DEVELOPMENT OF EFFECTIVE SPORE INDUCTION METHODS FOR ASPOROGENIC FUNGAL SPECIES USING THE STANDARD CULTURE MEDIUM

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DEVELOPMENT OF EFFECTIVE SPORE INDUCTION METHODS FOR ASPOROGENIC FUNGAL SPECIES USING THE STANDARD CULTURE MEDIUM

by

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LIST OF ABBREVIATIONS

CLS	Cercospora leaf spot
BLA	Banana leaf-piece agar
PDA	Potato dextrose agar
OA	Oatmeal agar
FWL 12:12	12 hours of fluorescent white light radiation and 12 hours of darkness
BLA (Awak)	Banana leaf-piece agar prepared from banana leaf of *M. acuminata* × *balbisiana* cv. Awak
BLA (Nangka)	Banana leaf-piece agar prepared from banana leaf of *Musa* × *paradisiaca*
BLA (Talun)	Banana leaf-piece agar prepared from banana leaf of *Musa acuminate* × *balbisiana* Colla 'Raja'
BLA (Wild)	Banana leaf-piece agar prepared from banana leaf of *M. acuminata* spp. malaccensis
CLA	Carnation leaf-piece agar
AAS	Atomic absorption spectrometry
CFP	Cercosporin facilitator protein
PDB	Potato dextrose broth
PCR	Polymerase chain reaction
S	Subunit
LD12:12	12 hours of near-UV radiation and 12 hours of darkness
ITS	Internal transcribed spacer
rDNA	Ribosomal deoxyribonucleic acid
TEF 1-α	Translational elongation factor 1-α
ACT	γ-actin gene
TOPI	DNA topoisomerase I gene
bp	Base pair

PGK	phosphoglycerate kinase gene
RPB2	RNA polymerase gene
mtSSU	Mitochondrial small subunit
CAM	calmodulin gene
DNA	Deoxyribonucleic acid
H3	Histone gene
OGTR	Office of the Gene Technology Regulator, Australia
rRNA	Ribosomal ribonucleic acid
NCBI	National Center for Biotechnology Information
f. sp.	Forma specialis
sp.	Species (Singular)
spp.	Species (plural)
SD	Standard deviation
WA	Water agar
OGA	Oat grain agar
ANOVA	Analysis of variance
WGA	Wheat grain agar
BGA	Barley grain agar
elf1f / ef2r	TEF primers
ITS1F/ ITS4	ITS primers
CMA	Cornmeal agar
FSSC	*Fusarium solani* species complex
CZK3	MAP Kinase Kinase Homolog
CTB	Cercosporin toxin biosynthesis
PL	Pectatelyase
PG	Polygalacturonase
B5	Calcium pantothenate, $C_{18}H_{32}CaN_2O_{10}$

LIST OF SYMBOLS

Cu	Copper
Fe	Ferum
Zn	Zinc
Mg	Magnesium
K	Potassium
Ca	Calcium
h	hour
p	p value/ calculated probability
nm	Nanometre
°C	Degree celcius
n	Haploid number
ml	Millilitre
g	Gram
mm	Millimetre
%	Percentage
TM	Trade mark
µl	Microlitre
w/v	Weight per volume
α	Alpha value/ significance level
mM	Millimolar
mg	Milligram
l	litre
µm	Micrometre

PEMBANGUNAN KAEDAH ARUHAN SPORA YANG BERKESAN UNTUK SPECIES KULAT ASPOROGENUS MENGGUNAKAN MEDIUM KULTUR PIAWAI

ABSTRAK

Faktor yang memperkuatkan penghasilan spora anamorph telah dicabar. Kesan-kesan jenis medium kultur dan keadaan pencahayaan pada pengaruhan konidium bagi pencilan *Cercospora citrullina* Cooke serta kesan-kesan medium kultur agar daun pisang (BLA) pada pengaruhan makrokonidium bagi spesies *Fusarium* telah dikaji. *C. citrullina* yang dipencilkan daripada daun labu manis yang dijangkiti penyakit bintik daun Cercospora (CLS) telah didapati tidak boleh mengeluarkan konidium apabila dikultur pada agar dekstosa kentang. Ujian kepatogenan telah dijalankan pada beberapa tumbuhan perumah kukurbit sebelum kajian kesan medium kultur agar dan substrat dalam keadaan pencahayaan yang berbeza pada penghasilan konidium bagi *C. citrullina* telah dijalankan. Kultur agar serbuk oat yang boleh didapati secara komersial, kultur agar yang diperbuat daripada infus bijirin oat, gandum dan barli serta substrat yang disediakan daripada daun kering spesies *Trichosanthes* (Cucurbitaceae), bijian oat, bijian gandum dengan bijian barli telah didapati berkesan untuk mengaruhkan penghasilan konidium *C. citrullina* ($p = 0.05$). Penghasilan konidium *C. citrullina* telah didapati berkesan apabila diinkubasi selama 12 jam bawah sinar lampu putih berpendarfluor dan 12 jam kegelapan berterusan serta dalam keadaan kegelapan sepenuhnya. Sebanyak enam pencilan *Fusarium* telah dikultur pada enam jenis agar kultur kepingan daun pisang yang diperbuat daripada daun-daun pisang *Musa* × *paradisiaca* (Nangka and Tanduk), *Musa acuminata* ×

balbisiana Colla ‘Raja’ (Toman and Talun), *M. acuminata* × *balbisiana* cv. Awak dan *M. acuminata* spp. malaccensis (Wild) untuk mengaji kesan-kesan agar kultur BLA terhadap penghasilan makrokonidium *Fusarium*. Perbandingan rupa bentuk serta saiz makrokonidium yang terhasil pada BLA yang berlainan dengan makrokonidium yang terhasil pada agar kultur pengaruhan makrokonidium yang piawai, iaitu agar kultur kepingan daun bunga teluki telah dijalankan. Walaupun terdapat perbezaan yang ketara pada kepanjangan dan kelebaran makrokonidium yang terhasil pada BLA dan CLA, tetapi, morfologi makrokonidium yang terhasil pada kedua-dua jenis agar kultur adalah sama ($p = 0.05$). Tambahan pula, pemerhatian pembentukan sporodokium telah dilakukan. Pembentukan sporodokium didapati lebih banyak pada BLA berbanding CLA, terutamanya daripada BLA yang disediakan dengan daun pisang kultivar Nangka, di mana medium tersebut dapat mengaruhkan penghasilan sporodokium pada keeenam-enam pencilan *Fusarium* yang dikaji. Analisis elemen telah dilakukan melalui spektrometri serapan atom (AAS). Dalam kalangan enam jenis unsur (Cu, Fe, Zn, Mg, K, Ca) yang dikaji, daun kering bungah teluki didapat mempunyai kandungan magnesium dan kalsium yang lebih tinggi. Keberkesaan agar kultur BLA yang ditambah dengan garam kalsium dengan CLA dalam penghasilan makrokonidium *Fusarium* serta pembentukan sporodokium telah dikaji. Makrokonidium yang terhasil pada BLA yang ditambah dengan garam kalsium telah didapati mempunyai rupa bentuk yang sama secara umum apabila dibanding dengan makrokonidium yang terhasil pada CLA, kecuali pencilan K2K2 dan K2L4. Perubahan dalam morfologi makrokonidium telah didapati pada pencilan K2K2 dan K2L4 apabila dikultur pada BLA yang ditambah dengan garam kalsium. Perbezaan yang ketara telah didapati pada kepanjangan dan kelebaran makrokonidium yang terhasil pada BLA yang ditambah dengan garam kalsium serta makrokonidium yang terhasil pada CLA ($p = 0.05$).

