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Full Length Research Paper

Morphological, pathological and molecular variability of *Colletotrichum capsici* causing anthracnose of chilli in the North-east of Thailand

Aphidech Sangdee*, Sarawut Sachan and Surasak Khankhum

Department of Biology, Faculty of Science, Mahasarakham University, Thailand 44150, Thailand.

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Anthracnose disease is one of the major economic constraints to chilli production in tropical and subtropical regions. Ten isolates of *Colletotrichum capsici* causing chilli anthracnose were collected from 10 provinces in the northeast of Thailand. The isolates were evaluated for their morphological and cultural characteristics, pathogenic variability on chilli fruits and genetic characterization using random amplified polymorphic DNA (RAPD-PCR). Based on the morphological traits and cultural characteristics of the *C. capsici* populations, 10 isolates were categorized into six groups. These were designated, respectively, as CC-I, CC-II, CC-III, CC-IV, CC-V and CC-VI. In Potato Dextrose Agar culture, most of the isolates produced cottony colonies. However, differences were obtained in colony color, shape and size of conidia. Based on the effect of carbendazim, 10 isolates were classified into two groups designated as highly resistant group (<40% inhibition) and highly sensitive group (>90% inhibition). Three virulence degrees of 10 isolates on chilli fruits were evaluated. Molecular polymorphism generated by RAPD confirmed the variation of the different isolates and they were grouped into two clusters. However, morphological, pathological and RAPD grouping of isolates suggested no correlation among the test isolates.

Key words: *Colletotrichum capsici*, morphological and cultural characteristics, pathogenic variability, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Chilli (*Capsicum annuum* L.) is one of the most popular and widely grown vegetables in the world and the most popular in Asia (Makari et al., 2009). However, a major problem for chilli production in tropical and subtropical areas is anthracnose disease (Sharma et al., 2005). Anthracnose, caused by *Colletotrichum capsici* is one of the most serious fungal pathogens of chilli in Asia. In Thailand, *C. capsici* is reported to be an important pathogen in chilli crop production areas (Than et al., 2008; Montri et al., 2009). This disease produces symptoms on leaves, stem and fruits and causes severe damage to mature fruits in the field. Moreover, during transit and storage, this disease also causes severe damage to chilli fruit (Mehrotra and Aggarwal, 2003). The virulence degree of disease symptoms on host plants depends on the fungal pathotype. Sharma et al. (2005) reported the existence of 15 pathotypes of *C. capsici* based on disease symptom development on inoculated fruit of *C. annuum* genotypes. Montri et al. (2009) showed virulent pathotype differences within *C. capsici* isolates based on percent lesion size, appearance of necrotic or water-soaked tissue and presence of acervuli on *Capsicum* species. Therefore, information on the distribution of race or pathotype in chilli growing areas and an accurate method for identification and characterization of *C. capsici* is necessary for effective disease management and development of host resistance in breeding programs

*Corresponding author. E-mail: aphidech_sangdee@yahoo.com.

Table 1. The list of *Colletotrichum capsici* isolated from chilli fruits in the north-east of Thailand.

Isolate	Location	Colony color	Colony morphology	Conidia shape and size	Morphology group	Growth reduction (%)	Virulence group	RAPD group
NK	Nongkhai	White	Zigzag cottony colonies	Fusiform, large	CC-I	9.81	CCP-II	A2
NB	Nongbualamphu	White	Zigzag cottony colonies	Fusiform, medium	CC-II	100.00	CCP-III	A2
SI	Sisaket	White	Zigzag cottony colonies	Fusiform, medium	CC-II	5.56	CCP-II	A1
SK	Sakhonnakon	White	Zigzag cottony colonies	Fusiform, small	CC-III	17.32	CCP-I	A2
KR	Nakornratchasima	Grey	Zigzag cottony colonies	Fusiform, large	CC-IV	100.00	CCP-I	B
KS	Kalasin	Grey	Zigzag cottony colonies	Fusiform, large	CC-IV	100.00	CCP-III	B
KK	Khonkaen	Grey	Zigzag cottony colonies	Fusiform, medium	CC-V	100.00	CCP-III	B
MK	Mahasarakham	Grey	Zigzag cottony colonies	Fusiform, medium	CC-V	22.16	CCP-II	A1
UD	Udon Thani	Grey	Zigzag cottony colonies	Fusiform, medium	CC-V	100.00	CCP-III	A1
RL	Roi-et	White	Circular cottony colonies	Fusiform, large	CC-VI	100.00	CCP-II	A2

(Sharma et al., 2005; Freeman et al., 1998; Kim et al., 2010).

