Powdery mildew on common bean (*Phaseolus vulgaris* L.) in Northern Sinaloa, Mexico.

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Powdery mildew of beans is a disease in Northern Sinaloa state in Mexico, however, the identity of the causal agent, which is an obligate parasite, has not yet been elucidated due to lacking formation on its teleomorph. The objective of this study was to identify the causal agent of the disease through morphometric studies of the anamorph, and molecular techniques based on the ITS (internal transcribed spacers) region of the ribosomal DNA. We collected ten samples of different bean varieties that exhibited symptoms of the disease in the Fuerte Valley during the 2006-2007 fall/winter growing season. Morphological characteristics of the anamorph and the molecular phylogenetic analysis revealed that the causal fungus belongs to the mitosporic genus *Oidium* subgenus *Pseudoidium*. All samples had conidiophores of the *Pseudoidium* type, whose conidia were mainly cylindrical, and when partially collapsed, their walls showed longitudinal wrinkling, except on their ends, where the wall appeared almost smooth. Phylogenetic analysis of the ITS1-5.8S-ITS2 rDNA region revealed that our collections of powdery mildew of beans are closely related to specimens of *Erysiphe diffusa* associated with soybeans and to an *Erysiphe* sp. associated with *Phaseolus vulgaris*.

Key words: anamorph, electron microscopy, morphology, fungal diseases, ITS rDNA

Bean production covers an area of 92 340 ha in Sinaloa state, Mexico with a yield of 151 209 tons (CAADES 2008) in recent growing seasons. However, the crop quality and yield of this legume has been

limited by fungal diseases, such as the white mold caused by *Sclerotinia sclerotiorum*, the root and stem rot caused by several species of *Fusarium*, *Rhizoctonia solani* and *Macrophomina phaseolina*, and the leaf rust disease locally named "roya" or "chahuixtle" caused by *Uromyces phaseoli* var. *phaseoli* Arth (Campos-Ávila 1987). Powdery mildew is a disease widely distributed in commercial bean fields during the fall-winter growing season in Sinaloa; although the impact on the crop yield has not been elucidated in this region, losses from 40 % to 50 % and from 17 % to 69 % in some bean varieties have been reported from Brazil (Ferreira-Arriel *et al*. 1991) and Colombia (Schwartz *et al*. 1981), respectively. *Erysiphe diffusa* (Cooke & Peck) U. Braun and S. Takam., previously known as *Microsphaera diffusa* Cooke & Peck (Takamatsu *et al*. 2002) has been implicated as the causal agent of this disease (Mignucci & Chamberlain 1978). In contrast, León-Gallegos (1988) suggests that *Erysiphe polygoni* DC. *s. lat* is the cause of the disease in Sinaloa. Up to this date, however, there has not been solid scientific evidence to support this statement, despite the fact that the disease is confined to the northern area of the state. Additionally, the identity of the pathogen is required to know in breeding programs for disease management. Consequently, the objective of this study was to identify the causal agent of powdery mildew of common bean in Sinaloa, Mexico.

Materials and Methods

Bean cultivars

We collected ten samples of symptomatic bean leaflets of the cultivars Azufrado 200 (from a 50-ha commercial field in Los Mochis area), Faba Blanco Español, Azufrado Amarillo 33, Pinto Bayacora, Pinto Mestizo, Pinto Saltillo, Pinto Durango 1616, Pinto Americano 114, Bill Z and Pinto Durango 1602 (from semi commercial plots ranging 2-3-ha, located in the Experimental Station of the El Fuerte Valley, belonging to the National Institute for Research on Forest, Agriculture and Cattle Management (INIFAP for its abbreviation in Spanish). The symptoms consisted of whitish powdery spots on the foliage. All samples were obtained from 20 Nov 2006 to 30 Jan 2007. Samples were placed into plastic (polyethylene) bags and taken to the laboratory, where they were maintained at 5-7 °C for their further analyses; subsamples were taken for molecular analyses and morphological studies which were done within 48 h.

Light microscopy of somatic and asexual structures.

