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Observation of mulberry flowers and fruits invaded by *Ciboria carunculoides* with resistance of mature seeds

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Abstract

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Popcorn disease is an important disease of mulberries (*Fructus mori*) due to invasion of *Ciboria carunculoides*, and losses are common in Chinese growing regions. This study was to investigate the process of pathogenic invasion, the pathological changes of cellular tissues of mulberry fruits and seeds, and resistance of seeds at different maturity levels using optical microscopes in combination with an electron microscope for observation. The results indicated 72.90% ascospore and hyphae adhered to female stigma with an ascosporic germination rate at 77.80%. After 10 days of hyphae inoculation, their invasion extended to the ovary, inner and outer perianths and interior of young seeds via stigmatic intercellular spaces. After 15 days, numerous filamentous haustoriums were generated with gradually complete disappearance of the host cell structure. In the following 20 to 30 days, few hyphae and numerous spherical haustoriums generated young sclerotium in mulberry fruit leading to the form of popcorn with the grey-white colour appearance of dying feature. The morbidity of mulberry fruits was at 96.53%. Embryo and endosperm of the mature seed coated by sclerotium showed normal cell morphology and that the plant heights of the seedling grown from the infected seeds showed no significant variations ($p > 0.05$) in comparison with the healthy seeds' seedling. Consequently, understanding that mulberry female flower stigma becoming the foremost invaded part avails the development of prevention and control against mulberry sclerotinose. Additionally, that mature seeds possessed excellent resistance to sclerotinose was highly possible due to compactness and the secondary metabolites with antibacterial activities in the seed coat. Moreover, there are uncertainty about the attachment of ascospores and stromatic ascospores.

Keywords: Mulberry (*Fructus mori*); *Ciboria carunculoides*; infection process; mature seeds; resistances

Introduction

Mulberry is a fast-growing hardy perennial woody plant which belongs to the genus *Morus* in the Moraceae family and is believed to have originated in the area of China-Japan and in the Himalayan foothills. Although mulberry has been widely cultivated to many countries under varied climatic conditions, ranging from tropical to temperate, China currently becomes the world-leading country in sericulture as a result of the largest silk production at 120 400 metric tonnes followed by Japanese silk production at 20 metric tonnes in 2018 (Commission, 2020). Furthermore, China possesses the largest area of mulberry at over 626 000 ha while there are only 280 000 ha mulberry fields in India as the largest consumer of silk production in the world (Sánchez, 2000).

Mulberry (*Morus alba* L.) fruits are not only normally edible as fresh or dried fruits, fruit jams and juices, or processed into wine for its delicious taste, pleasing color and

low calorie content, but also in possession of tremendous healthy benefits such as fever reduction, treatment of sore throat, liver and kidney protection, eyesight improvement, antioxidation, anti-obesity and ability to lower blood pressure on account of containing amino acids, fatty acids, mineral, polyphenolics, anthocyanins, rutin, quercetin, chlorogenic acid, and polysaccharides (Yuan & Zhao, 2017; He et al., 2018).

Mulberry sorosus parvulling sclerote disease, caused by the fungus *Ciboria carunculoides*, is currently the major sclerotinia of mulberry fruits (*Fructus mori*) in Chinese growing regions while the other three sclerotinias, *Ciboria shiraiana, Scleromitrula shiraiana and Sclerotinia sclerotiorum* were also reproted (Hu et al., 2011). The disease was originally reported with the name of "popcorn" disease of mulberry in the southern regions of America in 1903 (Siegler & Jenkins, 1922). The disease is also called as "white mulberry disease" or "white fruit disease" in Chinese literatures because the interior of infected mulberry fruits is sapless and off-white along with black granular sclerotium (Kuai & Wu, 2012). This fungus requires a period of approximately eleven-month duration for its complete development. There are two phases in its developmental cycle, a sclerotial phase and an apothecial phase but lack conidia (Whetzel & Wolf, 1945). Normally, its sclerotial stage functions for hibernation and for the initiation and nurturing of developing apothetcia. The apothecial stage functions for reproduction and dissemination. The infection occurs at the late March and early April as a result of ascospores discharged. The apothecial development is initiated about a month after the young fruits have been inoculated.

Guangxi Zhuang Autonomous Region is the biggest sericulture province bordering Vietnam and locates in China. The Dainippon Silk Foundation reported that the 3-years yield of Guangxi's silkworm cocoon is at 41.37% of total yield of China and equals to 32.19% of the world production in between 2012 and 2014. Moreover, the yearly average production of Guangxi Province is at 24.89% of the production of P. R. China and 20.46% of the world production, respectively (Shinbo, 2016). However, many commercial mulberry farms have been threat by sclerotinias, in particular *C. carunculoides* in Guangxi Province, and that the outbreak trend was observed at some local areas with a loss rate at from 60% to 90% (Lu et al., 2011).

While there are few literatures on the studies about the infection process of *C. carunculoides* on mulberry fruits, the past studies predominantly focused on not only the morphology and life history (Siegler & Jenkins, 1922; Whetzel & Wolf, 1945), but also the screening of reagents for prevention and cure with the studies of utilising methods (Li et al., 2003). A recent transcriptomic and proteomic study exposed the molecular mechanisms and dynamics of mulberry (*Morus atropurpurea*) fruits response to *C. carunculoides*, for example the changes of transcriptome and proteome, 145 differentially regulated genes, the stimulation of both plant hormone signalling and calcium-mediated defense signalling with the suppression of photosynthesis and cellular growth–related metabolism (Dai et al., 2019). Nevertheless, there is no histological study on the process of photogenic fungus infecting mulberry flower, fruit and seed, in particular *C. carunculoides*. In this study, both optical and electron microscopes were employed to observe not only how pathogenic fungus (*C. carunculoides*) contacting, sprouting and invading on the surface of mulberry (Guisang Superior 12), but also intracellular diffusion, pathological change and relevant response in different cellular tissue to discuss the pathogenic mechanism of mulberry sclerotinia fungus and interaction of mulberry in order to reveal the nature of pathogenic invasion, host being infected and self-defence for providing legitimately scientific evidence on the effective prevent and cure of mulberry sclerotinia.

