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A spreading colony formed method for rapid evaluation of dicarboximide fungicides resistance level of field tobacco brown spot disease

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Abstract - A new method (spreading colony formed method) for rapidly identifying and evaluating the dicarboximide fungicides resistance level of field tobacco spot brown disease caused by *Alternaria longipes* was developed. Two typical colonies with distinct differences in colony morphology on media containing 5 μg/ml dicarboximide fungicides dimethachlon (CAS registration number: 24096-53-5) were discovered by using this method. The two typical colonies were named spreading colony and dense pad colony, respectively. Isolates (250) of *A. longipes* were quickly separated by this method, and their growth properties (including the sensitivity to dimethachlon, the cross-resistance to phenylpyrroles fludioxonil and hyphal development) were examined. Our results indicated that (1) monospore isolates from spreading colonies and dense pad colonies were respectively resistant and sensitive to dimethachlon; (2) resistant and sensitive isolates formed respectively spreading colonies and dense pad colonies on dimethachlon media. Furthermore, molecular experiments confirmed the spreading colony formed method reliable. In conclusion, field resistant isolates and resistant situation in population level of field tobacco spot brown disease could be exactly and timely determined and evaluated by spreading colony formed method.

Key words: *Alternaria longipes*; spreading colony; dense pad colony; dicarboximide fungicides.

INTRODUCTION

Brown spot disease, caused by *Alternaria longipes*, as one of the most common and destructive diseases of tobacco, results in significant loss every year in Yunnan, China. The application of fungicides, such as dicarboximides, phenylpyrroles, carbamates, benzimidazoles and triazoles, is the most common tactic used to reduce losses to this disease. Though these fungicides can effectively control the spread of the disease especially in the beginning, the increasing prevalence of the disease is observed all over the world. The decline of fungicide in control effect might be related the emergence of fungicide resistance. To date, field resistance to dicarboximide fungicides (DCFs) has been reported in a wide range of phytopathogenic fungi including *Alternaria alternata* (Hutton, 1988), *A. longipes* (Laidou *et al*., 2000; Luo *et al*., 2008), *Botrytis cinerea* (Pommer and Lorenz, 1982; Beever *et al*., 1989), *Monilinia fructicola* (Ritchie, 1982), *Penicillium expansum* (Rosenberger and meyer, 1981), *Sclerotinia homoeocarpa* (Detweiler *et al*., 1983), and *Sclerotium cepivorum* (Littley and Rahe, 1984).

Rapid and effective methods for detecting field DCFs resistance are much necessary. At present, the molecular basis of DCFs resistance in fungi is not completely understood. In the basidiomycete *Ustilago maydis*, the *adr-1* gene, coding for a serine/threonine protein kinase, and the *ubc1* gene, coding for the regulatory subunit of a cAMP-dependent protein kinase, were thought to confer its DCFs resistance (Orth *et al*., 1995; Ramesh *et al*., 2001). In addition, various mutations in the six 92-amino-acid repeat domain (AARD) of the group III two-component histidine kinase (HK) were responsible for many filamentous fungi DCFs resistance (Fujimura *et al*., 2000; Cui *et al*., 2002; Yoshimi *et al*., 2004; Avenot *et al*., 2005; Luo *et al*., 2008). In light of the complexity of the DCFs-resistance mechanisms in phytopathogenic fungi, it is not easy to develop molecular methods for detecting DCFs-resistant fungal populations, and conventional techniques still have to be used. Currently, spore germination test or mycelial plug test (Beever *et al*., 1989) is adopted by many phytopathologists. However, these methods are tedious and time consuming if large numbers of samples need to be tested. Thus, the objective of this study was to develop a spreading colony formed method to detect rapidly and timely the DCFs-resistant level of field tobacco spot brown disease caused by *A. longipes.*

MATERIALS AND METHODS

Sample collection and spreading colony formed method. In 2007, 10 small pieces of tissue (0.5 x 0.5 cm) from each of five tobacco-producing regions of Yunnan province were taken from the margin of the tobacco

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TABLE 1 - Isolates and their dimethachlon-resistant levels distribution

Sample	Source	EC50	Numbers of isolates in different dimethachlon-levels*			
			S	LR	MR	HR
N-01 ~ N-50	Kunming, China	4.9 ~ 67.8	22	15	10	3
X-01 ~ X-50	Chuxiong, China	7.0 ~ 107.9	11	17	19	3
W-01 ~ W-50	Wenshan, China	4.1 ~ 71.4	27	18	4	1
D-01 ~ D-50	Dali, China	4.2 ~ 52.7	23	15	11	1
Q-01 ~ Q-50	Qujing, China	5.4 ~ 61.5	25	10	13	2
N-08	Kunming, China	33.7			1	
X-15	Chuxiong, China	107.9				1
W-03	Wenshan, China	4.1	1			
D-33	Dali, China	4.7	1			
Q-05	Qujing, China	9.4		1		