Conidiophores and conidia were prepared by touching the whitish lesions with clear adhesive tape, and then setting the tape over the microscope slides with a drop of distilled water, so the structures could be observed by light microscopy. We measured the width of the myc-

elium, the conidiophore length, the length and width of its foot cell, and also the length and width of the detached conidia. The hyphal width was determined in the second or the third adjacent hyphal cell from which the conidiophores emerged. According to Boesewinkel (1980), a conidiophore is defined as the series of cells from the foot cell to the generative cell, measured from the basal septum of the foot-cell to the septum delimiting the generative cell from the conidium (recognized by its swollen diameter). The width of the conidiophore was determined at its midpoint. Forty representative specimens per sample of each somatic or reproductive structure were measured and the average and the standard deviation for each variable were obtained.

Because some powdery mildews have conidia with inclusions called fibrosin bodies, conidia were mounted in a 3 % solution of potassium hydroxide (Kable *et al.* 1963).

To observe the germination of the conidia, they were transferred to sterilized slides from naturally infected tissue, using a brush of smooth bristles, and were incubated for at least 24 h at 100% of relative humidity, at a temperature of 23 ± 2 °C; the width of the germ tube was measured at its midpoint.

Scanning electron microscopy of conidia

Within three hours of making the collection in the field, leaf fragments (3.5 cm^2) of bean cv. Azufrado 200 with symptoms of powdery mildew were immersed in a fixing solution (FAA: 10 % formaldehyde, 5 % acetic acid, 50 % of a 96 % ethyl alcohol solution, and 35 % water). After 48 h, samples were washed with tap water for ten minutes, and immediately dehydrated by increasing concentrations of ethanol (Ruzin 1999). Samples were then dehydrated up to a critical point with CO_2 in a desiccant glass BAL-TEC CPD030. Samples were mounted in aluminum sample-holders over a conductive carbon strand, and sputtered with gold using an ionizer Denton Vacuum Desk II. Micrographs of conidia were taken using a JEOL JSM-53110 LV scanning electron microscope (Bozzola & Russel 1999).

DNA extraction, PCR, and sequencing

A mix of 50 mg of mycelium and conidia of infected tissue was removed from adaxial leaf surface from five out of the ten sampled cultivars (Azufrado 200, Pinto Durango 1602, Pinto Durango 1616, Pinto Mestizo and Bill Z) used in the morphometric study. From all samples, genomic DNA was extracted using DNAzol (DNAzol® genomic DNA isolation reagent, Cat. No. DN127, Molecular Research Center, Inc. Cincinnati, Ohio) following the manufacturer directions. The genomic DNA was re-suspended in 30 µL ultra pure water. The polymerase chain reaction (PCR) was set in a total volume of 25.0 µL including 0.4 µM of each primer (ITS1, ITS4; White *et al.* 1990), 1× of

PCR buffer, 2.5 mM $MgCl_2$, 500 µM dNTPs, 1.0 U Taq DNA polymerase (Platinum Taq DNA Polymerase High Fidelity, Cat. No. 11 304-029. Invitrogen, Carslbad, CA, USA), and 1 µL sample DNA (dilution 1:10). The amplification was done in a thermal cycler (BIORAD, Icycler, Mexico City, Mexico). Reactions were started by 4 min denaturation at 95 °C, followed by 30 cycles consisting of denaturation 1 min at 95 °C, annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min, with a final extension step at 72 °C for 5 min. The PCR products were separated through electrophoresis in a 1 % agarose gel, and stained with ethidium bromide (0.5 µg/mL). PCR products were purified by cutting the bands from the agarose gels and using a QIAquick gel extraction kit (Qiagen, Cat. No. 28706, Mexico City, Mexico). The cloning of the product was carried out using a pGEM-T Easy Vector System II® (Promega, Cat. A1380, Madison, WI, USA) cloning kit, following manufacturer directions. PCR products cloned into the pGEM-T Easy plasmid were transformed into JM-107 *Escherichia coli* competent cells. To purify the plasmid DNA a Qiaprep spin miniprep kit (Qiagen, Cat. No. 27106, Mexico City, Mexico) was used. To determine the presence of the cloned fragment a digestion of the plasmid DNA was done with the enzyme EcoRI (Invitrogen, Cat. No. 15 202-013, Oregon, USA). The plasmid DNA was quantified by fluorometry using the Quant-iT ds DNA HS kit (Invitrogen, Cat. No. Q32 854, Oregon, USA), following directions from the manufacturer. For detection, the multimodal detector DTX 880 (Beckman, Mexico City, Mexico) was used. The amplified and cloned fragments were sequenced using the dideoxy Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Two PCR products were sequenced in both directions with an ABI Prism 3 100 automated DNA sequencer (Applied Biosystems, Mexico City, Mexico). The sequences were blasted against the NCBI database (http://www.ncbi.nlm.nih.gov/, Wheeler *et al.* 2000; BLASTN v. 2.2.22, Zhang *et al.* 2000). The nucleotide sequences derived from this research were deposited in GenBank (accession numbers: GU570595, HQ444194-HQ444198).

