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Full Length Research Paper

Host range evaluation and morphological characterization of *Pseudoperonospora cubensis*, the causal agent of cucurbit downy mildew in Malaysia

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A total of 29 isolates of *Pseudoperonospora cubensis* were collected from various cucurbit farms in West Malaysia. Sporangia of 13 isolates had the ability to germinate at 14°C and were used for host range (pathotype) study using leaf disc assay on a set of twelve cucurbit cultivars. Twelve different pathotypes of *P. cubensis* were determined and this demonstrated that *P. cubensis* isolates from cucurbit farms in West Malaysia are highly variable. Based on the host range study, majority of *P. cubensis* isolates were categorized into medium and high pathogenicity groupings and this fact shows the potential of this pathogen in invading cucurbit fields in the tropical regions. However, the pathotypes could not be differentiated based on morphological characterization of the sporangia and sporangiophores. Therefore, molecular characterization of the 13 isolates of *P. cubensis* will be useful to study the relationships among them.

Key word: Oomycete, differential hosts, compatibility rating, pathogenic variation, sporangial germination.

INTRODUCTION

Pseudoperonospora cubensis (Berk. et Curt.) Rostow is the causal agent of cucurbit downy mildew. It is among the most well-known downy mildew pathogens, especially in wet and temperate regions, with worldwide distribution (Adam, 2008; Balass et al., 1992; Palti, 1974; Thomas, 1996). Over 50 cucurbit species are known as hosts of this oomycete (Lebeda and Widrlechner, 2003). Despite some success in controlling downy mildew in cucumber (*Cucumis sativus*), it has remained a serious problem for cucumber production (Shetty et al., 2002). It was suggested that the existence of different pathogenic races could have overcome the defense system of available resistant cultivars. Efforts to identify host range and pathotypes of *P. cubensis* are important to the management of downy mildew, especially for breeding resistant cultivars (Colucci, 2008).

This oomycete belongs to a group of "the highest risk pathogens" with high evolutionary potential (Sarris et al., 2008). Pathogenic and morphological variation of this oomycete appear to be correlated with host and environmental conditions (Lebeda and Widrlechner, 2003), and significant variation has been found at both the individual and population levels. Pathotype and race identification or host-parasite interaction of *P. cubensis* has been conducted by many researchers and various pathotypes of *P. cubensis* have been recorded in cucurbits in different countries (Cohen et al., 2003; Colucci, 2008; Shetty et al., 2002; Thakur and Mathur, 2002; Thomas et al., 1987). Thomas et al. (1987) and Thomas and Jourdian (1992) revealed five distinct pathotypes in the U.S. and Asia based on highly compatible reactions in producing symptoms on representatives of three cucurbit genera (*Cucumis sativus*, *Cucumis melo* var. reticulates, *C. melo* var. conomon, *C melo* var. *acidulous*, *Citrullus lanatus* and *Cucurbita* spp.). A sixth pathotype has been identified,

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Table 1. List of locations where *P. cubensis* isolates were collected.

Isolate number	State	Host
A1	Selangor	Cucumber
A7	Selangor	Cucumber
A9	Selangor	Luffa
A10	Selangor	Luffa
B1	Ipoh	Luffa
B2	Ipoh	Luffa
C1	Negri sembilan	Cucumber
C2	Negri sembilan	Luffa
C4	Negri sembilan	Cucumber
D1	Johor	Cucumber
D2	Johor	*Trichosanthes cucumerina*
D3	Johor	Melon
E1	Melaka	Melon

with a high compatibility rating on all hosts except *C. lanatus* (Cohen et al., 2003). Genetic knowledge of the interactions between an individual *P. cubensis* isolate and most important genera of cucurbits remain limited. Moreover, it was found that the populations of *P. cubensis* in Europe were very variable, 13 pathotypes were detected between 22 isolates of *P. cubensis* (Lebeda and Gadasova, 2002), and they usually did not match with the preliminary set designed by Thomas et al. (1987). Finally, Lebeda and Widrlechner (2003) proposed an improved differential set (twelve species) of cucurbit accessions for the characterization of pathogenic variability of *P. cubensis* isolates. However, they revealed that the pathogenic variation is still lacking at the race level.

