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Full Length Research Paper

Characterization of powdery mildew in cucumber plants under greenhouse conditions in the Culiacan Valley, Sinaloa, Mexico.

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Powdery mildew is an important disease of cucumber and melon caused by the species of the order Erysiphales. In 1991, *Erysiphe (Golovinomyces) cichoracearum* was reported as the only causal agent of powdery mildew in Cucurbitaceae and other plant families in the Culiacan Valley center of Sinaloa Mexico. Due to the prevalence of powdery mildew on cucumbers grown in greenhouses and problems related to its control, the objective of this research was to characterize the causal agent of the disease. This research consisted of the light microscopy analysis of both its anamorph and teleomorph; the molecular analysis of the nucleotide sequence obtained from the region comprising the ITS1, 5.8 S rDNA and the ITS2, amplified by PCR, and the assessment of physiological races by the use differential melon cultivars growing in greenhouse and growth chamber. The powdery mildew growing on cucumber showed euoidium type conidiophore, crenated marginal outline and fibrosin bodies. Chasmothecia had large peridial cells, filamentous appendages, one ascus with ocular opening diameter of 20 µm, containing six to eight ascospores. These characteristics and the sequenced PCR amplified fragments matched with those described for *Podosphaera xanthii* that was composed of the physiological races 1, 2F, 4 and 5.

Key words: *Podosphaera xanthii*, powdery mildew, chasmothecia, *Golovinomyces cichoracearum*, physiological race.

INTRODUCTION

The state of Sinaloa, located in the North Western region of Mexico, is recognized for its high horticultural productivity mainly destined for exportation. Protected agriculture under greenhouse and shadow nets increased in the past ten years by a rate of 36% a year, from 224 ha in 2000 to 3389 ha in 2009 (CAADES, 2010). In 2009, cucumber was grown in 926 ha under protection and in 2100 ha in the open fields (CAADES, 2010). Even though greenhouse culture increases the production of cucumber, it also favors the development of fungal diseases such as powdery mildew (Jarvis et al., 2002; Lebeda et al., 2008). Powdery mildew is a disease caused by fungi of the order Erysiphales. These fungi cover the surfaces of leaves, petioles and stems, mainly in dicots (Takamatsu, 2004) forming white powdery colonies that reduce yield (Bolay, 2005), limit photosynthetic activity, cause plant senescence, and premature fruit ripening (Braun, 1987).

The most common Erysiphales species that infect cucurbits are *Podosphaera xanthii* (sect. *Sphaerotheca*) (Castagne) U. Braun and Shish. Comb. Nov. (Braun et al., 2001; Shishkoff, 2000); formerly referred as *Sphaerotheca fuliginea* (Schltdl: Fr.) Poll. Sensu lato (s. lat.), and two species of *Golovinomyces* namely; the plurivorous *G. orontii* (Castagne) Heluta (≡ *Erysiphe*

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orontii Castagne, = *E. polyphaga* Hammarl.) and *G. cucurbitacearum* (Zheng and Chen 1981), Vakal and Kliron (Vakalounakis and Klironomou, 2001), both previously referred to as *G. cichoracearum* (DC.) Heluta (≡ *Erysiphe cichoracearum* DC) (Jahn et al., 2002). These species need distinct ecological requirements (Lebeda et al., 2009).

Based on anamorphic characteristics, *G. cichoracearum* was previously identified as the only causal agent of powdery mildew in cultivated Cucurbitaceae and wild plants of diverse families in the Culiacan Valley at the center of the state of Sinaloa, Mexico (Cebreros et al., 1991). Later, *P. xanthii* was reported on Cucurbitaceae in Northern Sinaloa (Félix-Gastélum et al., 2005) and in the Culiacan Valley (Pérez-Ángel et al., 2010). However, no *G. cichoracearum* has been again reported in this state. The characteristics of the ascocarp (chasmothecium) (Braun et al., 2002) have mainly been used to identify and classify the species of the order Erysiphales. As it is not common to find such surviving structures in tropical and subtropical areas, (Álvarez and Torés, 1995; Lebeda et al., 2008), the Erysiphales have also been classified based on their anamorphic characters (Boesewinkel, 1980) that include their conidial germination patterns (Cook and Braun, 2009). Based on the analysis of the internal transcribed spacer region sequences (ITS) of ribosomal DNA (rDNA), including the 5.8S subunit, Saenz and Taylor (1999) found that the Erysiphales form six evolutionary taxa, more strongly correlated with the anamorph than with the ascoma morphology. These authors restructured the taxonomic classification of this group of fungi and situated the genera *Cystotheca*, *Podosphaera,* and *Sphaerotheca* in the same evolutionary branch due to the presence of fibrosin bodies in their catenate conidia (Braun, 1987). More recently Mori et al. (2000) based on the sequence of nucleotides of the ribosomal subunits 18S, 5.8S, 28S and the two ITS regions, found that this order was divided in five major lineages, having the genus *Uncinula* as the base. Hirata and Takamatsu (1996) found that the rDNA of the Erysiphales can be studied either on conidia or chasmothecia, because the nuclear rDNA is highly conserved between the teleomorph and the anamorph. Now the identification of *P. xanthii* using molecular techniques can be done without the presence of chasmothecia (Saenz and Taylor, 1999). Based on the nucleotide sequence of the ITS rDNA, Chen et al. (2008) designed the specific primers pairs S1/S2 and G1/G2 to identify the species *P. xanthii* and *G. cichoracearum* after the amplification and sequencing of the specific PCR obtained fragments of 454 and 391 base pairs (bp), respectively.

The name *Podosphaera fuliginea* (≡ *S. fuliginea*) was previously used in a very broad morphological sense covering the hole complex of *Podosphaera* (*Sphaerotheca*) species with large peridial cells (=*Podosphaera* sect, *Sphaerotheca* subsect. *Magnicellulatae*), but now this species is confirmed to collections on *Veronica* species as hosts (Shishkoff, 2000; Braun et al., 2001) which are morphologically, biologically and genetically quite distinct from collections referred to as *P. xanthii*.

Braun et al. (2001) differentiated *P. fusca* from *P. xanthii* based on the ocular diameter of their asci, which averaged 10 and 19 µm, respectively. However, some authors continued to report *P. fusca* as synonym with *P. xanthii* (Pérez-García et al., 2009), although, molecular sequence analyses clearly confirmed that at least two species are involved (Ito and Takamatsu, 2010).

In Israel, the United States of America, and Mexico, powdery mildew of cucurbits was often identified as *G. (E.) cichoracearum* s. lat., but it was actually *P.* (*S.*) *fuliginea* s. lat. (McCreight, 2004). However, both species may occur on cucurbits, as is reported in the Czech Republic (Lebeda et al., 2009) as well as, in other plants such as sunflower in Taiwan (Chen et al., 2008). *G. cichoracearum* s. lat. predominates in temperate or cold environments, whereas *P. fuliginea* s. lat. is more common in tropical environments and in greenhouses (Lebeda et al., 2009; Del Pino et al., 2002).