Pembentukan sporodokium telah dipertingkatkan dengan penambahan garam kalsium kepada BLA (Awak), disebabkan pembentukan sporodokium telah didapati pada pencilan *Fusarium* setelah BLA (Awak) ditambah dengan garam kalsium. Kesimpulannya, medium dan substrat yang disediakan dengan bijirin didapati berkesan untuk mengaruhkan penghasilan konidium *C. citrullina* apabila diinkubasi bawah kondisi FWL 12:12 dan keadaan kegelapan sepenuhnya. Di samping itu, BLA yang diperbuat daripada daun pisang kultivar Nangka didapati standing dengan CLA dalam mengaruhkan penghasilan makrokonidium *Fusarium*.

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ABSTRACT

Factors enhancing anamorph spore production were challenged. Effects of types of media and lighting conditions in the induction of *Cercospora citrullina* Cooke conidiation as well as effects of banana leaf-piece agar (BLA) in the induction of *Fusarium* spp. macroconidia production were studied. *C. citrullina* isolated from pumpkin leaves with Cercospora leaf spot disease (CLS) was found unable to produce conidia when cultured on potato dextrose agar. Pathogenicity test was carried out on several cucurbit host plants prior to the study on effect of culture medium and substrates under different lighting conditions in inducing the conidiation of *C. citrullina*. Commercially available oatmeal agar; homemade agar prepared from the infusion of cereal grains such as oat grain, wheat grain and barley grain in addition to substrates prepared from dried *Trichosanthes* sp. (Cucurbitaceae) leaves, oat grain, wheat grain and barley grain were effective in inducing conidia production in *C. citrullina* ($p = 0.05$). Conidia production was prominently found when the *C. citrullina* cultures were incubated under 12 h fluorescent white light irradiation plus 12 h darkness and complete darkness condition ($p = 0.05$). A total of six isolates of *Fusarium* were cultured on six types of banana leaf-piece agar (BLA) prepared from the leaves of *Musa* × *paradisiaca* (Nangka and Tanduk), *Musa acuminate* × *balbisiana* Colla 'Raja' (Toman and Talun), *M. acuminata* × *balbisiana* cv. Awak and *M. acuminata* spp. malaccensis (Wild) respectively to learn about the effects of BLA in the macroconidia production of *Fusarium*. Comparisons of shape and size of

macroconidia produced on different types of BLA with standard macroconidia inducing medium, carnation leaf- piece agar (CLA) were made. Although significant difference was found between the length and width of macroconidia produced on BLA and CLA, however, the morphology of macroconidia produced on both types of agar was generally similar ($p = 0.05$). Besides that, observation of sporodochium formation was also conducted. Sporodochium production was much more prominent in BLA compared to CLA, particularly BLA prepared from cultivar Nangka induced sporodochium production in all six isolates of *Fusarium* tested. Analysis of elements was conducted using atomic absorption spectrometry (AAS). Among the six elements (Cu, Fe, Zn, Mg, K, Ca) tested, carnation dried leaf possessed higher amount of magnesium and calcium ($p = 0.05$) than banana dried leaf. Effectiveness of calcium salts amended BLA and CLA in term of *Fusarium* macroconidia and sporodochium induction was studied. Macroconidia produced on calcium salts amended BLA were generally similar in shape compared to those produced on CLA, except for isolates K2K2 and K2L4, in which morphological changes in macroconidia were found on calcium salts amended BLA cultures. Length and width of macroconidia were significantly different when macroconidia produced on BLA amended were compared with calcium salts and CLA ($p = 0.05$). Sporodochium formation was enhanced in calcium salts amended BLA (Awak), which was unable to induce sporodohium formation in any of the six *Fusarium* isolates was found capable of inducing sporodochium formation in at most of the *Fusarium* isolates. In general, conidia production of *C. citrullina* was successfully induced by using media and substrates prepared from cereal grains and host plant leaf under FWL 12:12 and dark condidtion. Whereas, BLA prepared from banana leaf Nangka has comparable ability in term of *Fusarium* macrocondia induction as CLA.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background study

Production of conidia is an act of reproduction in most deuteromycetes and conidia are asexual spore which form at the conidiogenous cell (Alexopoulos *et al*., 2007). Conidia are essential in the survival of fungi under harsh conditions as they are able to withstand stress and germinate when the environment is appropriate (Wyatt *et al*., 2013).

Observation of conidial morphology is crucial step in morphological identification of fungi. Even until today, morphological identification is still play an important role in research. For example, it is useful in the study of plant pathogenicity which involves the observation of cultural characteristic and morphological identification to decide whether Koch's Postulates are fulfilled (Agrios, 2005).

Unfortunately, induction of conidia in fungi can be hard to achieved in laboratory. For instance, some members from the genus *Cercospora* are known to be incapable of producing conidia on commercial culture media and only vegetative growth could be observed (Djebali *et al.*, 2010). Manipulation of media composition and alteration of factors such as pH of culture media, temperature and lighting conditions for incubation are common means of artificial conidia induction (Burgess *et al*., 1994; Winder, 1999; Tyagi & Paudel, 2014).

In this study, filamentous fungi from the genera *Cercospora* and *Fusarium* were selected to study the induction of conidia under laboratory conditions. An isolate

C. citrullina was not able to produce conidia on commercially available potato dextrose agar (PDA). Efforts to induce the conidia production in *C. citrullina* included manipulating the lighting conditions for incubation and culturing the species on different types of agar and substrates. Apart from that, induction of *Fusarium* macroconidia was also carried out by using banana leaf-piece agar (BLA) to find out whether the morphology of the macroconidia would be the same as the macroconidia produced on carnation leaf-piece agar (CLA) which is widely used in the morphological study of *Fusarium*.