Materials and Methods

Test mulberry trees and collection of pollen

Ten female parent mulberry trees (Guangdong mulberry, *Morus atropurpurea* Roxb, named "Sha 2") were collected as the first-time selected samples from the seed mulberry field (free of mulberry sclerotinia disease) of "Guisang Superior 12" in Guangxi Zhuang Autonomous Region Sericulture Research Institute in January of 2013. Semitransparent parchment bags (Sheng Bang Nong Yue Plastic) were used to cover the flower buds of mulberry sprouting stage for the preparation of inoculation pathogen test. Five of the ten female parent mulberry trees were used in inoculation zone and the other five were used as controlled zone. The distance was at 5 metre between the inoculation zone and controlled zone. The pollen of the male parent mulberry trees (hybrid species "Gui 7722": "Taiwan Green Skin" (*Morus alba* L.) X "Lun 109" (*Morus atropurpurea* Roxb)) from the seed mulberry field of "Guisang Superior 12", was collected by using clean Chinese traditional brush pens (Wang Xiao Yuan Pen Manor). The ten covered branches were selected when the male flowers were in full bloom at sunshine and then the pollen was kept in plastic boxes (Grand Hyatt Home Design) stored in the fridge (BCD-649WDVC, Haier) at 10°C.

Two of the original selected female parent mulberry trees were used for the second time samples in January of 2014. Similarly, part of the mulberry branches was covered by semitransparent parchment bags for the preparation of the

inoculation pathogen test when they were sprouting. One of the above two trees was used for inoculation and the other was used as control.

Fungal material

In the first-time inoculation, *Ciboria carunculoides* was supplied by the Laboratory of Mulberry Protection in Guangxi Zhuang Autonomous Region Sericulture Research Institute. The sclerotium was cultivated by potato dextrose agar (Becton Dickinson China) and then the hypha generated was isolated and proliferated. The hypha produced was selected and grounded prior to centrifugation (TDZ5-WB centrifuge, Cence) at 4000 r/min for 10 min and then 5% hyphae suspension with sterilized water (generated using UPSL-120L, Chengdu Youpu Equipment) was prepared for future use (Chen, 1997). In the second inoculation, Harada's mulberry sclerotium ascospore inoculation method was developed (Harada, 1980). In January of 2014, the sprouting sclerotium was collected from the mulberry diseased fruits which were scattered on the ground last year and then cultivated in culture dish (Baike Medical Equipment) at natural temperature with daylight in laboratory. The sclerotium which grew apothecium was kept for future use.

Chemicals and reagents

A 1:1 mixture of 95% ethanol (Jinzhou Chemicals): glacial acetic acid (Tianjing Third Chemical Plant) was used to fix and clear mulberry fruit (note: it is mulberry female flower at the pre-developmental stage; mulberry fruit is at the late period of development; from here, in the following text, all mulberry flower is female flower). The floating carrier solution of 0.02% trypan blue (Shanghai Regal Biology Technology) was prepared by adding 40 mg trypan blue to the mixture of 50 ml lactic acid (9 Ding Chemicals), 100 ml glycerol (9 Ding Chemicals) and 50 ml distilled water for dying and sealing the mulberry sclerotium pathogen (Fang, 1998).

Inoculation and sampling

The first inoculation was conducted in February of 2013. The sterilized brush pen was dipped to the hyphae suspension before the parchment bags were removed from 5 mulberry trees in the inoculation zone. And then the bag was used to cover the young flower again after their surface were gently spotted by the dipped brush pen. A controlled group (5 mulberry trees) was established by using sterilized brush pen without hyphae at the same time in 2013. In three days later, the procedure of artificial pollination was conducted after the parchment bags were removed and pollen were dropped to young flower. And then the parchment bags were used to cover the young flowers for 30-days. The mulberry flowers inoculated were collected from 2 of 5 trees at days 1, 2, 3, 4, 5, 10, 15, 20 and 30 respectively. But only the flowers of two of five controlled mulberry trees were collected using the above process.

Both the diseased and healthy mulberry flowers were taken photos (DSLR Camera D7, Cannon; Card Digital Camera MDC-TZ11GK, Panasonic) prior to their being fixed and stored in the prepared mixture of 95% ethanol and glacial acetic acid.

The numbers of infected and healthy fruits were counted in the rest of 3 trees in inoculation and 3 trees in controlled zones at days 10, 20 and 30, respectively. At the initial stage of infection, the fruits were swell with indigo colour. At the middle stage of infection, they were swell with light grey colour. At the final stage of infection, the unhealthy fruits were swell with greyish white and white colour. But those healthy fruits were firm with cyan, red and atropurpureus colours.

The second inoculation was conducted in February of 2014. Two healthy branches with young flowers about to bloom, were clipped from the healthy mulberry trees. Both of the two branches were cultivated in glass bottles filled with distilled water in the laboratory. One is for inoculation and the other one is controlled purpose. The process of ascospore infecting mulberry flower via air flow was simulated due to its ejection nature (Luo et al., 2015). The culture dish loaded with mature apothecium was taken close to the young flower at 1cm distance, and then ascospore ejected and adhered to the young flower stigma under the direction of air flow produced by utilising a rubber suction bulb to gently blow. The parchment bags were used to cover the infected flowers for 10 days. A relevant controlled group was established at the same time with the same process. After the inoculation, both the diseased mulberry flower and the healthy flower were clipped at days 1, 2, 3 and 10 respectively, and then they were fixed, cleared and stored by the method mentioned above.