P. xanthii is a complex of physiological races, mainly identified by the differing responses of melon cultivars to the pathogen (Bardin et al., 1997; Cohen et al., 2004; McCreight, 2006). Worldwide, there are at least 28 reported physiological races of *P. xanthii* (McCreight, 2006). Races 1 and 2 are the prevalent races in Sudan (Mohamed et al., 1995) and California (Coffey et al., 2007), where race 1 was the only one found in summer time; race 1 infected only the more susceptible differential melon plants and the cucumber, but race 2 is the most extended race in the world (Cohen et al., 2004; Del Pino et al., 2002; McCreight, 2006), it has replaced race 1 and more aggressive races such as 2 France (2F), 4, and 5 have derived from it (Del Pino et al., 2002).

A new race of *P. xanthii*, termed race S, surged in the melon producer area of California and Arizona, and overcame the defense of 19 differential cultivars for identification of physiological races (Coffey et al., 2007; McCreight and Coffey, 2011). These areas seems to be the places of appearance of new physiological races, and the resistance of the powdery mildew to fungicides myclobutanil and trifloxystrobin, members of the DMI and strobilurin groups with different mode of action that before best controlled the powdery mildew. Another concern is the recent presence of chasmothecia, product of sexual reproduction that has the potential to generate variation in the powdery mildew population (Coffey et al., 2007).

Differences in *P. xanthii* physiological races prevalence are common and depend on the environment at the time of monitoring (Cohen et al., 2004; McCreight, 2006), but only "*S. fuliginea"* race 2 was found in melon farms in Northern Mexico (Hernández and Cano, 1990). To our knowledge, no studies on physiological races of powdery mildew have been reported for the state of Sinaloa. Due

to the uncertainty about the species causing powdery mildew in cucumber crops in the Culiacan Valley, and given the lack of studies on powdery mildew, physiological races in this region, the objectives of this work were to determine the etiology of the powdery mildew species that affect cucumber crops in greenhouses in the Culiacan Valley and to define the physiological races of these mildews.

MATERIALS AND METHODS

Plant material and inoculum

Samples of 24 cucumber or squash plants infected with powdery mildew were collected from the open fields or in greenhouses along the state of Sinaloa, (Table 1), in order to identify by light microscopy and by molecular techniques, the powdery mildew species causing the cucurbits powdery mildew. The melon cultivars Edisto 47, Iran H, MR-1, Nantais oblong, PI 124112, PI 414723, PMR 45, PMR 5, Védrantais, and WMR 12, donated by Dr. Michel Pitrat from the Institut National de la Recherche Agronomique (INRA), France, and PI 313970, donated by Dr. James D. McCreight from the United States Department of Agriculture USDA-ARS at Salinas, California, were used for the determination of physiological races of *P. xanthii*, (Bardin et al., 1997; Cohen et al., 2004; McCreight, 2006; Mohamed et al., 1995; Pitrat et al., 1998). These plants were allowed to grow in greenhouses or growth chambers located at the CIAD, A. C., in Culiacan Sinaloa, Mexico, in order to observe the reaction of the cultivar differentials in presence of cucumber powdery mildew in the prevailing environmental conditions in the local greenhouses, as well as, in controlled conditions in growth chambers. The plants in the greenhouses were naturally infected (McCreight, 2006) and no fungicide was applied in order not to disturb the fungus growth on the cucurbit plants. Infected plants growing in the greenhouse were used as the source of inoculum for the first two experiments in the growth chamber for the determination of the physiological races. For the third experiment on physiological races in the growth chamber, conidia were obtained from a single colony at the beginning of the actively growing stage from an infected cucumber plant growing in a shadow net protected area at CIAD, Culiacan in July 23rd and increased on cucumber plantlets Estrada (Fito® S. A., Barcelona, Spain). The differential plants on the two true extended leaves stage were inoculated by shaking infected plantlets over them.

Morphological characterization

The holomorphic structure of the fungi was observed on cucumber and some differential plants. Conidia of the fungus growing on the cucumber Estrada were lyophilized and preserved at 4°C; the sampled leaves containing conidia or chasmothecia (Figure 1A) were dried and preserved in newspaper sheets. Additionally, leaves with sporulating colonies were collected for immediate analysis. All the samples were observed under a light microscope (Olympus model BX41TF, Olympus Corporation, Tokyo, Japan), images were taken with a digital Olympus DP20 camera, and its software was used to measure hyphae, appressoria, conidiophores, conidia, chasmothecia, asci, and ascospores. The dried samples were rehydrated with a 3% KOH solution as needed. The morphological characterization of the species was made by comparing the holomorphic characteristics of the fungus obtained from cucumber Estrada growing in the greenhouse at CIAD, with those described in the monographs of Erysiphales (Bolay, 2005; Braun, 1987), and the conidia germination pattern was assessed according to the key proposed by Cook and Braun (2009).

DNA extraction and purification

DNA was extracted from 3 to 10 mg of conidia obtained from the 24 fungus infected samples, according to the technique described by Chen et al. (2008) and was modified by the addition of 2.5 µL of RNase 8 $mg \cdot mL^{-1}$ and incubation at 37°C for 30 min prior to DNA precipitation (Möller et al., 1992). The integrity of the extracted DNA was assessed by electrophoresis by adding 5 µL of the DNA suspension plus 1 µL of blue-orange 6x charge buffer to an electrophoresis chamber (Thermo EC320, Minicell, Primo®) in a 1% agarose gel stained with 1 µL ethidium bromide (10 $\mu g\ mL^{-1}$) at 65 volts for 40 min. The DNA purity and concentration were measured in an Eppendorf model 22331 biophotometer (Eppendorf, Hamburg, Germany), and part of the concentration was adjusted to 10 $ng \cdot \mu L^{-1}$ for PCR amplification (White et al., 1990).

