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Studies of a *Cercospora Nicotianae* Strain on Tobacco in China

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Abstract

A tobacco leaf spot disease first prevalent in Shandong province of China was investigated according to Koch's postulates. Fungal colonies on potato dextrose agar (PDA) were grey and velvet-like. The mycelium was initially colourless, but gradually turned dark brown and was septate. No spores were produced on PDA but the cultured mycelium was pathogenic to tobacco and produced reverse stick-shaped conidia with 3-20 diaphragms and its length was 256–806 μm × 4.1–19.5 μm. The conidiophore was straight, with 1–5 diaphragms and its length was 88–627 μm × 10.6–20.5 μm. Production of conidia was induced by adding tobacco leaf juice to oat agar culture and incubating in darkness at 27℃. Based on the morphological characteristics and ribosomal DNA-internal transcribed spacer (rDNA ITS) sequence analysis, the fungus was identified as *Cercospora nicotianae* (KM485926.1). The DNA (G+C) mol % was 51.86%, and fatty acid composition in cells was also examined. In bioassay, bordeaux, carbendazim and metalaxyl mancozeb showed good control against the pathogen.

Keywords: Cercospora Nicotianae; Identification; Control; Tobacco Frog Eye

1 INTRODUCTION

Tobacco is an important economic crop in China with a long history of cultivation. Fungal disease is one of the important factors causing yield and quality decline on tobacco. The State Tobacco Monopoly Bureau of China brief to "investigate harmful organisms living on tobacco" encouraged us to study the leaf disease first prevalent in Qingdao Tobacco Farm since 2012. The plant leaf disease first appeared in water-stained dark green spots, then gradually extended to circular, polygonal or irregular brown spots surrounded with a narrow yellow-green halo and finally became grey-white round spots with narrow and deep brown edges, scattered with small black spots. The disease symptoms were similar to the 'frog eye' disease, but differed from the typical 'frog eye' lesions (Zhu et al. 2001).

Tobacco frog eye disease generally occurs during the harvest period and seriously affects crop yield and quality. The morphological and biological characteristics of the pathogen have been reported in Nigeria, Zaire and India (Stavely and Nimmo, 1969; Alasoadura and Fajola, 1970; Pululu and Corbaz, 1989; Shamarao and Hundekar, 2010). Tobacco frog eye disease is prevalent in Southwest China including Hunan, Yunnan and Taiwan Provinces but less occurring in Northeast China (Fan and Chen 1994; He et al. 1996). The morphological characteristics of tobacco frog eye disease have not been reported in Shandong Province.

The disease has shown an increasing trend in recent years. During 2012 and 2014, the leaf symptom was more serious and occurred earlier in greenhouse than in field. The spots were then spread quickly from the bottom of leaves, lesion density was increased and consequently yield was lost.

To determine the pathogenicity of the disease, pure cultures from diseased leaves were isolated and 'Koch's rule' was used. Three fungicides were conducted in bioassay and control trials. Morphological characteristics and rDNA-ITS sequence of the disease are important information to provide early warning of the disease.

2 MATERIALS AND METHODS

2.1 Isolation and Culture of the Pathogen

Diseased plants of *Nicotiana tabacum* var. NC89 were collected in Qingdao Tobacco Farm of China using the tissue isolation method. The diseased leaves were washed and 2–5 μm junction tissues between the infected and uninfected tissue were cut out with a scalpel. These samples were sterilized in 75% alcohol for 10–15 s. After three-time washings in sterile water, the isolated tissue was cultured on PDA medium at 25℃ in the dark. The morphological characteristics were observed with time.

2.2 The Detection of Pathogenicity

To make an inoculation suspension, mycelia growing well on PDA plates were collected in sterile water (one 10-day colony of 35 mm in diameter with 20 mL water). All six expanding leaves of *N. tabacum* var. NC89 and *N. tabacum* var. Gexinsanhao plants were punctured with sterile syringes and were daubed with inoculation suspension. Uninoculated plants were used as a control. All plants were cultured at 25℃ with natural sunlight. The spot symptoms were recorded and the pathogen was isolated and cultured on PDA.