Staining and microscopic observation of inoculated and intact mulberry flowers

Stigma, inner & outer perianth and seed isolated from inoculated mulberry flowers which were stored at different dates using free-hand microtomy (Blade, Gillette Shanghai), were sectioned into very thin sections with stereomicroscope, respectively. The sections were mounted on grooves of glass slides (Changde BKMAN) and stained with 0.02% Trypan Blue in lactoglycerol before they were placed in a pan heated by boiled water for 3 min. And then fresh lactoglycerol solution was used to de-staining. If necessary, the fresh lactoglycerol solution was used one more time until the pathogen and host tissue were dyed dark blue and light blue or colourless and transparent, respectively, before the samples were sealed

for observation and taking photographs using biological microscope (Eclipse Ci-E, Nikon) (Luo et al., 2015). The same process was applied to the intact mulberry flowers. Ascospore spread over stigma papilla areas was observed.

Preparation and ultramicro observation of inoculated and intact mulberry flowers

Both of outer perianths isolated from inoculated and intact mulberry flowers, were immersed using 2.5% glutaric dialdehyde (Shanghai Aladdin Bio-Chem Technology) for prefixation. 1% osmic acid (Electron Microscopy China) was used for postfixation before ethanol was used for the gradient of dehydration. Epoxy resin (Epon812, Electron Microscopy Sciences) was used for embedding. And then, an ultramicrotome (EMUC7, Leica) was employed to section the perianths prepared using double staining process described in the next section. A transmission electron microscopy (H-7650, HITACHI) was used to taking photos and observation.

Staining, preparation, microscopy and ultramicro observation of Sclerotium encased seeds

Sclerotium encased both young seeds (white yellow colour with flat shape) and mature seeds (auburnish yellow with full shape) were isolated from infected mulberry. Length cutting was used to isolate unilateral hull of sclerotium encased seeds at stereomicroscopy until the enough thin section was obtained. And then the process of staining, biological microscopy observation and taking photographs was repeated. The grey white tissues isolated and sectioned from the sclerotium encased young seeds were placed with 2.5% Glutaraldehyde solution for prefixation in finger tubes. The sclerotium encased mature seeds were processed the same as what young infected seeds were treated. However, under stereomicroscopy observation (Stereomicroscope SMZ-171TP, Motic), only the colour of the tissues the same as the colour of the healthy seeds' tissue were taken for the process of prefixation. And then the two different seeds samples were processed, observed and taken photos using the process described in the next section.

Germination test and microscopic observation of Sclerotium encased seeds

Sclerotium encased both young seeds (300 grains) and mature seeds (300 grains) were isolated from mulberry harvested in the seed mulberry field of "Guisang Superior 12" together with healthy mature seeds (300 grains). Each type of seeds was divided into 3 zones which contained 100 grains in each zone. The seeds were immersed in 0.1% Mercuric Chloride disinfectant (Huashen Chemicals) for 5 seconds and then 70% ethanol for 5 minutes. Sterilized water was used to rinse the seeds continuously for three times before the seeds were transferred to *Φ*120 mm culture dish (Huashen Chemicals) which fully wet filter papers (Shenzhen Liangyi Laboratory Instruments) were placed in respectively. Culture dish, water and filter papers were biocidal treatment. The seeds were placed on the filter papers in an intelligence *artificial climate box (RXZ-260BZ, Shangdong Hengmei Electronic Technology)* for germination at 28°C and 80–90% relative humidity with photoperiod at 12L:12D. In the experiment of comparing germination, germination percentage (GP) of different type of seeds were investigated and calculated. The GP formula was showed as follows:

$$GP = \text{number of germinated seeds / total number of seeds involved in germination x 100\%}$$

During the healthy seeds germinated to slender seedlings, the first both sclerotiums encased seeds (10 grains) and healthy mature seeds (10 grains) to sprout were collected and placed on the sterilized filter paper dipped with sterile water in plastic boxes sterilized, respectively. The climate and environmental conditions of germination were the same as the above section mentioned. In the 5 days of germination, the height of the seedlings was measured daily using an electronic digital vernier caliper (DL91150, Ningbo Deli Group) in order to calculate their absolute growth rate (AGR) for understanding the growth of height of individual seedling in a defined time frame. Relative growth rate (RGR) was calculated by formula transformation to study the net growth rate in a defined time unit and growth efficiency (Zhang & Yang, 2007). The formula of calculating AGR and RGR were showed as follows (Wang, 2000):

$$AGR = (W_{i+1} - W_1)/(T_{i+1} - T_1)$$
$$RGR = (\ln W_{i+1} - \ln W_1)/(T_{i+1} - T_1),$$

where W_i and W_{i+1} were the representatives of the seedling heights (mm) while T_i and T_{i+1} were defined as time units (day) while ln is natural logarithm. According to their AGR and RGR, the significant difference between the two different seedlings were determined using *t*-test with software Excel 2003. In the test of germination, healthy seedling of sclerotium encased mature seeds were visualized and taken photos under stereomicroscope.

Results

Process of Sclerotinia sclerotiorum invading mulberry fruit

The process of *Ciboria carunculoides* ascospore and mycelium invading mulberry fruit is consisting of the stages of

adhesion, germination, intruding, extension and host paroxysm.

