



THESIS

IDENTIFICATION OF PATHOTYPES OF *COLLETOTRICHUM CAPSICI* CAUSING CHILLI ANTHRACNOSE IN THAILAND

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**GRADUATE SCHOOL, KASETSART UNIVERSITY
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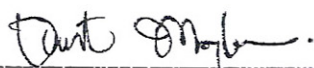
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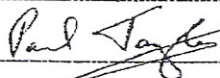
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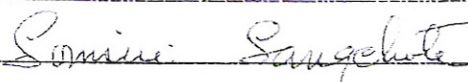
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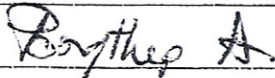
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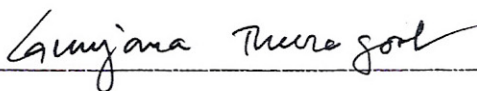


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THESIS

**IDENTIFICATION OF PATHOTYPES OF
COLLETOTRICHUM CAPSICI CAUSING CHILLI ANTHRACNOSE
IN THAILAND**

PAWEENA MONTRI

**A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Agricultural Biotechnology)
Graduate School, Kasetsart University
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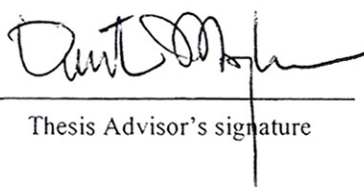
Paweena Montri 2008: Identification of Pathotypes of *Colletotrichum capsici* Causing Chilli Anthracnose in Thailand. Master of Science (Agriculture Biotechnology), Major Field: Agriculture Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Orarat Mongkolporn, Ph.D. 74 pages.

Genetic diversity of 45 *C. capsici* isolates in Thailand was studied based on colony growth rate, surface mycelium, colony color, mass conidial color, conidial and appressoria size in relation to three different levels of location including within farm, within districts in western area, and within Thailand. Cluster analysis based on colony growth rate, surface mycelium, colony colors, and mass conidial colors divided the 45 isolates into 16 groups. However when the data was combined with conidia and appressoria data, the isolates were not be able to group indicating a large genetic diversity based on variation of conidia and appressoria size. Based on the clusters of the isolates, there was no correlation to geographic location.

Eleven isolates of *C. capsici* were inoculated on nine chilli genotypes derived from four cultivated species of *Capsicum*: *C. annuum*, *C. baccatum*, *C. chinense* and *C. frutescens* using microinjection on red fruit, the host reaction was assessed nine days after inoculation. A set of disease scales were developed based on % lesion size in relation to fruit size; and assigned disease scores from 0 to 9. Three pathotypes, PCC1, PCC2 and PCC3 were identified according to differential qualitative infection of the fruit of the *C. chinense* genotypes 'PBC932' and 'C04714'. PCC1 was the most virulent pathotype that infected all genotypes whereas; PCC3 was the least virulent pathotype that infected only the genotypes of *C. annuum* and *C. frutescens*. Quantitative infection occurred in all chilli genotypes except for genotypes of *C. baccatum* where no infection occurred, thus demonstrating various levels of aggressiveness of isolates within pathotypes.

Paweena Montri

Student's signature



Thesis Advisor's signature

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IDENTIFICATION OF PATHOTYPES OF *COLLETOTRICHUM CAPSICI* CAUSING CHILLI ANTHRACNOSE IN THAILAND

INTRODUCTION

Anthracnose caused by *Colletotrichum* spp. is a serious problem for chilli production in the tropics and subtropics worldwide. *Colletotrichum* also causes anthracnose in a wide range of hosts including cereals, legumes, vegetables, perennial crops and tree fruits (Bailey *et al.*, 1992). Chilli anthracnose is mainly a problem on mature fruits causing severe losses due to both pre- and post-harvested fruit decay (Hadden and Black 1989). In Thailand chilli anthracnose can cause fruit yield losses greater than 50 up to 100% (Pakdeeveraporn *et al.*, 2005). Typical anthracnose symptoms on chilli fruit include sunken necrotic tissues, with concentric rings of acervuli.

At least three species of *Colletotrichum* including *C. capsici*, *C. acutatum* and *C. gloeosporioides* have been identified as the most important pathogen causing chilli anthracnose in Thailand (Than *et al.*, 2008). In western Thailand, *C. capsici*, and *C. gloeosporioides* have been reported to be the most important pathogens causing anthracnose of chilli however *C. capsici* was predominant and appeared to be virulent more than *C. gloeosporioides*. This study was aimed to investigate genetic diversity of *C. capsici* in Thailand based on morphological characters in relation to different geographic location. The outcomes from the study would provide a better understanding of the causal agent, would benefit the chilli breeding program.

Control of chilli anthracnose has been through the use of fungicides however, fungicide applications are only partially effective under environmental conditions that are favorable for pathogen infection. Furthermore, fungicides are not sustainable especially in small holder farming systems in Thailand due to the cost and safety to the environment. Breeding for resistance has mainly focused on introgression of resistance from the related *Capsicum* species: *C. chinense* and *C. baccatum*, into elite susceptible genotypes of *C. annuum* (Pakdeeveraporn *et al.*, 2005). For breeding programs to successfully develop resistant genotypes knowledge of the variability and pathotype structure within the pathogen population is important.

Sharma *et al.* (2005) reported the existence of 15 pathotypes of *C. capsici* from the Himachal Pradesh area of northern India based on quantitative differences in lesion development on inoculated fruit of *C. annuum* genotypes. Than *et al.* (2008) showed pathotype differences within *C. acutatum* isolates from infected strawberry and chilli fruit. Isolates from chilli were able to infect inoculated fruit of the resistant *C. chinense* genotype 'PBC932', whereas isolates from strawberry were unable to infect this genotype. Both isolates were able to infect the susceptible *C. annuum* chilli genotype 'Bangchang'.

The objectives of the present study therefore were to study morphological diversity of *C. capsici* in relation to three different geographical location in Thailand, to develop a set of disease scales for efficient, accurate measurement of the severity of infection in a range of *Capsicum* species; and to identify pathotypes of *C. capsici* based on qualitative differences of infection in differential genotypes of *Capsicum* spp.

OBJECTIVES

1. To study morphological diversity of *C. capsici* in Thailand at three levels including within farm, within western region and within country.
2. To identify pathotypes of *C. capsici* in Thailand using differences in qualitative levels of infection on nine *Capsicum* spp. genotypes.

LITERATURE REVIEW

1. Chilli anthracnose

Anthracnose caused by *Colletotrichum* spp. is an important disease of Chilli (*Capsicum annuum* L.) and many kinds of plants in tropical areas. It occurs as a pre-harvest or post-harvest fruit rot, causing extensive losses in chilli pepper grown during the warm, wet season in tropical and subtropical climates (AVRDC, 1998). Anthracnose attacks on a wide range of plants including cereals, legumes, grasses, vegetable, and fruit plants. The pathogenic fungi *C. capsici* E. J Butler & Bisby and *C.gloeosporioides* (Penz) Penz. & Sacc. in Penz, were reported as chilli anthracnose in Thailand.(Sangchote *et al.*, 1998; Sangchote, 1999). They are also the main causal in the tropical Asia (Manandhar *et al.*, 1995; Kim *et al.*, 1989). Apart from these species, *C. graminicola*, and *C. atramentarium* had been reported in India (Verma, 1973), and *C. cocodes* in Florida (Roberts *et al*, 2001). *Colletotrichum* spp. is capable of causing disease on many of the chilli plant parts during any stages of growth. However, fruit lesions are the most economically important aspect of this disease. *C. capsici* generally infected ripe red fruit, while *C. gloeosporioides* infected both green and ripe fruits (Kim *et al.*, 1989; Sangchote *et al.*, 1998). Concentric rings of the acervuli within the fruit spots are common symptoms. In some cases, the lesions are brown, not orange, and then turn black from the formation of setae and sclerotia (Roberts *et al.*, 2001).

2. Causal agents of chilli anthracnose

In *Colletotrichum* pathosystem, different *Colletotrichum* species can be associated with anthracnose of the same host (Simmonds, 1965; Freeman *et al.*, 1998; Cannon *et al.*, 2000). *Colletotrichum* species causing anthracnose of chilli have been reported from different countries.

Although these species have been the subject of numerous investigations, there remain many gaps in the knowledge of the disease process and understanding of the complex relationships between the species involved. Kim *et al.*, (2004) reported that different species cause disease of different organs of the chilli plant; for example, *C.*

acutatum and *C. gloeosporioides* infect chilli fruits at all developmental stages, but usually not the leaves or stems, which are mostly damaged by *C. coccodes* and *C. dematium*. Leaf anthracnose of chilli seedlings caused by *C. coccodes* was first reported in chilli growing in a field in Chungnam Province of Korea in 1988 (Hong & Hwang, 1998). Different *Colletotrichum* species may also play important roles in different disease of mature stages of chilli fruit as well. For example, *C. capsici* is widespread in red chilli fruits whereas *C. acutatum* and *C. gloeosporioides* have been reported to be more prevalent on both young and mature green fruits (Kim *et al.*, 1999; Hong & Hang, 1998). Anthracnose caused by *C. coccodes* does not result in the severe epidemic in chilli fruits (Hong & Hwang, 1998). Within a species, *C. gloeosporioides*, which was previously the predominant species in Korea, Park *et al.* (1987) successfully differentiated it into G strain and R strain by isozyme pattern of esterase, leucine amino peptidase, phosphatase and glutamine oxalocetic trasminase.

Colletotrichum species can survive in and on seeds as acervuli and micro-sclerotia (Pernezny *et al.*, 2003). Survival of mycelia and stomata in colonized chilli seeds had been reported (Mananadhar *et al.*, 1995b). It has been shown that the pathogen readily colonizes the seed coat and peripheral layers of endosperm even in moderately colonized seeds. Heavily colonized seeds had abundant inter- and intra-cellular mycelium and acervuli in the seed coat endosperm and embryo, showing disintegration of parenchymatous layers of the seed coat and depletion of food material in endosperm and embryo (Chitkara *et al.*, 1990).

Fungi can overwinter on alternative hosts such as other solanaceous or legume crops, or plant debris and rotten fruits in the field. Micro-sclerotia are naturally produced by *Colletotrichum* species to allow the fungus to lie dormant in the soil during the winter or under stressed conditions and they can survive for many years (Ping *et al.*, 1995). During warm and wet periods, conidia from acervuli and micro-sclerotia are splashed by rain or irrigation water from diseased to healthy fruit and foliage. Diseased fruit acts as a source of inoculum, allowing the disease to spread from plant to plant within the field.

The initial infection processes of *Colletotrichum* species involve the attachment of conidia to plant surfaces, germination of conidia, production of adhesive appressoria, penetration of plant epidermis, growth and colonization of plant tissue and production of

acervuli and sporulation (Bailey *et al.*, 1992, Prusky *et al.*, 2000). Anthracnose is mainly a problem on mature fruits, causing severe losses due to both pre-and post-harvest fruit decay (Hadden and Black, 1989; Bosland and Votava, 2003) exhibiting the phenomenon of quiescence in which symptoms do not develop until the fruit ripens (Jeffries *et al.*, 1990). Appressoria that formed on immature fruits may remain quiescent until the fruits mature or ripen.

3. Disease cycle and epidemiology of anthracnose caused by *Colletotrichum* spp.

Environmental factors play a major role in the development of disease epidemics. The relation between rainfall intensity, duration and crop geometry and the dispersal of inoculum possibly lead to different levels of disease severity (Dodd *et al.*, 1992). The effects of temperature often interact with other factors such as leaf surface wetness, humidity, light or competitive microorganisms (Royle and Butler, 1986). The duration of the surface wetness, however, appear to have the most direct influence on the germination, infection and growth of the pathogen on the host. Generally infection occurs during warm, wet weather. Temperature around (27° C) and high humidity (a mean of 80%) are optimum for anthracnose disease development (Robert *et al.*, 2001).

Species of *Colletotrichum* use diverse strategies for invading host tissues, intracellular hemibiotrophy to subcuticular and necrotrophy (Bailey *et al.* 1992). *Colletotrichum* develops a series of specialized infection structures, including germ tubes, appressoria, intracellular hyphae, and secondary necrotrophic hyphae (Perfect *et al.*, 1999). Anthracnose pathogens infect plants by either colonizing subcuticular tissues intramurally or being established intracellularly. The preinfection stages of both are very similar; conidia adhere to and germinate on the plant surface, producing germ tubes that form appressoria, which penetrate the cuticle directly (Bailey *et al.*, 1992). Following penetration, the pathogens that colonize the intramural region beneath the cuticle invade in a necrotrophic manner and spread rapidly throughout the tissues (O'Connell *et al.*, 1985). There is no detectable biotrophic stage in this form of parasitism. By contrast, most anthracnose pathogens exhibit a biotrophic infection strategy initially by colonizing between the plasmalemma and cell wall intracellularly. After the biotrophic state, which varies in duration, intracellular hyphae colonize one or two cells and subsequently develop secondary necrotrophic hyphae (Bailey *et al.*, 1992). Therefore these pathogens are

considered hemibiotrophs or facultative biotrophs. For example, *C. gloeosporioides* infects avocado, chilli and citrus via both types of colonization, producing intracellular biotrophic hyphae in the early stages and forming intramural necrotrophic hyphae at the same time or soon afterwards (O'Connell *et al.*, 2000).

Although prepenetration events of *Colletotrichum* species appear similar, there are differences between species in the mechanisms of spore adhesion and the relative importance of melanization and cutinase for penetration of the plant cuticle by the appressoria. For example, the host-pathogen interaction of *C. acutatum* appears to be more biotrophic than that of some other species such as *C. gloeosporioides* (Wharton & Diéguez-Uribeondo, 2004). Based on studies with *C. acutatum* on specific hosts, four types of interactions or infection strategies were described by Peres *et al.*, (2005):

(1) Biotrophic growth of *C. acutatum* with secondary conidiation in which conidia germinate to form appressoria and quiescent infections, and secondary conidia are formed after germination of the appressoria (e.g., predominantly biotrophic disease cycle on citrus leaves).

(2) Subcuticular intramural necrotrophy with hyphal development within periclinal and anticlinal walls of epidermal host cells which are swollen and wider apart (e.g., predominantly necrotrophic disease cycle on strawberry).

(3) Hemibiotrophic interaction with infection vesicle and broad primary hyphae within host cells. Inter- and intracellular hyphal growth could be seen as the subsequent necrotrophic phase (e.g., combination of bio- and necrotrophy but mostly biotrophic disease cycle on blueberry fruits).

