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Biological characteristics and mating type distribution of *Phytophthora capsici* from China

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ABSTRACT. *Phytophthora capsici* from seven provinces of China were investigated for their mating type, hyphal growth, zoospore production, and virulence. All of the morphological characteristics and the results of polymerase chain reaction confirmed that these isolates were indeed *Phytophthora capsici*. The test of mating type showed that the mating types of 19 representative isolates from China varied. The hyphal growth and the amount of zoospores produced from these isolates differed and there was no evident relationship between them, which indicated the existence of genetic diversity among the isolates in China. Also, the isolates that were more virulent on the pepper cultivars that we checked produced more zoospores than other isolates.

Key words: *Phytophthora capsici*; Biological characteristics; Mating type; Polymerase chain reaction identification

INTRODUCTION

Phytophthora capsici is one of the most destructive plant pathogens, which caused billion dollar losses in crops in America (Tyler, 2002; Oelke et al., 2003). It is not host-specific to pepper; instead, it has a broad range of hosts including tomato, eggplant, cucurbits, and lima beans (Lamour et al., 2012). *P. capsici* has been reported worldwide; consequently it draws increasing efforts to understand the epidemiology, genetics, and mechanisms of virulence (Quesada-Ocampo et al., 2011). *P. capsici* is a diploid oomycete that is heterothallic, and reproduction between A1 and A2 mating types produces oospores for propagation and long-term survival. It is reported that the oospores in the field can persist for many years and are resistant to harsh environmental conditions (Bowers et al., 1990; Drenth et al., 1995; Lamour and Hausbeck, 2003). *P. capsici* produces a large number of sporangia on the surface of the infected host tissue when the conditions are favorable. The sporangia can spread during rain or irrigation and release zoospores under cold conditions, which can swim towards the host plants and colonize them. The disease symptoms differ according to the host and environment. However, on peppers, the disease symptoms appear mostly on the roots and crown and result in black or brown lesions at the soil line. Although reports of *P. capsici* in China are emerging (Zuo, 2001; Sun et al., 2008; Zhang et al., 2009), most of the reports are about isolates that were collected from a certain province, and no reports about the representative isolates throughout China exist. In order to investigate the diversity of *P. capsici* isolates in China, the mating types, hyphal growth, production of zoospores, and virulence were investigated in 19 representative isolates from different parts of China. Results from this study showed that there were isolates with the A1A2 mating type in some provinces like Yunnan. We also found that there was no evident relationship between the hyphal growth and the amount of zoospores produced, which indicated the existence of genetic diversity among the isolates in China.

MATERIAL AND METHODS

P. capsici isolates

P. capsici isolate YD was isolated from Qinghai Province and provided by Professor Quanhong Xue (College of Resources and Environment, Northwest A&F University); P3 was from Guizhou Province and was provided by researcher Xuehui Yang; ZLT was from Guangzhou Province and was provided by Dr. Zhijun Li (Seed and Seedling Centre, Guangdong Academy of Agricultural Sciences, Guangzhou); N-1 and N-4 were collected from the Province of inner Mongolia; P16 was from Sichuan Province; PS and P15 were from Shaanxi Province and were from our research group; Pc, which was used as a control in this study, was from South Korea and was from Plant Protection College of Henan Agriculture University; Yn-1, Yn-2, Yn-7, Yn-9, and Yn-10 were from Yunnan Province and were provided by Associate Professor Zhijian Zhao (Yunnan Academy of Agricultural Sciences); Pg-1 and Pg-3 were from Beijing; and Hx-5, Hx-9, and Hx-11 were from Hebei Province and were provided by Dr. Yang Bi (China Agriculture University).

Medium

Potato dextrose agar (*PDA*) *medium*

Two hundred grams of peeled and chopped potato were boiled for 30 min with about 600 mL Milli-Q water, 20 g glucose and 17 g agar were added, and the final volume was 1 L.

Carrot agar (*CA*) *medium preparation*

For 1 L CA medium, 200 g fresh carrots were peeled, chopped into pieces, and boiled for 1 h before centrifuging. Twenty grams glucose and 1 g agar were added to the supernatant and autoclaved.

PDA selection medium

Penicillin (50 mg/L) and rifampicin (100 mg/L) were added to PDA medium after the PDA medium was autoclaved and cooled to 60°C.

Purification and characterization of *P. capsici* isolates

Isolation of pure cultures of P. capsici

The isolates that we collected were purified according to the method described by Zhang et al. (2009). We grew them first on the selection PDA medium at 28°C for 3 days and then selected the mycelium at the tip to grow on PDA medium and preserve.