To date, no systematic research in pathotype identification of *P. cubensis* has been reported in Malaysia. Hence, the aim of this study is to determine the pathotype of *P. cubensis* isolates collected from cucurbit farms in West Malaysia using the new set of differential cucurbit hosts proposed by Lebeda and Widrlecher (2003). In addition, the collected isolates were characterized based on morphological characters and the ability of the sporangial germination was evaluated.

MATERIALS AND METHODS

Collection of *P. cubensis* isolates

Leaves with typical symptoms of downy mildew were collected from the commercial production areas or greenhouses from Perak, Selangor, Melaka, Negri Sembilan and Johor states in West Malaysia. During the growing season between November 2008 and March 2009, 29 infected leaf samples were collected from *C. sativus*, *C. melo*, *Luffa cylindrica* and *Trichosanthes cucumerina* in the five states of West Malaysia and 13 of 29 isolates of *P. cubensis* had the ability to germinate and were used for subsequent studies (Table 1). The field samples were placed in plastic bags and stored in a cold container and transferred to the laboratory. The samples were then placed in moist conditions for sporulation in the dark at 14°C for 24 h.

Inoculum preparation

Sporangia were dislodged by spraying cool (4°C) purified water from the back of the infected leaves. The concentration of the sporangia was adjusted to 10^5 sporangia ml^{-1} and stored at 4°C before use.

Plant materials

The pathogenic variation of each individual isolate was conducted according to Lebeda and Widrlechner (2003) under greenhouse conditions and using twelve different cultivars including; four cultivars of *Cucumis* spp., four cultivars of *Cucurbita* spp., *C. lanatus*, *Benincasa hispida*, *L. cylindrical* and *Lagenaria siceraria* (Table 2). They were planted in 1L plastic pots filled with a sterile mixture of soil, sand and peat (3:2:1, v/v/v) maintained in the greenhouse under optimal conditions (27°C/15°C day/night cycle, daily watering, and weekly fertilization). No other chemicals were used except pesticide Decis (2.5% Deltamethrin EC) with 1 ml /L to control whitefly.

Reaction of differential cucurbit genotypes to *P. cubensis* isolates

A leaf disc assay was used to evaluate the differential cucurbit genotype responses to various *P. cubensis* isolates following the method described by Lebeda and Widrlechner (2003). Leaf discs were prepared from the oldest leaves of 3-6 true leaf stage (6-8 weeks old) using a 20 mm cork borer. Five discs from one leaf of each plant and three plants per isolate were tested. The leaf discs were placed abaxial surface facing upward on moist filter paper in Petri dishes. Four leaf discs were inoculated with four droplets (10 µl) of inoculum suspension. The fifth leaf disc, inoculated with water, served as a control. The Petri dishes were placed in an incubator at 14°C and in darkness to induce production of zoospores. After 24 h, the droplets containing sporangia were removed from the leaf discs using a sterile pipette (Colucci, 2008). The Petri dishes were then transferred to the same condition as mentioned above but in 12 h photoperiod. The evaluation of infection was rated five times, every two days from 6 through 14 days after inoculation using

Table 2. Differential set of cucurbit taxa for determination of *P. cubensis* pathogenic variability*.

No.	Taxon	Accession number		Cultivar name	Country of origin
		Donor	EVIGEZ		
1	*Cucumis sativus*		H39-0121	Marketer 430	USA
2	*C. melo* subsp. *melo*	PI 292008	H40-1117	Ananas Yoqne´am	Israel
3	*C. melo* subsp. *agrestis* var. *conomon*	CUM 238/1974	H40-0625	Baj-Gua	Japan
4	*C. melo* subsp. *agrestis* var. *acidulous*	PI 200819	H40-0611		Myanmar
5	*Cucurbita pepo* subsp. *Pepo*	PI 171622	H42-0117	Dolmalik	Turkey
6	*C. pepo* subsp. *texana*	PI 614687	H42-0130		USA
7	*C. pepo* subsp. *fraterna*	PI 532355	H42-0136		Mexico
8	*Cucurbita maxima*		H42-0137	Goliáš	Czechoslovakia
9	*Citrullus lanatus*		H37-0008	Malali	Israel
10	*Benincasa hispida*	BEN 485	H15-0001		USA
11	*Luffa cylindrical*		H63-0010		-
12	*Lagenaria siceraria*		H63-0009		-