(4) Hemibiotrophic and subcuticular, intra- and intercellular development of *C. acutatum* (e.g., combination of bio- and necrotrophy but mostly necrotrophic disease cycle on almond leaves and fruits).

However, on chilli there are only a few detailed studies on penetration and colonization by *Colletotrichum* species. Kim *et al.* (2004) noticed that there was no biotrophic infection vesicle found during the infection process of *C. gloeosporioides* in

susceptible chilli (*C. annuum* cv. Jejujaerae), However, they found that epidermal cytoplasm were condensed and small vacuoles increased and cell destruction extended to the subepidermal cells, which are likely to be damaged by pathogen enzymes. At the later stage of infection, tissues were colonized inter- and intracellularly by the fungus. They concluded that this structural feature indicated that the infection was governed by necrotrophic fungal growth.

4. Identification of *Colletotrichum* species

4.1. Morphology identification

Identification of *Colletotrichum* species and knowledge of populations responsible for epidemics are vital for developing and implementing effective disease control strategies (Freeman *et al.*, 1998). Traditionally, identification and characterization of *Colletotrichum* species has been based on morphological characters, such as size and shape of conidia and appressoria; existence of setae; the teleomorph state and cultural characters such as colony colour, growth rate and texture (von Arx, 1957; Smith and Black, 1990). These criteria alone however, are not always adequate for species identification due to overlap in morphological characters and phenotypic variation among species under different environmental conditions. Conidial shape provides a reliable means of discriminating certain species and has been used to elucidate the identity of *Colletotrichum* species pathogenic to strawberry (Denoyes and Baudry, 1994). However, in other cases, identification can be complicated because of overlapping ranges of conidial morphology and variation in colony characteristics (Adaskaveg and Hartin, 1997). Correct taxonomic identification is important in disease management such as choosing appropriate fungicides (Whitelaw-Weckert *et al.*, 2007). For example, unlike *C. gloeosporioides*, *C. acutatum* is generally moderately resistant to benzimidazole-based fungicides (Peres *et al.*, 2004).

4.2. Molecular genetics approach

To overcome the inadequacies of traditional morphology based identification schemes, DNA sequence analyses have been used to characterise and analyse the taxonomic complexity of *Colletotrichum* (Sreenivasaprasad *et al.*, 1996; Moriwaki *et al.*, 2002; Photita *et al.*, 2005; Du *et al.*, 2005). Cannon *et al.* (2000) stated that data derived

from nucleic acid analyses should provide the most reliable framework to build a classification of *Colletotrichum*, as DNA characters were not directly influenced by environmental factors. Most fungal phylogenetic studies utilised sequences from the ribosomal gene cluster, since they were present in large numbers as tandem repeats and evolved as a single unit. In particular, sequence analysis of the internal transcribed spacer (ITS) regions, which lie between the 18S and 5.8S and, 5.8S and 28S genes, have proved useful in studying phylogenetic relationships of species of *Colletotrichum* because of their comparative variability (Sreenivasaprasad *et al.*, 1994, 1996; Moriwaki *et al.*, 2002; Photita *et al.*, 2005). Apart from rDNA, sequence analysis of protein coding genes such as partial β -tubulin gene, have also been applied to resolve phylogenetic relationships among *C. acutatum* species complexes (Sreenivasaprasad and Talhinhas, 2005). Sequences of introns from two genes (glutamine synthase and glyceraldehyde-3-phosphate dehydrogenase) were also used to evaluate a diverse collection of isolates of *C. acutatum* (Guerber *et al.*, 2003). Yun *et al.* (1999) stated that because of the high intra-species variability and the low inter-species variability, *MAT1*-2 mating type sequences gave strong support for branches, allowing differentiation of closely related *Cochliobolus* spp. whose relationships were not resolved by ITS sequences alone. Consequently, Du *et al.* (2005) confirmed that *MAT1*-2 mating type was useful in differentiating the groups of isolates from the species complexes (*C. graminicola*, *C. gloeosporioides* and *C. acutatum*). Recently, Ratanacherdchai *et al.* (2007) could separate two species of *Colletotrichum* from chilli anthracnose observed in Thailand that is *C. capsici* and *C. gloeosporioides* by RAPD makers.

A combined application of molecular diagnostic tools along with traditional morphological characterization is an appropriate and reliable approach for studying *Colletotrichum* species complexes (Cannon *et al.*, 2000). Than *et al.* (2007) differentiated isolates of chilli anthracnose from Thailand into three species viz: *C. acutatum*, *C. capsici* and *C. gloeosporioides* based on morphological characterization, sequencing based on rDNA- ITS region and beta tubulin gene and pathogenicity testing. Hong and Kim (2007) reported that from analysis of the sequences in partial beta-tubulin 2 (exons 3-6) Korea isolates were phylogenically separated from the global groups of *C. acutatum* A1 to A8. Restriction fragment length polymorphisms (RFLP) of ITS region resulting from AluI, RsaI and BamHI digestion have also been employed to differentiate *Colletotrichum* species from chilli anathracnose in Taiwan (Sheu *et al.*, 2007). Four species of *Colletotrichum*

were identified by ITS-RFLP fingerprinting and observation of undistinguishable isolates of *Colletotrichum* from their study indicated the various inter- and intra-species variations in *Colletotrichum* species.

5. Pathogenic variability of *Colletotrichum* species

When anyone of the progeny exhibits a characteristic that is different from those present in the ancestry individuals or descent individuals, this individual is called a variant. Although the variant may vary from the ancestors and the other progeny in more than one characteristic, the simplest case is that in which one change may appear (Agrios, 2005). This may involve a change in any conceivable biological characteristic, such as colour, shape, rate of growth and rate of reproduction. In the case of pathogens, changes in host range may occur, *i.e.*, it may be able to infect a variety of the host plant or cultivar not previously infected by the ancestor, or in virulence, *i.e.*, it may produce a milder or the much more severe disease than the ancestors (Agrios, 2005). Some pathogen populations are known to be pathogenicity diverse, and that diversity seems to be due to continuous generation of novel pathogenic variations (Taylor and Ford, 2007).

Genetic population structure refers to the amount and distribution of genetic variation within and between populations (Abang, 2003). The genetic structure of plant pathogen populations, the potential for gene flow and long distance dispersal, and the relative contribution of sexual and asexual reproduction have direct implications for agricultural ecosystems (McDonald and McDermott, 1993; McDonald, 1997). For example, the amount of genetic variation maintained within a population indicates how rapidly a pathogen can evolve, and this information may eventually be used to predict how long a control measure is likely to be effective (McDonald *et al.*, 2002).

Pathogens with large genetic variations are likely to adapt faster to fluctuating environments (e.g. resistant genes and fungicides). Quantifying the impact of the different evolutionary forces that shape the fungus pathogen populations can provide a risk assessment framework for predicting their evolutionary potential (Abang, 2003).

In general, organisms reproduce by means of a sexual process. Variation in progeny is introduced primarily through segregation and recombination of genes in meiotic division of the zygote (Agrios, 2005). However in the case of anamorphic fungi such as *Colletotrichum*, reproduction is mainly or exclusively vegetative (Katan, 2000). Parasexual reproductions, by which a system of genetic recombination can occur within fungal heterokaryon, will likely lead to variation. The new variants come into existence, which may be identical in appearance to that of the ancestral types, but behave differently as far as disease production is concerned.

The appearance of the new pathogen variants is made even more dramatic when the change involves the host range of the pathogens. If the change in the variant pathogen enables it to infect a plant variety cultivated because of its resistance to the ancestral strains, the variant individual grows and multiplies on the new variety without any competition, soon after that they produce large populations that spread and destroy the resistant variety. This is the way that resistance of a plant variety is said to be 'broken down' (Agrios, 2005). The spread of a resistant genotype capable of escaping a current prevalent pathogen will be challenged by a new parasitic strain that harbors a virulent gene which is capable of overcoming that resistance (McDonald *et al.*, 2002). Similarly, a host with a new resistant gene, possibly at another locus, will be able to restore resistance against the same pathogen (Agrios, 2005). On account of this, compatibility of plant-pathogen interaction is often governed by the gene-for-gene model in many pathosystems (Flor, 1971). This suggests a continuous co-evolutionary change in both host and parasite. Information on pathogen diversity and the geographic distribution of the pathogen population is therefore a prerequisite for accurate assessment of durable resistant germplasm in breeding programs.

6. Disease management of chilli anthracnose

Bailey (1987) recommended integrated management techniques, as no single specific management program could not eliminate the chilli anthracnose. Effective control of *Colletotrichum* diseases usually involves the use of a combination of cultural control, biological control, chemical control and intrinsic resistance (Wharton and Diéguez-Uribeondo, 2004).

Cultural practices: Pathogen-free chilli seed should be planted and weeds eliminated. Crops should be rotated every 2-3 years to crops that are not alternative hosts of *Colletotrichum*. Transplants should be kept clean by controlling weeds and solanaceous volunteers around the transplant houses. The field should have good drainage and be free from infected plant debris. If disease was previously present, crops should be rotated away from solanaceous plants for at least 2 years (Robert *et al.*, 2001). Sanitation practices in the field include control of weeds and volunteer chilli plants. Choosing cultivars that bear fruit with a shorter ripening period may allow the fruit to escape infection by the fungus. Wounds in fruit from insects or other means should be reduced to the extent possible because wounds provide entry points for *Colletotrichum* spp. and other pathogens like bacteria that cause soft rot. At the end of the season, infected plant debris from the field must be removed or deep ploughed to completely cover crop diseases (Agrios, 2005).

Use of resistant cultivars: Agrios (2005) mentioned that the use of resistant varieties not only eliminated losses from diseases, but also eliminated chemical and mechanical expenses of disease control. Some genetic resources resistant to anthracnose in chilli have been independently reported from different countries (AVRDC, 1999; Hong and Hwang, 1998; Kim and Park, 1988; Kim *et al.*, 1986; Kim *et al.*, 1987; Pae *et al.*, 1998; Yoon and Park, 2001). In particular, some lines of *C. baccatum* show strong resistance to the pathogen, and pathogen inoculation resulted in no or limited lesions on the chilli fruits (Yoon *et al.*, 2003). However, to date, no high resistance has been found in *Capsicum annuum*, which is the only species grown worldwide (Park, 2007). Recently Voorrips *et al.* (2004) found one main quantitative trait locus (QTL) with high significance and large effects on resistance and three other QTL with smaller effects on the F2 population (cross between *C. annuum* and *C. chinense*) on the traits they tested such as infection frequency, the true lesion diameter and overall lesion diameter after inoculation with *C. gloeosporioides* in the study of resistance to anthracnose disease in Indonesia.

Use of chemicals: Chemicals are the most common and practical method to control anthracnose diseases. However, fungicide tolerance often arises quickly, if a single compound is relied upon too heavily (Staub, 1991). The fungicide traditionally recommended for anthracnose management in chilli is Manganese ethylenebisdithiocarbamate (Maneb) (Smith, 2000) although it does not consistently control the severe form of anthracnose on chilli fruit. The strobilurin fungicides azoxystrobin (Quadris),

trifloxystrobin (Flint), and pyraclostrobin (Cabrio) have recently been labeled for the control of anthracnose on chilli, but only preliminary reports are available on the efficacy of these fungicides against the severe form of the disease (Alexander, 2002). The disease can be controlled under normal weather conditions with a reasonable spray program. However, there are numerous reports of negative effects of using chemicals on farmer income and health, and toxic contamination to the environment, particularly in developing countries (Voorrips *et al.* 2004).

Use of biofungicides: The control of chilli anthracnose fruit rot has, for many years, relied on chemicals and resulted in many undesirable problems. There is a need to incorporate alternative control components that are effective in field. Biological control of fruit rot and die-back of chilli with plant products tested in many laboratories and field trials showed that the crude extract from rhizome, leaves and creeping branches of sweetflag (*Acorus calamus* L.), palmarosa (*Cymbopogon martinii*) oil, *Ocimum sanctum* leaf extract, and neem (*Azadirachia indica*) oil could restrict growth of the anthracnose fungus (Jeyalakshmi and Seetharaman 1998, Korpraditskul *et al.* 1999). Among the biofungicides used against the fungus *Colletotrichum* spp. on chilli fruit, Nalinee (2000) found that the most effective control was sweetflag crude extract when applied in two intervals when the majority of the plants were at the first bloom stage and at the mature bloom stage.

MATERIALS AND METHODS

1. Morphological diversity of *Colletotrichum capsici* in Thailand

1.1. Collection of *Colletotrichum* isolates

1.1.1. Sampling of *Colletotrichum* isolates

Infected chilli fruits were randomly collected from chilli farms in the western Thailand in July 2004. Hierarchical sampling (McDonald, 1997) was performed, of which within a farm an area of 30x30 m was randomly selected to collect infected chilli samples. Five anthracnose infected chilli fruits were collected from each of the five spots located at the four corners and the middle of the selected area (Fig 1). Each spot (within 2-3 m in diameter) was within 2-3 m in diameter. The five fruits collected from each spot were labelled A, B, C, D and E respectively.

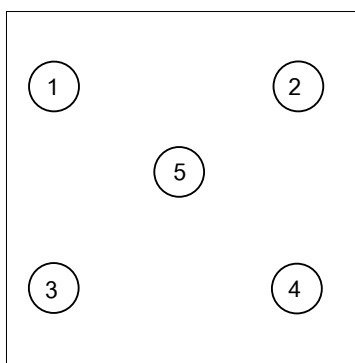


Fig 1 Hierarchical sampling within a randomly selected 30x30 m area in a chilli farm.

1.1.2. Sources of *Colletotrichum* isolates

Samples of anthracnose infected chilli fruits were collected from eight chilli farms designated as F1, F2, F3, F4, F5, F6, F7 and F8, which were located in four provinces in the western Thailand as follows: Kanchanaburi, Nakhon Pathom, Ratchaburi and Suphanburi (Table 1).

Table 1 Eight chilli farms in the western Thailand, where 37 *Colletotrichum capsici* isolates were collected from.