* S (sensitive): < 5 μg/ml, LR (low resistant): 5-10 μg/ml, MR (moderate resistant): 10-50 μg/ml, HR (high resistant): > 50 μg/ml (Shi *et al*., 2000).

brown spot lesion, and 50 pure cultures of the *A. longipes* were obtained according the method of Ma *et al*. (2003). In order to quickly detect whether resistant single spore isolate existed in a pure culture and evaluate the DCFs-resistant condition of field tobacco spot brown disease, a spreading colony formed method was developed. The method was carried out as follows. After the pure cultures had been incubated on Potato Dextrose agar (PDA) media for 12 days, their conidia were harvested and conidial suspension with a concentration of 1 x 10^6 conidia/ml was prepared. The conidia from each sample were spread on the surface of the PDA media containing 5 μg/ml DCFs dimethachlon (CAS registration number: 24096-53-5). After incubation at 28 °C for 5 days in dark, dishes with conidial suspension were examined with naked eyes and microscope, and colony characteristics were described. After 7 days of incubation, conidia were collected and 250 monospore isolates were obtained (Table 1). Two sensitive (W-03 and D-33) and three resistant (N-08, Q-05 and X-15) strains were selected randomly and submitted to the following molecular experiments.

Fungicides resistance testing. The sensitivity of the isolated pathogen (*A. longipes*) population to dimethachlon and fludioxonil was determined using mycelial growth assays. Mycelial plugs (6 mm diameter) of each isolate were removed from the edge of 7-day old culture and transferred onto PDA plates containing 1, 5, 10, 40, 80, 100 μg/ml dimethachlon or 1, 5, or 10 μg/ml fludioxonil, respectively. After incubated at 28 °C for 3 days in dark, the diameters of colonies were measured. Each experiment for a strain was carried out in triplicate. The 50% effective concentration (EC50) for each isolate was calculated using the method of Ma *et al*. (2006).

Determination of the relationship between DCFs resistance and colony morphology. Four grades, S (sensitive, $EC50_S$ < 5 μg/ml), LR (low resistant, $EC50_S$ ≥ 5 μg/ml and < 10 μg/ml), MR (moderate resistant, $EC50_S$ ≥ 10 μg/ml and < 50 μg/ml), and HR (high resistant, $EC50_S$ ≥ 50 μg/ml), were used to evaluate the isolates resistant levels according to the standard of Shi *et al*. (2000). Each of 5 isolates of S, LR, MR and HR were spread on 3, 5, 10, 30, 50, 80 μg/ml dimethachlon PDA media, respectively, and their colony morphologies were observed and characterized. Twenty single spore isolates from the dense pad colony and 30 single spore isolates from the spreading colony (each 10 strains from N, X, W, D and Q series in Table 1) were selected randomly and spread on 1, 5, 10, 30, 50, 80 μg/ml dimethachlon plates and their resistant levels and grades were evaluated.

Molecular experiments confirm the resistant and sensitive strains. W-03, D-33, N-08, Q-05 and X-15 were inoculated onto sterile cellophane placed on the surface of PDA media and incubated until mycelia covered the cellophane. The mycelia were then scraped off the cellophane and frozen in liquid nitrogen. Genomic DNA was extracted according to our previous study (Luo *et al*., 2008). A primer set (HKFwd 5′-CATGGCATTGAGGACATCG-3′ and HKRev 5′-CGAGCGTAAGTTGGGTCAT-3′) was designed according to the whole length *AlHK1* gene of the sensitive strain C-00 (GenBank accession numbers: DQ887538) and amplified the AARD of the *A. longipes AlHK1* gene using the pfu high-fidelity DNA polymerase (BBI, Canada). PCR products were cloned into a pMD18-T vector (Takara, Japan) and sequenced. Using the DNAman software package (Version 5.2.2, Canada), sequence of AARD was blasted with the whole length *AlHK1* gene of strain C-00 and their introns were confirmed. Finally, their deduced amino acid sequences were blasted.