Sequencing and Phylogenetic analysis

Sequence and phylogenetic analysis were performed with the Molecular Evolutionary Genetics Analysis Software (MEGA 4 Beta) (Tamura *et al.* 2011). Homologous sequences reported by Almeida *et al.* (2008) and Limkaisang *et al.* (2006) were downloaded from the NCBI database and aligned with the queries using MUSCLE multiple sequence alignment software (Edgar 2004) implemented in MEGA 4. Sequences variable in length were used (553 to 598 bp). Models of nucleotide substitution were selected by the Akaike information criterion (AIC), using jModelTest (Posada 2008). A phylogenetic tree was constructed by using the neighbor-joining method (NJ) (Saitou & Nei

1987) and the Tamura-Nei substitution model. Among-site rate variation was modeled by a gamma distribution, four rate categories and invariable sites were considered (TN93+G+I). Gaps were treated as missing data. The robustness of NJ topology was evaluated by 1000 bootstrap replicates.

Results

Morphology of somatic and asexual structures of the fungus associated with powdery mildew in common bean

The teleomorph was not observed during the present study. Mean values and standard deviations of somatic and different asexual structures of the fungus are given in Table 1. The superficial and hyaline mycelium had a mean diameter of 5.5–6.5 µm; hyphae were substraight to flexuous, branching mainly at right angles. Appressoria on hyphae were single and lobed (Fig. 1). Conidiophores (*Pseudoidium* type) consisted of 1-4 cells (mostly 3-celled) with conidia formed singly from the apex. The average length of the conidiophores varied from 49.4 to 66.1 µm; the foot cell of the conidiophores was straight, cylindrical and flared from the base, without any swellings and with a diameter from 9.7 to 11.1 µm across its medium part. Conidia were mostly cylindrical, from 33.4 to 39.1 µm in length and 18.5 to 21.0 µm in width, without fibrosin bodies. Conidia from fresh infected tissue under the compound microscope exhibited a slight constriction at one end. In contrast, when the conidia were incubated in a moist chamber (to ensure 100% relative humidity) during 24 h at room temperature (19-23 ˚C), they became turgid and without the constriction observed before. The germ tubes emerged from a shoulder of the conidia; they were short, lobed, and slightly longer than the diameter of the conidia; their diameter varied from 3.2 to 3.8 µm. Under the scanning electron microscope, the surface of the conidia was not smooth, they appeared slightly collapsed with longitudinal wrinkling (Figs. 2 A and B). The surface of the terminal end of the conidia was always smooth (Fig. 3).

Molecular identification of the causal agent of bean powdery mildew

The symptomatic samples analyzed by PCR of the ITS1-5.8S-ITS2 ribosomal DNA regions from varieties Azufrado 200, Pinto Mestizo, Pinto Durango 1616, Bill Z and Pinto Durango 1602 showed a band of 648 nt. This sequence showed the highest degree of homology to an *Erysiphe* sp. (AY7391092) specimen associated with *Phaseolus vulgaris* and with reports of fungi identified as *Oidium* sp. and *Erysiphe diffusa* associated with soybeans and wild soybeans. Two different PCR product clones were sequenced and found identical to each other. This was performed with all five bean varieties from Northern Sinaloa; and they showed 99.2 to 100 % homology between each other. Blasting