Molecular characterization of the fungal specie by PCR

DNA extracts from the 24 collected samples were subjected to PCR amplification with the PCR Core system II Go Taq kit (Promega, Madison, WI, USA), using the primer pairs PN23/PN34 (Bardin et al.,1997), S1/S2, G1/G2 (Chen et al., 2008) and ITS1/ITS4 (White et al., 1990) to amplify a specific internal transcribed spacer region, including the ITS1, ITS2 and the 5.8S subunit of the ribosomal DNA (rDNA) of *Erysiphales* (Bardin et al., 1997), *P. xanthii* and *G. cichoracearum*, (Chen et al., 2008) and the universal ITS region (White et al., 1990), respectively. The PCR reaction mixture contained the following: 0.15 mM dNTPs, 0.4 µM primers, 1 U Taq polymerase, 1X PCR buffer, 1.5 mM $MgCl_2$ and 10 ng of fungus DNA as template. The final volume was adjusted to 25 µL with nanopure water. For the PCR reaction, an Eppendorf Mastercycler thermocycler (Eppendorf Scientific, Westbury, NY) was used with the following conditions: 5 min at 95°C for DNA polymerase activation; 30 cycles of 40 s for denaturation at 94°C; 1 min of annealing at 62°C for primers PN23/PN34, S1/S2 and G1/G2, and 60°C for ITS1/ITS4; 1 min of DNA synthesis at 72°C and a final extension of 5 min at 72°C. *P. xanthii* and *G. cichoracearum* DNA from *Carica papaya* and *Helianthus annuus,* respectively, were used as positive controls, whilst water was used as the negative control. The obtained PCR products were purified with the Wizard® Gel and PCR clean-up System (Promega, Madison, WI, USA) and sequenced at the Instituto de Fisiologia Celular at the Universidad Nacional Autonoma de Mexico and McLab, San Francisco, CA, USA. The obtained sequences were registered in the GenBank under the accession numbers included in Table 1. The sequence HQ316143 for *P. xanthii* Culiacan was blasted with the data bank of the National Center for Biological Information (NCBI) (www.ncbi.nlm.nih.gov/) and compared with the sequence accessions: 2) EU367960.1 *P. xanthi,* 3) EU294368.1 *P. fuliginea,* 4) AB040336.1 *P. fusca,* 5) AB040309.1 *P. fusca,* 6) AB040306.1 *P. euphorbiae,* 7) AF011319.1 *P. fusca,* 8) EU327327.1 *P. xanthii,* 9) AB040347.1 *Podosphaera sp.,* 10) AY450961.1 *P. xanthii,* 11) D84387.1 *P. xanthii,* 12) EU424056.1 *P. fusca,* 13) AB040316.1 *P. xanthii,* 14) EF442023.1 *P. fusca,* 15) AB046989.1 *P. fusca,* 16) FJ625796.1 *P. balsaminae,* 17) EF010913.1 *P. xanthii* and 18) EF137856.1 *P. fusca*. These sequences showed homology from 99 to 100% with the blasted nucleotide sequence of HQ316143. These sequences were then aligned and a phylogenetic tree was constructed using the Molecular and Evolutionary Genetic Analysis (MEGA) software version 4, by the Neighbor Joining method(Tamura et al., 2007), with the *G. cichoracearum* sequence 19(EF010914) as the out-group species sequence.

Table 1. Sources of powdery mildew collected in Sinaloa, location, date of collection and DNA database accession numbers.

Host	Location			Date (M Y)	Code	PCR	GenBank Accession no.
	Municipality	Lat N	Long W				
Cucurbita sp.	Ahome	25° 48' 49"	108° 58' 14"	03 2009	CCLMPx23	+	
Cucurbita sp.	Ahome	25° 48' 41"	109° 00' 39"	03 2009	CCLMPx22	-	
Cucurbita sp.	Guasave	25° 42' 04"	108° 42' 42"	03 2009	CCRCPx21	+	
Cucurbita sp.	Guasave	25° 30' 59"	108° 21' 27"	03 2009	CCBUPx20	+	
Cucurbita sp.	S. Alvarado	25° 27' 54"	108° 03' 42"	01 2010	CCGUPx07	-	
Cucurbita sp.	Angostura	25° 20' 10"	108° 07' 09"	03 2009	CCANPx19	+	
Cucumis sativus	Sinaloa	24° 49' 33"	108° 12' 55"	05 2011	CCSIPx13	+	
Cucumis sativus	Culiacan	24° 45' 13"	107° 28' 41"	05 2011	CCCUPx02	+	JN640299
Cucumis sativus	Culiacan	24° 44' 01"	107° 27' 13"	05 2011	CCCUPx05	+	
Cucumis sativus	Culiacan	24° 44' 01"	107° 27' 13"	05 2010	170410	+	HQ242711
Cucumis sativus	Culiacan	24° 44' 00"	107° 27' 16"	05 2010	170410	+	HQ316143
Cucumis sativus	Culiacan	24° 38' 02"	107° 26' 20"	03 2009	CCCUP × 14	+	
Cucumis sativus	Culiacan	24° 38' 02"	107° 26' 20"	03 2009	CCCUP × 16	+	
Cucumis sativus	Culiacan	24° 38' 02"	107° 26' 20"	03 2009	CCCUP × 17	+	JN653000
Cucurbita sp.	Culiacan	24° 38' 01"	107° 26' 18"	05 2011	CCCUP × 01	+	JN640298
Cucurbita sp.	Culiacan	24° 37' 57"	107° 26' 32"	03 2009	CCCUP × 18	+	
Cucumis sativus	Culiacan	24° 37' 06"	107° 26' 55"	06 2011	CCCUP × 24	+	
Cucumis sativus	Culiacan	24° 34' 32"	107° 24' 24"	03 2009	CCCRP × 15	+	
Cucurbita sp.	Culiacan	24° 17' 59"	107° 16' 30"	01 2010	CCEAP × 08	+	JN652997
Cucurbita sp.	Culiacan	24° 16' 55"	107° 15' 20"	01 2010	CCECP × 11	-	
Cucurbita sp.	Elota	23° 59' 51"	106° 58' 28"	05 2011	CCLCP × 03	+	JN640300
Cucurbita sp.	Elota	23° 47' 40"	106° 51' 47"	01 2010	CCCGP × 09	+	JN652998
Cucurbita sp.	San Ignacio	23° 44' 00"	106° 47' 10"	01 2010	CCDIP ×10	+	
Cucurbita sp.	Escuinapa	22° 49' 48"	105° 47' 37"	01 2010	CCESP ×12	+	JN652999

Powdery mildew of *Cucurbita* sp., collected from open fields, and *Cucumis sativus* from greenhouse and open fields, along the state of Sinaloa. Most of the collections rendered a PCR product with S1S2 primer, but not all of them could be sequenced.

Identification of physiological races in greenhouse conditions

Three experiments were conducted in two commercial type greenhouses EURO (Euronovedades agrícolas S. A. de C. V., Culiacan, Sinaloa, Mexico), located at CIAD, Culiacan, in March to June, 2009, October, 2009 to January, 2010, and February to May, 2010. Temperature (T), relative humidity (RH), and illumination varied with ranges of 19 to 37°C, 30.5 to 81.1% RH and 25000 to 50000 lux, respectively, in February to May and March to June, 2009; in October, 2009 to January, 2010, these ranges were 14.7 to 28°C, 45.8 to 100% RH and 15000 to 25000 lux, respectively, due to seasonal differences and greenhouses facilities.