Farms	Location	Provinces
F1	Don Cha-em (ดอนชะเอม)	Ratchaburi
F2	Border of Ratchaburi and Kanchanaburi	Ratchaburi
F3	Kanchanaburi	Kanchanaburi
F4	Thaa Muang (ท่าม่วง)	Kanchanaburi
F5	Baan Nong Phai (บ้านหนองไผ่)	Kanchanaburi
F6	Baan Sapungkoen (บ้านสะปึงเกิน.)	Suphanburi
F7	Nong Sala (หนองศาลา)	Nakhon Pathom
F8	Saengsaam (แสงสาม)	Suphanburi

1.1.3. Isolate nomenclature

The collected *Colletotrichum* isolates were named using the combination of the farm number, spot location within the farm, and the sample order. For example ‘F6-1A’ was isolate from the fruit (A) was collected from spot location 1 of Farm 6.

1.2. Isolation of *Colletotrichum* from infected chilli fruit

1.2.1. Culturing *Colletotrichum* from anthracnose infected chilli

One piece (~2-5 mm) of infected tissues was cut from the edge of the anthracnose lesion on the collected infected chilli fruit using sterilized sharp scalpel. The cut tissue was surface sterilized by dipping the tissue in 1 % (w/v) sodium hypochlorite for

3 min followed by washing twice with distilled water. The surface sterilization of the tissue was to eliminate bacteria and other saprophytes that contaminated on the fruits. The tissue was then wiped dry with sterilized paper towel, and placed on the surface of potato glucose agar (PGA; 10 g/l potato, 15 g/l glucose, 15 g/l agar). The tissue was cultured for 3 days under near UV light at 28 °C. Newly growing edges of any hypha developing from the diseased tissue were then transferred onto a new PGA and incubated further for 7 days under near UV light at 28 °C to let fungus sporulated.

1.2.2. Single conidium isolation

Single conidium was isolated from each of the sporulating cultures. Conidia masses were touched with a sterilized wire loop and streaked onto the surface of water agar (WA; 15 g/l agars) and then incubated overnight at room temperature (~ 28-30°C). A single conidium was picked out of the WA under microscope with a sterilized needle and transferred to a new PGA. The isolated single conidium was incubated at room temperature for 7 days under alternative near UV light and fluorescence light (12/12 hrs).

1.2.3. Identification of *Colletotrichum* spp.

The *Colletotrichum* isolates were identified their species based on their conidia shape and size (Sutton, 1980) as characterized from the initial cultures of the single conidium that sporulated on the PGA in 1.2.2.

1.3. Morphological characterization of *C. capsici*

1.3.1. *C. capsici* isolates in this study

The *C. capsici* isolates used in this study were randomly selected from two collections including 1) Collection of *C. capsici* isolates from the western Thailand, by Associate Professor Dr. Orarat Mongkolporn, Department of Horticulture, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand; and 2) Collection of *C. capsici* from the northern and north-eastern Thailand, by Ms. PoPo Than, Department of Agronomy, Maejo University, Chiang Mai, Thailand.

The total 45 isolates (Table 2) comprised 37 from the west and six from the north and north-east collections, and two references including ‘158ci’ (donated from Associate Professor Dr.Somsiri Sangchote, Department of Plant Pathology, Kasetsart University, Bangkok, Thailand) and ‘Indian isolate’ which was *C. capsici* from Coimbatore in India.

Table 2 Forty-five *Colletotrichum capsici* isolates used in the study of morphological diversity.

Diversity level	<i>Colletotrichum</i> isolate	Location
Within farm (Farm 6)	F6-1A, F6-1B, F6-1C, F6-1D, F6-1E, F6-2A, F6-2B, F6-2C, F6-2D, F6-2E, F6-3A, F6-3B, F6-3C, F6-3D, F6-3E, F6-4A, F6-4B, F6-4C, F6-4D, F6-4E, F6-5A, F6-5B, F6-5C, F6-5D, F6-5E	Farm 6, Suphanburi, western Thailand
Within region (Western)	F1-2E, F2-4B, F2-1E F3-1B, F4-2C, F5-2C F7-3C, F7-4B, F7-5A F8-3A, F8-4C, F8-5A	Ratchaburi, western Thailand Kanchanaburi, western Thailand Nakhon Pathom, western Thailand Suphanburi, western Thailand
Within country (Thailand)	SKP16, Ccmj9, R11 Mit135, U5, 254(3) F1-2E, F2-4B, F2-1E F3-1B, F4-2C, F5-2C F7-3C, F7-4B, F7-5A F8-3A, F8-4C, F8-5A	Chiang Mai, Northern Thailand Udon Thani, North-eastern Thailand Ratchaburi, western Thailand Kanchanaburi, western Thailand Nakhon Pathom, western Thailand Suphanburi, western Thailand
Reference	158ci, “Indian isolate”	Unknown, Thailand Coimbatore, India

1.3.2. Morphological characterization

Each *C. capsici* isolate was cultured on PGA for five days. The cultures were grown under dark 12/12 hr light cycles at 28 °C in an incubator. Three replications were performed by taking three pieces of 5 mm diameter plugs from the culture edge of each isolate and placing each of them onto a new PGA plate (9 cm diameter). The new cultures were grown under the same conditions as described above. Morphological characteristics of colony, conidia and appressoria were investigated as follows.

1.3.2.1. Colony growth rate and characteristics

Colony diameter of each isolate was measured at 8 days after inoculation (DAI), and mean colony growth rate was determined. Colony characteristics including surface mycelium, color and mass conidia color was recorded.

1.3.2.2. Conidia shape and size

Conidia suspension was made from 8-day-old colony of each isolate. To investigate its conidia shape and size, 15 conidia were randomly selected from each replicate to measure their length and width under compound microscope with 100x magnifier.

1.3.2.3. Appressoria shape and size

Appressoria shape and size were investigated using modified slide culture technique (Johnston and Jones, 1997). A culture was prepared by placing a piece of 10x10 mm WA and dropping conidia suspension of each isolate at the edge of the WA. The slide culture was covered with a sterile cover slip and was held in a petri dish to serve as a moisture chamber for 24 hrs at room temperature. 15 randomly chosen appressoria length and width were measured from each replicate.

1.4. Morphology diversity analysis

Data were analyzed using analysis of variance ($P < 0.05$) with Duncan's multiple range tests (DMRT) and analysis of variance (ANOVA) was performed using Tukey's pairwise comparison test ($P < 0.05$) (MINITAB release 13, Minitab Inc., Boston, MA, USA). Cluster analysis was with the unweighted pair group arithmetic mean method (UPGMA) in the SAHN program of NTSYSpc package (NTSYS version 2.20e, Applied Biostatistic Inc, 1986-2005) and dendrogram best fit to similarity matrix was generated in different morphology diversity study levels. Morphological diversity was analyzed at three levels including 1) within farm 6, 2) within the western regions, 3) within country as described in Table 2.

2. Identification of pathotypes of *C. capsici* in Thailand

2.1. *Colletotrichum capsici* materials

Total of 11 isolates of *C. capsici* were selected from the collections as described in chapter 3. Ten of which were from western Thailand and one was from northern Thailand (Table 3).

Table 3 Eleven *Colletotrichum capsici* isolates used in the study.

No	Isolate	Location
1	F4-2C	Kanchanaburi
2	F4-5A	Kanchanaburi
3	F7-3A	Nakhon Pathom
4	F8-1A	Suphanburi
5	F8-2A	Suphanburi
6	F8-3A	Suphanburi
7	F8-3B	Suphanburi
8	F8-4C	Suphanburi
9	F8-5A	Suphanburi
10	F8-5B	Suphanburi
11	SKP16	Chiang Mai

2.2. Plant materials

Nine chilli varieties with different degrees of resistance to anthracnose derived from four cultivated species included *Capsicum annuum*, *C. baccatum*, *C. chinense* and *C. frutescens* were used for pathotype testing. (Table 4) Ten plants of each chilli variety were grown in a shade house from April to December 2007 at the Tropical Vegetable Research Center (TVRC), Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand.

Table 4 Nine chilli genotypes used in the pathotypes study of the *Colletotrichum capsici*.

No.	<i>Capsicum</i> species	<i>Capsicum</i> varieties
1	<i>C. annuum</i> L.	Bangchang
2		Jinda
3		83-168
4	<i>C. baccatum</i> L.	PBC80
5		PBC81
6		PBC1422
7	<i>C. chinense</i> Jaq.	PBC932
8		C04714
9	<i>C. frutescens</i> L.	Kee Noo Suan

2.3. Inoculation method

2.3.1. Inoculum preparation

Conidia suspension of each *C. capsici* isolate was prepared from mass conidia were harvested from 7-day-old cultures grown on potato glucose agar (PGA; 10 g/l potato, 15 g/l glucose, 15 g/l agar) under dark/light 12/12 hrs at room temperature (~28-30°C). The *C. capsici* culture plate was flooded with sterilized water, and the conidia were gently scraped from the culture plate. The conidia were counted under the microscopes 10x magnification using a haemocytometer and adjusted to 10⁶ conidia/ml.

2.3.2. Chilli fruit inoculation

Five fruits at ripe red stage were harvested from each variety, and had their pedicels and calyces removed. The fruits were surface sterilized with 1 % (w/v) sodium hypochlorite for 5 min, were washed twice with distilled water and then wiped dry with paper towel. Each chilli fruit was injected once with 1 µl of the prepared conidial suspension in the middle of the fruit. The injection was performed using a microinjector which comprised of a Micro Syringe™ model 1705 TLL, dispenser PB600-1 (Hamilton, Switzerland), and a needle with 1mm diameter and 1 mm depth. The microinjector delivered a control number of conidia in each injection (1,000 conidia per 1 µl) at a depth of 1 mm onto the pericarp. The inoculated chilli fruits were placed in a plastic box 20 x30 x10 cm³ containing 500 ml distilled water and were incubated at room temperature (~28-30°C), 100% RH dark/ light 12/12 hrs for three days and 70 % RH dark/ light 12/12 hrs until 9 days.

2.4. Evaluation of anthracnose symptoms

The disease was evaluated by visual measurement of lesions that developed on the fruit at the inoculation site at 3, 5, 7 and 9 DAI and by the description of the anthracnose symptoms at 9 DAI. Disease scores ranging from 0 to 9 were given based on percentage of lesion size to fruit size as described in Table 5. Disease diagrams representing degrees of infection from 0 to 9 of different chilli fruit types are displayed in Fig 2-7.

Table 5 Anthracnose scores ranging from 0 to 9 and their symptom descriptions
modified from Than *et al* (2008).

Score	Degree of resistance	Anthracnose symptom
0	HR –highly resistant	no infection
1	R – resistant	>1-2% of the fruit shows necrotic lesion or a larger water soaked lesion surrounding the infection site
3	MR – moderately resistant	>2-5% of the fruit shows necrotic lesion, acervuli may be present /or water lesion up to 5% of the fruit surface
5	MS - moderately susceptible	>5-15% of the fruit shows necrotic lesion, acervuli present/or water soaked lesion up to 25% of the fruit surface
7	S – susceptible	>15-25% of the fruit shows necrotic lesion with acervuli
9	HS – highly susceptible	>25% of the fruit shows necrosis, lesion often encircling the fruit, abundant acervuli

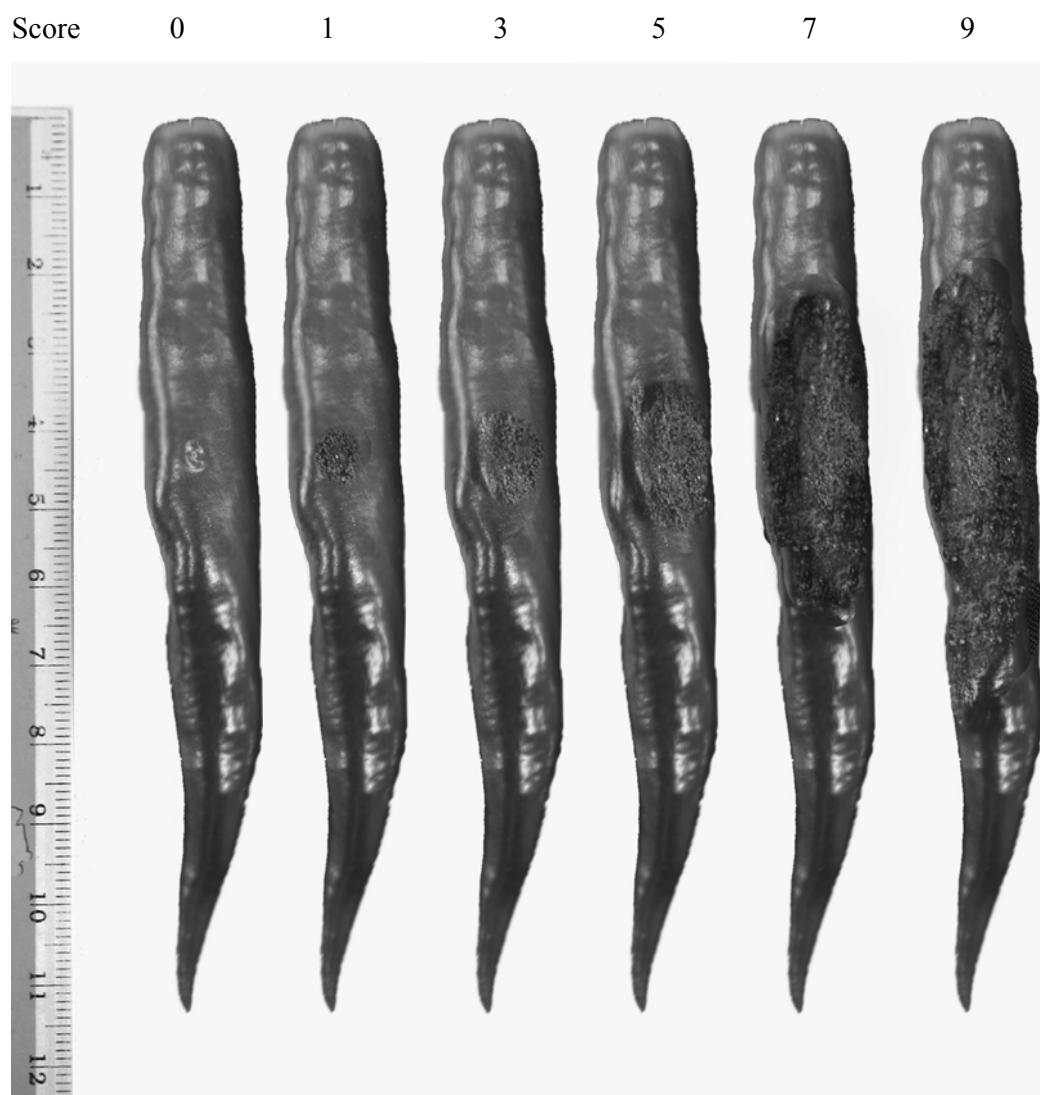


Fig 2 Disease diagrams of anthracnose symptom ranging from score 0 to 9 on ‘Bangchang’ representing elongate large fruit chilli type.