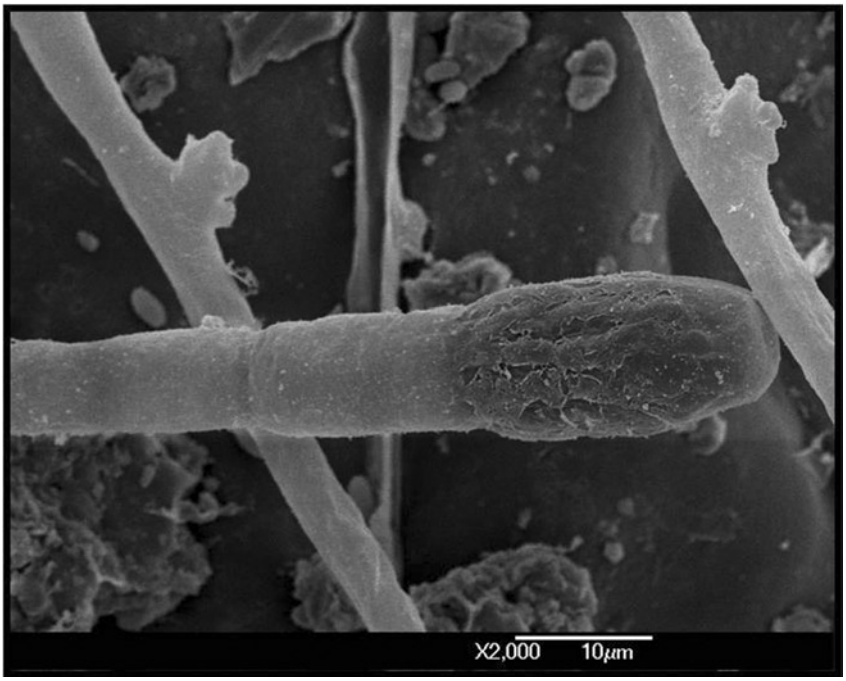


Fig.1. – Hyphae with a single, moderately lobed appresorium and immature conidium attached to the conidiophore showing linear wrinkling.

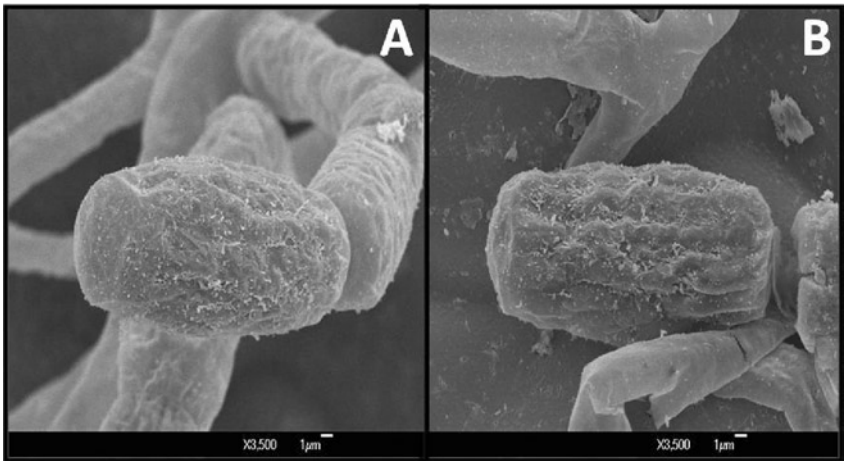


Fig. 2. – Scanning electron micrographs of outer walls in: A) a partially dehydrated mature conidium attached to the conidiophore and B) conidium with a greater degree of dehydration and linear wrinkling.