Morphological observation

Sporangia were induced by changing water intermittently as described by Zuo (2001). *P. capsici* were grown on PDA medium for 5 days, and 8-mm culture plugs were cut and transferred to a 7-cm plate before adding 20 mL distilled water. The water was changed 5 times every 30 min, and then 20 mL Petri nutrient solution was added and incubated at 25°C for 18-24 h. The morphology of the colonies and sporangia were observed, and the zoospores that were released by sporangia were used for the infection assay.

Confirmation of *P. capsici*

Preparation of mycelia

Nineteen *P. capsici* isolates were collected from 9 different pepper fields in China between 2006 and 2009 (Table 1). In order to confirm the genetic uniformity of these 19 *P. capsici* field isolates together with the two mating type reference isolates A1 (P_{991}) and A2 (P_{732}), cultures were grown on PDA medium by the method described by Bonnie (2008). *P. capsici* isolates were grown on PDA medium for 5 days, mycelia were picked and grown in 50 mL sterile liquid PDA medium at 27°C and 170 rpm for 72 h in the dark. The mycelia were

then dried and stored at -70°C before DNA extraction.

DNA extraction

The method used was described previously (Yue et al., 2000; Bonnie, 2008). The mycelia were ground by liquid nitrogen, and 0.1 g was added to 1 mL extraction buffer [7 M urea, 50 mM Tris-HCl, pH 8.0, 62.5 mM NaCl, 1% sodium dodecyl sulfate (SDS)] and mixed well before centrifuging at 12,000 rpm for 10 min. The supernatants were collected and centrifuged again at the same speed for another 10 min. Phenol, chloroform, and isoamyl alcohol were added (25:24:1) to the same volume ratio of the supernatant, mixed well, and centrifuged for 10 min at 12,000 rpm. The same volume of isopropanol and 1/10 volume of 3 M NaAc, pH 5.2, were added to the supernatant and placed at -20°C for 20 min before centrifuging at 12,000 rpm for 5 min. The DNA was collected and dissolved in 40 µL Milli-Q water, 1 µL 10 g/L RNase A was added, and the DNA was placed at 37°C for 30 min before storing at -20°C. DNA was used as template in PCR with the *P. capsici*-specific primers according to the method described by Ristaino et al. (1998).

Specific primers

The primers that were used in this study were described by Ristaino et al. (1998); we used the PCAP (5'-TAATCAGTTTTGTGAAATGG-3') and ITS1 (5'-TCCGTAGGTGAACC TGCGG-3') primers. The total volume of the PCR was 15 µL and included the following: 1.5 µL 10X 750 buffer, 0.3 µL 2.5 mM dNTP, 0.12 µL *Taq* DNA polymerase, 1 µL ITS1/PCAP (10 µM), and 1 µL template DNA (10 µM). The PCR procedure used in this study was the following: 94°C for 3 min; 35 cycles of 94°C for 50 s, 56.0°C for 40 s, and 72°C for 1.5 min; and 72°C for 7 min. PCR products were run on a 1% agarose gel. A no template control was included as an empty control.

Biological characterization of *P. capsici* isolates in China

Detection of hyphal growth

P. capsici were grown on PDA plates for 7 days before being punched on a 5-mm disk and grown on fresh PDA plates. *P. capsici* were grown at 28°C in the dark, and diameters of the mycelia were measured each day (Guo et al., 2002). The average scores from 5 replicates were used as results.

Productivity of zoospores

P. capsici isolates were grown on PDA for 7 days and then were used to induce zoospores as described by Hou et al. (2007). The PDA plates, which were used to grow *P. capsici*, were then punched on 8-mm disks. We took 5 disks per plate and 3 plates per isolate. The 5 disks from 1 plate were then moved to a tube with 1 mL Milli-Q water and kept at 4°C for 30 min before being filtered. One droplet 0.1% aniline blue was added to the zoospore suspensions, and they were counted with a globulimeter.

The virulence of different *P. capsici* isolates

Cultivars

The P1, P12, P89, B9, B20, and A30 cultivars that were used in this study were provided by College of Horticulture, Northwest A&F University.