Fig 3 Disease diagrams of anthracnose symptom ranging from score 0 to 9 on 'Jinda' representing large fruit Khee noo chilli type.

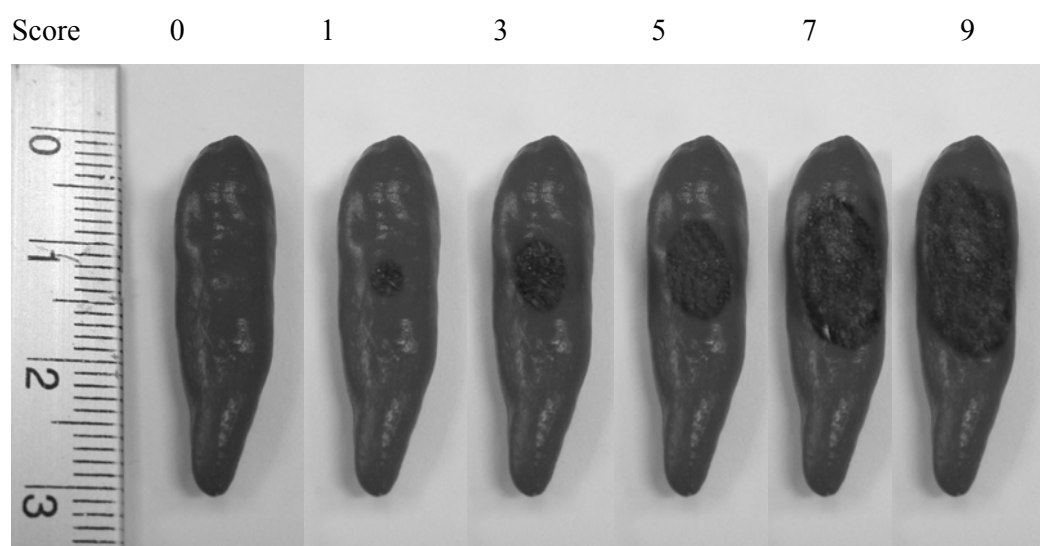


Fig 4 Disease diagrams of anthracnose symptom ranging from score 0 to 9 on '83-168' representing small fruit Khee-noo chilli type.



Fig 5 Disease diagrams of anthracnose symptom ranging from score 0 to 9 on 'PBC80', 'PBC81' and 'PBC1422' representing baccatum chilli type.

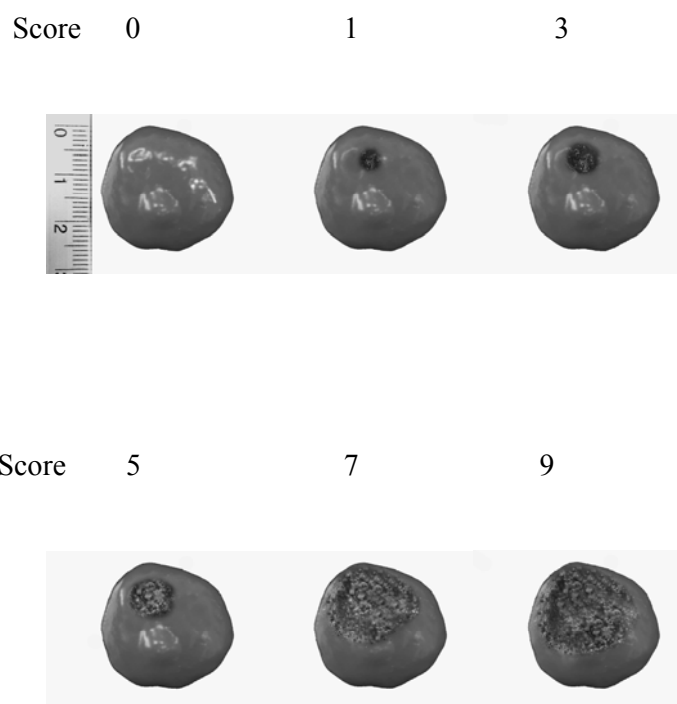


Fig 6 Disease diagrams of anthracnose symptom ranging from score 0 to 9 on 'PBC932' representing cherry chilli type.

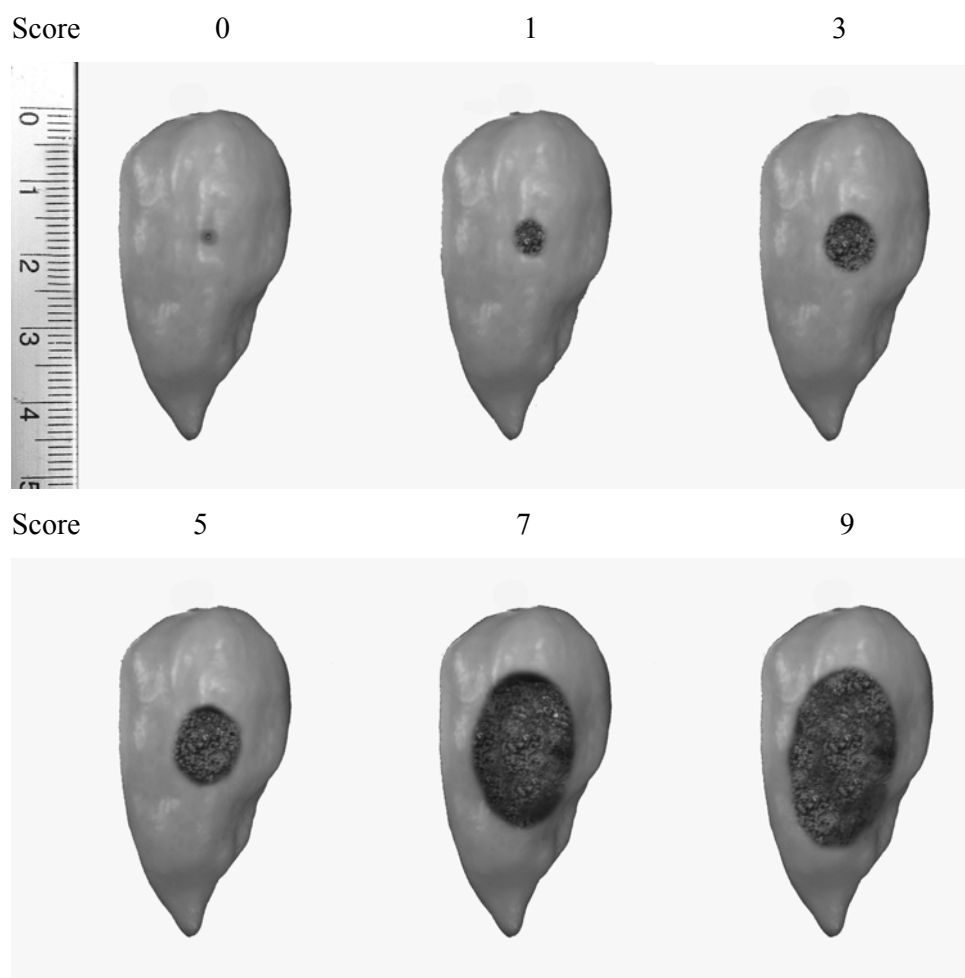


Fig 7 Disease diagrams of anthracnose symptom score 0 to 9 on 'C04714' representing conical chilli type.

2.5. Identification of pathotypes of *C. capsici*

Pathotypes of *C. capsici* were identified based on qualitative differences between isolates to infect a specific variety. Highly resistant (0) host reaction was when there was no evidence of infection by the isolate on the variety. Differences in aggressiveness were based on quantitative differences in the level of infection a host reaction from resistant (1) to highly susceptible (9) resulting from infection by isolates on a specific variety.

RESULTS AND DISCUSSION

Results

1. Morphological diversity of *Colletotrichum capsici* in Thailand

1.1. Identification of *Colletotrichum* spp.

A total of 84 single conidium spore isolates of *Colletotrichum* spp. were established from anthracnose infected chilli fruits collected from eight farms in western Thailand. Identification of *Colletotrichum* species was based on spore shape and size. The conidia of *C. capsici* were falcate whereas *C. gloeosporioides* were oblong with obtuse ends. Sixty three *Colletotrichum* isolates were identified as *C. capsici* and 21 as *C. gloeosporioides* (Table 6)

Table 6 Identification of *Colletotrichum* spp. collected from eight chilli farms in the west of Thailand.

Farm	Isolates		Provinces
	<i>C. capsici</i>	<i>C. gloeosporioides</i>	
1	2	5	Ratchaburi
2	4	1	Ratchaburi
3	2	-	Kanchanaburi,
4	8	5	Kanchanaburi
5	5	3	Kanchanaburi
6	25	-	Suphanburi
7	5	7	Nakhon Pathom
8	12	-	Suphanburi
Total	63	21	

1.2. Morphological characteristics of 45 *C. capsici* isolates.

The morphological characteristics of 45 *C. capsici* isolates were studied to characterize and compare isolates from three levels of sampling 1) *C. capsici* isolates which is within one farm (Farm 6) from Suphanburi, 2) *C. capsici* isolates within region and 3) *C. capsici* isolates within country

1.2.1. Colony growth rate and characteristics

Colony characteristics were classified based on 1) colony growth rate 2) surface mycelium description 3) colony color 4) conidia mass color

1.2.1.1. Colony growth rate

Growth rate of 45 *C. capsici* isolates on PGA ranged from 6.0 to 11.1 mm/day, mean = $8.5 \pm 33.4 \mu\text{m}$ (Fig 8). There was significant ($p = 0.05$) difference in growth rate among the 45 *C. capsici* isolates. The 45 isolates could be divided into three groups according to colony growth rate, the slow growing with growth rate less than 7.5 mm/day, medium growing with growth rate more than 7.5 to 9.5 mm/day and the fast growing with growth rate faster than 9.5 mm/day. Thirty three *C. capsici* isolates belonged to the medium growing group and six isolates of *C. capsici* belonged to the slow and six isolates of *C. capsici* belonged to fast growing group.

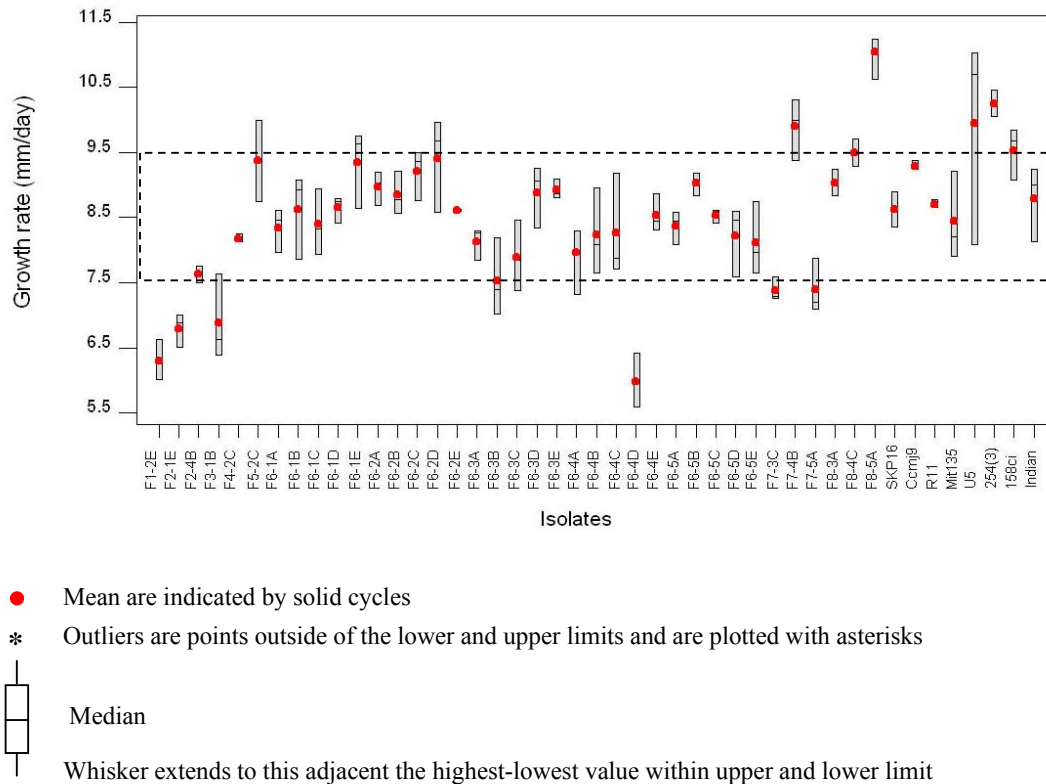


Fig 8 Colony growth rate of the 45 *Colletotrichum capsici* isolates: slow rate <7.5 mm/day; medium rate 7.5-9.5 mm/day; and fast rate > 9.5 mm/day.

1.2.1.2. Surface mycelium

The *C. capsici* isolates could be divided into four groups according to the surface mycelium: uniform (U), concentric rings (C), sector (Se) and irregular (I) (Fig 9). Surface mycelium of 26 *C. capsici* isolates were uniform, 10 were concentric rings, one was sector and eight were irregular.