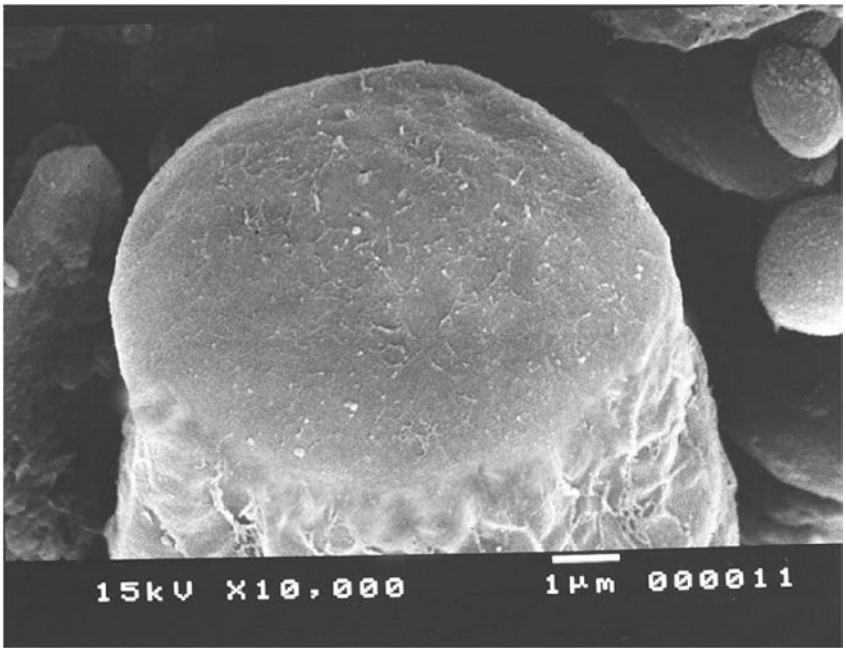


Fig. 3. – Scanning electron micrograph of smooth septum in a conidium of *Oidium* sp.

against GenBank the highest homology was found with *Erysiphe* sp. AY739109.2 (99.2–100 %) isolated from *Phaseolus vulgaris* (Almeida *et al*. 2008). In the phylogenetic tree (Fig. 4) this sequence clustered and sequences from our isolates formed one clade together with sequences from *E. diffusa* isolated from soybean (*Glycine max*; Almeida *et al*. 2008), and *Oidium* sp. isolated from soybean and wild soybean (*G. soja*; Takamatsu *et al*. 2002). *E. howeana* and *E. pisi* – the latter is also known to infect leguminous plants formed a sister clade to the clade mentioned above. Other powdery mildews associated mainly with legumes such as *E. baeumleri, E. trifolii*, *E. glycines*, and *E. friesii* grouped together but distant from the *E.-diffusa*-clade.

Discussion

The asexual stage of the fungus associated with powdery mildew of bean was identified as *Oidium* (Barnett and Hunter 1972, Yarwood 1973) subgenus *Pseudoidium* (Cook *et al*. 1997). The teleomorph of the fungus was not found in the present study. The morphological characteristics as dimensions, type and form of the conidiophores and conidia, as well as the presence of one germ tube per conidium fit with the description for *Oidium* (subgenus *Pseudoidium*) anamorphic stage of *Erysiphe diffusa* (Takamatsu *et al*. 2002).

Table 1. – Morphological characteristics and measurements [µm] of somatic and asexual structures of *Oidium* sp. on ten cultivars of common bean (*Phaseolus vulgaris*) in Northern Sinaloa, Mexico.

Bean varieties/ location	Mycelium	Conidiophore		Foot cell		Conidia		Germ tube	Cells in the conidiophore
	Width	Length	Width	Length	Width	Length	Width	Width	
Azufrado 200 commercial field, Los Mochis Sinaloa	2.5–9.0[1]	30.3–85.2	3.8–19.0	14.5–34	6.5–12.6	29.5–45.0	12.5–24.8	2.5–5.0	1.0–3.0
	5.8[2]	57.2	11.7	24.25	9.6	37.1	18.4	3.8	2.0
	1.4[3]	14.6	1.6	6.7	1.3	4.4	1.9	0.9	0.5
Faba Blanco Español, semi commercial plot experiment station (INIFAP)	3.7–8.7	11.5–78.7	3.7–20.0	12.5–35.0	7.5–18.7	26.2–43.7	11.2–28.7	2.5–5.0	1.0–4.0
	6.4	49.4	10.9	24.0	9.8	36.3	18.7	3.8	2.1
	1.3	14.2	2.3	4.9	2.0	3.7	3.0	1.0	0.5
Azufrado Amarillo 33, semi commercial plot experiment station (INIFAP)	2.5–10.0	25.0–76.2	6.2–13.7	10.0–37.5	6.2–15.0	3.5–57.5	13.7–23.7	2.5–6.2	1.0–4.0
	5.7	52.4	10.0	23.1	8.8	35.0	19.4	3.5	2.8
	1.6	9.2	1.6	6.4	1.5	6.4	2.2	1.0	0.6
Pinto Bayacora, semi commercial plot experiment station (INIFAP)	2.5–8.7	36.2–101.2	6.2–12.5	18.7–43.7	6.2–11.5	10.0–43.7	16.2–22.5	–	2.0–4.0
	6.2	53.8	9.8	28.7	8.7	33.4	19.9	–	2.5
	1.8	15.4	1.3	5.7	1.3	6.7	1.5	–	0.6
Pinto Mestizo, semi commercial plot experiment station (INIFAP)	2.5–10.0	31.2–91.2	8.7–12.5	20.0–43.7	6.2–12.5	25.0–45.0	17.5–22.5	–	2.0–4.0
	6.0	56.3	10.0	28.6	8.7	34.3	20.0	–	2.3
	2.1	14.1	1.1	6.9	1.3	4.3	1.2	–	0.5
Pinto Saltillo, semi commercial plot experiment station (INIFAP)	2.5–7.5	21.5–98.7	6.2–12.5	12.5–47.5	6.2–10.0	28.7–43.5	16.2–22.5	2.5–5	1.0–4.0
	5.5	54.6	9.7	28.1	8.1	36.0	19.3	3.4	2.3
	1.4	15.6	1.5	7.1	1.3	3.3	1.3	0.8	0.6