Detached leaf assay

The detached leaf assay was performed according to the method described by Li et al. (2007). The leaves that were used in this assay were from 1-month-old peppers. The zoospore suspensions were prepared as described by Hou et al. (2007). Ten microliters zoospore suspension with a concentration of 4 x 10^6/mL was inoculated on the lower side of pepper leaves. The leaves were kept in moisture with a temperature of 25°C and light of 3000 lx for 12 h/day. Disease rating was performed according to the method of Li et al. (2007): 0, no lesion; 1, lesion is limited within a diameter of 0.5 cm; 2, lesion diameter is larger than 0.5 cm but smaller than 1/3 of the leaf area; 3, lesion size between 1/3 and 1/2 leaf area; 4, lesion size between 1/2 and 3/4 leaf area; 5, lesion size larger than 3/4 leaf area. The average disease rating was calculated and used to classify the resistance: immune (I), disease rating (DR) = 1; highly resistant (HR), $0 \leq DR \leq 0.5$; resistant (R) $0.5 < DR \leq 1.5$; middle resistant (MR), $1.5 < DR \leq 2.5$; sensitive (S), $2.5 < DR$.

Mating type pairing test

The mating type of the 14 *P. capsici* isolates were determined by pairing them (Zheng and Lu, 1990; Zheng, 1995; Wang and Lu, 1997; Ma et al., 2003; Liu et al., 2008) with reference isolates A1 (P_{991}) and A2 (P_{732}), which were kindly provided by Dr. Yonggang Liu (Gansu Provincial Academy of Agricultural Sciences, Plant Protection Research Institute). The 8-mm disks of *P. capsici* isolates were placed on 1 side of the Petri dish with CA medium, and the reference isolate was placed on the opposite side. The plates were incubated at 18°C in the dark for about 20-25 days before observing the presence or absence of oospores by light microscopy. If there were oospores, the mating type of the tested isolate was recorded as the opposite of that of the reference isolate. There were 3 replicates for each isolate, and the experiments were repeated twice with the same results.

RESULTS

Confirmation of *P. capsici* isolates

In order to confirm that the 21 isolates (19 isolates listed in Table 1 together with the reference isolates A1 and A2) used in this study were indeed *P. capsici*, morphology characterization and PCR analysis were performed. Results showed that *P. capsici* grew rapidly on both PDA and CA plates, and many aerial hyphae were present. The shape of sporangia was mostly ovate or pyriform. Oospores were brown and round or oval. These morphology characteristics were typical of *P. capsici*. To further confirm these isolates, we isolated DNA

from them, and *P. capsici*-specific primers PCAP/ITS1 were used for PCR analysis. Results showed that all 21 isolates yielded the same 172-bp product, indicating that these isolates were *P. capsici* (Figure 1).

Table 1. Resistance of peppers to *Phytophthora capsici* isolates detected by the detached leaf assay.

Isolates	P1	P12	P89	B9	B20	A30
Yn-1	3.60S	1.75MR	3.87S	2.18MR	2.00R	3.70S
Yn-2	0.25HR	1.17R	2.31MR	2.58S	2.47MR	1.18R
Yn-7	1.30R	2.61S	2.36MR	1.46R	1.95MR	2.81S
Yn-9	1.10R	2.90S	4.00S	2.18MR	1.40R	3.85S
Yn-10	1.50R	4.30S	4.00S	4.30S	1.60MR	3.60S
N-1	3.00S	3.70S	3.18S	4.07S	1.50R	3.72S
N-4	4.11S	3.80S	3.70S	2.82S	2.60S	3.82S
Hx-5	1.78MR	2.40MR	3.30S	3.33S	2.00MR	3.60S
Hx-9	3.79S	1.77MR	2.4MR	1.40R	2.33MR	3.73S
Hx-11	2.01MR	1.56MR	2.84S	1.42R	1.50R	2.94S
Pg-1	3.55S	1.60MR	4.90S	3.20S	1.69MR	4.58S
Pg-3	2.85S	2.41MR	3.54S	2.68S	1.43R	2.97S
Pc	1.43R	2.38MR	2.73S	2.75S	2.31MR	3.51S
P3	1.52MR	1.69MR	3.67S	2.56S	1.95MR	2.87S
Ps	4.55S	3.50S	5.00S	4.50S	2.90S	4.30S
P15	4.00S	1.70MR	4.91S	1.56MR	2.80S	1.60MR
P16	0.78R	1.09R	3.01S	1.00R	1.10R	4.27S
YD	3.41S	3.90S	5.26S	2.64S	2.41MR	3.42S
ZLT	3.98S	2.65S	4.03S	4.57S	3.54S	2.59S

Immune (I), disease rating (DR) = 1; highly resistant (HR), $0 \leq DR \leq 0.5$; resistant (R), $0.5 < DR \leq 1.5$; middle resistant (MR), $1.5 < DR \leq 2.5$; and sensitive (S), $2.5 < DR$.