*This set was provided by Prof. Lebeda, Czech Republic EVIGEZ - Czech GeneBank number- unknown.

a scale of 0-4 (0, no sporulation; 1, sporulation covering ≤ 25% of disc surface; 2, >25% to ≤50% ; 3, >50% to ≤75%; 4, >75%) (Lebeda 1991; Lebeda and Widrlechner, 2003). Percent of disease severity was expressed based on maximum scores according to the Towsend and Heuberger formula quoted by Urban and Lebeda (2007), as follows:

$$P = \sum \frac{(n \times v) \times 100}{x \times N}$$

Where, P is percent of the disease severity (DS), n the number of discs in every category of infection, v the category of infection (0-4), x the extent of infection scale and N the total number of evaluated discs.

Pathotype identification was done based on unique numerical codes (tetrade) for *P. cubensis* as described by Lebeda and Widrlechner (2003), and number of pathogenicity factor was determined based on the method by Lebeda and Urban (2004).

Determination of sporangial germination and measurement of sporangia and sporangiophores

The aim of this experiment is to determine the optimum temperature and media for germination of sporangia. Sporangia from isolate A1 were collected by spraying infected leaves with cool (4°C) purified water and the concentration was adjusted to 10^5 sporangia ml^{-1}. An aliquot of this suspension (100 µL) was loaded onto cavity-slides and incubated in Petri dishes on distilled water-saturated filter paper in the dark at temperature of 6, 10, 14, 18, 22, 28 and 32°C. Evaluation of germination was recorded after 4 h incubation. Sporangia were considered germinated if their colors changed from brown to light brown (translucent sporangia) or their cytoplasmic content was emptied (Kanetisac et al., 2009) with the persistence of the discharge pore at its end (Palti and Cohen, 1980). Germination percentage was then expressed as: number of germination percentage/total number of sporangia assessed) 100, using a light microscope.

To screen the best medium for sporangium germination, suspensions of sporangia were prepared by spraying the back of infected leaves with different sources of water, which is tap, rain, purified, distilled and pond water in separate containers. The percentage of sporangia that germinated was determined as described above. The experiment was repeated five times with three units per replicate. The length and width of 25 sporangia and sporrangiophores were measured using a light microscope and ocular micrometer.

Statistical analyses

All analyses were conducted using the statistical analysis system (SAS) version 9. Data was analyzed using standard analysis of variance (ANOVA). When the F values were significant, comparison of means was performed using the least significant difference value (LSD) at the significant level of P=0.05.

RESULTS

Reaction of differential cucurbit genotypes to *P. cubensis* isolates.

Table 3 showed the responses of differential cucurbit hosts to the 13 isolates of *P. cubensis*. Isolates D1 and D2 from Johor collected from Cucumber and *Trichosanthes cucumerina*, respectively, showed similar compatibility to all differential hosts except *C. lanatus*. Isolate E1 showed no symptoms with most differential genotypes except (1), (2), (4) and (5). *C. sativus* (differential genotype 1) and *C. lanatus* (differential genotype 9) presented the highest and the lowest compatible responses to all isolates tested, respectively. Thus, twelve pathotypes of *P. cubensis* were identified from 13 different isolates based on different genotype responses.

The degree of pathogenicity occurred for each isolate is shown in Table 4. Three obvious categories could be characterized based on responses to differential cucurbit genotypes. Isolate E1 was placed in low pathogenicity category (1-4), seven isolates (A7, A9, A10, B2, C2, C4 and D3) were in medium category (5-8) and five isolates (A1,

Table 3. Responses of differential cucurbit genotypes to isolates of *P. cubensis* two weeks after inoculation.