Table 1. - Continued

Bean varieties/ location	Mycelium	Conidiophore		Foot cell		Conidia		Germ tube	Cells in the conidiophore
	Width	Length	Width	Length	Width	Length	Width	Width	
Pinto Durango 1616, semi commercial plot experiment station, Los Mochis Sinaloa	3.7–10.0 5.8 1.5	32.5–78.7 56.1 10.9	7.5–13.7 10.1 1.5	17.5–65.0 29.7 9.2	6.2–12.5 8.5 1.4	32.5–38.7 36.0 2.1	17.5–26.2 20.1 2.0	2.5–5.0 3.2 0.9	1.0–3.0 2.1 0.4
Pinto Americano 114, semi commercial plot experiment station (INIFAP)	2.5–10.0 6.5 1.7	41.2–96.2 66.1 13.6	7.5–15 11.1 1.7	17.5–55.0 32.4 8.6	6.2–11.5 9.1 1.3	23.7–47.5 39.1 5.4	17.5–25 21.0 1.8	– – –	2.0–3.0 2.4 0.4
Bill Z, semi commercial plot experiment station (INIFAP)	2.5–10.0 6.4 1.8	28.7–88.7 55.3 14.1	7.5–13.7 10.6 1.3	12.5–38.7 26.1 6.4	6.2–15.0 9.3 1.7	28.7–48.7 39.1 4.5	16.2–22.5 18.5 1.6	– –	2.0–3.0 2.3 0.5
Pinto Durango 1602, semi commercial plot experiment station (INIFAP)	2.5–7.5 5.8 1.3	26.2–91.2 56.5 15.3	8.7–13.7 11.0 1.3	13.7–37.5 24.8 5.4	7.5–12.5 9.8 1.4	30.0–46.2 39.0 3.9	16.2–23.7 20.2 1.9	– – –	2.0–3.0 2.2 0.4
Mean of ten samples	6.0	56.1	10.3	26.9	8.9	36.5	19.7	3.5	2.0

The first line presents the maximum and minimum length (1) of the structures; the second line indicates the means (2) of the 40 structures in each sample, while the third line refers to the standard deviation (3) of the data.