1.2 Problem statements

1. To develop suitable culture media and lighting conditions for *C. citrullina* conidia induction.

2. To compare the performance of banana-leaf piece agar (BLA) with carnation leaf-piece agar (CLA) in the induction of *Fusarium* macroconidia.

CHAPTER 2

LITERATURE REVIEW

2.1 The genus *Cercospora*

The genus *Cercospora* Fresen. is known as one of the largest anamorph genus associated with the teleomorph genus *Mycosphaerella* (Crous, 2009). Up to 2003, around 3000 names have been recorded in this genus (Crous and Braun, 2003). The genus comprised of many pathogens which target various economically important crops such as *Sphaerulina oryzina* Hara (= *Cercospora janseana* (Racib.) Constant)

which causes Narrow brown leaf spot in rice (*Oryza sativa* L.), *Cercospora beticola* Sacc. which causes Cercospora leaf spot disease in sugar beet (*Beta vulgaris* L.) and *Cercospora kikuchii* (Tak. Matsumoto & Tomoy) M.W. Gardner which is the causative agent of Cercospora leaf blight in soybeans (*Glycine max* (L.) Merr.) (Setiawan *et al*., 2000; Soares *et al*., 2015; Mani *et al*., 2016). The name of this genus *Cercospora* is derived from two Greek words; *kerkos* which means a tail and *spora* which means a spore. Although not all species from this genus can produce cercosporin, a photoactivated perylenequinone toxin; however, the production of it is considered as one of the key to recognised species from the genus *Cercospora* (Assante *et al*., 1977; Daub and Chung, 2007). According to Solheim (1930), conidiophores of *Cercospora* spp. can either branch in sympodial or monopodial form while the conidia were described as acicular, obclavate, fusiform or cylindrical. A more detailed description was provided by Chupp (1954), in which the shape and form of the conidia base is regarded as more dependable compared to the tip in identifying the *Cercospora* species. The base of conidia is described to be either truncate or obconic in shape while the

side of it can be classified to be straight, concave or convex. In addition, colour of conidiophores was also given much attention by Chupp (1954). According to To-Anun *et al*. (2011), reclassification of cercosporoid fungi was made by Deighton (1987) through the approach of determining the conidial scars at the conidiogenous cell and the hilum at the conidia base at to be thickened or unthickened using light microscope. Thickened conidial scars and hilum were found in the *Cercospora* species while species from the allied genera possess the unthickened type. Revision and reclassification of this complex genus have been continued through many publications. For instance, *Cercospora pittospori* Plakidas which has unthickened and undarkened conidiogenous loci as well as hilium has been shifted to the genus *Pseudocercospora* followed the justification of Braun and Crous (2007). Apart from that, advancement in molecular technique enables the compilation of first phylogenetic results and the revision of the cercosporoid genera (Bakhshi *et al*., 2015).

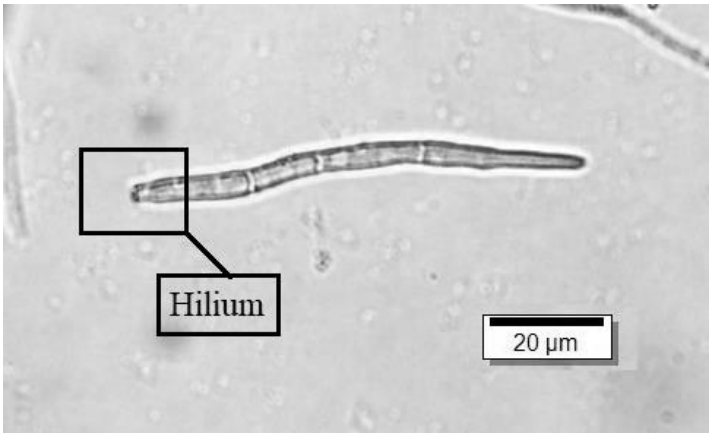

Figure 2.1: Conidium of *Cercospora* sp. (Black box indicates the hilium at the base of conida)

2.2 *Cercospora citrullina* Cooke

Cercospora citullina Cooke is responsible for the Cercospora leaf spot disease in the plants from the family of Cucurbitaceae, including squash, muskmelon,

cucumber and water-melon (Chupp, 2006). Apart from the cucurbits, it was also reported that morning glory (*Ipomoea nil*) is the new host of *C. citrullina* (To-Anun *et al*., 2011). Production of redish coloured toxin, cercosporin is a significant feature of the members in the genus *Cercospora* (Daub and Chung, 2007). This species is also producing the toxin to infect the host plant, bitter gourd (*Momordica charantia* L.) (Donayre and Minguez, 2014). This organism can be found worldwide. Recent findings have reported the presence of this organism in Korea, New Caledonia and some Pacific Islands as well as Pakistan (Braun *et al*., 1999; Mukhtar *et al*., 2013; Hong *et al*., 2014). The morphological particularity is recognized such as conidiophore. The conidiophores of *C. citrullina* are septated with light brown to dark brown colour. They arise from stromata and can either be single or many with fasciculate arrangement (Chupp, 1954; To-Anun *et al*., 2011). To-Anun *et al*. (2011) described that the conidiogenous cells are proliferating in sympodial style while the conidiogenous loci are thickened and darkened. The conidia are multi-septated, hyaline and acicular. The base of the conidia has subtruncate to truncate shape with darkly pigmented hilium (Mukhtar *et al*., 2013; Hong *et al*., 2014).