Isolate number	Differential genotype											
	1*	2	3	4	5	6	7	8	9	10	11	12
A1	+++	+	+	-	+	++	+	+	+/-	+++	++	-
A7	+++	+	-	na	-	+++	-	+	-	-	+++	-
A9	+++	+	-	-	+/-	-	-	++	-	+	+++	-
A10	+++	+/-	++	-	+++	+	-	-	na	-	+++	+++
B1	+++	-	++	+	+++	+++	+	+++	-	++	+++	++
B2	+++	+	+	-	+	-	-	+	-	+++	+++	-
C1	+++	+	+++	-	+++	+++	+++	+++	-	+++	+++	-
C2	+++	+	-	-	+++	+++	++	+++	-	-	+++	+
C4	+++	+	-	-	+	++	-	++	-	+	++	-
D1	+++	+	+++	+++	+++	+++	+++	+++	-	++	+++	++
D2	+++	+	+++	+++	++	+++	++	+++	-	++	+++	+++
D3	+++	+++	+++	++	-	+++	-	+	-	-	-	+++
E1	+++	+++	-	++	++	-	-	-	-	-	-	-

*Differential set of 12 genotypes of cucurbitaceous plants: 1, *C. sativus*; 2, *C. melo* subsp. *Melo*;, 3, *C. melo* subsp. *agrestis* var. *conomon*; 4, *C. melo* subsp. *agrestis* var. *acidulous*; 5, *C. pepo* subsp. *Pepo*; 6, *C. pepo* subsp. T*exana*; 7, *C. pepo* subsp. *Fraternal*; 8, *C. maxima*; 9, *C. lanatus*; 10, *B. hispida*; 11, *L. cylindrical*; 12, *L. siceraria*; - = no symptom; +/- = low compatible (1%<DS< 5%); + = sparse sporulation present (6%<DS<20%); ++ = moderate sporulation (21%<DS<49%); +++ = highly compatible more than 50% of leaf disc; na = not tested because of unavailable leaf at the time of assay.

Table 4. Responses of isolates of *P. cubensis* based on degree of pathogenicity.

P. F.***	Differential genotypes host												Tetrade code of pathotypes	Isolate
	1*	2	3	4	5	6	7	8	9	10	11	12		
	1**	2	4	8	1	2	4	8	1	2	4	8		
4	+	+	-	+	+	-	-	-	-	-	-	-	11.1.0	E1
5	+	+	-	na	-	+	-	+	-	-	+	-	3.2.4	A7
5	+	+	-	-	-	-	-	+	-	+	+	-	3.8.6	A9
6	+	-	+	-	+	+	-	-	na	-	+	+	4.3.12	A10
7	+	+	+	-	+	-	-	+	-	+	+	-	6.9.14	B2
7	+	+	-	-	+	+	-	+	-	+	+	-	3.12.6	C4
7	+	+	+	+	-	+	-	+	-	-	-	+	15.10.8	D3
8	+	+	-	-	+	+	+	+	-	-	+	+	3.15.12	C2
9	+	+	+	-	+	+	+	+	-	+	+	-	7.15.6	A1
9	+	+	+	-	+	+	+	+	-	+	+	-	7.15.6	C1
10	+	-	+	+	+	+	+	+	-	+	+	+	8.15.14	B1
11	+	+	+	+	+	+	+	+	-	+	+	+	15.15.14	D1
11	+	+	+	+	+	+	+	+	-	+	+	+	15.15.14	D2

*Differential set of 12 genotypes of cucurbitaceous plants: 1, *C. sativus*; 2, *C. melo* subsp. *Melo*;, 3, *C. melo* subsp. *agrestis* var. *conomon*; 4, *C. melo* subsp. *agrestis* var. *acidulous*; 5, *C. pepo* subsp. *Pepo*; 6, *C. pepo* subsp. T*exana*; 7, *C. pepo* subsp. *Fraternal*; 8, *C. maxima*; 9, *C. lanatus*; 10, *B. hispida*; 11, *L. cylindrical*; 12, *L. siceraria*; ** value of each genotype of cucurbitaceous plant (Lebeda and Urban, 2004). - = resistant, + = susceptible reaction, na= not tested because of unavailable leaf at the time of assay; ***, number of pathogenic factor.

B1, C1, D1 and D2) were in high category (9-12).

Determination of sporangial germination and measurement of sporangia and sporangiophores

Sporangial germination at different temperatures is shown in Table 5. The best temperature for sporangial germination was 14°C which also produced the highest germination rate at 56.3%. Zoospores were released from sporangia via discharge pore after 105 min (1 h and 45 min) and each sporangium produced 2-10 zoospores. Zoospores with two lateral flagellae remained active and swam for 120 to 180 min and then encysted.