Adhesion, germination and invasion of ascospore on stigmata of mulberry flower

The stigmatic papilla of mulberry flower excrete sticky adhesives such as saccharides in order to stick pollen (Ke, 1997) for promoting pollen germination. After one day of air flow inoculation, ascospores felled to the stigmatic papilla were adhered by sticky material and some ascospores suspending on the stigmatic papilla showed right angle between each other even if there is only a small top area of ascospore in touch with stigmatic papilla with light microscopic observation. It suggested that the stigmatic papilla possess strong viscidity (Figure 1a). The ascospores adhered to the stigmatic papilla have never been deciduous although the mulberry flower samples went through a series of preparation process such as being harvested, immersed in chemical reagents, physically squeezed for freehand slice method and heat treatment. This result further demonstrated that pathogen spore successful invasion is benefit from the strong adhesive power of the stigmatic papilla. After inoculation of 2 days, slender germ tube and slightly thicker appressorium grown were downward to the stem base of stigmatic papilla due to germination of ascospores (Figure 1b). After inoculation of 3 days, the germ tube, appressorium and infect hypha grown from one of the ascospores which took the lead were observed. The fine tip of the infect hypha was downward and this demonstrated that the infect hypha has been successfully penetrated to the gap of epidermal cells. And that the extension of the infect hypha was towards the stigmatic interior showed the trend of invasion. The body of this ascospore displayed that the blue colour of left side was much shallower than the blue colour of right side due to the consumption of internal materials for growth of the appendages (Figure 1c-a). In addition, comparing the germinated ascospore at stigma with morphology of mulberry pollen, the germ tube was obviously slenderer than pollen tube (Figure 1c-b). According to the statistical survey of light microscopic observation (Table 1), most of ascospores were adhered to stigmatic papilla and germination there with relevant rates at 72.9% and 77.8%, respectively.

The numbers of protuberant conglutination and germination of ascospores at the surrounding areas of stigmatic papilla significantly decreased at 26.3% and 41.2%, respectively. Moreover, there were only two ascospores of not germination at the perianth which locates far from the stigmatic papilla. According to the analysis of the above figures and Table 1, the secretions of female stigmatic papillas and surrounding small papillas possessed very strong adhesive action. Therefore, this is not only beneficial to capture pollen but also in favour of adhering to ascospores of mulberry sclerotinia sclerotiorum pathogen. On the other hand, the germination percentage of ascospore at the stigmatic papilla was much higher at one time than that at the surrounding areas.

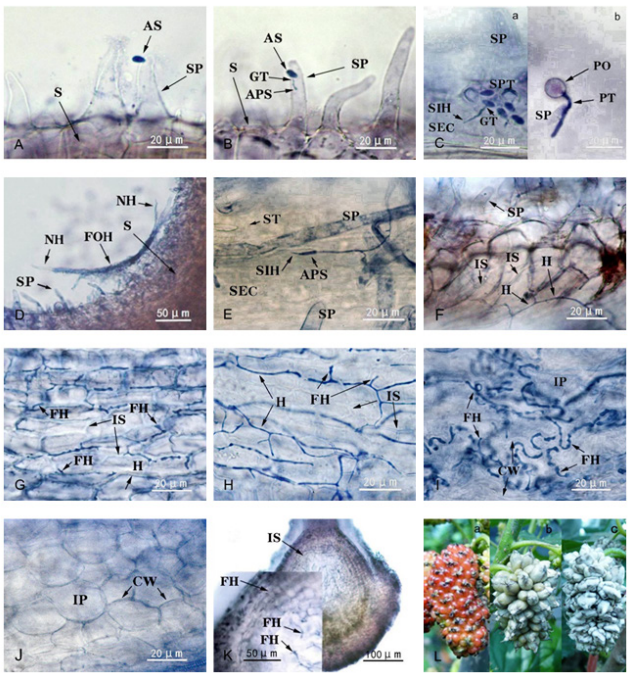

Fig. 1

Table 1. The variation of adhering location and germination for the ascospores on mulberry female flower with observation using light microscope.

Location	Number of photos observed	Number of ascospores adhesion	Spore adhesion to total adhesion, %	Number of ascospore germination	Spore germination to total adhesion, %
Stigmatic papilla	147	189	72.90	147	77.80
Stigmatic papilla surrounding areas	39	68	26.30	28	41.20
Surface of outer perianth	23	2	0.80	0	0
Controlled group	4	0	0	0	0

The pathogen ascospores indicated well growth and that the stigmatic papilla displayed as the primary part of producing the secretions. For the above description and deduction, the secretions provided good nutrition for the ascospore germination and that the stigmatic papilla facilitated the upgrowth of ascospores. Hence, while the stigmatic papilla is the predominantly part of ascospore invasion, the surrounding areas with small papillas are minor parts.

Adhesion, germination and invasion of hyphae at stigmatic papilla

After 1 day of hypha suspension inoculation, the parts of old hyphae were sticked by the numerous papillas and small protuberances on the stigmatic surface. And fresh hypha grew up from both sides of the old hypha (Figure 1d). After 3 days of inoculation, that the front part of new hyphae indicated parallel growth with stigmatic epidermal cells was convenient for the hyphae invading into the internal part of stigma and that expanding downward with growing branches. The tip of hyphae consisted of inflatable appressorium and fine infect hyphae which showed pale blue colour due to its successfully penetrating into stigmatic epidermal cell and that preparing to move toward the gap of left side cells. The hypha did not invade via the surrounding stomas (Figure 1e). After 5 days of inoculation, a piece of stigmatic sectional infect hypha which was very fine with branches and separation, came off from intercellular space due to sectioning. Therefore, according to the analysis of hypha branch morphology and expanding trend downward, the hypha was apparently extending toward the style (Figure 1f).