2.3 Cercospora leaf spot disease

Cercospora leaf spot (CLS) disease is a foliar disease affecting various plants worldwide. For example, *Cercospora armoraciae* Sacc. has been reported as the causal agent of CLS on horseradish (*Armoracia* rusticana P. Gaertn., B. Mey. & Scherb. in Serbia, *Cercospora* cf. *alchemillicola* has been reported as the culprit which causes CLS on *Toona ciliate* M. Roem. in Brazil and *Pseudocercospora fici* (Heald & F.A. Wolf) X.J. Liu & Y.L. Guo (= *Cercospora fici* Heald & F.A. Wolf) (Schubert *et al*.,

1999; Trkulja *et al.*, 2015; Silva *et al.* 2016). CLS disease is signified with the formation of circular spot with dark margins on host foliage. The centre of the spot appears to be white to light brown in colour (Kehinde, 2013). Schlösser (1971) described that spots surrounded with yellowish, discoloured tissues will form at the outer region of the leaf surface and these spots will later fuse together to form a larger necrotic region. The disease brings detrimental effects to the yield and quality of crops thus resulting in the destruction of economy. For instance, CLS disease in sugar beets (*B. vulgaris*) has resulted in the decrease of sugar concentration, higher concentration of impurities as well as decrease in root weight of the plant (Shane and Teng, 1992). Besides that, CLS disease caused by *Mycosphaerella coffeicola* (Cooke) J.A. Stev. & Wellman (= *Cercospora coffeicola* Berk. & Cooke) on Arabica coffee (*Coffea arabica* L.) has led to the defoliation of host plant thus resulted in the reduction of photosynthetic leaf area and ultimately caused crop loss (Nelson, 2008). The disease cycle of CLS is initiated with the germination of conidia. Once the hyphae arrived at the stomata on the host plant leaf, it will differentiate into appressoria to invade into the host followed by the induction of necrotic lesions. Lastly, conidiophores will emerge from the stomata and produce conidia which will be dispersed by wind or water and infecting the nearby host plants (Kim *et al.*, 2011). Production of toxin, cercosporin is known to be important in the pathogenesis of many species from the genus *Cercospora*. Study by Callahan *et al*. (1999) has demonstrated that the disruption of cercosporin facilitator protein (*CFP*) which results in the reduction of cercosprorin production will eventually lead to the reduction of virulence in *C. kikuchii* to the host plant, soybeans. Crop rotation, deep ploughing and fungicide application are some of the methods to handle the disease (Naqvi, 2004). Although the application of fungicide is a popular choice of disease management but resistance against

fungicide has become an issue. Study by Karaoglanidis *et al*. (2002) has consolidated that statement by the finding of some *C. beticola* strains which resist the effect of sterol-demethylation-inhibiting fungicides.

2.4 *In vitro* induction of conidiation in *Cercospora citrullina*

Induction of conidiation is one of the crucial step in many laboratory experiments. However, induction of conidial production of *Cercospora* spp. is often challenging as some of them only grow vegetatively on the standard commercial medium. As reported by Vathakos and Walters (1979), *C. kikuchii* was found to demonstrate vegetative growth on standard media, agar prepared by using the decoctions of carrot leaves and agar made from the tissues of immature and senescent corn, alfalfa, wheat, cotton. As for *C. citrullina*, Goode and Brown (1970) reported that the organism was not able to produce conidia in artificial medium despite the approaches of culturing the organism under different nutritional, temperature and lighting conditions have been implemented. Culture medium is found to be crucial in inducing sporulation. Djebali *et al.* (2010) demonstrated that production of conidia of *Cercospora* sp. was selective on the culture medium. No conidia production was found when four strains of *Cercospora medicaginis* Ellis & Everh. were cultured on PDA, half-strength PDA and *Medicago* leaf juice agar but at least one strain of the species could produce conidia on synthetic nutrient-poor agar, carrot juice agar, *Medicago* crushed seed agar, wheat bran juice agar and V8 juice agar. On the other hand, study of Suto (1985) emphasized the role of lighting condition in induction of sporulation in several *Cercospora* species. The study stated that continuous irradiation from the black-light fluorescent lamp with approximately 310 to 410 nm wavelength was

effective in inducing conidia production in 15 *Cercospora* isolates cultured on potato sucrose agar, while some isolates preferred the cyclical irradiation which exposed the isolated to 12 h of irradiation and 12 h of darkness. However, conidia produced under such conidiation come either directly or singly rather than in tuft of conidiophores as observed on the host plants. Besides that, conidia produced by some of the isolates were found to be longer and more septated than the conidia produced on the host plants. Although, there are not many research about the induction of conidiation specifically on *C. citrullina* has been done but studies of conidia induction in other species of *Cercosprora* can provide some hints about the subject.

In short, conidiation of *Cercospora* spp. is influenced by the culture medium used as well as the lighting condition during the incubation period.

2.5 The genus *Fusarium*

Fusarium is a genus houses many pathogens which are not only harmful to plant but also human and animal. *Fusarium* belongs to the phylum Ascomycota, class Sordariomycetes, order Hypocreales and family Nectriaceae (NCBI, 2017). Teleomorphic stage of *Fusarium* is often associated with genera such as *Gibberella*, *Haematonectria* and *Albonectria* (Leslie and Summerell, 2006). Many members of this genus are known to be able to produce secondary metabolites which are detrimental to the host (Moretti, 2009). Examples of these secondary metabolites include moniliformin, fumonisins, deoxynivalenol, zearalenone and trichothecenes (Nesic *et al*., 2014). Several *Fusarium* spp. are recognized as the causal agents of Fusarium head blight and Fusarium ear rot on cereal grain and maize, respectively, worldwide (Bottalico, 1998). In Malaysia, economically important crops are the victims of *Fusarium* spp. For instance, *F. oxysporum* f. sp. *cubense* caused the

Fusarium wilt, also known as Panama disease in banana and *F. proliferatum* was reported to cause mango malformation in Malaysia (Ploetz and Pegg, 1997; Nor *et al*., 2013).

According to Summerell and Leslie (2011), the genus was first introduced by Link (1809) as *Fusisporium* which comprised of numerous plants and soil pathogens, endophytes and saprobes. Improvisation was made in the identification of *Fusarium* through the publication of Wollenweber and Reinking in 1935 which became the pillar in the establishment of taxonomy and systematics of this genus and sorted out a sub-generic system based on 16 sections, 65 species and 77 subspecific varieties and forms. Studies carried out by Snyder and Hansen (1940) has modified the taxonomy system proposed by Wollenweber and Reinking by reducing 65 *Fusarium* species to 9. They also suggested that only single spore cultures could be considered reliable for species identification. The study of *Fusarium* taxonomy has been continued and numerous studies has been contributed by researchers. For instance, works of Gerlach and Nirenberg (1982) and Nelson *et al*. (1983), both established and improved from the Wollenweber and Reinking's system, have significantly helped in understanding the *Fusarium* taxonomy (Leslie and Summerell, 2008).

Burgess *et al*. (1994) described that, keys include the shape of macroconidia, the formation of microconidia and their shape, the presence of structures such as chlamydospores are useful in the primary identification of *Fusarium* species. The apical cell and the base of the basal cell of *Fusarium* macroconidia are given much attention in the identification process. In addition, colony morphology and growth rates on PDA are deemed useful as secondary criteria for *Fusarium* identification.