Conventional methods for identification and characterization of *Colletotrichum* species are based on morphological characteristics such as size and shape of conidia, existence of setae or presence of a telomorph, and cultural characteristics such as colony color, growth rate and texture (Smith and Black, 1990). These criteria are not clear enough to differentiate *Colletotrichum* species because morphological and phenotypic variation among species is recognized under different environmental conditions (Martinez-Culebras et al., 2002). The random amplified polymorphic DNA (RAPD) technique has been used successfully to differentiate between *Colletotriichum* species infecting different hosts. Shin et al. (2000) used RAPD-PCR to detect variation among the *Colletotriichum* species, *Colletotriichum gloeosporioides, Colletotriichum coccodes, Colletotriichum dematium* and *Colletotriichum acutatum* isolated from capsicum in Korea and China.

Moreover, the genetic relationship bet-ween five morphological groups of *Colletotriichum capsici* was classified using RAPD analysis (Sharma et al., 2005).

Molecular techniques coupled with conventional methods have been developed for separating closely related species and providing new insight into their identification. rDNA sequences along with morphological/cultural data have been shown to provide the best approach to subgeneric classification within *Colletotrichum* among New Zealand fruit-rotting isolates (Johnston and Jones, 1997). Thus, the objective of the present work was to investigate the variability in *C. capsici* isolates infecting chilli in the north-east of Thailand based on morphological traits, cultural characteristics and molecular approaches.

MATERIALS AND METHODS

Collection and isolation of *C. capsici*

Samples were collected from anthracnose lesions on chilli (*C. annuum* L.) in 10 provinces from the north-east of Thailand. Isolation was carried out using the tissue transplanting technique. The samples were cut (5 × 5 mm^2) from the margins of infected tissue, surface sterilized by dipping in 1% sodium hypochloride for 2 min, and rinsed several times with sterile distilled water before being transferred onto the surface of water agar. The mycelium growing out of the plant tissue was sub-cultured to potato dextrose agar (PDA) and incubated at ambient temperature for 7 to 10 days. After confirming *C. capsici* by microscope examination, one monoconidial culture from each isolate was prepared and used in this study (Table 1).

Examination of morphological characteristics

The isolates were cultured on PDA at ambient temperature for 3 days, after which mycelial disks were cut from the colony margins and transferred to the center of a new PDA medium. The colony features of each isolate on PDA medium were examined daily for 7 days. After sporulation, the conidia were harvested from each isolate and mounted in water. The size and shape of twenty conidia were measured under a light microscope.

Effect of carbendazim on mycelial growth

The mycelial disks of all isolates were cut from colony margins and transferred to the center of PDA medium and and PDA medium containing 1,000 µg ml^{-1} of the active ingredient of carbendazim. The mycelial growth rate was calculated after 10 days. The percent reduction in

mycelial growth on PDA medium containing carbendazim was calculated using the procedure described by Kumer et al. (2007). All tests consisted of four replicates.

Pathogenicity test

Five chilli fruit, at a mature stage from a local market were surface sterilized in 5% sodium hypochloride solution for 5 min, washed with sterile distilled water, blotted dry on sterilized filter paper and pin pricked gently with a sterilized needle prior to inoculation. Inoculum of the isolates was prepared by culturing on PDA medium until sporulation. Conidia were harvested by flooding the cultures with sterile distilled water. The concentration of propagules in suspension were standardized with the aid of a hemocytometer to 1 × 10^6 conidia ml^{-1}. Twenty microlitre of the conidia suspensions were applied to a pin pricked wound. The inoculated fruits were incubated in a humidity chamber and kept at ambient temperature. The disease was estimated by visual observation based on the acervulus development time on inoculated fruits. The disease was scored on a 0 to 3 point scale, where 0 was no infection (non-virulence), 1 was a small lesion and no acervulus development 10 days after inoculation (mild virulence), 2 was a large lesion with tissue collapse and acervulus production 5 to 9 days after inoculation (moderate virulence) and 3 was a large lesion with tissue collapse and acervulus production 1 to 4 days after inoculation (severe virulence).

Random amplified polymorphic DNA (RAPD) analysis

The isolates were cultured in potato dextrose broth (PDB) at ambient temperature for 5 days. Mycelia harvested from the PDB were homogenized in a microcentrifuge tube and genomic DNA was extracted using the procedure described by Abd-Elsalam et al. (2003). The final DNA pellet was re-suspended in 4 0µl of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.4) and stored at -20°C.