Extension of hypha in the stigma, ovary wall, perianth of mulberry flower

After 10 days of inoculation, hypha morphology showed significant change from stigma to intercellular space of ovary wall. Most hyphae became thick and irregular surfaces with extending short branches into cells, namely filamentous haustorium with various morphology such as filamentous, strip, club and globosity. The intercellular spaces of ovary wall which had not been invaded by haustorium extension revealed white colour at refraction with regular line weight, and that the cellular original profile was still explicitly observed due to the intact cellular walls (Figure 1g). After 10 days of inoculation of ascospore, the hyphae and filamentous haustorium infected to cell of ovary wall (Figure 1h) but the status of infection was similar to that in Figure 1g. After 15 days of inoculation, the filamentous haustorium invaded into the internal cells of the inner perianth with irregularly rapid growth and being full of the cell body. That the state of invasion, proliferation and expansion occurred in a single cell or a local tissue resulted in the indistinct cell shape, cytoderm changing to scattered folding with dysfunction for supporting cell. The above descriptions speculated that lytic enzyme from haustorium was the primary reason for disintegrating the cellular structure (Figure 1i). In the controlled group, at the days 15, the perianth cell of the intact mulberry flowers displayed full form and clear profile of cellular wall (Figure 1j). On the section of the immature seed inoculated at the same day, there was an amount of filamentous haustorium without any sign of early albuminous cell in the seed centre coated by ovary. Although there were some regularly arranged early embryonic cells beside the seed centre, but the profile of the cells was inexplicit with few filamentous haustorium (Figure 1k).

External symptom and internal tissue change of mulberry fruit infected by Sclerotinia sclerotiorum

After water spraying treatment was using for 30-days in the controlled zone, the healthy and near mature mulberry fruit showed that the appearance was spontaneous full with red colour in visual study but the mature fruit was atropurpureus colour (Figure 1L-a). At 10 days of hypha suspension inoculation, the small fruits started to swell with healthy cyan. However, after 20 days of hypha suspension inoculation, those small fruits infected were swelling significantly like popcorn appearance with changing to light grey colour instead of the

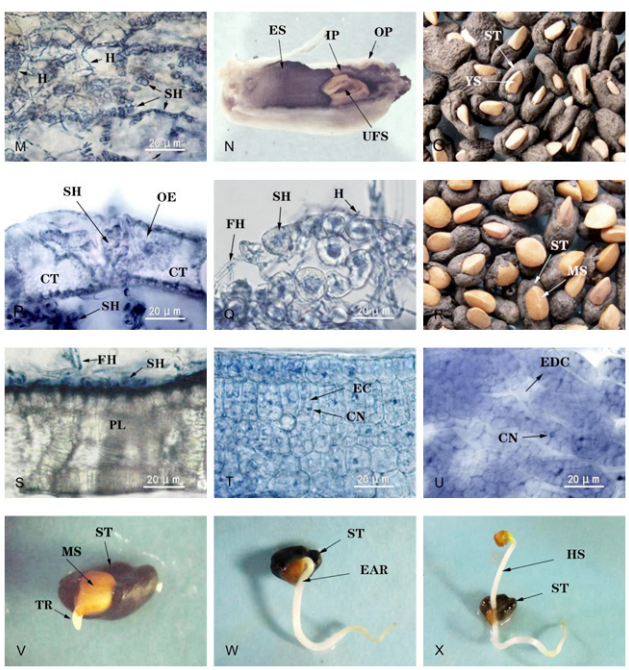

Fig. 2

healthy cyan colour (Figure 1L-b). There were few hyphae with a mass of spherical haustoriums which formed "grid phenomenon" due to cluster and array in the swelling outer perianth with light microscopic observation (Figure 2-M).

There was not any host cell organelle presented except the large-scale spherical haustoriums with few hyphae in the swelling outer perianth under transmission electron microscopic observation (Figure 3A). However, there were not only cell nucleus, small vacuoles and mitochondria presented in the healthy perianth cell but also the plasma lemma and cellular walls in the exterior (Figure 3B).

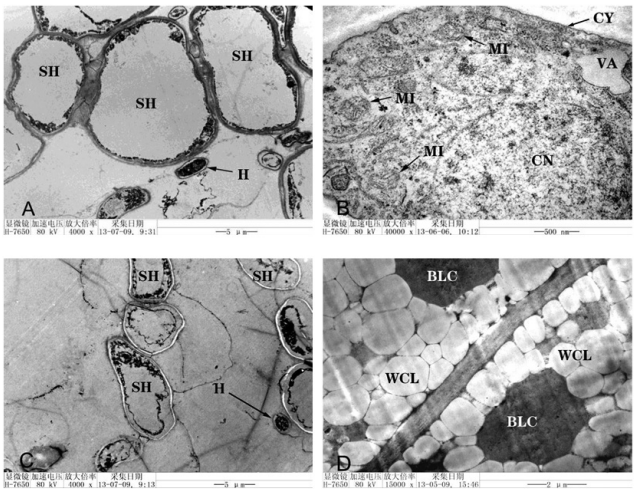

Fig. 3

There were probably two reasons for the "grid phenomenon" indicated in the infected mulberry perianth. One was that in the last stage of invasion, the nutrient materials of the host cell had been completely exhausted and the pathogen produced large-scale spherical haustoriums with big superficial area in order to sequentially enhance the power of intake for survival purpose. The other was that for feeding purpose the haustoriums had to stay together as array and adhere to the only remained material which was the cell walls with difficulty to decompose for absorption. After 30 days of inoculation, the whole diseased mulberry fruit revealed not only still swelling appearance but also its colour changed to grey-white or white (Figure 1-L-c). The longitudinal section of the infected mulberry fruit showed that the centre of seed was empty, disappearance of ovary walls, the inner perianth was black colour with the outer perianth presenting white colour. Furthermore, while the sclerotium which consisted of haustoriums and lumps only occupied two-third small fruit space.