Table 5. Effect of different temperatures on *Pseudoperonospora cubensis* isolate A1 sporangial germination after 4 h of incubation in purified water.

Temperature (°C)	Start of germination (min)	Germination (%)
6	0d	0d
10	145 ± 9.3b	30.4±9.7bc
14	105 ± 5.2a	56.3±9.5a
18	135 ±10.6b	48.7±9.4ab
22	170 ±18.7b	27.1±11.3c
28	240 ±21.3c	25±7.2c
32	0d	0d

Means followed by the same letter within a column are not significantly different at P = 0.05 using LSD. Data represented the mean of n=15 ± SD.

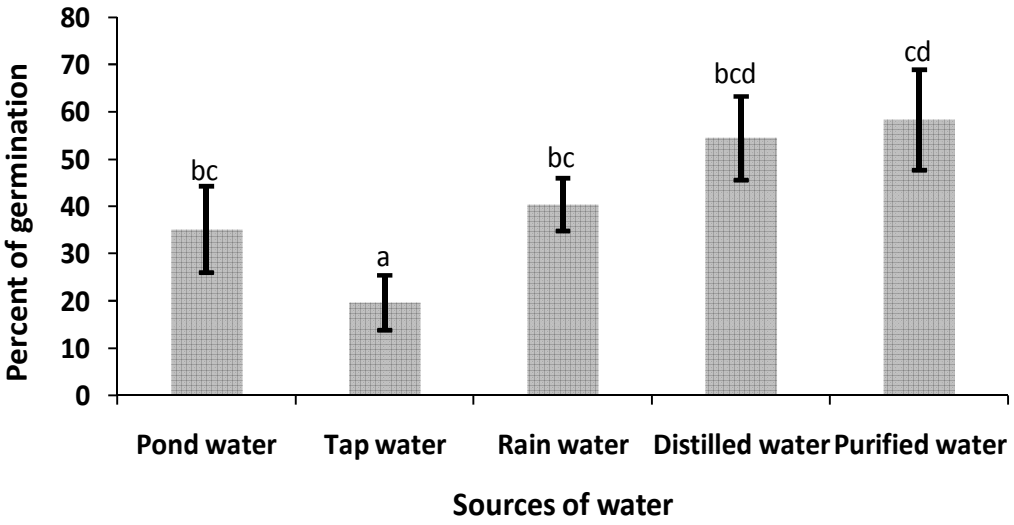

Figure 1. Effect of different sources of water on germination of *P. cubensis* isolate A1 sporangia at 14°C after 4 h of incubation. The same letter are not significantly different at P = 0.05 using LSD (n=15). Error bars represent the standard deviations.

Figure 1 shows the result of sporangial germination of isolate A1 with different sources of water. Sporangial germination was lowest in tap water (19.53 ± 5.8) and was significantly higher in purified (58.27 ± 10.62) and distilled (54.4 ± 8.85) water as compared to tap water. Germination in pond water (35.07 ±9.1) and rain water (40.35 ± 5.57) did not differ significantly from distilled water. The mean of sporangial germination in purified water was higher than distilled water with no significant difference between them.

There were significant differences in sporangiophore length among different *P. cubensis* isolates (Table 6). Isolates C1 and D3 had significantly, the longest (425.3 µl) and shortest (201.3 µl) sporangiophore, respectively, than that of the other isolates. However, there was no significant difference in the size of sporangia among different *P. cubensis* isolates (Table 6).

DISCUSSION

Most ecological and morphological studies on downy mildew species reveal that they are very host-specific; however, rare exceptions exist such as Pseudo-peronospora (Göker et al., 2007). The taxonomic and nomenclatural status of the genus *Pseudoperonospora* was reported rather controversial (Choi et al., 2005). The host range of *P. cubensis* is about 60 cucurbit species (Sarris et al., 2008). One of the important aspects of cucurbit downy mildew population biology study is the finding of host specificity among different cucurbits because host-restricted species are very dependent on

Table 6. Average measurement of sporangia and sporangiophores of *P. cubensis* isolates.