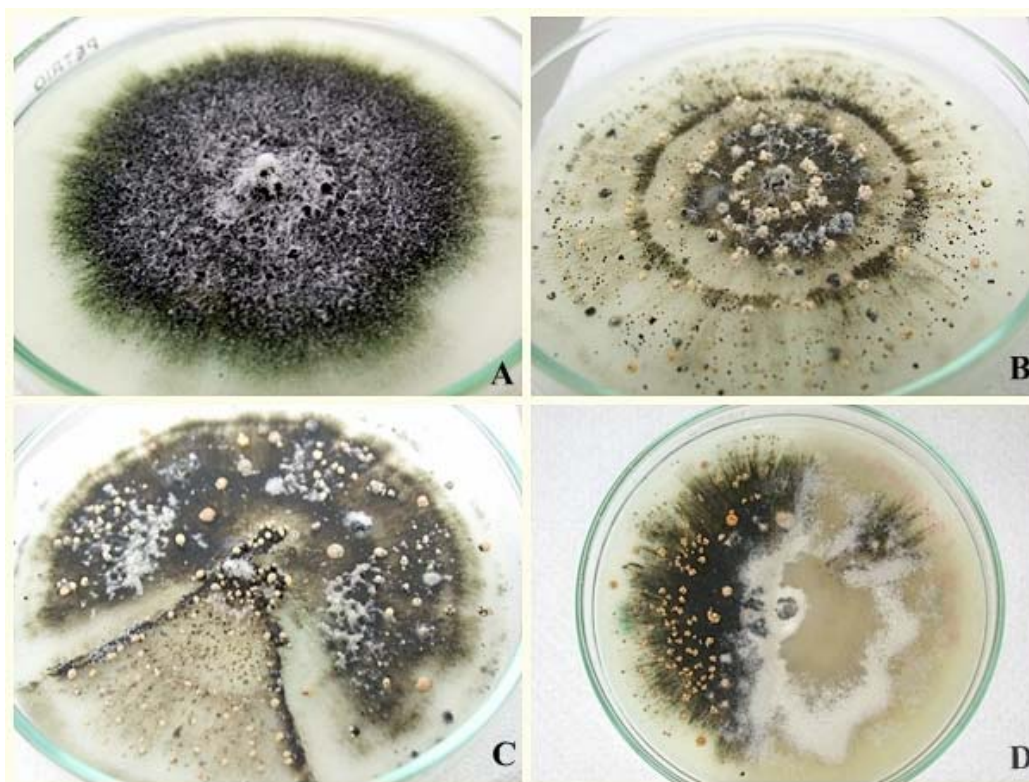


Fig 9 Surface mycelium of the 45 *Colletotrichum capsici* isolates after 8 days on potato glucose agar A. uniform (U), B. concentric rings (C), C. sector (Se), D. irregular (I).

1.2.1.3. Colony and conidia mass color

Colony color was divided into 2 groups whitish to grey (G) and whitish to brown (B) (Fig.10). Forty *C. capsici* isolates were whitish to grey and five were whitish to brown colony color. Conidia mass color was divided into 2 groups 1) whitish to grey (W) and orange (O) Fig 10. Three *C. capsici* isolates had whitish, 42 had orange conidia mass color.

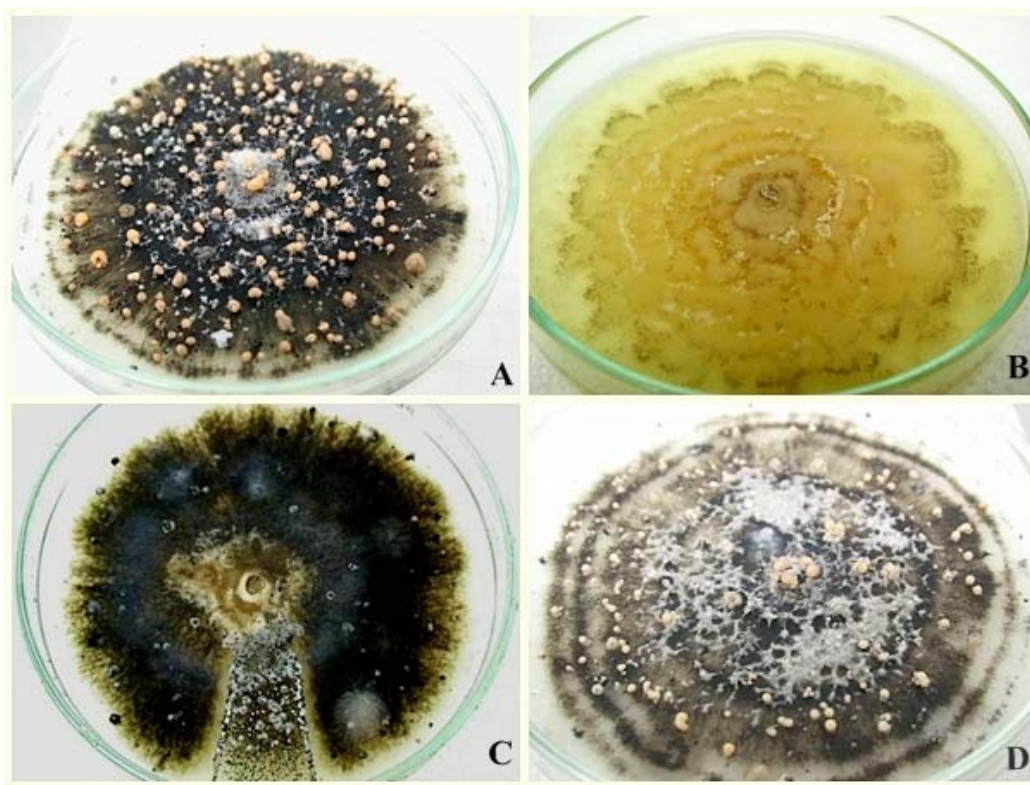


Fig 10 Colony and mass conidia colors of the 45 *Colletotrichum capsici* isolates after 8 days on potato glucose agar. Colony colors: A. whitish to grey (G), B. whitish to brown (B) and conidia colors: C. whitish mass conidia (W), D. orange mass conidia (O).

Combining all classes of the three colony characters (growth rate x3, surface mycelium x4; mass conidia color x2). There were possible 24 groups as categorized in Table 7.

Table 7 Colony growth rate and characteristics of the 45 *Colletotrichum capsici* isolates.

Growth rate	Surface mycelium	Colony color	Mass conidia color	Isolates
Slow(S)	Uniform (U)	Whitish to grey (G)	Whitish (W)	-
			Orange (O)	F6-4D, F7-4B
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	-
	Concentric rings (C)	Whitish to grey (G)	Whitish (W)	F1-2E
			Orange (O)	F2-4B
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	F8-3A
	Sectors (Se)	Whitish to grey (G)	Whitish (W)	-
			Orange (O)	-
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	-
	Irregular (I)	Whitish to grey (G)	Whitish (W)	-
			Orange (O)	F7-3C
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	-

Table 7 (continued).

Growth rate	Surface mycelium	Colony color	Mass conidia color	Isolates
Medium	Uniform (U)	Whitish to grey (G)	Whitish (W)	Indian
			Orange (O)	Mit135, F6-1A, F6-1C, F6-1D, F6-1E, F6-2A, F6-2B, F6-2C, F6-2D, F6-2E, F6-3A, F6-3C, F6-3D, F6-3E, F6-4B, F6-4C, F6-5A, F6-5B, F6-5C, F8-5A
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	-
	Concentric rings (C)	Whitish to grey (G)	Whitish (W)	-
			Orange (O)	F6-4A, F6-4E, F7-5A
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	F6-3B, F8-4C, R11
	Sectors (Se)	Whitish to grey (G)	Whitish (W)	-
			Orange (O)	F6-1B
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	-
	Irregular (I)	Whitish to grey (G)	Whitish (W)	-
			Orange (O)	F3-1B, F6-5D, F6-5E, SKP16, Ccmj9

Table 7 (continued).

Growth rate	Surface mycelium	Colony color	Mass conidia color	Isolate
Fast (F)	Uniform (U)	Whitish to grey (G)	Whitish (W)	F5-2C
			Orange (O)	F4-2C, 158ci
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	-
	Concentric rings (C)	Whitish to grey (G)	Whitish (W)	-
			Orange (O)	F2-1E
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	-
	Sectors (Se)	Whitish to grey (G)	Whitish (W)	-
			Orange (O)	-
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	-
	Irregular (I)	Whitish to grey (G)	Whitish (W)	-
			Orange (O)	U5
		Whitish to brown (B)	Whitish (W)	-
			Orange (O)	254(3)

1.2.2. Conidial size

Colletotrichum capsici conidia were falcate ranging from 18.1-24.7x 3.1-4.1 μm . There was significant ($p = 0.05$) difference in conidia length and width among the 45 *C. capsici* isolates (Fig 11).

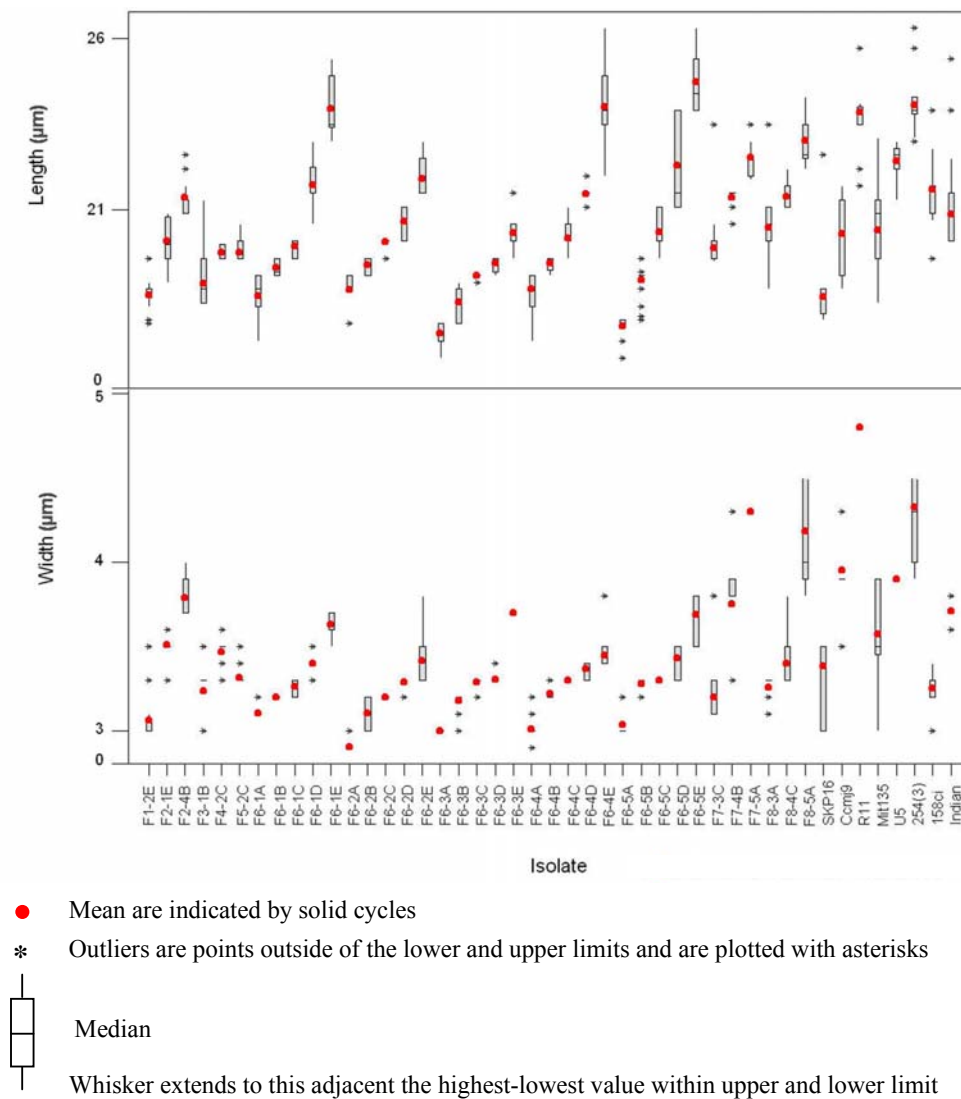


Fig 11 Conidia size of the 45 *Colletotrichum capsici* isolates.

1.2.3 Appressoria size

Obclavate and ovoid appressoria forms were commonly observed. Appressoria length ranged 6.0-10.5 μm (mean 8.1 μm) and width ranged 5.2-8.3 μm (mean 6.4 μm). There was significant ($p = 0.05$) different in appressoria length and width among the 45 *C. capsici* isolates (Fig 12).

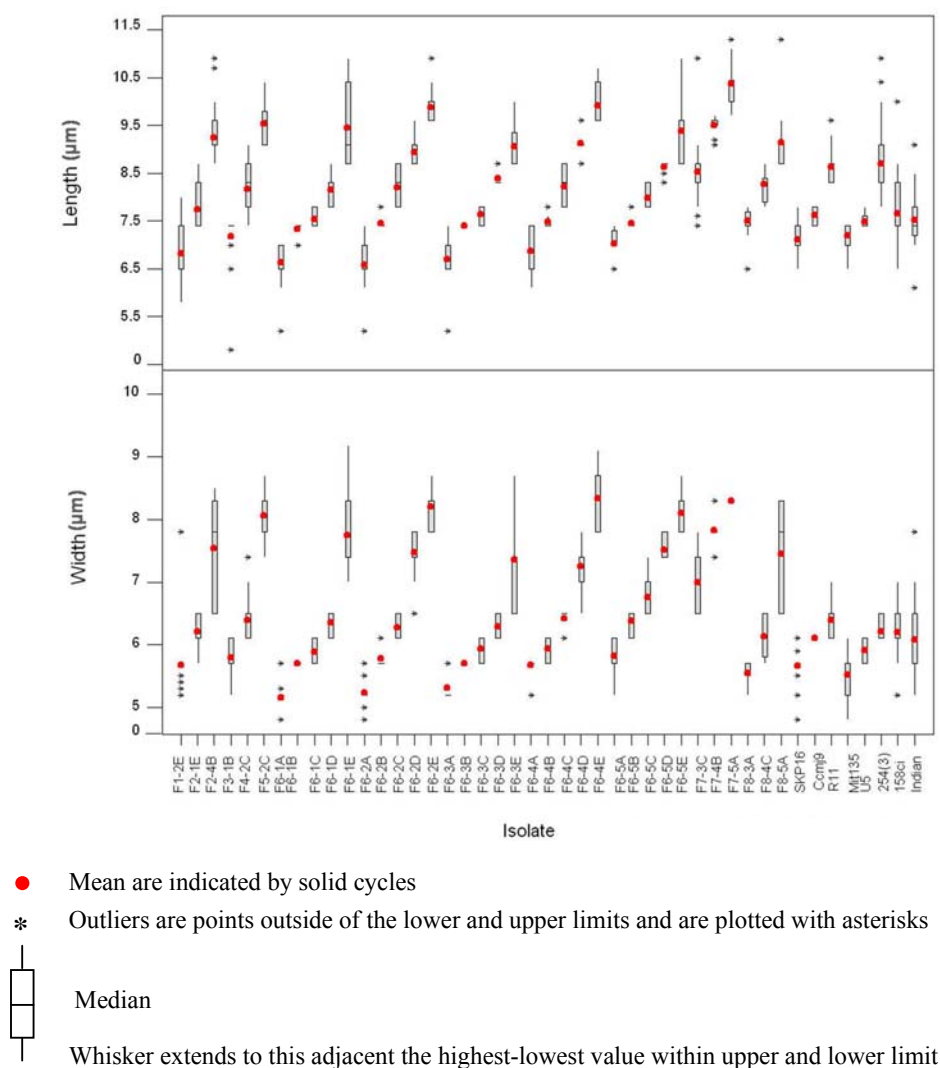


Fig 12 Appressoria size of the 45 *Colletotrichum capsici* isolates.