2.6 *Fusarium* macroconidia

Members from the genus *Fusarium* produce two types of airbone conidia, namely macroconidia and microconidia, which are distinguished by their respective sizes (Alexopoulos *et al*., 2007). Macroconidia are signified by their crescent-shaped structures and they are multiseptated; whereas microconidia are generally smaller and one-celled with spherical or oval shape (Alexopoulos *et al*., 2007).

In morphological identification of *Fusarium*, shape of macroconidia and microconidia are taken into consideration but greatest weighting are given to the shape of macroconidia (Leslie and Summerall, 2006). Burgess *et al*. (1994) noted that macroconidia produced on aerial hyphae are deemed unsuitable to be used in morphological identification as their shape are often varied. Description of macroconidia shape involves the observation of apical and basal cells (Burgess *et al*., 1994). The apical cells of macroconidia can either be blunt, papillate, hooked or tapering while the basal cells of macroconidia are either in foot-shaped or notched (Leslie and Summerall, 2006). Figure 2.1 shows the macoconidium and microconidium of *Fusarium* sp. The apical and basal cells of macroconidium are also indicated on the figure.

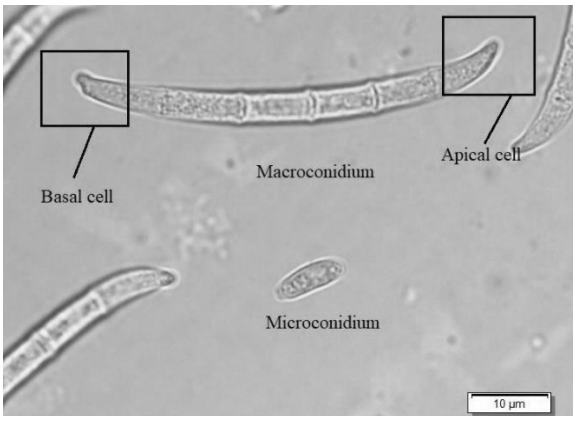

Figure 2.2: Macroconidia and microconidia of *Fusarium* sp. (Apical and basal cells of macroconidium are indicated in black boxes).

2.7 *Fusarium* sporodochium

Sporodochium is a structure which bear closely-packed conidiophores on a cushion-shaped stroma (Alexopoulos *et al*., 2007). Formation of sporodochium is crucial in the morphological study of *Fusarium* as the macroconidia produced on sporodochium are more consistent in terms of shape and size compared to those produced on isolated phialides (Leslie and Summerall, 2006). Under the laboratory condition, sporodochium are more readily formed when cultured on CLA rather than PDA (Leslie and Summerall, 2006). Sporodochia of *Fusarium* could either be found on leaf substrate or on agar surface and depending on the species, sporodochia with various colours such as purple, orange or yellow could be observed (Leslie and Summerall, 2006). Figure 2.2 shows the microscopic observation of *Fusarium* sporodochium.

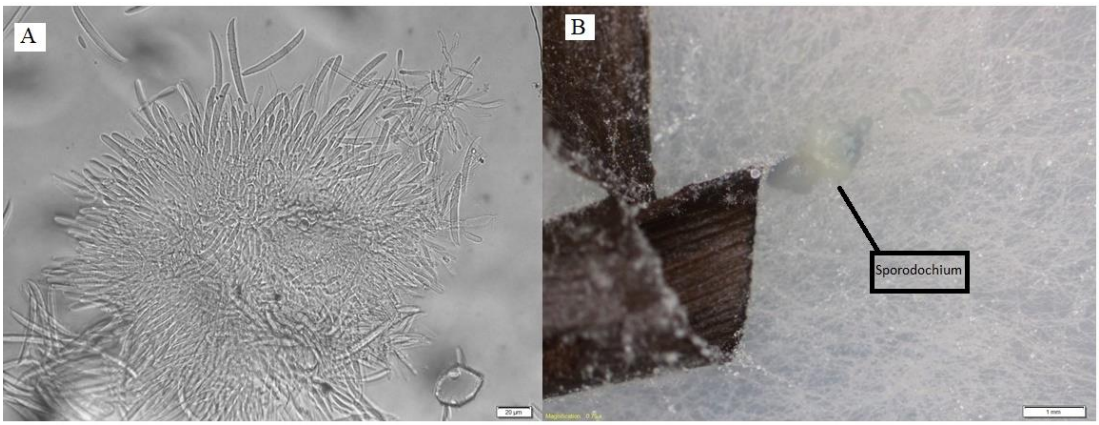

Figure 2.3: Sporodochium of *Fusarium* sp. **A**: Sporodochium of Fusarium sp. observed under light microscope (40 x magnification) **B**: Sporodochium of *Fusarium* sp. observed under Stereomicroscope (1.5 x magnification)

2.8 *In vitro* induction of macroconidia production in *Fusarium* spp.

Shape of macroconidia produced by *Fusarium* spp. is very important in the morphological identification (Burgess *et al*., 1994). Efforts have been contributed to

induced macroconidia production in *Fusarium* in the laboratory. Culture media used to culture *Fusarium* isolates can affect the macroconidia production. In fact, type of substrates seems to be crucial in *Fusarium* macroconidia induction. For instance, it was found that 19 isolates from *Asparagus officinalis* which were identified as *F. moniliforme* could produce abundant macroconidia on sterilized cereal grains while 17 out of 19 isolates could only produce a few macroconidia on cornmeal agar (Goth and Johnston, 1981). Furthermore, a study by Mansour *et al*. (2012) demonstrated that macroconidia formation in *F. graminearum* 3- and 15-acetyldeoxynivalenol (ADON) chemotypes was prominent and uniform when culture on sucrose-water medium. In addition, *F. graminearum* which infects wheat could give 10 to 100-fold more macroconidia on yeast malt extract agar compared to carboxymethyl cellulose liquid media (Rittenour and Harris, 2012).