That the interior of sclerotium displayed grey-black colour while the exterior was black with the low part exhibiting lighter colour suggested that was rudiment sclerotium (Figure 2-N). However, at the initial stage of infection at days 10, the diseased fruits were swelling and cyan colour at 73.30% of the total fruits in the inoculation zone. At the middle stage of days 20, the diseased fruits were swelling and light grey colour at 57.73%. At the final stage of days 30, the diseased fruits were swelling with ashen and white colour at 67.68%. The total morbidity was at 96.53% (Table 2). In the controlled zone, none of diseased fruit was found. The external change of infected fruits demonstrated that the infection could seriously influence the whole mulberry fruits as time goes by rather than single case observed by anatomical observation.

Expression of seed resistance to Sclerotinia sclerotiorum

External seed shell possesses the function of resisting external harmful influences for the self-protection purpose. The capacity of resisting hypertrophic sclerotia invasion depends on the maturity of seed shell.

Most seeds were failure to maturation due to invasion of pathogen hyphae and haustoriums

The young seed coated by sclerotium which were grey-black colour with the shape of rat feces exposed half or part

Table 2. External change of mulberry fruit inoculated by *Ciboria carunculoides*

Treatment	Days	Total amount[a)]	Healthy amount[b)]	Initial stage[b)]	Middle stage[b)]	Final stage[b)]	Total unhealthy[b)]
Inoculation	10	367	70 (19.07)	269 (73.30)	28 (7.63)	0 (0)	297 (80.93)
	20	414	34 (8.21)	114 (27.54)	239 (57.73)	27 (6.52)	380 (91.79)
	30	461	16 (3.47)	30 (6.51)	103 (22.34)	312 (67.68)	445 (96.53)
Controlled	10	346	346 (100)	0 (0)	0 (0)	0 (0)	0 (0)
	20	379	379 (100)	0 (0)	0 (0)	0 (0)	0 (0)
	30	418	418 (100)	0 (0)	0 (0)	0 (0)	0 (0)

[a)] Each fruit is defined as one unit. Total amount of fruits includes the number of new fruits.

[b)] Unit is % in brackets

of body with flat and not full appearance, light yellow or white colour, and soft texture (Figure 2-O). The cross section of seed shell coated by sclerotium revealed that there were many cavities, few support structure and thin texture with aggregated spherical haustoriums invading into inside of seed shell by directly penetrating from its exterior (Figure 2-P). There were numerous large-scale spherical haustoriums, few filamentous haustoriums and hyphae without embryo and endosperm tissue in the young seeds infected (Figure 2-Q). The large-scale spherical haustoriums and hyphae were observed in the interior of the invaded seeds with original host cell tissue being entire decomposition and absorption applying transmission electron microscopic observation (Figure 3C).

Part of the seeds were successful to maturation due to resistance for invasion of pathogen hyphae and haustoriums

The shape of the mature seeds coated by sclerotium was full and enrichment with deep tawny colour and firm seed shell the same as what the healthy mature seeds exhibited (Figure 2-R). Seed shell contains the external epidermis of thick and firmness, and the internal epidermis which is thin and soft. The section of seed shell showed that the surface of the external epidermis was colour depth and there was bulky pillar supporting structure, namely palisade layer which was much tighter and higher density than that of young seeds. The haustoriums which had already invaded into the surface of seed shell had not been observed in both the cross section of the palisade layer and interior. This illustrated that the morphological structure of seed shell for the resistance expression was correlation to the failure of haustoriums' invasion (Figure 2-S) (Zhang, 2003). That the embryonic cell in the mature seeds coated by sclerotium displayed the shape of round or cylinder, staggered up and down, stagger of rhombus, tidiness and tightness, and uniformity of cell size with cell nucleus presenting dark colour, revealed the characteristics of healthy cell (Figure 2-T).

Although the endosperm cell sparsely arranged, that the cell body were full with appearing irregular oval or striper and cell nucleus of dark colour were considered as healthy cellular morphology (Figure 2-U). In the endosperm of the mature seeds coated by sclerotium, the black large-scale globular material with high electron density was lipoprotein complexus which was surrounded by many white colour liposomes of low electron density. The liposomes not only were polygon with mosaic array extending to cell walls but also stayed close without any gap between each other the same as presentation of healthy rapeseed and tallow seed's endosperm cells (Figure 3D) (Fu, 1993; Liao, 2014). Notwithstanding, most part of mature seed were coated tightly by sclerotium for long time, germination could still occur if the growth environment were satisfactory for example suitable temperature and humidity together with enough water. The milk-white radicle tip grew up from the rupture of seed shell (Figure 2-V). The snow-white threadlike embryonic stem and radicle continuously grew up from the mature seeds (Figure 2-W). Eventually, the healthy seedling was grown up with straw yellow cotyledon, white slender hypocotyl, light brown radicle. The above description demonstrated that mature seeds still possibly grew up seedling in spite of sclerotium invasion (Figure 2-X).

Comparison of germination rates for mature and young seeds coated by sclerotium and healthy mature seeds

The germination rates were a great difference for the seeds from three different sources at room temperature with high humidity environment (Figure 4). While the germination rate of infected mature seed was at 36%, the same rate of young unhealthy seeds was at 3.3%. However, the germination rate of healthy mature seeds was the highest one at 90.3%. Though the germination rate of long-term infected mature seeds was not very high as only approximately one third, that part of them could geminate illustrated that these mature seeds still possessed strong survival capacity and resistance when they were long-term invaded by sclerotium, hyphae and haustoriums**.**

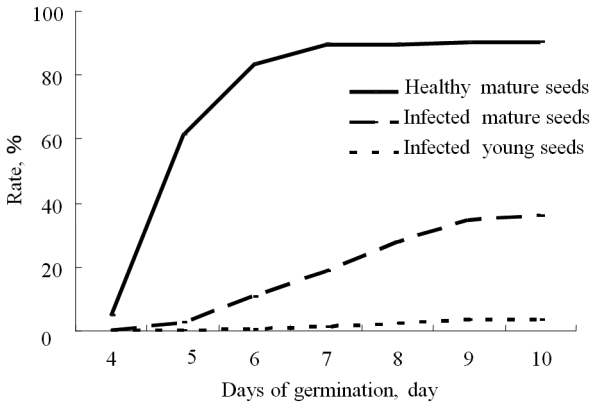

Fig. 4. Comparison of average germination rates for different mulberry seeds

Comparison of between absolute growth rate and relative growth rate for plant heights of both infected and healthy seedlings