1.3. Morphological diversity analysis

Morphological diversity based on colony growth rate and characteristics, conidia and appressoria size was analyzed at four levels including 1) overall 45 isolates, 2) within Farm 6, 3) within the western region, 4) within country. *C. gloeosporioides* (F7-4C) was the out group isolate.

1.3.1. Cluster analysis of 45 *C. capsici* isolates

Six morphological characters including colony growth rate, surface mycelium, colony colors, mass conidia colors, conidia size, and appressoria size were investigated in 45 *C. capsici* isolates, showing significant different ($P < 0.001$). NTSYS analysis using UPGMA revealed high variation of the *C. capsici* isolates which were not be able to form group (Fig 13). Based on the clusters of the 45 isolates, there was no correlation to geographic location.

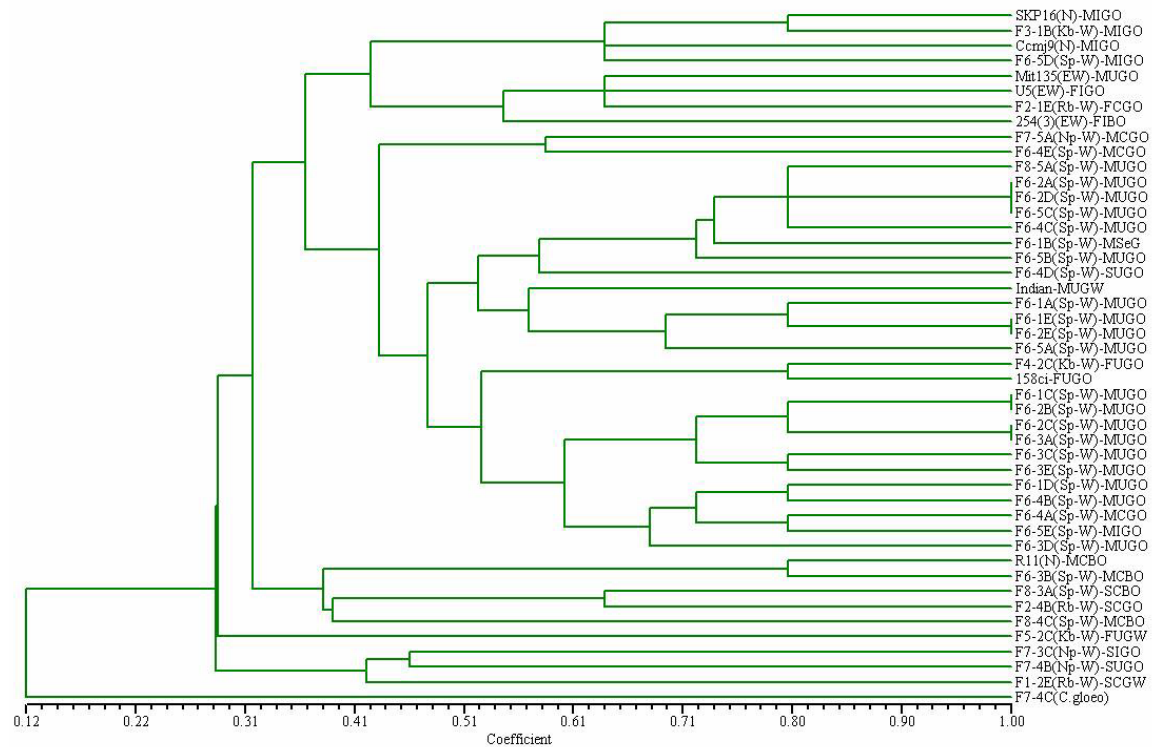


Fig 13 Dendrogram of 45 *Colletotrichum capsici* isolates generated by unweighted pair group method arithmetic mean (UPGMA) analysis using the Jaccard's similarity coefficient based on combination of colony characteristics including colony growth rate, surface mycelium, colony colors, mass conidia colors, conidia size, and appressoria size. Scale at the bottom depicts the similarity coefficient value.

However marked differences between 45 *C. capsici* isolates were observed in growth rate and colony characteristics. Consequently, fungal isolates were categorized into 16 groups (Fig 14) as follows 1) medium -growing colony and irregular surface mycelium producing whitish to grey colonies with abundant orange conidia masses (MIGO) 2) medium-growing colony and sectors surface mycelium producing whitish to grey colonies with abundant orange conidia masses (MSeGO) 3) medium -growing colony and uniform surface mycelium producing whitish to grey colonies with abundant orange conidia masses (MUGO) 4) fast-growing colony and uniform surface mycelium producing whitish to grey colonies with abundant orange conidia masses (FUGO) 5) slow-growing colony and irregular surface mycelium producing whitish to grey colonies with abundant orange conidia masses (SIGO) 6) slow-growing colony and concentric rings surface mycelium producing whitish to grey colonies with abundant orange conidia masses (SCGO) 7) slow-growing colony and uniform surface mycelium producing whitish to grey colonies with abundant orange conidia masses (SUGO) 8) medium -growing colony and concentric rings surface mycelium producing whitish to brown colonies with abundant orange conidia masses (MCBO) 9) medium -growing colony and concentric rings surface mycelium producing whitish to grey colonies with abundant orange conidia masses (MCGO) 10) slow-growing colony and concentric rings surface mycelium producing whitish to brown colonies with abundant orange conidia masses (SCBO) 11) fast-growing colony and uniform surface mycelium producing whitish to grey colonies with abundant whitish conidia masses (FUGW) 12) medium-growing colony and uniform surface mycelium producing whitish to grey colonies with abundant whitish conidia masses (MUGW) 13) fast -growing colony and irregular surface mycelium producing whitish to grey colonies with abundant orange conidia masses (FIGO) 14) fast-growing colony and irregular surface mycelium producing whitish to brown colonies with abundant orange conidia masses (FIBO) 15) fast-growing colony and concentric rings surface mycelium producing whitish to grey colonies with abundant orange conidia masses (FCGO) 16) slow-growing colony and concentric rings surface mycelium producing whitish to grey colonies with abundant whitish conidia masses (SCGW)

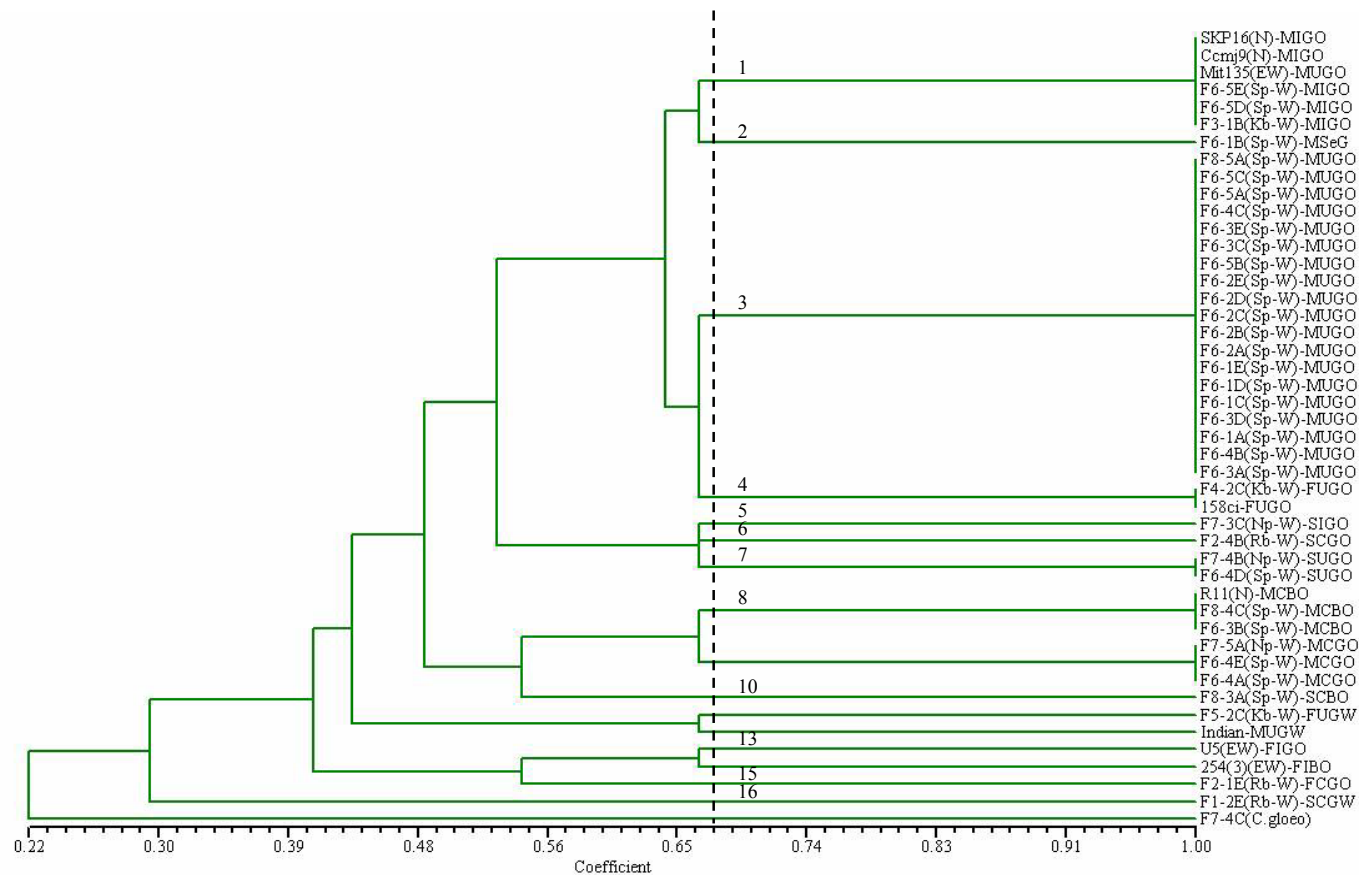


Fig 14 Dendrogram of 45 *Colletotrichum capsici* isolates generated by unweighted pair group method arithmetic mean (UPGMA) analysis using the Jaccard's similarity coefficient based on combination of colony characteristics including colony growth rate, surface mycelium, colony and mass conidia colors. Scale at the bottom depicts the similarity coefficient values.

Most of 45 *C. capsici* isolates belonged to MUGO group (20 isolates, which 19 form Farm 6 and one was form Farm 8), 5 were isolates MIGO, 3 isolates were MCGO, 3 isolates were MCBO, 2 isolates were SUGO, 2 isolates were FUGO, 1 isolate was SCGW, 1 isolate was SCGO, 1 isolate was SCBO, 1 isolate was SIGO, 1 isolate was MUGW, 1 isolate was MSeGO, 1 isolate was FUGW, 1 isolate was FCGO, 1 isolate was FIGO, 1 isolate was FIBO. Distinctive characters of the propose groups are showed in Table 7.

1.3.2. Cluster analysis of *C. capsici* within farm

Twenty five isolates of *C. capsici* from Farm 6, Suphanburi province showed significant difference ($P < 0.001$). NTSYS analysis using UPGMA revealed high variation of the *C. capsici* isolates within the farm. Twenty five *C. capsici* isolates were divided into six main groups at ranges of 0.57-0.59 similarity coefficient (Fig 15). Three pairs and a group of three isolates were identical including F6-1E and F6-2E (group 1); F6-2A, F6-2D and F6-5C (group2); F6-1C and F6-2B (group4); and F6-2C and F6-3A (group4) (Table 8). Based on the clusters of the isolates, there was no correlation with the sampling position (1-5, Fig 1) within the farm.

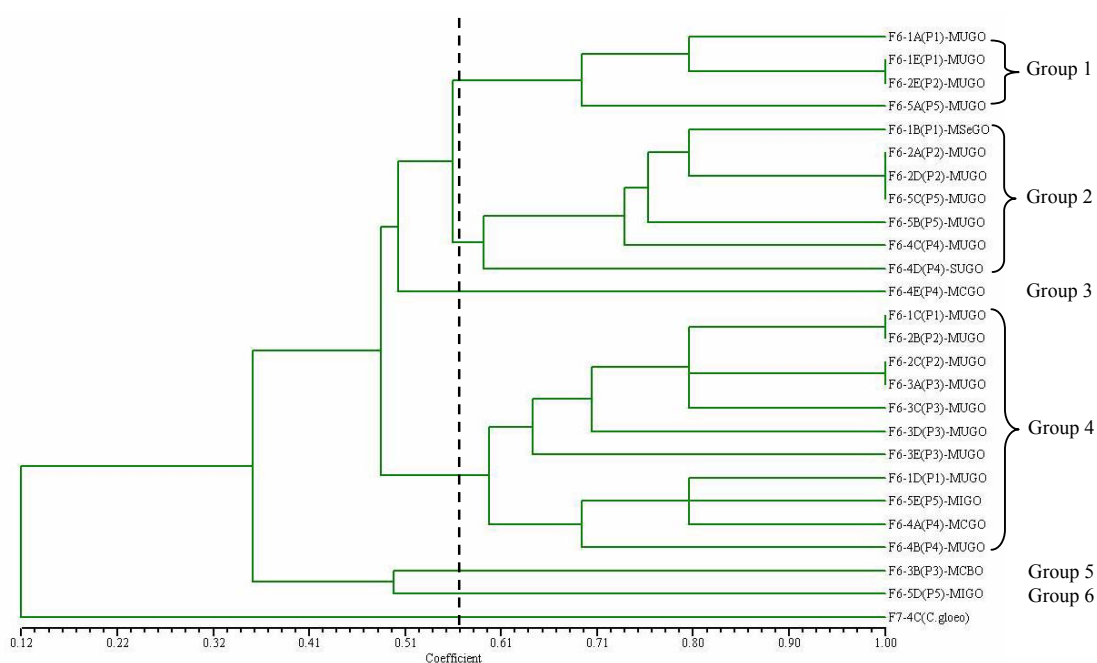


Fig 15 Dendrogram derived from a UPGMA cluster analysis using the Jaccard's similarity coefficient based on colony characteristics, spore and appressoria size of the 25 *Colletotrichum capsici* isolates from Farm 6 Suphanburi. Scale at the bottom depicts the similarity coefficient values.