RESULTS AND DISCUSSION

The characteristics of two types of colony

Through spreading colony formed method, we observed two types of colony were with distinct differences in colony morphology on the same plate (Fig. 1). The dense pad colony formed mainly by conidia and short-inflated hypha (B). Hyphal development was badly inhibited, and the mycelium was hardly observed under the light microscope, and there were many black spots on the surface of the plates when we examined with naked eyes. In contrast, the spreading colony formed mainly by mycelium, and hyphal development or colony morphology was normal (A). The main reason for this phenomenon would be that the DCFs play a role in inhibiting sensitive isolates mycelial growth but did not kill their conidia or prevent their conidial germination (Orth *et al*., 1994; Zhang *et al*., 2002; Iacomi-Vasilescu *et al*., 2004), while DCFs have no

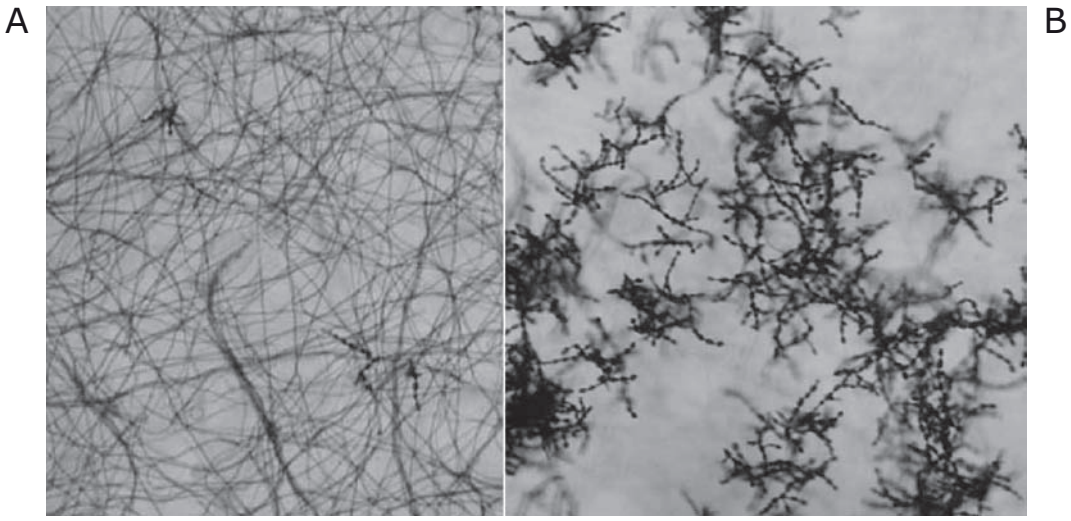

FIG. 1 - Mycelial characteristics of the two types of colony (10×). A: spreading colony, B: dense pad colony.

effect on whatever resistant isolates mycelial development and conidial germination.

Rapid identification different resistance to DCFs levels of *Alternaria longipes* isolates

EC50s in Table 1 showed that most field *A. longipes* isolates were resistant to dimethachlon, and some isolates' dimethachlon level even reached 100 μg/ml. In addition, all the resistant isolates showed a cross-resistance to fludioxonil, and can grow normally on media containing 5-10 μg/ml fludioxonil, whereas the sensitive isolates could not grow even on media containing 1 μg/ml (data not shown). Figure 2 showed the colony characteristics of single spore isolates from the two types of colony on the PDA media with 10 μg/ml dimethachlon and 5 μg/ml fludioxonil, respectively. All single spore isolates (represented by N-08, Q-05, and X-15) from spreading colony had an ability to grow and reproduce on media with dimethachlon up to 10 μg/ml. In contrast, W-03 and D-33 which from dense pad colony were completely inhibited by 5 μg/ml fludioxonil. In order to analyze the detail relationship between fungicide resistance and colony characteristics, two experiments was carried out as follows: 1) sensitive and resistant isolates were spread on various concentrations dimethachlon PDA media, and their colonies were observed under the light microscope; 2) isolates from two types of colony were tested for their dimethachlon resistance levels by mycelial growth assays. Interestingly, all S isolates formed spreading colony when exposed to less than 5 μg/ml dimethachlon, and dense pad colony was formed above 5 μg/ml dimethachlon, moreover, all R isolates formed spreading colony in the media containing above 5 μg/ml dimethachlon. This result indicated that 5 μg/ml dimethachlon was the dose differentiating the S from the R isolates. In addition, 10 μg/ml and 50 μg/ml were the EC_{50} values characterizing the increasingly resistant isolates among LR, MR, and HR isolates. That was, LR formed spreading colony on media containing less than 10 μg/ml and formed dense pad colony on media containing above 10 μg/ml; MR formed spreading colony under 50 μg/ml and formed dense pad colony above 50 μg/ml; HR formed spreading colony in all tested concentrations. Besides, isolates from dense pad colony and spreading colony were respectively sensitive and resistant to dimethachlon. These results showed that using the spreading colony formed method, DCFs-resistant levels (S, LR, MR or HR) of field tobacco brown spot disease could be evaluate rapidly according to the types of colony by spreading their spores on media containing 3, 5, 10, 30, 50, 80 μg/ml dimethachlon, respectively.