2.3 Identification of the Pathogen

2.3.1 Morphological Characteristics of the Pathogen

Pure pathogen isolates from the first disease spots and the inoculated diseased spots were selected. The fruiting bodies were placed on glass slides to observe the colony morphology, conidial fructification and conidial morphology under Nikon microscope.

2.3.2 Sporulation Condition Analysis

Purified cultured isolates on PDA were inoculated onto five different media (① PDA; ② PDA with 2% new tobacco leaf juice; ③ oat agar; ④ oat agar with 2% new tobacco leaf juice; ⑤ agar with 2% new tobacco leaf juice) (He et al. 1996). There were four replicates each treatment. The isolates were cultured at 27℃ in the dark for 7 d and colony growth and sporulation were observed. Then, one replicates of four plates from each treatment was placed in the dark at 18℃ for 7 d and growth and sporulation were observed regularly (Stavely et al. 1969). Colony diameters in different culture media were recorded after 14 d.

2.3.3 DNA-ITS Sequence Analysis of the Pathogen

Three well-growing pathogen samples (2012, 2013 and 2014) were cultured on 50 mL PDB medium (potato dextrose without agar) at 27℃ on one shaker incubator operated at 120 rpm/min for 7 d. The liquid culture was filtrated to obtain mycelia for genomic DNA extraction. Fungal ITS universal primers (5′-TCCGTAGGTGAACCTGCGG-3′) and (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the pathogen rDNA-ITS by PCR (Anjeneyulu et al. 1985). The PCR products were confirmed by 1% agarose gel electrophoresis, and sequenced by Shanghai Sangon Biological Engineering Technology Services Co. Ltd. The sequences were analyzed using the GenBank database. When rDNA-ITS sequence similarity reaches more than 99%, sequences can be identified as the same kind (Landeweert et al. 2003; White et al. 1990). A phylogenetic tree was constructed according to the sequence homology of the three sequences and other *Cercospora* spp. sequences from GenBank by the MEGA6.06 software.

2.3.4 FAME Analysis and G+C Content of Genomic DNA

The cultured strains in PDA medium were extracted for FAME (fatty acid methyl ester) analysis using an HP6890 gas chromatograph system (Agilent Technologies Co. Ltd., USA). The FAME composition was analyzed using the MIDI (Microbial Identification) Sherlock automatic fungi identification system (Newark, DE, USA), and the results were compared with the database information for the standard strain.

The (G+C) mol % was determined on Lambda35UV/VIS Spectrometer by using the melting temperature (T_m) method, with *Escherichia coli* (K12, CGMCC1.365) as the reference group. The temperature was control by the

PTP-1 digital temperature control instrument (PerkinElmer Co. Ltd., USA).

2.4 Chemical Control Experiments

Currently no registered fungicide for tobacco frog eye disease is available in China. Here, three fungicides registered for controlling fungal diseases of tobacco were tested to control the frog eye disease. The three fungicides were 80% Bordeaux wettable powder 600 times, 50% carbendazim WP 500 times and 68% metalaxyl mancozeb WG 500 times. Distilled water was used as a control.

2.4.1 Bioassay Experiments

In vitro bioassay experiments were used to determine the mycelial growth rate. Culture colonies on PDA at 10 d were first selected to punch 4 mm samples, and then switched to oat medium with a different fungicide. There were four replicates per treatment, each consisting of four plates cultured at 27℃ in the dark for 14 d. The diameter of each colony was recorded.

2.4.2 Field Experiments

Field experiments were performed on *N. tabacum* var. NC89 plants. Three replicates were arranged in a randomized block design. Tobacco leaves were sprayed with fungicides, twice at intervals of 7 d. The symptom grade of each leaf was recorded for all treatments 7 d after the last spraying. The symptoms were rated using the following scale with leaf blades as the grading unit: 0, leaves completely free of disease; 1, lesion area < 5% of the total leaf area; 3, lesion area 6–10% of the total leaf area; 5, lesion area 11–20% of the total leaf area; 7, lesion area 21–40% of the total leaf area; 9, lesion area > 40% of the total leaf area.