A completely randomized design with one level (cultivar) was arranged with three plants of each cultivar in the first experiment and five plants in the next two experiments. Melon seeds were sown in 1 m "Riococo"® coconut panels (Ceyhinz Link International Inc. Irving, TX 75038 USA). Powdery mildew inoculum in March to June, 2009 came from naturally infected cucumber plants grown in the same greenhouse. In the remaining two experiments, naturally dispersed inoculum in the greenhouse was relied on to produce infections. The European hybrid cucumber Estrada ® (Semillas Fitó, Barcelona, Spain) was used as a control. Nutritive Steiner solution (11.0 NO_3^-, 1.5 $H_2PO_4^-$, 3.8 SO_4^{2-}, 4.5 Cl^-, 1.0 NH_4^+, 7.5 K^+, 10.0 Ca^{2+} and 4.0 Mg^{2+} me·L^{-1}) with an electric conductivity (E. C.) of 2.1 dSm^{-1} was applied with a pressurized drip irrigation system Xilema NP35 (Novedades agrícolas S.A., Murcia, Spain). Drainage water E. C. was maintained below 4 dS·m^{-1}.

The infection severity was evaluated 62 to 64 days after sowing. In March to June, 2009 and October, 2009 to January, 2010, disease severity on leaves fourth, seventh, and tenth was evaluated; in February, 2010, severity was evaluated on leaves from one to ten. The infection severity was considered as the average of the evaluations obtained according to the 1 to 9 scale proposed by McCreight (2006). Plants with severity average values <4 (hyphae growth was restricted and no sporulation detected), were considered resistant, whereas those with values ≥4 (hyphae growth with sporulated colonies), were considered susceptible.

Identification of physiological races under growth chamber conditions

Three experiments were conducted in order to identify the powdery mildew physiological races. In March and June 2010, five plants of each 10 melon cultivars used for identification of physiological race test (Mohamed et al., 1995; Pitrat et al., 1998), were grown in the five true leaves stage. In September, 2011 the cultivar, PI 313970 was included for a new physiological race evaluation (McCreight and Coffey, 2011); five plants of each of the 11 differential cultivars were grown in the second true leave stage. The plants for the three experiments were grown in potting substrate Premier Sogemix® VT-M (Quebec Canada), supplemented with 30% perlite. The

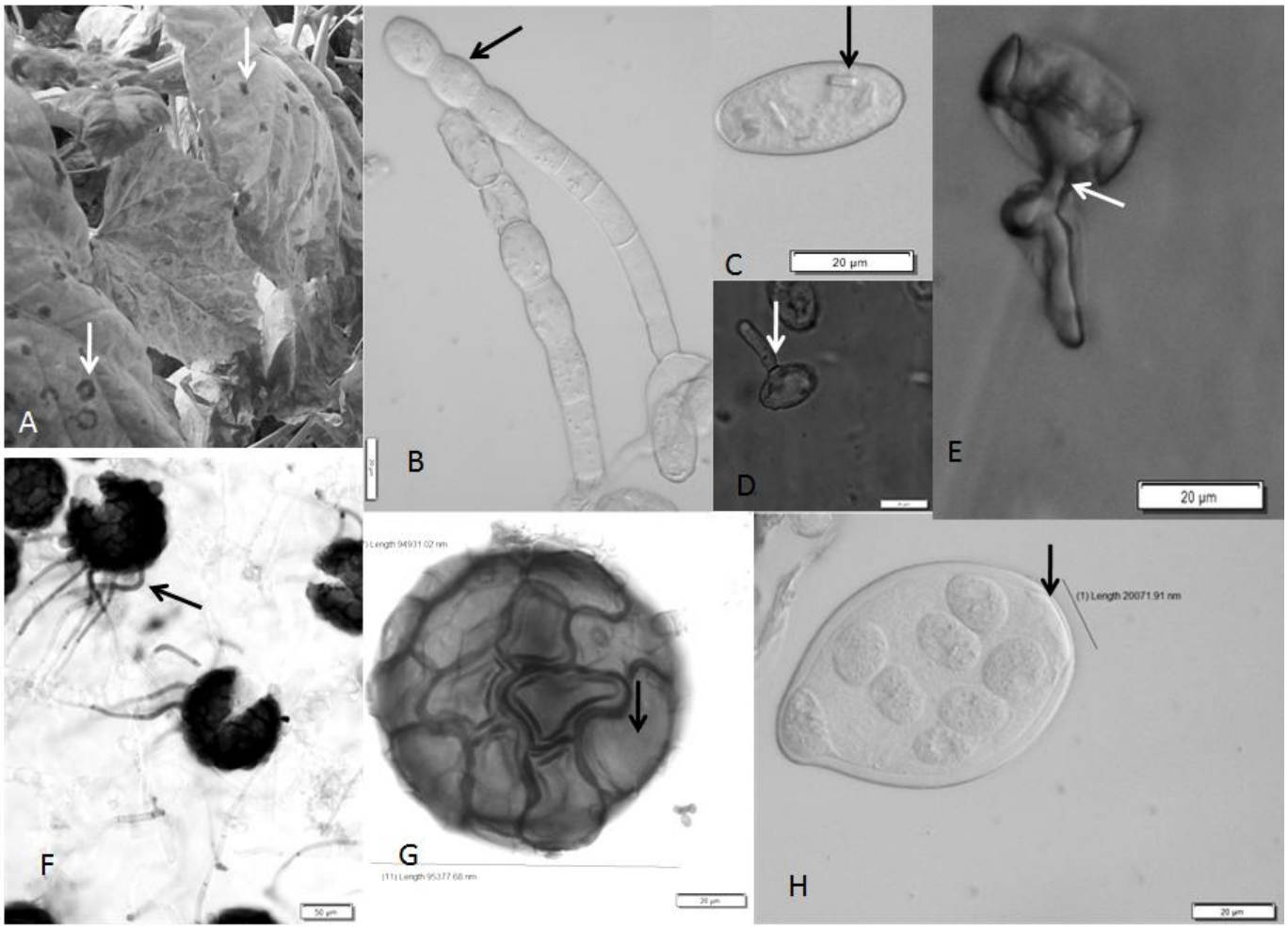

Figure 1. Principal *Podosphaera xanthii* morphological characteristics (indicated by arrows). A) chasmothecia groups on melon Iran H leaves; B) euoidium type conidiophore with crenated (rounded teeth) margins, straight or slightly bend basal cell, with a slight constriction at the base; C) ellipsoidal conidium with the presence of fibrosin bodies; D and E) lateral germination with thick simple or forked germ tube; F) chasmothecia with mycelial appendages; G) chasmothecium with large peridial cells; H) Ascus with eight ascospores and ocular opening 20 µm in diameter. Scale bar: B, C, D, E, G and H, 20 µm; F, 50 µm.

growth chamber was set at 23°C, with a 14 h day/10 h night photoperiod at 3500 lux illuminance using fluorescent and incandescent lights. The plants were watered every other day with 50 mL of 50% Steiner solution. In the first two experiments, the plants were inoculated by manually spraying 50 mL of a $4 \cdot 10^4$ conidia·mL^{-1} suspension (Floris and Álvarez, 1996) in .01% Tween 20 (Cohen et al., 1993), made from plant infected leaves growing in the greenhouse. For the third experiment, the inoculation was made by shaking leaves infected with conidia obtained in July, 23rd from a single colony and increased in a growth chamber in the aforementioned described conditions. Disease severity in the first two experiments was evaluated in the first five leaves of each one of the 10 varieties. For the third experiment, the evaluation was made in two leaves of each one of the 11 varieties (McCreight, 2006). The evaluation of the disease severity was made 10 days after inoculation, according to the 1 to 9 scale used by McCreight (2006), where mean disease rating <4 was considered resistant and mean rating ≥4 susceptible. Each one of the three experiments in growth chamber was once repeated. In some cases, appeared problems by inadequate watering, presence of other disease like damping off or failure in the illumination or air conditioner was occasionally made to lose some leaves or plants. As the obtained mean of both results for each repetition coincided in the range for resistance or susceptibility of the cultivars, determining the same race, the results presented here are from a single experiment in which plants had a more uniform growth and less or no plants were lost.