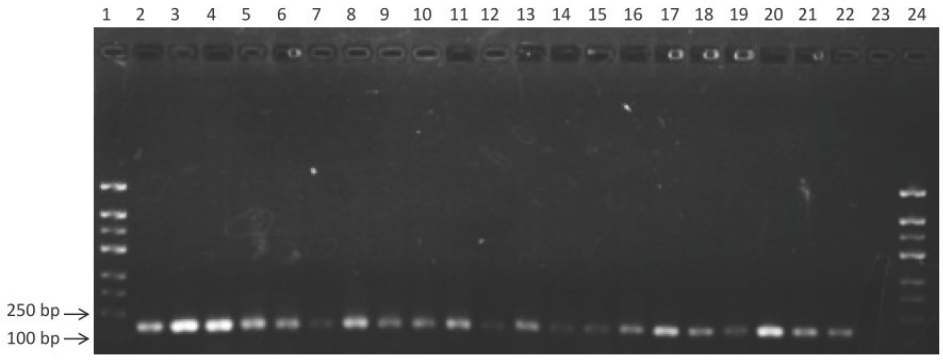

Figure 1. DNA amplified with the PCAP primer and ITS1 from *Phytophthora capsici*. *Lane 1* = molecular ladder; *lanes 2-22* = *P. capsici* isolates Yn-1, Yn-2, Yn-7, Yn-9, Yn-10, N-1, N-4, Hx-5, Hx-9, Hx-11, Pg-1, Pg-3, Pc, P3, Ps, P15, P16, YD, ZLT, A1, A2; *lane 23* = no template control; *lane 24* = molecular ladder.

Biological characterizations

Hyphal growth of different isolates

P. capsici isolates were grown on PDA plates for 4 days, and the diameter of the colony was measured. The diameter of colonies varied between 28.3 and 49.3 mm (Figure 2). Additionally, the colony diameters were different even for isolates from the same province (Figure 2).

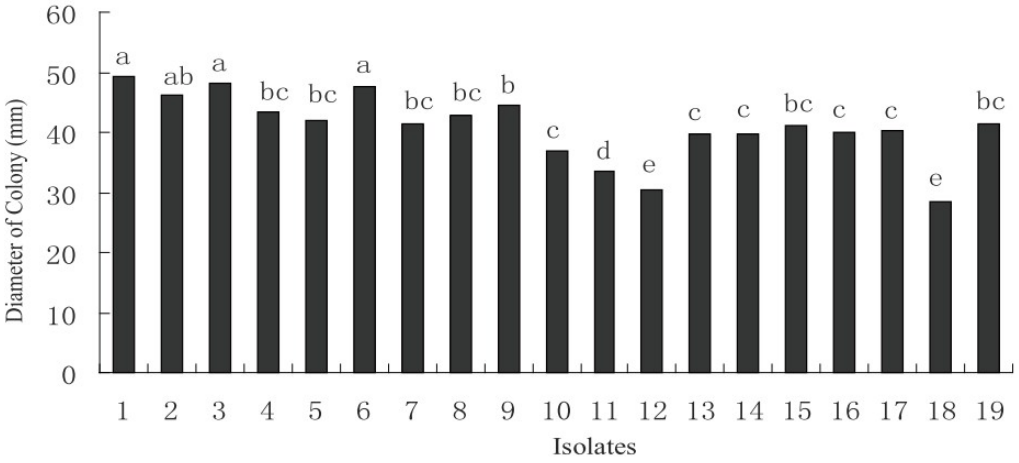

Figure 2. Colony sizes of various *Phytophthora capsici* isolates on PDA at 25°C for 4 days. Columns 1-19 = *P. capsici* isolates Yn-1, Yn-2, Yn-7, Yn-9, Yn-10, N-1, N-4, Hx-5, Hx-9, Hx-11, Pg-1, Pg-3, Pc, P3, Ps, P15, P16, YD, ZLT. Different characters above columns indicate the significant differences between isolates.

Production of zoospores

The zoospores from the 19 *P. capsici* isolates were counted, and there were significant differences between the isolates. Between 20,000 and 95,600 zoospores/mL were produced, and there was no relationship between zoospore production and hyphal growth (Figure 3).