Apart from nutrient, adequate light exposure is often needed for macroconidia production in *Fusarium*. Burgess *et al*. (1994) mentioned that 12 h of photoperiod was mimicked by using white fluorescent light and black light irradiation for the sporulation of *Fusarium* cultures. Light exposure is affecting not only the production but also morphology of macroconidia. Study by Harter (1939) showed that not only maximum number of macroconidia could be found under illumination but the length of the conidia was much longer under light condition compared to dark condition. Molecular study also supported that exposure of *Fusarium* culture to light does alter the macroconidia formation in *Fusarium*. A study by Castrillo *et al*. (2013) proved that Δ*cryD* mutants of *Fusarium fujikuroi* produced large septated macroconidia, which was not found in the wild-type, under light irradiation and nitrogen-starved condition. The *cryD* gene is photoinduced and it encodes enzymes in the carotenoid pathway of *F. fujikuroi*. Moreover, formation of macroconidia under illumination and nitrogen-

starved conidition suggested that CryD is also a nitrogen-dependent repressor in the microconidia formation. Besides illumination and nutrient condition, pH is another factor which give an impact in the macroconidiation of *Fusarium*. Maximum macroconidia production in *F. oxysporum* was found to be 5.06 spore/ 100 ml of Potato Dextrose Broth (PDB) medium with pH 6.5 as compared to PDB with pH ranging from 2.0 to 7.5 (Tyagi and Paudel, 2014). Temperature for *Fusarium* cultures incubation is another parameter which has been given much attention. Study by Winder (1999) found out that *F. avenaceum* achieved higher macroconidiation under higher daytime temperature, which is 30 °C rather than 20 °C, when cultured under 12 h diurnal photoperiod.

2.9 Morphological identification of filamentous fungi

Identification of fungi using morphological approach is often deemed as the classical or traditional method and it involves direct observation of fungi. Advancement in the molecular analysis technique does provide convenience to researchers in the identification of fungi, however, there are many advantages of morphological identification which makes it still applicable and useful until today.

Variation in the morphology and growth of the fungi happens results from these few factors, such as the inoculum type, content of medium and pH (Pazouki and Panda, 2000).

According to Schmit and Lodge (2005), the morphological identification technique is the only way to distinguish which fungi could reproduce in certain cultural conditions or on a particular type of substrate provided. Besides that, it also mentioned that this method is generally less expensive and it requires less specialized equipment.

Morphological identification of fungi involves the observation of features. For example, study conducted by Cano *et al*. (2004) shown that clinical interest *Collectotrichum* spp. were identified using features such as the presence of sclerotia, shape of appressoria formation as well as shape and size of conidia produced.

Although morphological identification is a rather easy to apply method, however, some species may not be able to grow or unable to demonstrate reproductive structures which are used as key in the morphological identification (Schmit and Lodge, 2005). Thus, such species cannot be identified using the morphological identification and should apply alternative such as molecular technique to identify them.

2.10 Molecular identification of filamentous fungi

Comparative sequencing strategy is commonly applied in the identification of fungi. This method is a polymerase chain reaction, PCR-based method in which a target genomic DNA is amplified and sequenced followed by comparing the sequence obtained against an existing data library for evaluation (Balajee *et al*., 2009). However, which gene regions to be used is appropriate to apply in the identification is now a crucial question. For instance, 5.8S gene and flanking internal transcribed spacers (ITS1 and ITS2) regions of rDNA was sequenced to identify endophytic fungi isolated from *Livistona chiensis* (Chinese Fan Palm) (Guo *et al*., 2000). According to Balajee *et al*. (2009), the nuclear ribosomal internal transcribed spacer (ITS) region can be regarded as universal marker as it gives satisfactory results in the identification of *Aspergillus* and most *Mucorales* species as well as *Fusarium*. The translational elongation factor 1-α (TEF 1-α) gene is also very useful in the molecular identification as it consistently gives single-copy in *Fusarium* besides showing high level

polymorphism in the sequence (Geiser *et al*., 2004). Other than that, γ-actin (*ACT*), DNA topoisomerase I (*TOPI*), phosphoglycerate kinase (*PGK*) genes are the potential candidates to be incorporated in the study (Stielow *et al*., 2015). These target gene regions serve as DNA barcodes for identification and classification. Besides that, incorporation of other loci such as the second largest subunit of the RNA polymerase gene (RPB2), the mitochondrial small subunit (mtSSU) rRNA gene and the calmodulin gene (CAM) in the DNA sequence analysis was conducted by Wang et al., (2011) to identify 20 *Fusarium* spp.

As for the identification of *Cercospora* spp. ITS region is often used. However, it was found that ITS and TEF could not perform well in distinguishing the clades of *Cercospora* spp. and thus it is necessary to include three other genes, they are actin, calmodulin, histone (H3), to give a clearer picture of DNA phylogeny and more accurate species identification (Groenewald *et al*., 2013).

Even though the molecular technique is now playing a crucial part in the identification process of fungi, but it is still incompetent in terms of pathogenic and non-pathogenic strains differentiation. In this case, researchers can only rely on the pathogenicity test to solve the problem (Saikia and Kadoo, 2010).

2.11 Taxonomy of carnation plant

The carnation, or scientifically known as *Dianthus caryophyllus* L. is native in southern Europe and can be found abundantly in Normandy, France (Ward, 2008). OGTR (2006) stated that *D. caryophyllus* L. can be found in the wild in some Mediterranean countries as its natural distribution is restricted to that region.

The name of the genus, *Dianthus* is originated from Greek which carries the meaning of divine flower while the common name of the flower is also originated from Greek word Cornotion because the flower is used to adorn the crowns of Greek athletes (Sheela, 2008). Other common names of the species include Gilly flower and Pinks; while in Malay, it is called Bunga Teluki (Lim, 2012). It is placed under the phylum Streptophyta, subphylum Streptophytina, order Caryophyllales, family Caryophyllaceae, tribe Caryophylleae and genus *Dianthus* (NCBI, 2017). As the second largest genus under Caryophyllaceae, *Dianthus* comprised of about 300 species including *Dianthus barbatus* and *Dianthus chinensis*. It is also considered to be most diverse in southwestern Asia as well as southeastern Europe (APG III, 2009).

D. caryophyllus L. has a chromosome number of 2n = 30 and it is known that economically important varieties are those triploids carnations (Sato *et al*., 2000; OGTR, 2006). Efforts have been contributed by breeders and researchers in breeding carnations with better quality and high disease resistance and hence many cultivars have been bred. Study conducted by Maitra and Roychowdhury (2013) compared 10 cultivars of carnation such as Dark Red, Yellow with Red, C. Rimo, Orange Isac and so on in terms of criteria like the earliest flower bud initiation, *in situ* longevity of flowers, ability to produce highest number of flower and vase-life longevity to select cultivar with better quality. Apart from that, study has shown that carnation cultivar named Novada generate secondary metabolites, flavonoids known as kaempferide triglycoside could have contributed to the resistance of cultivar Novada against the infection of *Fusarium oxysporum* f. sp. *dianthi* (Curir *et al*., 2003). Galeotti *et al*. (2008) demonstrated chemical structure of another flavonoid glycoside and demonstrated that it could exhibit antifungal effect against *F. oxysporum* f. sp. *dianthi*.