Table 8 Similarity matrix of morphology characteristic of 25 *Colletotrichum capsici* isolates collected from Farm 6, Suphanburi.

	F61A	F61B	F61C	F61D	F61E	F62A	F62B	F62C	F62D	F62E	F63A	F63B	F63C	F63D	F63E	F64A	F64B	F64C	F64D	F64E	F65A	F65B	F65C	F65D	F65E	F74C		
	(C.gloeo)																											
F61A	1.00																											
F61B	0.38	1.00																										
F61C	0.50	0.38	1.00																									
F61D	0.50	0.38	0.64	1.00																								
F61E	0.80	0.50	0.64	0.64	1.00																							
F62A	0.50	0.80	0.50	0.50	0.64	1.00																						
F62B	0.50	0.38	1.00	0.64	0.64	0.50	1.00																					
F62C	0.64	0.38	0.80	0.80	0.80	0.50	0.80	1.00																				
F62D	0.50	0.80	0.50	0.50	0.64	1.00	0.50	0.50	1.00																			
F62E	0.80	0.50	0.64	0.64	1.00	0.64	0.64	0.80	0.64	1.00																		
F63A	0.64	0.38	0.80	0.80	0.80	0.50	0.80	1.00	0.50	0.80	1.00																	
F63B	0.29	0.20	0.29	0.50	0.29	0.20	0.29	0.38	0.20	0.29	0.38	1.00																
F63C	0.50	0.38	0.80	0.64	0.64	0.50	0.80	0.80	0.50	0.64	0.80	0.29	1.00															
F63D	0.50	0.50	0.64	0.80	0.64	0.64	0.64	0.80	0.64	0.64	0.80	0.38	0.64	1.00														
F63E	0.64	0.29	0.64	0.50	0.50	0.38	0.64	0.64	0.38	0.50	0.64	0.29	0.80	0.50	1.00													
F64A	0.38	0.38	0.50	0.80	0.50	0.38	0.50	0.64	0.38	0.50	0.64	0.64	0.50	0.64	0.38	1.00												
F64B	0.38	0.38	0.64	0.80	0.50	0.50	0.64	0.64	0.50	0.50	0.64	0.38	0.64	0.64	0.50	0.64	1.00											
F64C	0.50	0.64	0.50	0.50	0.64	0.80	0.50	0.50	0.80	0.64	0.50	0.20	0.50	0.50	0.38	0.38	0.50	1.00										
F64D	0.38	0.50	0.38	0.38	0.50	0.64	0.38	0.38	0.64	0.50	0.38	0.13	0.38	0.50	0.29	0.29	0.50	0.50	1.00									
F64E	0.50	0.50	0.64	0.38	0.64	0.50	0.64	0.50	0.50	0.64	0.50	0.29	0.50	0.38	0.38	0.50	0.38	0.50	0.38	1.00								
F65A	0.80	0.50	0.38	0.50	0.64	0.64	0.38	0.50	0.64	0.64	0.50	0.29	0.38	0.64	0.50	0.38	0.38	0.50	0.50	0.38	1.00							
F65B	0.50	0.64	0.50	0.50	0.64	0.80	0.50	0.50	0.80	0.64	0.50	0.20	0.64	0.64	0.50	0.38	0.50	0.64	0.64	0.50	0.64	1.00						
F65C	0.50	0.80	0.50	0.50	0.64	1.00	0.50	0.50	1.00	0.64	0.50	0.20	0.50	0.64	0.38	0.38	0.50	0.80	0.64	0.50	0.64	0.80	1.00					
F65D	0.50	0.38	0.29	0.50	0.50	0.38	0.29	0.38	0.38	0.50	0.38	0.50	0.29	0.38	0.29	0.50	0.38	0.38	0.29	0.38	0.50	0.38	0.38	1.00				
F65E	0.38	0.38	0.50	0.80	0.50	0.38	0.50	0.64	0.38	0.50	0.64	0.50	0.50	0.64	0.38	0.80	0.64	0.38	0.29	0.38	0.38	0.38	0.38	0.64	1.00			
F74C	0.13	0.06	0.20	0.13	0.13	0.13	0.20	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.06	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.06	0.06	1.00		
(C.gloeo)																												

1.3.3. Cluster analysis of *C. capsici* in western Thailand

The 12 isolates of *C. capsici* from four districts in the western area of Thailand showed significant difference ($P < 0.001$). NTSYS analysis using UPGMA revealed high variation of the *C. capsici* isolates from the four districts in the western area. Twelve *C. capsici* isolates were divided into seven main groups at ranges of 0.40-0.45 similarity coefficient (Fig 16). Two isolates F8-3A and F2-4B were identical (Table 9, Fig16). Based on the clusters of the isolates, there was no correlation with districts in western area of Thailand.

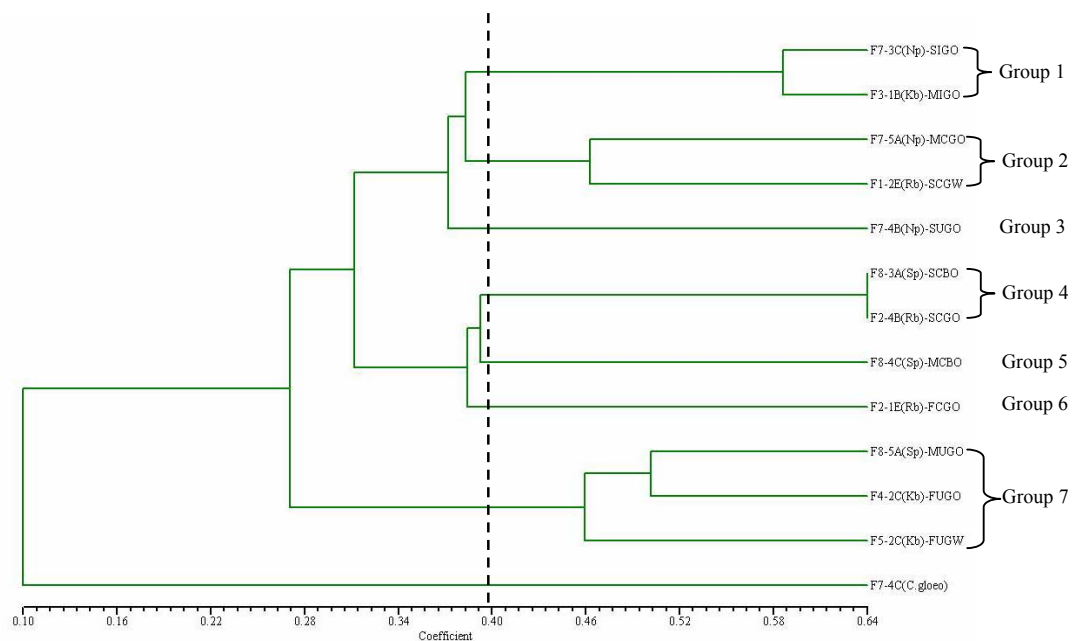


Fig 16 Dendrogram derived from a UPGMA cluster analysis using the Jaccard's similarity coefficient based on colony characteristics, spore and appressoria size of the 12 *Colletotrichum capsici* isolates collected from western Thailand. Scale at the bottom depicts the similarity coefficient values.

[illegible]

1.3.4. Diversity of *C. capsici* in Thailand

Twenty isolates of *C. capsici* from seven district areas Thailand showed significant difference ($P < 0.001$). NTSYS analysis using UPGMA revealed high variation of the *C. capsici* isolates within Thailand. Twenty *C. capsici* isolates were divided into 14 main groups at ranges of 0.57-0.63 similarity coefficient (Fig 17). Two pairs of isolates appeared to be identical including SKP16 and F3-1B (group 1) and F4-2C and 158ci (group 9) (Fig 17, Table 10). Based on the clusters of the isolates, there was no correlation with seven districts in Thailand and the Indian isolate formed a separate group to other isolates.

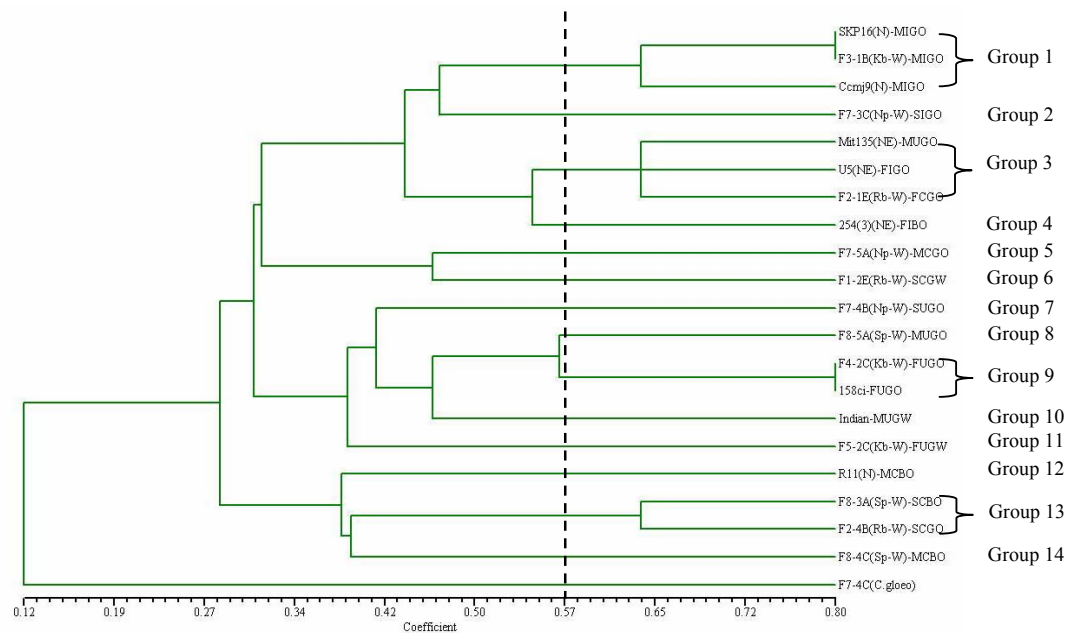


Fig 17 Dendrogram derived from a UPGMA cluster analysis using the Jaccard's similarity coefficient based on growth rate, colony characteristics and spore shape showing the associations among 21 *Colletotrichum capsici* isolates in Thailand.

Table 10 Similarity matrix of morphology characteristic of 20 *Colletotrichum capsici* isolates collected from Thailand.

	SKP16	Ccmj9	R11	Mit135	U5	254(3)	F73C	F74B	F75A	F83A	F84C	F85A	F12E	F21E	F24B	F31B	F42C	F52C	158ci	Indian	F74C
	(C.gloeo)																				
SKP16	1.00																				
Ccmj9	0.64	1.00																			
R11	0.29	0.29	1.00																		
Mit135	0.50	0.64	0.20	1.00																	
U5	0.38	0.38	0.20	0.64	1.00																
254(3)	0.38	0.29	0.20	0.50	0.64	1.00															
F73C	0.46	0.36	0.12	0.46	0.46	0.46	1.00														
F74B	0.38	0.29	0.20	0.29	0.38	0.20	0.46	1.00													
F75A	0.36	0.46	0.27	0.36	0.27	0.19	0.43	0.36	1.00												
F83A	0.20	0.29	0.38	0.29	0.38	0.38	0.27	0.38	0.36	1.00											
F84C	0.50	0.29	0.38	0.38	0.29	0.50	0.27	0.29	0.36	0.50	1.00										
F85A	0.50	0.64	0.29	0.38	0.29	0.20	0.27	0.38	0.46	0.29	0.29	1.00									
F12E	0.20	0.20	0.13	0.29	0.29	0.20	0.46	0.38	0.46	0.29	0.20	0.20	1.00								
F21E	0.29	0.38	0.20	0.64	0.64	0.50	0.36	0.29	0.36	0.38	0.38	0.29	0.38	1.00							
F24B	0.20	0.29	0.38	0.29	0.38	0.20	0.27	0.38	0.36	0.64	0.29	0.29	0.29	0.38	1.00						
F31B	0.80	0.64	0.31	0.64	0.50	0.50	0.58	0.29	0.36	0.20	0.38	0.50	0.29	0.38	0.21	1.00					
F42C	0.29	0.38	0.38	0.20	0.38	0.20	0.19	0.38	0.27	0.29	0.13	0.50	0.13	0.29	0.50	0.31	1.00				
F52C	0.13	0.21	0.13	0.13	0.21	0.13	0.20	0.31	0.29	0.13	0.06	0.42	0.31	0.21	0.29	0.14	0.50	1.00			
158ci	0.38	0.50	0.29	0.29	0.50	0.29	0.27	0.50	0.36	0.38	0.20	0.64	0.20	0.38	0.38	0.38	0.80	0.42	1.00		
Indian	0.38	0.38	0.29	0.38	0.38	0.20	0.27	0.38	0.27	0.20	0.20	0.50	0.38	0.29	0.20	0.50	0.38	0.31	0.50	1.00	
F74C	0.06	0.13	0.20	0.13	0.13	0.20	0.06	0.13	0.06	0.13	0.13	0.13	0.00	0.20	0.06	0.06	0.20	0.13	0.20	0.06	1.00
(C.gloeo)																					

2. Identification of pathotypes of *C. capsici* in Thailand

2.1. Differential reactions of anthracnose infection on the chilli varieties

All eleven *C. capsici* isolates infected *C. annuum* and *C. frutescens*, but none could infect *C. baccatum* (Table 11). In *C. chinense* ‘PBC932’ was infected by six isolates (F8-1A, F8-2A, F8-3A, F8-3B, F8-4C and F8-5B); and ‘CO4714’ was infected by all isolates except for F7-3A and SKP16 (Fig 18). The most susceptible *Capsicum* species were *C. frutescens* and *C. annuum*, as all isolates could infect the varieties of these species with disease scores 7-9. *C. chinense* was less susceptible than the *C. annuum* and *C. frutescens*, as not all isolates infected the two varieties of this species however, *C. chinense*, ‘PBC932’ appeared to be more resistant than ‘CO4714’ (Table 11).