RESULTS

Morphological characterization

Powdery mildew-infected plants exhibited white colonies that covered the leaves of the more sensitive squash, cucumber and melon cultivars (Figure 1 A); petioles and stems were partially infected, but not the fruit. The powdery mildew samples had hyphae with simple,

Table 2. Morphological characteristics of *Podosphaera xanthii* obtained from cucumber plants grown under greenhouse conditions in the Culiacan Valley, Mexico.

Morphological characteristics		Mean*	Std. Dev*	Range*	N
Chasmothecia	Diameter	91	8.7	74-121	104
Peridial cells	Long	29	9.9	10-67	104
	Wide	14	4.1	6-31	104
Appendages	Number	7	1.1	5-9	31
	Wide	6	1.0	4-8	54
Asci	Long	70	8.0	53-97	104
	Wide	61	6.3	47-90	104
Ocular opening	Diameter	20	2.6	14-26	104
Cells/conidiophore	Number	5	0.9	3-7	129
Basal cell	Long	49	11.9	28-84	129
	Wide	11	1.1	9-14	103
Conidiophore 4 cells	Long	106	17.0	73-169	129
Conidiophore chain	Long	135	31.1	73-238	129
Conidium	Long	30	2.7	25-41	129
	Wide	18	1.9	13-24	129
Conidium long/wide	Index	1.7	0.2	1.3-2.3	129

(*) in micrometers. N = number of observations.

indistinct or nipple-shaped appresoria; conidiophores were of the euoidium type (conidia in chains), with crenated margins (Figure 1B); conidiophore basal cells were straight or slightly bent, measuring 28 to 84 × 9 to 14 µm (Table 2), and were slightly constricted at the base (Figure 1B). Conidia were ellipsoidal-ovoid to doliiform-shaped and measured 25 to 41 × 13 to 24 µm, and had fibrosin bodies present (Figure 1C). The resulting index from the length/width ratio of conidia was 1.7 on the average. Conidia germination pattern was the brevitubus sub-type of fibroidium germinated laterally by a simple, or less frequently, a forked germ tube (Figure 1D and E). Chasmothecia (Figure 1A, F and G) were abundantly found in December, 2009 on leaves of cucumber Estrada hybrid and in the differential melon cultivars Iran H, Nantais oblong, Védrantais, but some of them were found in PMR 45, WMR 29, Edisto 47 and PI 414723. In May, 2010, only plants of Iran H and Nantais oblong scarcely produced chasmothecia. Chasmothecia were spherically shaped with diameters ranging from 74 to 121 µm (Table 1); containing five to seven septate brown-colored filamentous appendages (Figure 1F) and had irregular peridial cells (Figure 1G), measuring 6 to 67 µm in diameter. The chasmothecia contained only one ascus with six to eight ascospores. The ocular opening of the ascus (Figure 1H) was 20 µm in diameter (Table 2). These characteristics fit with those of *Podosphaera xanthii*.

Molecular characterization

PCR products of 750 and 454 bp were amplified from fungal DNA samples using the primers PN23/PN34 and S1/S2 (Figure 2), specific for Erysiphales and for *P. xanthii*, respectively. No product was obtained with the primers G1/G2, designed to amplify a 391 bp PCR product specific for *G. cichoracearum.* A product of 500 bp was obtained in some of the samples with the pair of primers ITS1/ITS4; however, no specific sequence was obtained. The sequences of the PCR product amplified with the primer pair S1/S2, using cucumber powdery mildew DNA as template, showed 99 to 100% homology with the sequences of *P. xanthii*, *P. fusca, P. balsamineae, P. euphorbiae and S. fuliginea* when compared to reported sequences in NCBI GenBank (Figure 3). No amplification was obtained with the primer pair G1/G2 specific for *G. cichoracearum.*

Physiological races

Melon cultivars used to identify powdery mildew physiological races showed different disease severity in greenhouse and growth chamber experiments (Table 3).

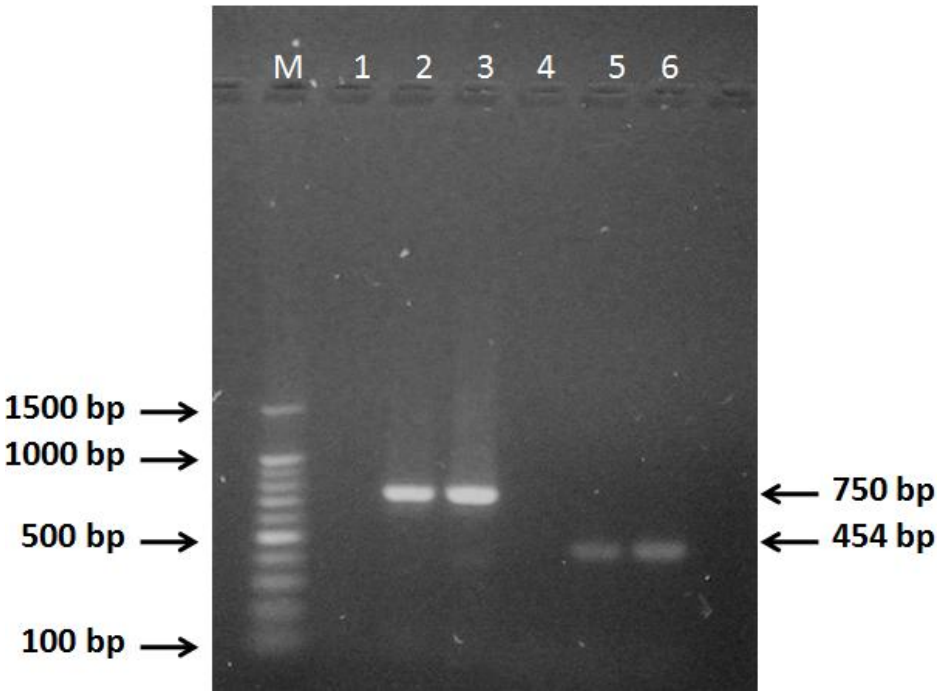

Figure 2. PCR products from *Podosphaera xanthii* DNA template, amplified with the primers PN23/PN34 and S1/S2 specific for the order Erysiphales and the species *P. xanthii*. M = 100 bp molecular weight marker; 1 = negative control for the primer pair PN23/PN34; 2 = positive control for PN23/PN34; 3 = DNA powdery mildew sample from cucumber leaves; 4 = negative control for the primer pair S1/S2; 5 = positive control for S1/S2 and 6 = DNA powdery mildew sample from cucumber leaves.