The plant height AGR of Seedling from seeds coated by sclerotium from day 2 to 5 increased day by day, and that there was no significant variation between the coated seed-

Table 3. Comparison of plant height AGR at between infected seedlings and healthy seedlings

Type of sample	Plant height absolute growth rate ($\bar{x}$±SD)(mm·plant^{-1}·d^{-1})			
	Germination			
	2nd day	3rd day	4th day	5th day
Infected mature seedlings	1.566±0.451a	2.254±0.803a	2.951±2.024a	3.621±4.632a
Healthy mature seedlings	1.664±1.429a	2.330±0.741a	3.198±1.655a	4.362±2.687a

Note: the same letter marked at the right side of figures meant that there was no significant variation with a t test at 0.05

Table 4. Comparison of plant height RGR at between infected seedlings and healthy seedlings

Type of sample	Plant height absolute growth rate ($\bar{x}$±SD)(mm·plant^{-1}·d^{-1})			
	Germination			
	2nd day	3rd day	4th day	5th day
Infected mature seedlings	0.696±0.088a	0.672±0.041a	0.616±0.029a	0.554±0.290a
Healthy mature seedlings	0.588±0.625a	0.808±0.070a	0.743±0.036a	0.702±0.017b

Note: the same letter marked at the right side of figures meant that there was no significant variation with a t test at 0.05

ling and the healthy one on a daily basis analysis (*t* test, Table 3). This above description may suggest that both the coated seedling and the healthy seedling grew up well due to no difference of biomass increase at between the two kinds of seedlings.

The RGR of the coated seedling decreased gradually every day while that of healthy seedling was mixed with up and down. However, the RGR of the above two kind seedlings were not significantly difference except on the fifth day (*t* test, Table 4). These results probably declared that there was no prominent variation on the RGR between each other in most of the time. The comprehensive analysis of the seedlings' AGR and RGR demonstrated that part of the invaded mature seeds not only germinated but also grew up with seedling as good as what healthy mature seeds did as a result of these part of seeds possessing high-calibre resistance to Sclerotium pathogen.

Discussion

Pollination is one of the key processes for spermatophyte to life extension. In this study, the used mulberry, called "Guisang Superior 12" belongs to Guangdong mulberry species (*Morus atropurpurea* Roxb.) whose pistil possesses wet type stigma. The epidermal cells of wet type stigmatic papillas, short hairs, small prominences and stigmatic expanding root, can secrete a mixture with water, saccharides, lipids, various vitamins, hormones for providing necessary nourishment for germination (Harada, 1980). Hence, pollen tube grows into ovary for fertilization through intercellular space channel of style at the same time absorbing nutrients, for example walnut, cotton and other plants (Cui, 2005).

This study manifested that the stigmatic papilla with stickum secreted were to the benefit of floated ascospore and hyphae being firmly adhered on the stigma, and that the secreted mixture could facilitate not only spore to germinate to germ tube, but also hyphae to grow appressoriums and infect hypha. Furthermore, the ascospore germ tube, infection hypha of hyphae and ascospore were much slenderer than pollen tube. This appearance was speculated that those very thin hyphae and filamentous haustoriums in the spaces between stigmas and cells of ovary walls with a high possibility of driving straight in the guidance tissue channel. It is not difficult to see ascospores and hyphae are the same as what ergot does, for example using all of the beneficial spaces and channels which were prepared for mulberry pollination for their adhesion, growth and expansion, and then easily made mulberry diseased by invasion (ABE & Kono, 1957). Moreover, mulberry fruits became useless at economics due to the high morbidity. Even though the infected seed became mature, it was still useless because the sclerotium could not be removed. The tremendous economic loss of mulberry farming was approved due to infection of *Ciboria carunculoides* in local areas in Guangxi Province

It is speculated that characteristics of both mulberry anatomical structure and biology is one of the key factors for the high incidence rate of mulberry sclerotiniose infection because ascospores and hyphae could easily invade into. Accordingly, effectively prohibiting ascospores and hyphae to invade mulberry flower stigma, become the key for prevention and control of mulberry diseases.

The shape and attachment of the ascospores were still uncertainty. Under high magnification the caruncles are seen to be composed of two bodies, one a body adjacent to the spore and more or less rhombic in shape and crescent-shaped as seen from above, the second a body adjoining the first and more or less hemispherical (Siegler & Jenkins, 1923; Whetzel & Wolf, 1945). However, the spores are occasionally re-

inform with light brown colour surface (Wang, 2009). It is not present in preserved specimens nor can the caruncles be found after preservation. Moreover, microconidia of species of Sclerotinia was firstly noted on the diseased fruit other than on culture media in 1923 (Siegler & Jenkins, 1923). But Whetzel described that *Ciboria carunculoides* lacks a conidial stage in 1945 while microspores (spermatium) and stroma were described in the black glutinous layer which was at between diseased droplet and the membrane in 2009 (Wang, 2009).