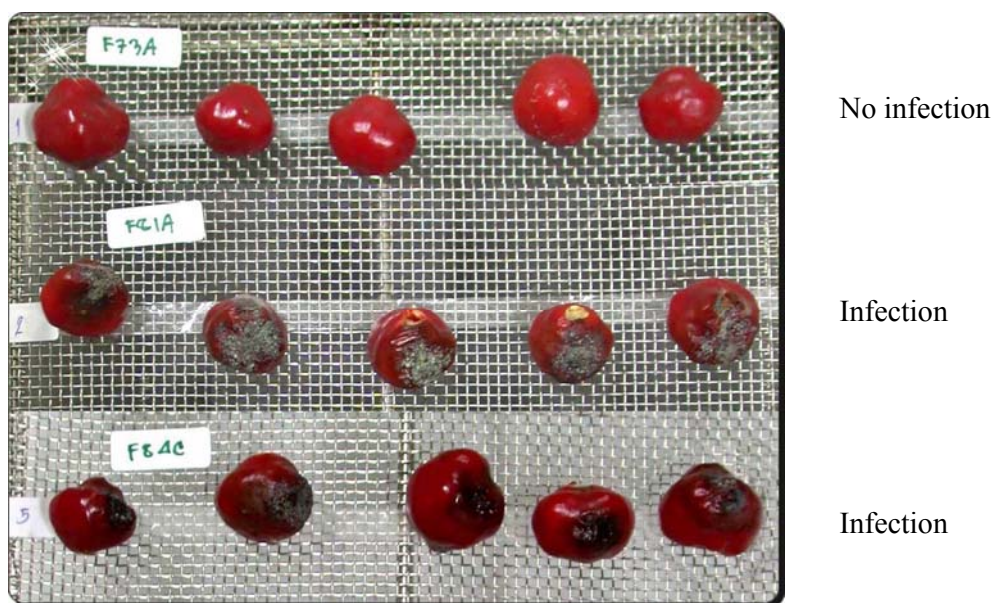


Fig 18 Qualitatively differential reactions on ‘PBC932’ as infected by of *Colletotrichum capsici* isolate ‘F7-3A’, ‘F8-1A’ and ‘F8-4C’ from top to bottom respectively.

Table 11 Differential reactions of nine chilli genotypes infected by 11 *Colletotrichum capsici* isolates in Thailand.

Isolates	Disease severity									Pathotypes
	<i>C. annuum</i>			<i>C. baccatum</i>			<i>C. chinense</i>		<i>C. frutescens</i>	
	Bang chang	Jinda	83-168	PBC 80	PBC 81	PBC 1422	PBC 932	C0 4714	Kee Noo Suan	
F8-1A	66 HS	28 HS	100 HS	0 HR	0 HR	0 HR	53 HS	55 HS	100 HS	PCC1
F8-2A	29 HS	26 HS	100 HS	0 HR	0 HR	0 HR	22 S	23 S	81 HS	
F8-3A	28 HS	25 HS	98 HS	0 HR	0 HR	0 HR	31 HS	21 S	100 HS	
F8-3B	57 HS	36 HS	100 HS	0 HR	0 HR	0 HR	18 S	27 HS	100 HS	
F8-4C	19 S	41 HS	100 HS	0 HR	0 HR	0 HR	59 HS	26 HS	100 HS	
F8-5B	35 HS	26 HS	43 HS	0 HR	0 HR	0 HR	34 HS	27 HS	100 HS	
F4-2C	14 S	28 HS	100 HS	0 HR	0 HR	0 HR	0 HR	15 S	100 HS	PCC2
F4-5A	32 HS	35 HS	100 HS	0 HR	0 HR	0 HR	0 HR	30 HS	100 HS	
F8-5A	68 HS	29 HS	100 HS	0 HR	0 HR	0 HR	0 HR	34 HS	100 HS	
F7-3A	36 HS	20 S	62 HS	0 HR	0 HR	0 HR	0 HR	0 HR	92 HS	PCC3
SKP16	12 S	15 S	65 HS	0 HR	0 HR	0 HR	0 HR	0 HR	45 HS	

HS, highly susceptible (score 9); VS, very susceptible (score 7); S, susceptible (score 5); MR, mediumly resistant (score 3); R, resistant (score 1); HR, highly resistant (score 0)).

2.2. Pathotypes identification of the *C. capsici*

Based on the differential reactions on different chilli varieties four pathotypes were identified (Table 14), PCC1, PCC2 and PCC3. The PCC1 group contained isolates that were able to infect all *C. annuum*, *C. frutescens* and *C. chinense*, but were not able to infect *C. baccatum* (F8-1A, F8-2A, F8-3A, F8-3B, F8-4C and F8-5B). The disease scores ranged from 5 to 9).

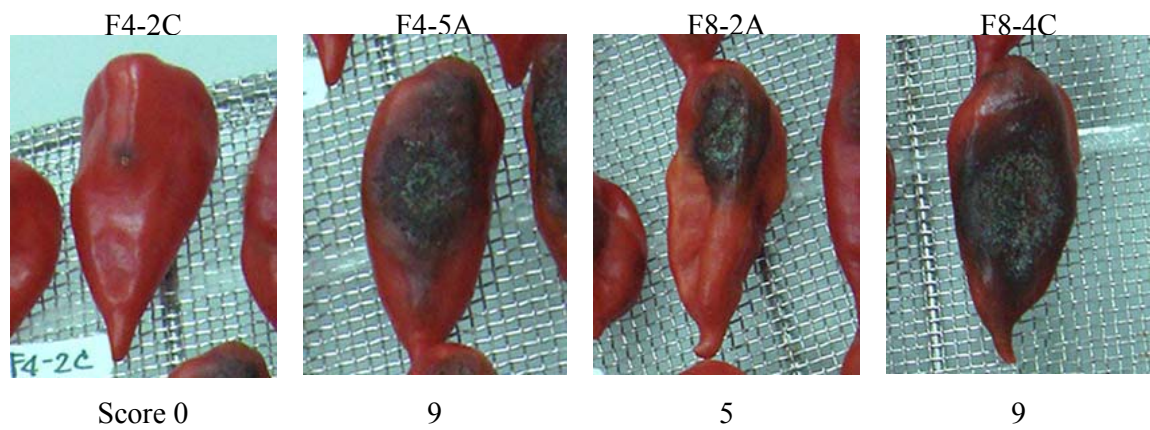
The PCC2 groups of isolates (F4-2C, F4-5A and F8-5A) were able to infect all *C. annuum*, *C. frutescens* and ‘C04714’ of *C. chinense*, but not the ‘PBC932’ nor all *C. baccatum*.

Finally, The PCC3 isolate F7-3A and SKP16 only infected *C. annuum* and *C. frutescens*, with a disease score of 7 to 9, but did not infect *C. baccatum* and *C. chinense*. Interestingly, Pathotype PCC1 was mainly composed of isolates originating from the one farm (Farm 8 from Suphanburi district), however another isolate from this farm was of Pathotype PCC2.

2.3. Aggressiveness of *Colletotrichum capsici* isolates

Differences in quantitative levels of infection occurred in all varieties except for *C. baccatum* species where no infection occurred (Fig 19). Two isolates SKP16 and F7-3A were generally the least aggressive producing smaller lesions 9 DAI compared to the more aggressive isolates e.g. F8-1A. The least aggressive isolates were classed as Pathotypes.

Fig 19 Quantitatively differential reactions on 'C04714' genotype as infected by *Colletotrichum capsici* isolates 'F4-2C', 'F4-5A', 'F8-2A', and 'F8-4C'.



DISCUSSION

1. Morphology diversity of *C. capsici* in Thailand.

High level of genetic diversity of 45 *C. capsici* isolates in Thailand were identified based on morphological characters including colony growth rate, surface mycelium, colony color, mass conidial color, conidial and appressoria size. The high level of genetic diversity could reflect the nature of *Colletotrichum* genus containing large amount of genetic variations (Baxter *et al.*, 1983, Katan, 2000). Other evidences based on both morphological and molecular studies also supported the high level of genetic diversity in *C. capsici*. Sharma *et al.* (2005) studied morphological characters and used molecular RAPD (random amplified polymorphic DNA) analysis in 35 *C. capsici* isolates collected from the north-western region of India and found a large diversity based on both data, although the RAPD did not congruent with morphological and virulence pattern. Recently, Insawang (2008) investigated the same 25 *C. capsici* isolates from Farm 6 as in this study using AFLP analysis, and found a similar high level of diversity of the isolates within a farm.

In addition, the high level of diversity of *C. capsici* in Thailand could indicate the well adaptability of this fungus to the environment. Although *Colletotrichum* rarely has sexual reproduction in tropical weather (Sariah, 1989; Shin *et al.*, 2000; Sharma *et al.*, 2005). like Thailand, the large genetic variations could be due to asexual reproduction. This is because *Colletotrichum* reproduction is mainly or exclusively vegetative (Katan, 2000). Parasexual reproductions, by which a system of genetic recombination and exchange of genetic material would be anastomosis and heterokaryosis can occur within fungal heterokaryon, will likely lead to variation, and vegetatively compatible isolates are expected to be more similar to one other, thereby constituting a district genetic subpopulation with the species complex (Pihalla, 1985; Leslie, 1993). Therefore *Colletotrichum* can contain large diversity without sexual reproduction.

The inability to group the 45 isolates when combined conidia and appressoria data, indicated a large genetic diversity based on variation of conidia and appressoria.

Based on cluster analysis, there were some isolates appeared to be identical (haplotype). However, all the identical isolates from Farm 6 were not identical with AFLP analysis (Insawang, 2008). Therefore, the identical isolates may not be certainly identified as haplotype without molecular confirmations.

Genetic groupings of *C. capsici* did not correlate with geographical distribution of the isolates. This could be due to large genetic variations within the population. Also there were a limited number of isolates used in the study which may have not represented the accurate genetic diversity within discrete areas (on farm, between districts etc). There should be a need to assess a wider geographic distribution of isolates including from other countries to assess the differences in diversity over a wide geographical area.

2. Identification of pathotype of *C. capsici* in Thailand.

Three pathotypes (PCC1, PCC2, PCC3) of *C. capsici* were identified from 11 isolates collected in western and northern Thailand, based on qualitative differences in infection on *C. chinense* ‘PBC932’, ‘CO4714’. In contrast, Sharma *et al* (2005) reported the existence of 15 pathotypes of *C. capsici* from 35 isolates collected in the Himachal Pradesh area of northern India. These isolates were screened on nine varieties of *C. annuum* where ‘pathotype’ differences were based on quantitative differences in the level of infection between varieties. However, according to Taylor and Ford (2007) a pathotype is defined as a group of isolates distinguished from others of the same species by a qualitative difference in disease severity; whereas a quantitative difference in infection reflects the natural distribution of aggressiveness within a population, ranging from low to high. Therefore, it is most likely that Sharma *et al* (2005) only observed differences in aggressiveness between varieties and did not identify pathotypes of *C. capsici*. Besides, they only used *C. annuum* varieties to screen for differences in infection and this species is known to be very susceptible to anthracnose disease (Le Thi *et al*, 2005).

Nevertheless, knowledge of both the existence of pathotypes and the level of aggressiveness of the pathogen isolates is extremely important when choosing appropriate isolates to screen for resistance in plant breeding programmes.

Further work should include screening of more isolates, particularly from other chilli growing regions of Thailand, on these differential varieties to know actually how many pathotype of *C. capsici* in Thailand.

CONCLUSIONS

1. Morphological diversity of *C. capsici* in Thailand.

Six morphological characters including colony growth rate, surface mycelium, colony colors, mass conidia colors, conidia size, and appressoria size were studied in 45 *C. capsici* isolates. Each character showed different phenotypes as follows: 1) colony growth rate: slow, medium, and fast 2) surface mycelium : uniform (U), concentric rings (C), sector (Se), and irregular (I) 3) colony colors : whitish to grey (G) and whitish to brown (B) 4) mass conidia colors whitish mass conidia (W) and orange mass conidia (O). 5) conidia size were $20.6 \pm 1.6 \times 3.45 \pm 0.4 \mu\text{m}$ 6) appressoria size $8.1 \pm 0.8 \times 6.5 \pm 0.8 \mu\text{m}$.

Cluster analysis based on four characters (colony growth rate, surface mycelium, colony colors, and mass conidia colors) divided the 45 isolates into 16 groups with no correlation to geographic location. Diversity levels were detected with a similarity coefficient at 0.22 to 0.66.

Finally, combination of all morphology characters with conidia and appressoria data resulted in the isolates not grouping, indicating a large genetic diversity and variation on conidia and appressoria size. Based on the clusters of the isolates, there was no correlation with geographic location in farm, within district in western area, and within Thailand.

2. Identification of pathotypes of *C. capsici* in Thailand

Based on differential qualitative reactions on nine genotypes injected with 11 *C. capsici* isolates, their pathotypes were identified. PCC1: 6 isolates contained isolates that were able to infect all *C. annuum*, *C. frutescens* and *C. chinense*, but were not able to infect *C. baccatum*, PCC2 : 3 isolates were able to infect all *C. annuum*, *C. frutescens* and

‘C04714’ of *C. chinense*, but not the ‘PBC932’ nor all *C. baccatum*, and PCC3 : 2 isolates only infected *C. annuum* and *C. frutescens*, but did not infect *C. baccatum* and *C. chinense*.

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SCHOLARSHIP/AWARDS	<p>-Center of Excellence on Agricultural Biotechnology, Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education, Kasetsart University, Kamphaeng Saen Campus.</p> <p>-National Center for Genetic Engineering and Biotechnology/Nation Science and Technology Development Agency</p> <p>Montri, P., C. Kanchana-udomkan., P.W.J. Taylor and O. Mongkolporn. 2005. Morphological diversity of <i>Colletotrichum</i> in the west of Thailand. Abstract of the 2nd AgBiotech Graduate Conference, Bangkok, Thailand, 123.</p> <p>Montri, P., P.W.J. Taylor and O. Mongkolporn. 2007. Identification of pathotypes of <i>Colletotrichum capsici</i> in Thailand. Oral presentation of the 3rd AgBiotech Graduate Conference, Bangkok, Thailand</p> <p>Mongkolporn, O., N. Chuenpom, N. Khampeng, P. Montri, P. Mahasuk, S. Wasee and P. Taylor. 2008. Anthracnose disease of chilli pepper-breeding and pathogenicity. Oral presentation of the 5th Taiwan-Thailand Bilateral Conference, Pingtung, Taiwan, 79.</p> <p>Taylor, P, O. Mongkolporn, Than. P, Montri. P, Mahasuk. C, Rananthunge. N, Ford. R, Pongsupasamit. S, Hyde. K. 2007. Anthracnose disease of chilli pepper-diversity and pathotypes. Asian Mycology Congress.</p>

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Capsicum species responding to
Colletotrichum capsici, *C. acutatum*
and *C. gloeosporioides* the causal
agents of chili anthracnose in Thailand,
Horticultural conference; 27 - 30
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