However, plants in greenhouse conditions showed higher severity indexes than those in growth chamber. In all the experiments in greenhouse or in growth chamber, cultivars PMR5, PI 414723, MR 1, PI 124112 and PI 313970 were resistant to the powdery mildew disease; Iran H, Védrantais and Nantais oblong were susceptible. A combination of resistance and susceptibility of the cultivars PR 45, WMR 29, and Edisto 47 (Table 3), was used to complement the identification of the physiological races 2F, 4 and 5. The resistance of cultivars PMR 5 and PI 414723 decreased respectively the presence of physiological races 3 and 2US, that is responsible for the outbreak of powdery mildew disease. In September 2011, using the inoculum derived from a single colony of powdery mildew, the severity indexes on the differential varieties showed that race 1 was present; this time, only the most susceptible of the differential melons and the cucumber Estrada hybrid were infected. In these experiments *Podosphaera xanthii* races 1, 2F, 4 and 5 were detected.

DISCUSSION

Morphological characterization

Reports on powdery mildew in Sinaloa dating before 1991, mentioned *Golovinomyces cichoracearum* as the only causal agent in cucurbits and wild plants of various other plant families (Cebreros et al., 1991). After that, *P. xanthii* (syn *S. fuliginea* s. lat.) was reported in Cucurbitaceae in 2005 and 2010 in the Northern and center of the state of Sinaloa (Félix-Gastélum et al., 2005; Pérez-Ángel et al., 2010). The morphology of the anamorphs of the collections from *Cucurbita* spp., ad from *Cucumis sativus* (Table 1) presented the characteristics described for *P. xanthii*, but no conidia from *G. cichoracearum* was found. In the greenhouse, cucumber hybrid Estrada showed to be very susceptible to powdery mildew, as were some of the melon cultivars (Table 3). In these plants, powdery mildew covered the leaves with sporulated hyphae, whilst on the resistant plants, no hyphae growth was observed. In greenhouse in December, 2009 at the stages of fruit development and ripening, chasmothecia were abundant on susceptible cultivars and a few of them were found in senescent plants of resistant cultivars. In May 2010, powdery mildew growth was less intensive than in December 2009, and only Iran H and Nantais oblong produced a few chasmothecia, probably due to the fact that after April 15 the maximum temperature reached 45°C in the greenhouse, surpassing the 35°C mentioned by Jarvis et al. (2002) as the maximum temperature for the growth of *P. xanthii*. This hot weather occurred at the time of fruit

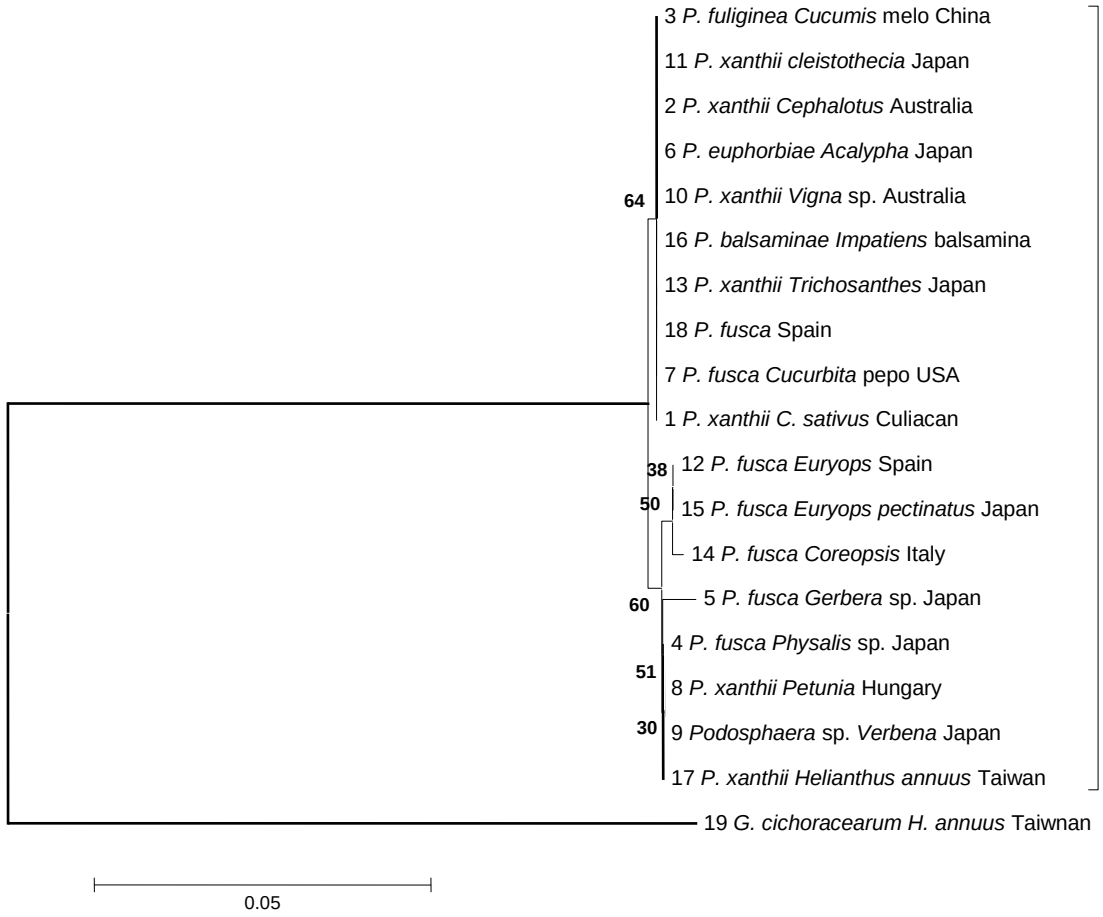

Figure 3. Phylogenetic tree, constructed based on the sequence of the 19 taxa PCR DNA products of the internal transcribed spacer regions (ITS), obtained with the primer pair S1/S2. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.20893310 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 407 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

ripening and plant senescence, when the chasmothecial presence is related to a bigger incidence and severity of powdery mildew at the end of the season. Chasmothecial formation indicates that two mating types were present because *P. xanthii* is a heterothallic species (McGrath et al., 1996). The presence and abundance of chasmothecia was related to plant susceptibility and coincided with temperatures, ranging from 13.3 to 26.4, averaging 19.3°C. Smith (1970) observed maximumascocarp production of *Erysiphe pisi* (as *E. polygoni)* on *Pisum sativum*, *E. ludens* var *lathyri* (as *Microsphaera penicillata*) on *Lathyrus ochroleucus* and *Golovinomyces cichoracearum* (as *Erysiphe cichoracearum*) on *Aster laevis*, when temperatures ranged from 10 to 20°C, while in North America and Europe ascocarps of *P. xanthii (= S. fuliginea* s. lat.) on cucurbits have been observed mainly in fall and winter time (McGrath et al., 1996).