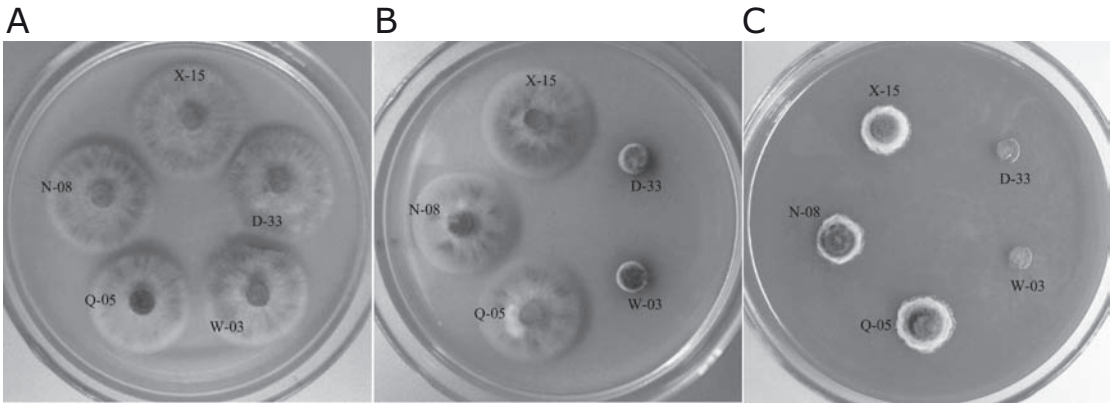

FIG. 2 - Morphological characteristics of resistant and sensitive isolates from the two types of colony on no fungicides (A), 10 μg/ml dimethachlon (B) and 5 μg/ml fludioxonil (C) PDA plates. D-33 and W-03 were from dense pad colony; N-08, Q-05 and X-15 were from spreading colony.

TABLE 2 - Comparison of the AARD of the *AlHK1* gene mutations among strains of *Alternaria longipes*

Strains	GenBank accession numbers	DNA sequence changed	Substitution type	Amino acid changed	Position in the AARD
W-03	FJ423766	c-t and a-t	Transition and transversion	—	—
D-33	FJ423765	t-c	Transition	—	—
N-08	FJ423767	g-a	Transition	G 239 S	44th in the 3rd repeat
Q-05	FJ423768	g-a	Transition	A 315 T	28th in the 4th repeat
X-15	FJ423769	g-a	Transition	E 332 K	45th in the 4th repeat

Sequence analysis of the AARD of the sensitive and resistant strains

A sequence comparison of the AARD of the represented strains (Table 2) revealed 2 and 1 bp differences between the sensitive isolates W-03, D-33, and C-00, respectively. However, all the base substitutions were silent, resulting in no amino acid changes among them. In addition, the resistant isolates showed a single base substitution in the AARD region, and these substitution cause one amino acid mutation. These results consisted with the DCFs-resistance molecular mechanism of many filamentous fungi and demonstrated the spreading colony formed method reliable.

Successful assessment and monitoring of the field overall resistance events of phytopathogenic fungi by spreading colony formed method

In our study, with the conventional mycelial plug test method, isolates from the samples with a 13-year history of DCFs applications were tested to be still very sensitive to DCFs (data not shown). This result seemed to be good news but it could not well explain why the tobacco brown spot disease showed a prevalent trend in the sample areas under the same farm management as the past. Using the spreading colony formed method, 142 DCFs-resistant isolates from the same sample was successfully obtained (Table 1). Why the two methods cause the results so much different? Many researches have described the difference in the ranges of fitness between the DCFs-sensitive and -resistant fungi (McPhee 1980; Northover 1983). In general, most studies showed that the resistant isolates can grow and form spores normally on the PDA plates containing up to 100 µg/ml DCFs, and their spore germination were beyond 90% while the spore germination percentages of the sensitive isolates were much lower. In contrast, on the free-DCFs media, the resistant isolates were not more competitive than the sensitive isolates in their ability to survive and reproduce. Thus, when the resistant mutations have occurred so seriously that affected the control effect but not increased to a dominant level in the population, the tissue isolation process on the free-DCFs media and the following monospore isolation process may result in a great increase of sensitive sub-colonies in frequency, which may lead to the above results using the conventional mycelial plug test method. However, the spreading colony formed method can successfully decrease magnifying the frequency of the sensitive sub-colonies. Using this method, people can not only obtain the resistant isolates but also monitor the resistance frequency on population level and offer early proofs for the field resistance assessment and management.

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