For RAPD analysis, the primer PELF (5'-ATA TCA TCG AAG CCG C-3'), URP1F (5'-ATC CAA GGT CCG AGA CAA CC-3') (Kang et al., 2002) and OPA03 (5′CAG GCC CTT C 3′) were used. PCR amplification was performed using a ABI thermal cycler and a 30 ∝l total volume containing 2.5 units of *Taq* polymerase (Promega, USA), 3 µl of 10X buffer, 2 µl of 2.5 mM $MgCl_2$, 1 µl of 2.5 mM dNTP, 3 µl of 10 ∝M primer, 5 µl of genomic DNA and sterile distilled water. The PCR conditions consisted of predenaturation for 4 min at 94°C followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 58°C (PELF and URP1F) or 36°C (OPA03) and extension for 2 min at 72°C with a final extension for 7 min at 72°C. The PCR products were electrophoresed on 1% agarose gels in TBE buffer (90 mM Tris-Borate, 1 mM EDTA, pH 8.0). The presence (1) or absence (0) of each RAPD band of particular molecular weight in all isolates was scored and entered in a computer file as a binary matrix. Similarity coefficients for all pairwise combinations were determined using Dice's coefficients in the SIMQUAL program of the NTSYS-PC package (Rohlf, 2009) and clustered by unweighted paired-group using arithmetic averages (UPGMA) by means in the SAHN program of the NTSYS-PC package.

RESULTS

Examination of morphological characteristics

The isolates of *Colletotrichum* sp. which were isolated from the lesions on chilli were identified as *C. capsici* based on size and shape of conidia. The isolates of *C. capsici* showed differences in morphological characteristics (colony color, colony diameter and conidial shape and size), ten isolates were assigned to six morphological groups (CC-I to CC-VI). Various isolates produced cottony colonies on PDA with a color of grayish-white to dark grey on the ventral surface whereas the reverse of the colonies was mainly black. The colony diameter of different groups ranged from 65 to 80 mm after 7 days incubation. The colonies of group CC-I to CC-V produced zigzag cottony colonies whereas the isolate in group CC-VI possessed circular colonies. The conidia shape of the different groups was fusiform with both their ends pointed. Average length and width of conidia varied between 23.5 to 35.0 µm and 2.5 to 3.75 µm, respectively (Table 1).

Effect of carbendazim on mycelial growth

The isolates were classified into two groups based on their reaction to carbendazim fungicide. The first group were highly resistant (<40% inhibition) and consisted of the isolates SK, MK, SI and NK whereas the second group were highly sensitive (>90% inhibition) and consisted of the isolates UD, KK, NB, KS, KR and RL (Table 1).

Pathogenicity test

The variable pathogenicity of *C. capsici* isolates to chilli fruit was examined. The isolates were pathogenic and produced typical anthracnose symptoms e.g. small lesions and tissue collapse, acervulus production and sporulation on chilli fruits after inoculation. Various disease scores based on the acervulus development time on inoculated fruits were observed and categorized into three groups (CCP-I, CCP-II and CCP-III). CCP-I was designed a mildly virulent strain consisting of 2 isolates, KR and SK. Four isolates, NK, SI, RL and MK, were assigned to group CCP-II (moderately virulence), with the remaining isolates assigned to group CCP-III (severely virulent) (Table 1).

Random amplified polymorphic DNA (RAPD) analysis

RAPD analysis of 10 monoconidial isolates of *C. capsici* was conducted with the primers PELF, URP1F and OPA03. The generated fingerprints were evaluated for overall clearness of the banding pattern. The primers showed polymorphism and consistently produced 6 to10 bands of 0.2-2.5 kb. The RAPD patterns obtained with primers PELF and OPA03 generated a set of major amplicons that were shared by almost all the isolates, whereas the RAPD fingerprint with the primer URP1F was indicative of genetic variability within the isolates.