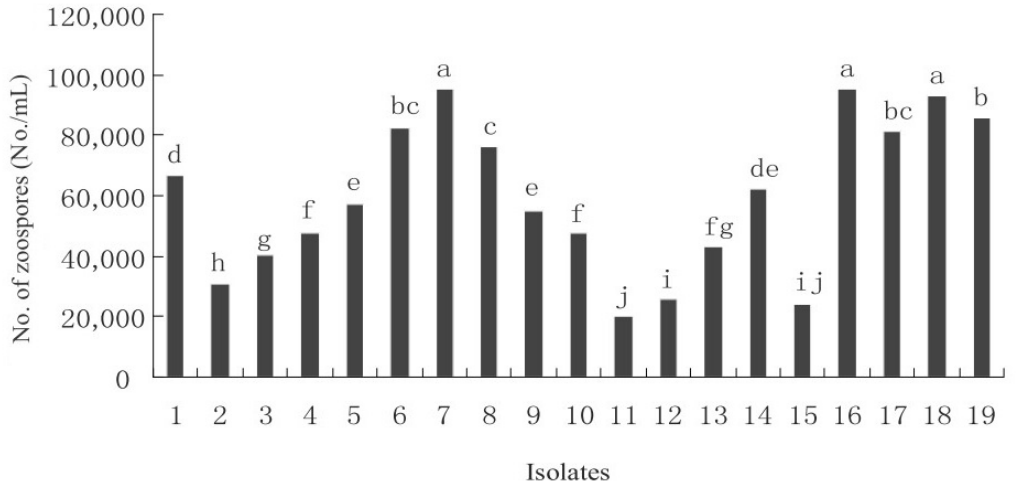

Figure 3. Zoospores production of various *Phytophthora capsici* isolates on PDA at 25°C for 7 days. Columns 1-19 = *P. capsici* isolates Yn-1, Yn-2, Yn-7, Yn-9, Yn-10, N-1, N-4, Hx-5, Hx-9, Hx-11, Pg-1, Pg-3, Pc, P3, Ps, P15, P16, YD, ZLT. Different characters above columns indicate the significant differences between isolates.

Virulence of different isolates

To investigate the virulence of the 19 isolates, 6 pepper lines were used for the detached leaf assay. Two isolates from Guangzhou Province, ZLT and YD, and 2 isolates from Inner Mongolia, N-1 and N-4, were virulent on most of the 6 pepper lines, while the other isolates were only virulent on some of the pepper lines (Table 1). Thus, we conclude that the most virulent isolates are from Guangzhou and Inner Mongolia. Additionally, we observed that isolates ZLT, YD, N-4, and N-1 also had higher zoospore production than other isolates.

Mating type determination

The results of the mating type determination showed that among the 14 isolates that we checked, 5 isolates were A2 type, 2 were A1 type, and 7 were self-fertile A1A2 type. All of the isolates from Yunnan were known to be self-fertile. These results showed that isolates of different mating types were present in a single province (like ZLT and YD from Guangzhou Province, Hx-5 and Hx-9 from Hebei Province, and N-1 and N-4 from Inner Mongolia). We also found that 18°C was the best temperature for this assay; when we increased the temperature to 28°C, no oospores were produced (Table 2).

Table 2. Origin and mating types of *Phytophthora capsici* isolates.

Isolates	Locality	Year	Mating type
YD	Guangzhou	2006	A2
ZLT	Guangzhou	2006	A1A2
P3	Qinghai	2006	A1A2
P15	Yangling	2007	A1A2
Ps	Yangling	2009	A1A2
P16	Sichuan	2006	A2
Pc	South Korea	2007	A1
N-1	Inner Mongolia	2007	A1A2
N-4	Inner Mongolia	2007	A2
Pg-1	Beijing	2007	A2
Pg-3	Beijing	2007	A2
HX-5	Hebei	2007	A1
HX-9	Hebei	2007	A1A2
HX-11	Hebei	2007	A1A2

DISCUSSION

P. capsici is a devastating oomycete pathogen that has a broad host range and is able to produce long-lived oospores during sexual production. It has extensive genotypic diversity and is easy to grow and mate in laboratory conditions. Consequently, this pathogen is becoming a model for investigating sexual reproduction, host range, and virulence.

In this study, we performed morphology characterization and *P. capsici*-specific PCR amplification to confirm that the isolates were indeed *P. capsici*. The biological characteristics demonstrated that these 19 isolates from 7 different provinces of China had different growth ability and zoospore production, which indicated a diverse genetic background. *P. capsici* is known to be a diploid oomycete that is heterothallic, and reproduction occurs between A1 and A2 mating types. However, 5 of 7 provinces in this study have self-fertile isolates. Among

these isolates that the mating type was checked, 50% were self-fertile isolates. The virulence of *P. capsici* was detected by the detached leaf assay, and results indicated that field isolates from Guangzhou Province and Inner Mongolia are more virulent than isolates from other places. However, because of the quantity of samples that was used in this study, further research needs to be done to collect more isolates throughout China to understand their genetic diversity, mating type, and virulence activities.

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