Data were calculated using the following formulae and analyzed with Duncan's new multiple range test using the DPS software.

$$\text{Morbidity percent} = \frac{number.of.diseased.plants}{number.of.total.plants} \times 100\%$$

$$\text{Disease severity} = \frac{\sum (disease.grage \times number.of.plants.in.each.grade) \times 100}{total.number.of.plants \times highest.disease.grade}$$

$$\text{Control effect \%} = \frac{disease.severity.of.CK - disease.severity.of.treatment}{disease.severity.of.CK} \times 100\%$$

3 RESULTS

3.1 The Pathogenicity of the Pathogen

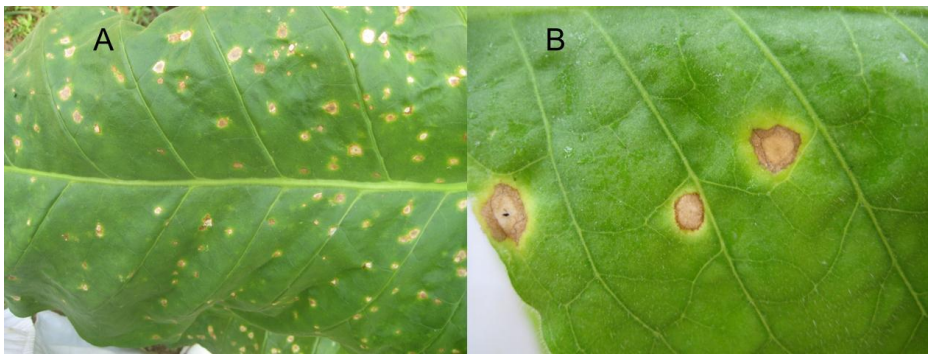

FIG. 1 SYMPTOMS OF TOBACCO FROG EYE DISEASE

(A) Symptoms on leaf; (B) magnification showing disease spots after re-inoculation with the mycelium.

The leaves of *N. tabacum* var. NC89 and *N. tabacum* var. Gexinsanhao were inoculated with hyphae cultured on PDA plates. The symptoms at 5 d after inoculating were the same as those first isolated in the diseased plants. The

leaves first showed water-stained dark green spots, then extended to circular, polygonal or irregular brown spots surrounded with a narrow yellow-green halo. Finally, the spots turned grey-white with narrow and deep brown edges. Small black dots were scattered in the centre of the spots. Disease spots were shown on all inoculated leaves and spots appeared earlier on the bottom of the leaves compared with the upper leaves (Fig. 1). The control plants were healthy. The black dots observed at the later stage in the disease spots were pathogenic conidia. The pathogen obtained from the re-inoculated leaves was consistent with the original pathogen.

3.2 The Morphology of the Pathogen

The 10-day colonies on PDA were 35 mm in diameter and appeared slow growth and grey and velvet-like colour in front view and grey-black in reverse side. The pathogen also expanded slowly on oat medium and appeared light grey. Initially, the hyphae were colourless and then turned dark brown, septate with a diameter of 6.2–14 μm. No conidia were observed on the two media but the hyphae had infectivity and caused identical symptoms. Identical conidial fructification and conidia in the inoculated spots were observed which was similar to *C. nicotianae*. The conidiophores were born in fascicles without branch but septate; the conidia had an inverted clavate form, slightly bent and tapering at the top, base truncate, with 3–20 diaphragms. The conidial length was 256–806 μm × 4.1–19.5 μm. The conidiophore was straight, with 1–5 diaphragms and its length was 88–627 μm × 10.6–20.5 μm (Fig. 2).

