1 nucleus

All mycelium, pycnium cells and spermatia in host Chaenomeles are monokaryotic, and mitotic happened normally in mother cells. The various mitotic pathways may be helpful for species survival under different environmental conditions. However, the genetic factors controlling the mitotic pathway and whether or not the newborn nuclei are homogenous are still unknown. Hacquard *et al.* (2013) believe nuclei fusion genes *Kar*5 and *Kar*9, meiosis genes *Rec*8、*Mre*11、*Rad*50、*Rad*51、*Spo*11、*Mnd*1 are highly expressed before diploid telia formed, and behavior of nuclear is regulated by a kind of mating gene (Shimomura *et al*., 2012). However, two nuclei in telia did not undergo fusion till germination, and directly develop 4 basidiospores, which infests this short of urediospore fungi are diploid in most life time，including telial stage.

An elongated and tapered stalk could be seen before the secondary spores formed. The matrix from the vacuum basidiospore concentrated at the stalk tip and formed a fluorescent septum. This phenomenon proved that one daughter nucleus dissolved before secondary spores matured. In the report of Mims and Richardson (1990), similar septa were found using electronic microscope technology; however, he did not explain its chemical trait.

Up to now, the conditions that trigger the basidiospore germination model and what guided the mitosis in the secondary spore formation are still unknown, however, from this paper, new born nucleus are tightly related to the dispel of the daughter nucleus. Mims and Richardson (1990) also found that only one daughter nucleus underwent mitotic division, and believed one disintegrated to prevent secondary spores from becoming tetranucleate, there is still something unknown which, how and why one daughter nucleus must dispel. Roncadori (1968) demonstrated that both secondary and tertiary spores were equally pathogenic as basidiospores, which is in contrast to the current research. Roncadori (1968) may not have considered the environmental conditions required for infection, especially humidity. Secondary and tertiary spores are only produced under high humidity, such as a spore landing on a water–film substrate. In general, the natural infection of this rust disease occurs after the first spring rains in China, under high humid conditions; thus, secondary spores may be the main source of primary infection. With mitotic division happening, new spores may have strong survival and infection ability, as the inoculation experiments indicated in this paper. Micro-environmental humidity was also vital for somatic mycelium survival and infection of *Melampsora larici-populina* (Yu *et al.*, 2011), germination in humidity ambient and mycelium hiding under the leaf cuticle layer may be an adaptable strategy of this kind leaf rust.

Sexual progress, with the formation of pycnium and aecium, happened on host *Chaenomeles lagenaria* in this paper, no difference of nucleus behaviors from other rust fungi was found, as reported by Olive (1994).

Conclusion

G. asiaticum Miyabe ex Yamada was a typical demicyclic rust fungus without uredia stage, but pycnium and aecium on host plant *Chaenomeles lagenaria,* telia and basidiospora on

alternative host *Sabina chinensis*, as showing in Fig. 7. However, nuclear behavior in *G. asiaticum* is distinctively different from the other rust fungi for its three secondary basidiospores developing pathways, with the dikaryotic basidiospores germinating by one sibling nuclei dispel and another taking one mitosis. We don't know whether this half-reserve mitosis is generally happened in other demicyclic rust fungi, or in micro and marcrocyclic rust fungi, but the stronger survival function of secondary spore of *G. asiaticum* was confirmed by artificial inoculation in this study.

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