Isolate	Sporangia		Sporangiophore length (µl)
	Length (µl)	Width (µl)	
A1	28.83a	16.8b	272.7cde
A7	28.25a	16.9 b	252.7ef
A9	28.30a	17.2ab	256.5def
A10	28.37a	17ab	257.3def
B1	28.4a	17.5ab	221.3fg
B2	28.2a	17.9ab	224.6fg
C1	29.48a	16.9b	425.3a
C2	29.93a	17.3ab	291.3cd
C4	28.67a	17.7ab	398.0ab
D1	29.83a	18.3a	296.6c
D2	28.63a	17.6ab	380.0b
D3	30.17a	18.3a	201.3g
E1	30.00a	17.7ab	244.7ef

Means followed by the same letter within a column are not significantly different at P = 0.05 using LSD. Data represented the mean of n=25.

the abundance of their hosts. The main objective of this study was to determine host specificity between various isolates and different cucurbit genotypes. In our study, the pathotype of *P. cubensis* from different hosts were identical based on different cucurbit genotypes (Lebeda and Widrlecher, 2003) and 12 pathotypes from 13 isolates were found in five states. Lebeda and Paris (2004) during investigations in Czech Republic found 43 different pathotypes in the group of highly, medium and low pathogenic pathotypes. In USA, Colucci (2008) identified 32 different host range patterns in 32 different isolates by artificial infection on 12 cucurbit genotypes. Cucumber was the most susceptible while *C. lanatus*, *L. siceraria*, *L. cylindrical*, *B. hispida* and *Cucurbita pepo* var. *pepo* were the least susceptible differential genotypes. A report from Italy indicated downy mildew has appeared on cucumber and melon but not on squash and watermelon. Lebeda and Urban (2007) showed that in Central Europe and the Czech Republic, *P. cubensis* causes disease mainly on cucumber, muskmelon (*C. melo*), squash, pumpkin, watermelon and *Benincasa* but not with *Luffa* spp. However, Cohen et al. (2003) quoted *Luffa* spp. infected under field conditions in India and in 1999; a strong epiphytotic of downy mildew on *Luffa acutangula* was reported in China. In this study, the most compatible response following *C. sativus* was detected in *C. melo* subsp. *melo*, *L. cylindrical* and *B. hispida*. However *C. lanatus* had the lowest compatible reaction to all isolates tested.

Morphological analysis on cucurbit downy mildew isolates revealed that variability is based on the specimens, host genera or species, season of collecting, part of the plant (Choi et al., 2005) and environmental conditions (Lebeda and Widrlecher, 2003). Comparative analysis of morphology on different isolates indicated no significant differences in the size of sporangia among different isolates, however, the length of sporangiophore was different. Cohen et al. (2003) demonstrated that there was no morphological difference between pathotypes 3 and 6 in Israel on cucumber and melon. The same results were obtained by Colucci (2008) and Thomas et al. (1987).

P. cubensis has indirect germination by producing and releasing zoospore from sporangia. Water and temperature are the most obviously necessary external factors required to bring about germination (Blackwell and Waterhouse, 1931). The process of zoosporogenesis is started with cold shock in different range from 5 minutes for *Phytophthora palmivora* and 30-60 min in *Phytophthora infestans* (Walker and West, 2007). In this study, the optimum temperature for germination was measured as 14°C for A1 isolate of *P. cubensis*.

To research sporangial germination, it is essential to use water favorable for indirect germination. Distilled water has been considered by many researchers to be favorable for indirect germination, but this is contrary to the results of Sato (1994) as well as the present results. Sato (1994) demonstrated that washing of sporangia in distilled water caused loss of ability of sporangial to germinate in *P. infestans* and the ability was recovered by suspending sporangia in a water solution containing appropriate concentrations of inorganic salts. In our study, the most probable explanation to the effect of different source of water in sporangial germination, is that, inorganic salts content was varied in different sources of water.

Conclusions

The results of this investigation showed that there were high variabilities of *P. cubensis* pathotypes in West

Malaysia (12 pathotypes of 13 isolates in five states). The majority of the isolates obtained in this study were categorized in medium and high pathogenicity groupings, and this fact shows the potential of this pathogen in invading cucurbit fields in tropical regions. Morphological characterization indicated that there is no relation between pathotype and the size and shape of sporangia. Therefore, molecular characterization of the 13 isolates of *P. cubensis* will be useful to study the relationships among them.

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