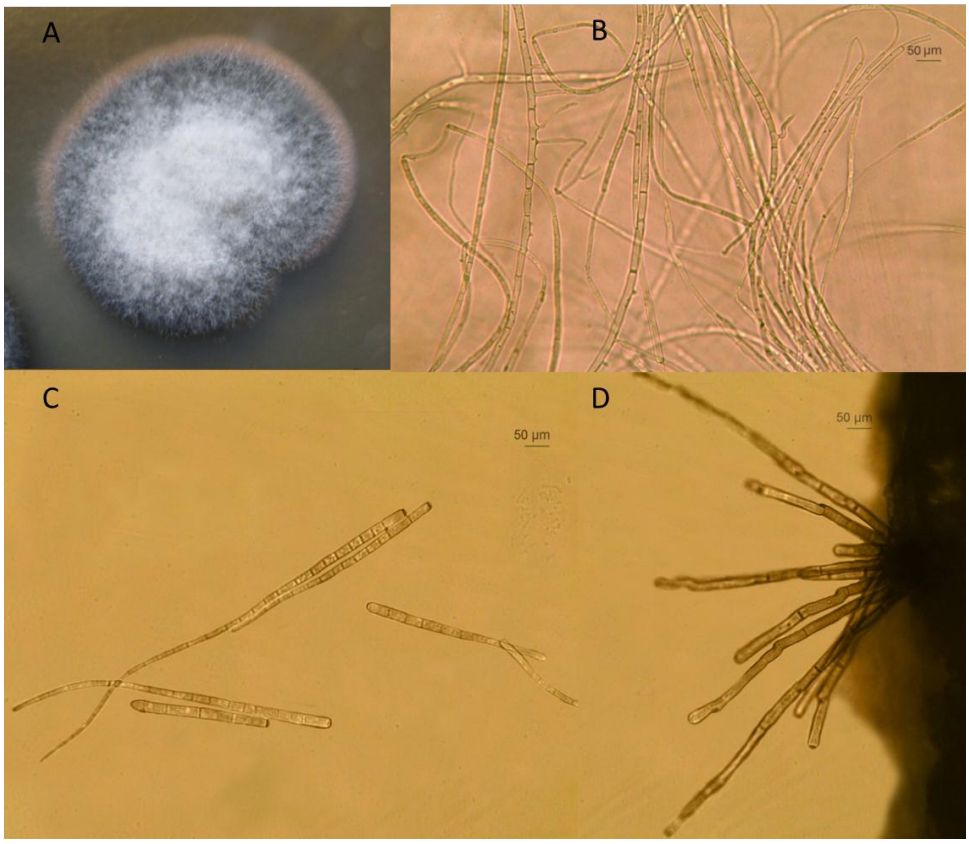

FIG. 2 MORPHOLOGY OF THE PATHOGEN CAUSING TOBACCO FROG EYE DISEASE

(A) Colony morphology on PDA; (B) mycelium on PDA; (C) conidia on diseased leaf, (D) conidiophore on diseased leaf.

3.3 Sporulation Conditions

Spores were produced only on ④ oat agar with 2% new tobacco leaf juice at 27℃ in the dark for 14 d, in which small light dots grew. No spores were observed on the other four media when cultured at 27℃ in the dark for 7 or 14 d. However, various colony morphology, size and the mycelial density were observed in different conditions. The diameter were 4.7 ± 0.1 cm, 5.2 ± 0.3 cm, 4.7 ± 0.2 cm, 4.3 ± 0.2 cm, 5.7 ± 0.1 cm at the five media (① PDA; ② PDA with 2% new tobacco leaf juice; ③ oat agar; ④ oat agar with 2% new tobacco leaf juice; ⑤ agar with 2% new tobacco leaf juice), respectively. The mycelium on PDA with 2% new tobacco leaf juice (media ②) grew the best,

with the largest colony and mycelial density, and with white velvet hyphae. The colony diameter on agar with 2% new tobacco leaf juice (media ⑤) was an average of 5.7 cm, significantly higher ($P < 0.05$) than on the other media (Fig. 3).