Chasmothecium characteristics (Table 2 and Figure 1F and G) were similar in size to those described by Félix-Gastélum et al. (2005) on cucumber plants with a diameter of 76.7 to 108.3 µm, but larger than those developed on sunflower plants, measuring 56 to 80 × 56 to 70 µm (Chen et al., 2008), indicating that the latter did not belong to *P. xanthii*. Each chasmothecium contained one ascus with six to eight ascospores. The ocular opening of the ascus (Figure 1H) was 20 µm in diameter (Table 2), which coincides with the described characteristics of *P. xanthii* (Bolay, 2005; Braun, 1987), whereas the ocular opening in *P. fusca* is 10 µm in diameter (Shishkoff, 2000; Braun et al., 2001). In this study, only fungal structures corresponding to *P. xanthii*

Table 3. Reactions of melon cultivars used as differentials for physiological races of *Podosphaera xanthii* and disease severity index*.

Cultivar	Expected reaction by physiological race							Disease severity index and (observed reaction)					
								I GH [u]	II GH [v]	III GH [w]	IGC [x]	II GC [y]	II GC [z]
Iran H	S	S	S	S	S	S	S	9.0 (S)	9.0 (S)	8.5 (S)	6.3 (S)	6.8 (S)	8.2 (S)
Védrantais	R	S	S	S	S	S	S	8.7 (S)	9.0 (S)	6.4 (S)	5.8 (S)	6.8 (S)	7.9 (S)
PMR 45	R	R	S	S	S	S	S	8.0 (S)	8.0 (S)	4.3 (S)	4.3 (S)	4.1 (S)	1.0 (R)
PMR 5	R	R	R	R	S	R	R	3.3 (R)	3.0 (R)	3.3 (R)	2.8 (R)	3.6 (R)	1.0 (R)
WMR 29	R	R	H	R	-	S	S	6.7 (S)	7.3 (S)	2.8 (R)	5.6 (S)	3.0 (R)	1.0 (R)
Edisto 47	R	R	S	R	S	R	S	6.0 (S)	2.3 (R)	3.7 (R)	4.8 (S)	3.4 (R)	1.0 (R)
PI 414723	R	R	S	R	R	R	R	2.3 (R)	3.3 (R)	1.8 (R)	2.0 (R)	2.4 (R)	2.1 (R)
MR-1	R	R	R	R	R	R	R	1.0 (R)	3.3 (R)	2.4 (R)	2.2 (R)	3.3 (R)	1.0 (R)
PI 124112	R	R	R	R	R	R	R	3.7 (R)	3.0 (R)	2.3 (R)	2.8 (R)	3.1 (R)	1.0 (R)
PI 313970	R	R	R	R	R	R	R	-	-	-	-	-	3.8 (R)
Nantais oblong	R	S	S	S	S	S	S	8.7 (S)	9.0 (S)	8.3 (S)	4.8 (S)	6.4 (S)	6.7 (S)
Race	0	1	2US	2F	3	4	5	5	4	2F	5	2F	1

u = first experiment in greenhouse in spring; v = second experiment in greenhouse in autumn; w = third experiment in greenhouse in spring; x = first experiment in growth chamber; y = second experiment in growth chamber; z = third experiment in growth chamber. (R) = resistant cultivar; (S) = susceptible cultivar; (H) = heterogeneous; (-) = not determined. Race 2 US= race 2 U.S. A.; race 2F= race 2 France.*evaluated according to the 1 to 9 scale proposed by McCreight (2006). The cut-off between R and S was < 4 = (R); ≥ 4 = (S).

were found, but not those of *G. cihoracearum*. These results is in line with the reports obtained by Bojorques-Ramos et al. (2011) who observed in a greenhouse in Salinas, California that conidia from *G. cichoracearum* growing on *Lactuca serriola* germinated but did not grow on cucumber plants very susceptible to *P. xanthii*. In Southern Italy (Miazzi et al., 2011) and the Southeastern coast of Spain (Del Pino et al., 2002), only *P. xanthii* was found on cucurbits reinforcing the hypothesis of Lebeda et al. (2009) that *P. xanthii* grows in tropical and sub-tropical areas and in greenhouses, and *G. cichoracearum* needs temperate or cooler temperatures in open fields.

Molecular characterization

Fragments of 750 and 454 bp were amplified by PCR from fungal DNA samples, using the primers PN23/PN34 and S1/S2 (Figure 2), specific for Erysiphales and for *P. xanthii*, respectively, but no product was obtained with the primers G1/G2, designed to amplify a 391 bp fragment specific for *G. cichoracearum*. These results indicated the presence of *P. xanthii* but not G. *cichoracearum* infecting cucumber plants in the greenhouses of the Culiacan Valley. The amplified products obtained with the PN23/PN34 and S1/S2 primers are located in the internal transcribed spacer region (ITS), including the 5.8S sub-unit of the ribosomal DNA (rDNA) that contains conserved sequences with enough variation to show differences at the species level (Saenz and Taylor, 1999; White et al., 1990). Hirata and Takamatsu (1996) demonstrated that the anamorph and teleomorph DNA sequences in the ITS region are highly conserved, such that the nucleotide sequence of any of the developmental stages of the powdery mildew species (anamorph or teleomorph) can be used in phylogenetic studies. Given that the primer pair PN23/PN34 not always renders specific PCR products Chen et al (2008) designed the primer pair S1/S2 to amplify a specific segment for *P. xanthii*. The pair of primers ITS1/ITS4 was used to amplify the ITS region and a product of about 500 bp was obtained in some of the samples. However, no specific sequence was obtained, may be due to biological contamination; when fragments of an unspecific sequence was blasted in the NCBI, they aligned with sequences found in *Cladosporium* sp., *Penicillium* sp. or uncultured fungus strains. Only 21 PCR products were obtained when the 24 samples were subjected to a PCR reaction with the primer pair S1/S2, it may be that old samples were contaminated with saprobic fungal species or that powdery mildew conidia were fed or damaged by mites during the samples storage. With the primers S1/S2 10 of 21, PCR products rendered specific and similar sequences that resulted in a 99 to 100 homology with *P. xanthii*, *P. fusca*, *P. phaseoli* and *P. balsaminae* (Figure 3), when they were compared with sequences reported in the NCBI GenBank; nine of these sequences were registered at the GenBank (Table 1). These results except for *P. balsaminae* were observed by Braun et al. (2001), who based his report on the phylogenetic analysis of the ITS rDNA region, considered *P. balsaminae*, *P. diclipterae*, *P. fuliginea*, *P. intermedia*, *P. pseudofusca* and *P. sibirica* to be different species; *P. fusca* and *P. xanthii* to form a complex, and *P. phaseoli* as a synonymous with *P. xanthii*. However, *P. balsaminae* is confined to collections of Impatiens noli-tangere, whereas collections on *Impatiens balsamina* and *I. textori* in Asia are genetically and morphologically