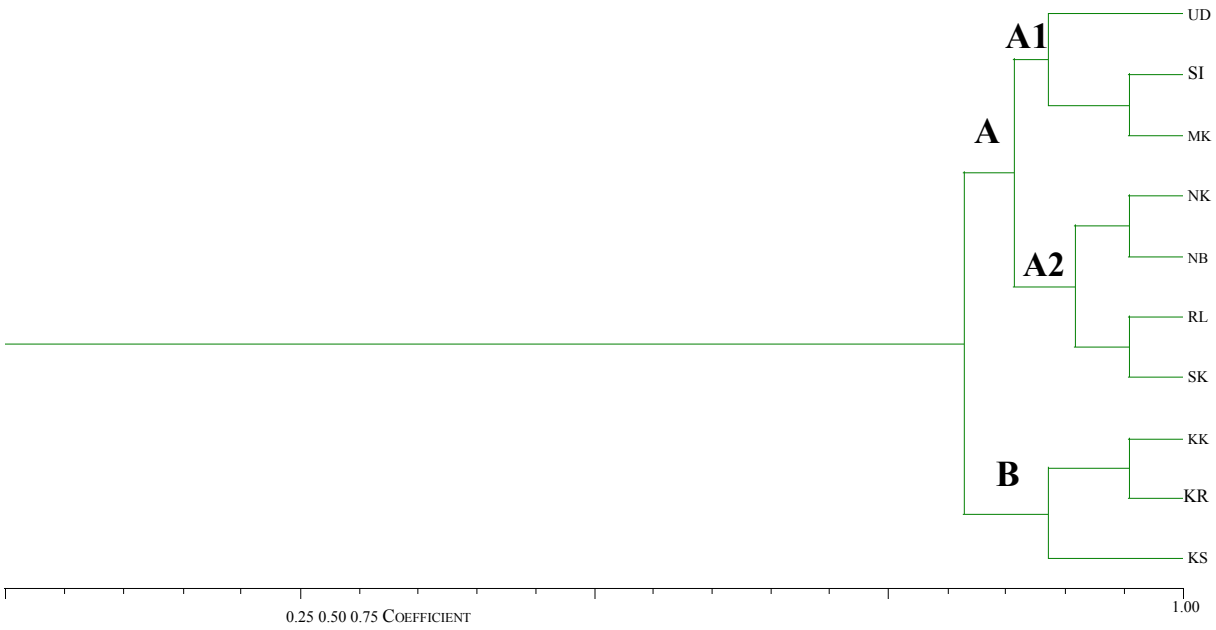

Figure 1. Dendrogram showing the relationship between the 10 *C. capsici* isolates. This was derived from cluster analysis of the RAPD allelic patterns.

The variable banding pattern for all the primers was used to determine genetic distance between isolates and to construct a dendrogram. In RAPD, the similarity coefficient of 0.81 was recorded among the tested isolates of *C. capsici* and two main clusters, groups A and B were obtained. RAPD group A was divided into 2 distinct groups, A1 and A2 at a similarity coefficient of 0.86. Group A1 consisted of isolates UD, SI and MK, whereas group A2 comprised of isolates NK, NB, RL and SK. RAPD group B contained the remaining isolates (Figure 1). However, 31.81% polymorphism was found, indicating that all isolates used in this study have approximately similar genetic identity.

DISCUSSION

Anthracnose disease is responsible for major economic losses in chilli production worldwide, especially in tropical and subtropical regions (Pakdeevaraporn et al., 2005). In this study, *C. capsici* was confirmed as the species responsible for chilli anthracnose in Thailand by pathogenicity test. The pathogenicity study showed that the behavior of *C. capsici* isolates were homogeneous with regard to disease symptoms. However, variation in virulence or the level of disease (measured quantitatively) within the isolates was observed. Differences in aggressiveness of *C. capsici* isolates have been reported previously by Taylor et al. (2007).

Morphological grouping of *C. capsici* based on cultural morphology, spore shape and size showed an overlap in colony color and conidial shape and size among the six groups studied. This result was in agreement with a previous study by Hindorf (1973), who found a morphometric overlap of conidial size within *Colletotrichum* species. Moreover, Lardner et al. (1999) and Baxter et al. (1983) observed differences in colony colors of *Colletotrichum* populations.

There was no clear relationship between the six morphological groups and pathological groups. For example, isolates NB and SI were classified in the same morphological group but different in virulence groups. Combination of these two characteristics has been successfully used to categorize *Colletotrichum* species in the part. Thind and Jhooty (1990) successfully used morphological and pathological characteristics to categorize 150 isolates of *C. capsici* and *C. gloeosporoides* causing chilli anthracnose.

The dendrogram derived from RAPD divided the isolates of *C. capsici* into two clusters. These did not correlate with the data from cultural morphology and virulence patterns. These results are similar to previous studies in which RAPD analysis was shown not to correlate with growth rates in culture and geographic region of different *Colletotrichum* sp. isolates (Thottappilly et al., 1999; Sharma et al., 2005). However, the RAPD approach has been useful for proper identification and categorization of *Colletotrichum* sp. isolates (Balardin et al., 1997; Mesquita et al., 1998; Thottappilly et al., 1999). We conclude that *C. capsici* in the northeast of Thailand consists of variable populations based on cultural morphology, reaction to carbendazim, virulence pattern and RAPD analysis. Molecular phylogenetic grouping obtained by RAPD analysis did not correlate with

morphological characteristics and virulence pattern. However, RAPD analysis can be used to classify *C. capsici* more rapidly than these other methods. Therefore, molecular phylogenetic grouping based on RAPD analysis represents a powerful tool for studying genetic diversity in *C. capsici*.

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