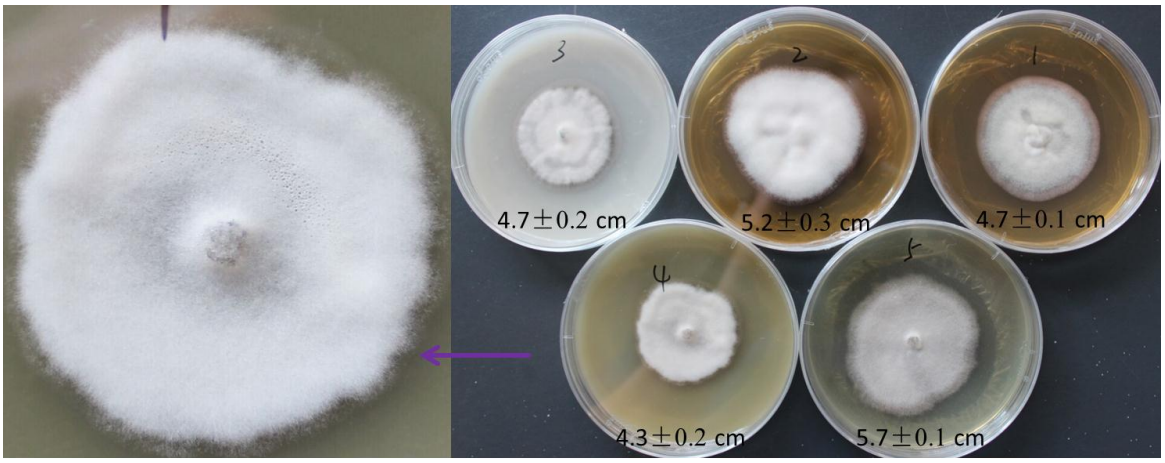

FIG. 3 COLONY MORPHOLOGY ON DIFFERENT CULTURES.

① PDA; ② PDA with 2% new tobacco leaf juice; ③ oat agar; ④ oat agar with 2% new tobacco leaf juice; ⑤ agar with 2% new tobacco leaf juice

3.4 Ribosomal DNA-ITS Sequence Analysis

The rDNA-ITS sequence length of the fungus was 540 bp. The blasted results in GenBank showed that the rDNA-ITS sequence had up to 100% homology with a variety of *Cercospora* spp. (*C. apii*, *C. zebrina*, *C. chrysanthemoides*, *C. kikuchii*, *C. achyranthis*, *C. beticola* and some other *Cercospora*. spp.).

Combined with its morphological characteristics in the host plant, the pathogen was determined to be *C. nicotianae*. The rDNA-ITS sequence of this strain was submitted to GenBank under the serial number KM485926.1. The phylogenetic tree showed that the identified strain KM485926.1 had very closest relationship with *C. nicotianae-2* and *C. nicotianae-3*. KM485926.1 and *Cercospora. apii* were in the same group, close to *C. kikuchii* and *C. chrysanthemoides*. The greatest distance was between KM485926.1 and the other *C. nicotianae*, possibly indicating different strains (Fig. 4). The DNA (G+C) mol % was 51.86%. The fatty acid profile showed 1.05% C16:1w7c/C16:1w6c, 38.72% C16:0, 42.74% C18:0antei/C18:2w6,9c, 1.52% C18:1w7c and 13.44% C18:0, respectively.