2.12 Taxonomy of banana plant

Banana is a monocotyledon plant placed in the genus *Musa* which is come from the family Musaceae, houses other genera such as *Musella* and *Ensete* (Perrier *et al*., 2011). The family Musaceae is placed under the order Zingiberales, class Liliopsida, phylum Streptophyta (NCBI, 2017). Originally, banana plants were seed-bearing, inedible, diploid plants found in the natural forest in South-east Asia and western Pacific regions while edible banana was produced because of intraspecific hybridization between various diploid subspecies of *Musa acuminata* Colla (Robinson and Saúco, 2010). Valmayor *et al*. (1999) illustrated the development of edible banana in a rather detailed way by stating that the hybridization of *Musa balbisiana* and *M. acuminata*, both wild banana with pure A and pure B diploid orientation has produced *Musa x patadisiaca*, which is AB diploid. Further hybridization resulted in triploid as well as tetraploid edible banana. Generally, the current varieties of banana are seedless triploid cultivars which either consisted of genome A solely such as *M. acuminata* (genome AAA) or both genomes A and B such as *M. acuminata* and *M. balbisiana* (genome AAB and ABB respectively) (Jain and Privadarshan, 2009).

Banana is known as Pisang in Malay and the popular cultivars of banana in Malaysia includes Pisang Mas (diploid AA genome); Pisang Nangka, Pisang Raja and Pisang Rastali (triploid AAB genome); Pisang Awak, Pisang Abu and Pisang Nipah (triploid ABB) (Molina *et al*., 2004).

CHAPTER 3

EFFECTS OF CULTURE MEDIUM AND LIGHTING CONDITION ON INDUCTION OF CONIDIATION IN *Cercospora citrullina*

3.1 Introduction

Cercospora citrullina Cooke causes disease known as Cercospora leaf spot (CLS) disease in plants from the family Cucurbitaceae, such as watermelon, cucumber and squash (Chupp, 2006). The disease is targeting the foliage of the plant by forming circular spots with darkly pigmented margin together with light coloured centre (Kehinde, 2013). Production of cercosporin, a redish coloured photoactivated perylenequinone toxin is found to be important in the pathogenesis. Photoactivated cercosporin can generate activated oxygen species which causes peroxidation of the host cell membrane thus destructs the host cell (Daub and Ehrenshaft 2000). The infection of this pathogen will eventually lead to defoliation of host plant and resulted in the decrease in yield (Nelson, 2008).

The study was first started with the isolation of *C. citrullina* from the pumpkin leaves with CLS disease symptoms. To ensure that *C. citrullina* was truly the culprit for the disease lesions found on the pumpkin leaves, pathogenicity was carried out on pumpkin plants (*Cucurbita maxima* Duch.). However, inoculation with conidia or mycelium on leaf was quite difficult in *C. citrullina* and re-isolation was prevented by other fungi due to the slow growth of this species. Therefore, fulfilling Koch's postulates is hard to achieve.

Apart from that, *C. citrullina* is known to produce acicular, hyaline and multi-septated conidia arisen from the conidigenous cells which are proliferating in sympodial style. The base of the conidia has darkly pigmented hilium (To-Anun et al., 2011; Mukhtar et al., 2013; Hong et al., 2014). Production of conidia is often observed on host plant, whereas some *Cercospora* species only demonstrate vegetative growth on standard commercial medium. *C. citrullina* was found to be not able to produce conidia in artificial medium although parameters such as nutrients, temperature and lighting conditions have been adjusted (Goode and Brown 1970). Preliminary study showed that the isolated *C. citrullina* was not able to exhibit any conidia production when culture on PDA. The morphology of *C. citrullina* conidia is important in the preliminary identification and the isolation of the pathogen during the pathogenicity test. Therefore, suitable media and culture condition need to be composed for the successful induction of conidia in *C. citrullina*.

3.1.1 Chapter objectives

1. To develop a technique which allows the inoculation and re-isolation of *C. citrullina* with lower contamination and higher successful re-isolation rate for the study of *C. citrullina*.