distinct and pertains to *P. xanthii* (Ito and Takamatsu, 2010). Ito and Takamatsu (2010) included the sequence of the D1/D2 domains of the 28 S rDNA region in order to improve the results on the phylogenetic studies on *P. xanthii* was based on the genetic sequence of the ITS region; they found that this region is too conservative for phylogenetic analyses of this group at the species level. Sequences of the 28 S rDNA have successfully been used in phylogenetic studies of the Erysiphales at the genus level, whilst ITS rDNA sequence is more useful at the species level (Ito and Takamatsu, 2010; Khodaparast et al., 2007). It is likely that reported species with different names but with homologies of 99 to 100% belong to the same species, but differences could be due to the changing nomenclature of this fungal group. Powdery mildew identity could be better assessed if morphological description of the holomorph were available (Glawe, 2008). Although, most of the compared sequences date from 2004 to 2009, it is possible that some proposed terms such as those proposed by Braun et al. (2001, 2002), had not been considered for the species identification. Uniformity in the use of concepts would help to establish real differences between species considered or not as synonymous.

In cucumber plants grown in the greenhouse in Culiacan, only *P. xanthii* (S. *fuliginea* s. lat.) was recently found (Pérez-Ángel et al., 2010) as it was the case in cucumber in Northern Sinaloa (Félix-Gastélum et al., 2005). Álvarez and Torés (1995) found that *P. xanthii* was the causal agent of powdery mildew in cultivated cucurbits on the Eastern coast of Malaga, Spain. *G. cichoracearum*, *E. polygonii* and a different *P. fuliginea* pathotype were found in wild plants. *G. cichoracearum* in Cucurbitaceae crops and wild plants was reported in the Culiacan Valley (Cebreros et al., 1991) but not recently.

In the Czech Republic, *P. xanthii* and *G. cichoracearum* were found mixed in Cucurbitaceae crops, with *P. xanthii* predominating in warm climates and greenhouses, and *G. cichoracearum* in temperate climates (Lebeda et al., 2009). Both of these species also infect sunflower plants in Taiwan, but *G. cichoracearum* is the predominant species (Chen et al., 2008). Bojorques-Ramos et al. (2011) found that *G. cichoracearum* from *Lactuca serriola* did not infect cucumber plants growing in a greenhouse at Salinas, California. Prior to 1973, the Cucurbitaceae powdery mildew in Israel was thought to be *E. cichoracearum*, when it was actually *P. xanthii* (McCreight, 2006). The USA literature referred exclusively to *G. cichoracearum* through 1963 when Kable and Ballantyne reported *P. xantii*. After that, the literature referred to one or both species was based on the presence of fibrosin bodies in the conidia (McCreight, 2004). Kontaxis (1979) reported the first observation of ascomata in the U.S. and suggested that the powdery mildew affecting Cucurbitaceae crops in the USA was *P. xanthii,* not *G. cichoracearum* as generally thought. Given that *G. cichoracearum* has not been reported recently in the Culiacan Valley, the causal agent of powdery mildew may have been replaced by *P. xanthii* as has occurred elsewhere (Glawe, 2008; Lebeda et al., 2009) or possibly it was mis-identified, given that only the anamorphic stage was used for the identification.

Physiological races

Data on susceptibility and resistance of the differential melon cultivars growing in greenhouses and growth chambers, inoculated with powdery mildew (Table 3), showed that races: 1, 2F, 4 and 5 of *P. xanthii* were present at the Culiacan Valley, Sinaloa, Mexico. Races 2F, 4 and 5 were found in greenhouses and races 1, 2F and 5 in growth chambers (Table 3). These results are in accordance with those obtained by Hernández and Cano (1990), who found *P. xanthii* race 2 in “La Comarca Lagunera” in Northern Mexico, using six of the differential melon varieties used in the present study. Lemus-Isla et al; (2005) found the races 4 and 5 of *P. xanthii* in Cuba during the farming period in 2002-2003, but only race 5 in 2003 to 2004. *P. xanthii* race 2 has been reported to have the widest geographical distribution of the physiological races (Cohen et al., 2004; Del Pino et al., 2002; McCreight, 2006) and has replaced race 1 and more aggressive races such as 2F, 4, and 5 have been derived from it (Del Pino et al., 2002). When the plants in the growth chamber were inoculated with the powdery mildew from cucumber plants growing in the greenhouse, races 5 and 2F were found, as was found in the greenhouse experiments. In the third experiment in a growth chamber in Sept. 2011, race 1 was found. It is probable that race 1 be present all year-round, but masked by other more aggressive powdery mildew physiological races since the inoculum in the third experiment was produced from a single colony. In Sudan, where races 1 and 2 were found (Mohamed et al., 1995), race 1 was the only one found in summer time. Race 1 infected only the more susceptible differential melon plants and the cucumber hybrid Estrada. Races 1 and 2 are the prevalent races in California and Arizona, where most of the melons in the United States are grown (McCreight, 2006). In the third experiment, melon cultivar PI 313970 showed a transient fungal growth and sporulation that was later confined to a blister like formation on the infected leaf, and the fungus stopped growing; this reaction in this cultivar is similar for races 1 and 2 (McCreight and Coffey, 2011; Sedlářová et al., 2009). PI 313970 is resistant to race S, that in 2003 overcame the defense of 19 differential cultivars for physiological races in California and Arizona (McCreight and Coffey 2011). In these places, concern is about the appearance of new physiological races, the resistance of the powdery mildew to the fungicides myclobutanil and trifloxystrobin, members of the DMI and strobilurin groups with different mode of action. Another concern is the

recent presence of chasmothecia, product of sexual reproduction that has the potential to generate variation in the powdery mildew population (Coffey et al., 2007). Differences in P. xanthii physiological races prevalence are common and depend on the environment at the time of monitoring (Cohen et al., 2004; McCreight, 2006). Based on the obtained results, it was confirmed that *P. xanthii* is responsible for the powdery mildew that infects cucumber plants grown under greenhouse and open field conditions in the Culiacan Valley and that the same species is extended throughout the state of Sinaloa. In this search no *G. cichoracearum* was found. Additionally, the physiological races in this valley were determined to be 1, 2F, 4, and 5. In Southern Italy, it was found that *P. xanthii* is also composed of a heterogeneous population physiological races 0, 1, 2FR, 3, 4 and 5 (Miazzi et al., 2011) as it is in Salinas, California (McCreight, 2006) and in Southeastern Spain (Del Pino et al., 2002). The variability of physiological races of powdery mildew and the suddenly appearance of new physiological races in the world and the capability of the powdery mildews to generate resistance to commercial fungicides (Coffey et al., 2007), justifies the continuous research on the prevailing physiological races of powdery mildew in order to better manage cucurbit crops and to prevent major damages by unexpected pathogen breakthroughs.

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