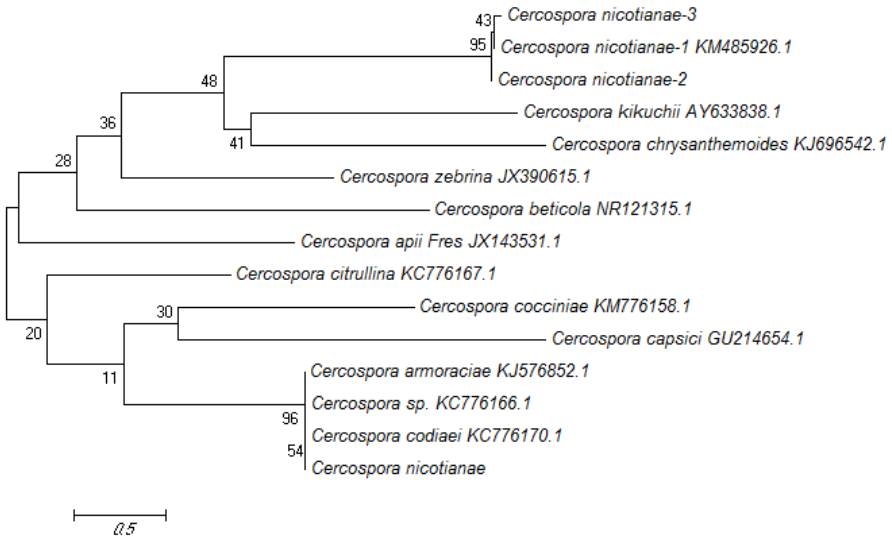

FIG. 4 NEIGHBOR JOINING PHYLOGENETIC TREE

3.5 Chemical Control

After *in vitro* bioassay experiments at 27℃ in the dark for 14 d, the average diameter of the control mycelium without fungicides was 39 mm. The mycelium did grow a little after chemical treatment with 80% Bordeaux wettable powder, 50% carbendazim WP or 68% metalaxyl mancozeb WG, showing the same colony diameter of 4 mm as the original. All three fungicides had a control effect on mycelium growth, with an inhibition rate of 100%. The average morbidity and disease severity with the three fungicides treatments were significantly lower than control treatment ($P < 0.05$). The control effects reached 62.6%, 64.1% and 67.3%, respectively (Table 1).

Table 1 Control effects of three fungicides on the disease

Treatments	Morbidity percent (%)	Disease severity	Control effect (%)
80% Bordeaux WP 600 times	72.9±8.1 b[z]	23.6±3.0 b	62.6±6.1 a
50% carbendazim WP 500 times	69.8±2.8 b	22.8±1.6 b	64.1±1.0 a
68% metalaxylmancozeb WG 500 times	65.8±2.8 b	20.7±3.5 b	67.3±1.3 a
The distilled water (control group)	98.2±0.8 a	63.4±3.5 a	-

[z] Data are the mean of three replicates ± standard deviation. Means ± SD within a row followed by the same letter are not significantly ($P<0.05$) different based on Duncan's new multiple range test.

4 DISCUSSION

Some controversy exists on the relationship between host range and classification in *Cercospora* spp. Some thought that the classification of *Cercospora* spp. should be mainly based on the host range, yet the limited host range causes that *Cercospora* spp. grows in a different genus within the same family (Guo, 1998; Guo and Liu, 2005). Some species or sprains in different plant hosts had the same morphology which can be determined as the same species. Up to 200 species of *Cercospora* were reported in China (Zhai, 2013).

In this study, the size of the conidia and conidiophores in different diseased leaf were various. Although the rDNA-ITS sequence in this study from tobacco showed 100% homology to *C. apii* and *C. zebrina* but only 99% to *C. nicotianae*, in combination with the morphological characteristics on host tobacco, it was still classified as *C. nicotianae*. The G+C mol % of pathogen DNA and FAME profile are important indexes for the identification of fungi. These data can be used as an important index to classify the pathogen.

The growth of *Cercospora* in culture was slowly. Although it was difficult to produce conidia, infectious hyphae could be produced. Production of conidia was induced by adding tobacco leaf juice to oat agar culture when incubated in darkness at 27℃. These results were consistent with the previous report (Alasoadura and Fajola, 1970; Fan and Chen 1994).

The fungi prefer an environment with high temperature and humidity. Mycelia can survive over winter in the soil, and conidia can spread by wind and rain (Liu and He, 2000; Fan and Chen, 1994). Our study also demonstrated that the symptoms showed up earlier in greenhouse than in the field, since the temperature and humidity in the greenhouse were more suitable for this fungi growth. There are no resistant or tolerate tobacco available so far. Twenty-two genus *Nicotiana* including *N. tabacum* var. NC89, Honghuadajinyuan and Gexinsanhao in our farm were susceptible to *C. nicotianae*. Anjeneyulu et al. (1985) found that *N. repanda* was highly resistant to frog eye disease by examining the resistance of 32 *N. repanda* varieties.

The frog eye pathogen is a weak parasite but with a wide host range and the disease could be effectively controlled with timely spraying of fungicides. Shamarao and Hundekar (2010) reported that fungicides hexaconazole, propiconazole or carbendazim could effectively control frog eye leaf spots on tobacco and improve the yield and quality of bidi tobacco. In this study, Bordeaux, carbendazim and metalaxyl mancozeb could potentially be sued to control the disease.

In summary, the tobacco leaf spot disease first prevalent in Qingdao Experimental Farm was identified as *Cercospora nicotianae* (KM485926.1) according to Koch's postulates and rDNA-ITS sequence analysis. It was difficult to produce spores, but the cultured mycelium was pathogenic to tobacco. Bordeaux, carbendazim and metalaxyl mancozeb showed good control against the pathogen.

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