2. To determine the effective culture media and lighting condition for conidia production of *C. citrullina.*

3.2 Materials and Methods

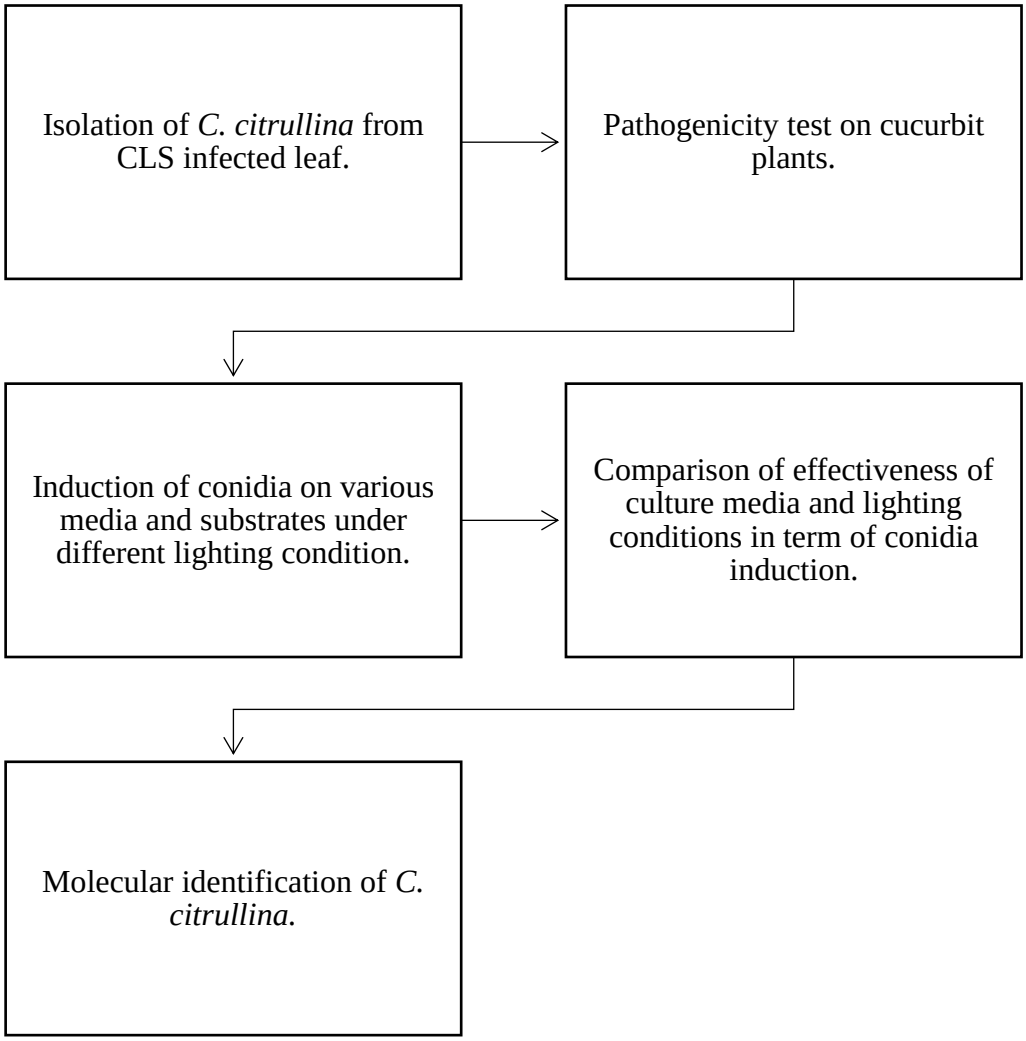

Figure 3.1: Flow chart for the works conducted in chapter three "Effects of Culture Media and Lighting Condition on Induction of Conidiation in *Cercospora citrullina*".

3.2.1 Media and substrates preparation

3.2.1(a) Potato dextrose agar (PDA)

Thirty-nine grams of potato dextrose agar (Oxoid CM0139, Basingstoke Hants, UK) was suspended in 1000 ml of distilled water. The medium was heated and stirred to dissolve the powder completely.

3.2.1(b) Oatmeal agar (OA)

Seventy-two grams of oatmeal agar (BD Difco™ Oatmeal Agar, New Jersey, USA) was measured and suspended onto 1000 ml distilled water. The mixture was heated and stirred until the agar was evenly distributed in the distilled water.

3.2.1(c) Oat grain agar (OGA)

Thirty grams of oat grains was weighted and added into a flask. Five hundred millilitres of distilled water was added into the flask. The flask was sealed and boiled at 100 °C for 45 minutes. The oat grains were filtered out and discarded. The broth was retained in a flask and distilled water was added to the broth until 1000 ml of broth was obtained. Eighteen g of agar (Oxoid LP0011, Basingstoke Hants, UK) was added into the flask containing the 1000 ml of broth. The method was modified from Djebali *et al.* (2010).

3.2.1(d) Wheat grain agar (WGA)

Thirty grams of wheat grains was weighted and added into a flask. Five hundred millilitres of distilled water was added into the flask. The flask was sealed and boiled at 100 °C for 45 minutes. The wheat grains were filtered out and discarded.

The broth was retained in a flask and distilled water was added to the broth until 1000 ml of broth was obtained. Eighteen grams of agar (Oxoid LP0011, Basingstoke Hants, UK) was added into the flask containing the 1000 ml of broth. The method was modified from Djebali *et al.* (2010).

3.2.1(e) Barley grain agar (BGA)

Thirty grams of barley grains was weighted and added into a flask. Five hundred millilitres of distilled water was added into the flask. The flask was sealed and boiled at 100 °C for 45 minutes. The barley grains were filtered out and discarded. The broth was retained in a flask and distilled water was added to the broth until 1000 ml of broth was obtained. Eighteen grams of agar (Oxoid LP0011, Basingstoke Hants, UK) was added into the flask containing the 1000 ml of broth. The method was modified from Djebali *et al.* (2010).

3.2.1(f) Cornmeal agar (CA)

Seventeen grams of cornmeal agar powder (Oxoid CM103, Basingstoke Hants, UK) was suspended in 1000 ml of distilled water. The medium was heated and stirred to dissolve the powder completely.

3.2.1(g) Oat grain substrate (OG)

Three grams of oat grains were measured and put into a universal bottle. Six millilitres of distilled water was added into the universal bottle containing the oat grain. This method was modified from Chand et al. (2013).

3.2.1(h) Wheat grain substrate (WG)

Three grams of wheat grains were measured and put into a universal bottle. Six millilitres of distilled water was added into the universal bottle containing the wheat grain. This method was modified from Chand et al. (2013).

3.2.1(i) Barley grain substrate (BG)

Three grams of barley grains was measured and put into a universal bottle. Six millilitres of distilled water was added into the universal bottle containing the barley grain. This method was modified from Chand et al. (2013).

3.2.1(j) *Trichosanthes* sp. dried leaves

Trichosanthes sp. (cucurbitaceae) leaves were collected in Universiti Sains Malaysia. The leaves were soaked in tap water overnight before cutting the leaves into small pieces of approximately 10 × 10 mm in size. The cut leaves were air dried for about 6 to 8 h under room temperature before being transferred into a 50°C incubator for 2 h. The leaves were then taken out form the incubator and air dried overnight to ensure that the leaves were completely dried.

Trichosanthes sp. dried leaves were measured to obtain 0.3 g of leaves in a universal bottle. Six-tenths millilitre of distilled water was added into the universal bottle containing *Trichosanthes* sp. dried leaves. This method was modified from Chand et al. (2013).

Culture media and substrates were sterilized by autoclaving at 121 °C for 20 minutes.

3.2.2 Isolation of *C. citrullina*

C. citrulllina was isolated from the pumpkin (*Cucurbita maxima* Duch.) leaves with CLS disease symptoms. The pumpkin leaves with CLS disease symptoms was collected from Kepala Batas, Penang, Malaysia. Single spore isolation was carried out to isolate pure culture of *C. citrullina*. The isolated pure culture of *C. citrullina* was maintained in PDA. The colony morphology, conidia and conidiophores formation of *C. citullina* were observed on OA.

3.2.3 Greenhouse inoculation of *C. citrullina* to the cucurbit plant

Three different pathogenicity tests were carried out in a greenhouse at School of Biological Sciences, USM. Only pumpkin (*Cucurbita maxima* Duch.) (Soon Huat Seeds Co. Sdn. Bhd.) plant was examined for two pathogenicity tests.

3.2.3(a) Spraying of *C. citrullina* conidial suspension

Inoculation was carry out by spraying conidial suspension (10^8 conidia /ml) prepared by using sterile 0.01% Tween™ 20 (ACROS Organics™, Thermo Scientific, MA, USA) on 2 pumpkin plants. Control was prepared by spraying sterile 0.01% Tween™ 20 onto a pumpkin plant. Both inoculated and control plants were covered in plastic bags for 2 days. The experiment was repeated twice.

3.2.3(b) Placing of *C. citrullina* agar block on leaf surface