In the literature, it is still uncertainty that microcondia and unknown microspore and stroma were yielded when the diseased fruits were on the tree or after the fruits fell down to ground. Izumi Saito published the study of the effect of temperature on the development of stipe primordia in *Sclerotinia sclerotiorum*. The results showed that if such sclerotia are transferred to the optimum temperature at 15°C rather than 4°C and 25°C, they germinate readily and synchronously resulting in a high percentage of germination to form apothecial stipe primordia. Then spermatiophores, spermatia and archicarps were generated (Saito, 1977). Although Whetzel & Wolf described that the mature sclerotia of *C. carunculoides* produced apothecium in next spring after hibernation. This is the same as *Sclerotinia sclerotiorum's* life cycle. However, they simultaneously considered that spermatia was on the surface of the infected droplets on the tree before hibernation. This result is contradiction. Nonetheless, in the current study, upon microscopical examination of the infected mulberry fruit collected from the tree in Guangxi Province, did not possess the black glutinous layer and any visible evidence of microconidia or conidium.

Plant seed coat innately possesses stress tolerance and resistance. The physical characteristics of seed coat are commonly solid density and hydrophobicity because of suberization, lignification and hydropenia due to disappearance of protoplasm. Hence, seed coat become a barrier as primary defense to protect seed life from external environmental disturbance (Yasseen et al., 1994). A study of *Vicia* reported that there were periostracum and palisade layer in its seed coat. The periostracum contained highly sophisticated fatty materials but this unique structure could impede access of water and microorganism. On the other hand, the suberization of palisade layer made the structure be densification to increase seed coast compactness and stiffness (Wen, 2013). Moreover, Whetzel & Wolf reported that the layers of sclerenchyma that constitute the outer portion of the achene are quite free from invasion, and consequently remain quite unchanged even in mature sclerotia (Whetzel & Wolf, 1945). Nonetheless, the current study indicated that the seeds in the infected mulberry fruits were still able to mature because the mature seed shell contained clear and obvious palisade layers which were solid, thick, firmness and densification due to suberization and lignification. Although sclerotium haustoriums had infected to the surface of epidermis, that they were hard to invaded into demonstrated that the physical structure of mature seed coast was in possession of excellent antifungal property. By contrast, young seeds were easily invaded into due to the thin epidermis, the lake of compact palisade layer and lots of intercellular spaces (Figure 2-P and S).

According to the chemical analysis of seed coat, the dead cells of the seed coat yielded and contained various secondary metabolites during the process of suberization, lignification and cutinization and that the generated special substances aggregated with cooperative interaction in the seed coast to establish chemical defense mechanism against invasion of pathogens (Wu, 2005). For instance, fatty compounds combining proteins made seed coat waterproof, lignin of phenolic polymers made seed coat toughest while polyphenols possessing antibacterial activities. In the literature, the dark colour part of mature seed shell abundantly contained polyphenols such as tannin and phenolic acids (Nakamura et al., 1959). Another report showed that tannin possessed inhibitory effect for fungus for example *Verticillium lecanii*, and other 30 fungus (Shi & Di, 2000). Additionally, phenolic acids isolated from *Ginkgo* biloba sarcotesta had inhibitory activity for *Sclerotinia allii* (Wang et al., 2009).

In the current study, the skin of the mature mulberry seed is snuffcoloured while the skin of the young seed is pale yellow and white. These colours suggested that there was a certain amount of polyphenolic substances in the seed skin. However, the question is that if mulberry mature seed coat contained the above secondary metabolites? This happens remains unclear and is not known, but those mature seeds coated by sclerotium were still able to grow into healthy seedling due to the high possibility of the existence of the secondary metabolites in the seed skin. Nowadays, there are two new and popular botanical fungiticides, Yintai (1-(4-Hydroxyphenyl)-1-butanone) and Yinguo (Ginkgo phenols) base on ginkgol successfully discovered and developed from *Ginkgo biloba* sarcotesta in Chinese pesticidal market (Meng et al., 2004). For the determination of the antifungal metabolites in the mature seed skin, chromatography in combination with other analytical techniques are suggested. Moreover, the antifungal and fungicidal activities of the natural products isolated from the seed coat will be determined in mulberry field drug trails.

Additionally, only few literatures are about the physiological anatomy of *Ciboria carunculoides*, and none of these described the stroma of this fungus in detail. Therefore, the aim of the further study is to provide theoretical foundation and technological support on comprehensive prevention and

control of mulberry sclerotiorum disease by understanding its rules and characteristic of invasion, in particular each disease stage and pathogenesis.

Conclusion

Ciboria carunculoides, is the major fungal disease for the mulberry fruit in Guangxi Province in P. R. China because its ascospore and hyphae can easily attach to mulberry female flower stigma due to viscosity of secretions on stigma. Then germinal tube, appressorium and infect hypha easily grew up and invaded to the stigma due to rich in nutrition of the secretions. Because infect hypha was much thinner than the pollen tube, it was free growth up and extension at the spaces between cells of stigmas and ovary walls. After the hyphae and filamentous haustoriums were generated, they invaded into stigma, ovary wall, cells of inner and outer perianth. At the final stage, the infected mulberry fruit was completely destroyed because of full filled with spheroidal haustoriums, and disintegration and disappearance of cellular walls. The incidence rate of mulberry fruit was very high so that the infected fruits were useless and caused significant loss of local economics. Therefore, effective means to suppression and avoidance of ascospore and hyphae invading into mulberry female flower stigma became the key for prevention and cure of *Ciboria carunculoides* invasion.

Mulberry seeds naturally possessed capacity at resistance of fungal pathogens. This reason could be the mature level of seed skin. At the mature seed shell, palisade layer was clearly observed. This demonstrated that the seed skin which had been not only suberification and lignification, but also stiffness, densification and hydrophobicity completely possessed physical characteristic resisted *Ciboria carunculoides*. Even if the seed was covered by sclerotium, germination and growth of young seedling occured also. However, young seeds were easily invaded by fungal pathogens because the skin was thin and lack of compact palisade layer. Finally, the deep colour of shell of mature seed was speculated that the existence of secondary metabolites which contribute to resistance of fungus but further chemical analysis and determination are suggested.

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