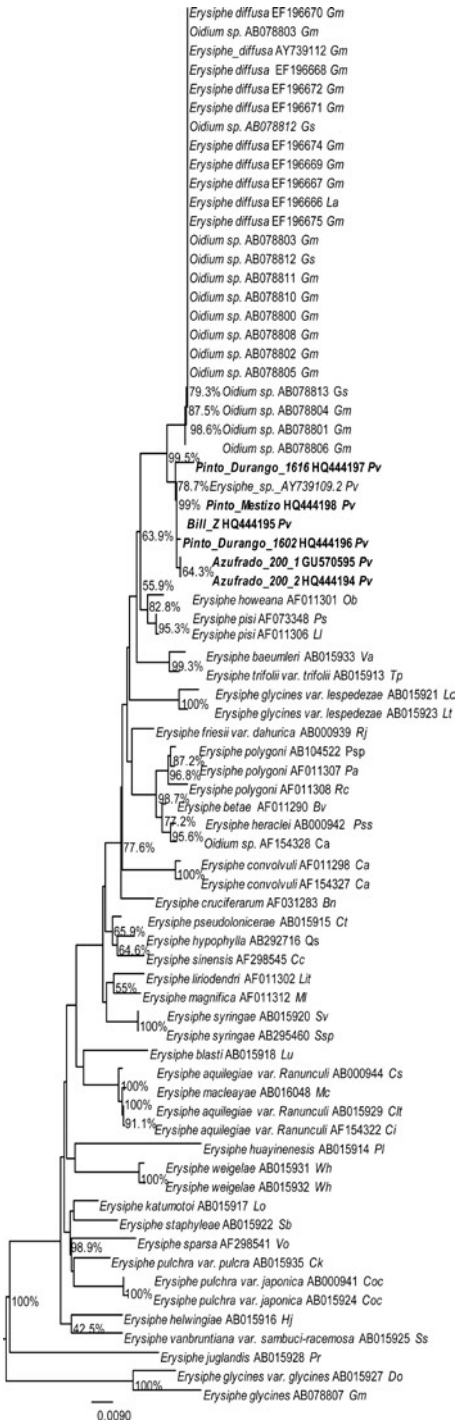
Applying scanning electron microscopy we could observe partially collapsed conidia with linear wrinkles and smooth ends. These observations are in agreement with the characteristics reported from *Erysiphe* species attacking leguminous plants (Cook *et al*. 1997).

Erysiphe polygoni DC. s. lat. has been previously reported causing powdery mildew of common bean in Sinaloa (León-Gallegos 1988) but no conclusive evidence or molecular proof was presented. Yáñez-Morales *et al*. (2009) reported the occurrence of *Erysiphe pisi* on *Phaseolus vulgaris* in central Mexico in Texcoco in 2008. The authors also discuss a collection of bean powdery mildew dating from 1953 which was referred to as *E. polygoni* previously. According to current concepts *E. polygoni s. str*. is confined to strains infecting Polygonaceae (Braun 1987). Collections on hosts of the Fabaceae previously assigned to *E. polygoni s. lat*. are now placed in *E. pisi* and *E. trifoliorum*, two common and widespread powdery mildew species on a wide range of legumes.

E. diffusa is reported to infect common bean in other parts of the world causing different symptoms than the ones observed in Sinaloa. These include necrosis, defoliation, discoloration, water-soaked lesions and wilting of leaves in some bean varieties; additionally, conidia of this species produce one true germ tube and up to 5 "germ tubes" which should be regarded as secondary hyphae (Mignucci & Chamberlain 1978). These symptoms are in contrast with those observed in Northern Sinaloa where the leaves of the bean plants exhibited small powdery spots which enlarged, coalesced, and covered the entire leaves.

Both *E. polygoni* and *E. diffusa* have been described by ITS rDNA analyses (Almeida *et al*. 2008). Our molecular study, based on the ITS1-5.8 rDNA–ITS2 rDNA region, supports the morphologic identification at the genus level, since the highest homology of the sequences of the

Fig. 4. – Phylogenetic tree based on ITS1, 5.8 rDNA and ITS2 sequences showing the relationships among six specimens of powdery mildew from Sinaloa state on different bean cultivars and 80 specimens reported previously (Almeida *et al*. 2008, Limkaisang *et al*. 2006). The tree was constructed by the neighbor joining method in Mega 4 Beta. Bootstrap support values are shown. Data includes microorganism species, Genbank number and host plant. Host plants: *Bn = Brassica napus*, *Bv = Beta vulgaris*, *Ca = Convolvulus arvensis*, *Cc = Castanea crenata*, *Ci = Clematis integrifolia*, *Ck = Cornus kousa*, *Clt = Clematis terniflora*, *Coc = Cornus controversa*, *Cs = Cimicifuga simplex*, *Ct = Cocculus trilobus*, *Do = Desmodium oxyphyllum*, *Gm = Glycine max*, *Gs = Glycine soja*, *Hj = Helwingia japonica*, *La = Lupinus albus*, *Lc = Lespedeza cuneata*, *Lit = Liriodendron tulipifera*, *Ll = Lathyrus latifolius*, *Lo = Ligustrum obtusifolium*, *Lt = L. thunbergii*, *Lu = Lindera umbellata*, *Mc = Macleaya cordata*, *Ml = Magnolia liliflora*, *Ob = Oenothera biennis*, *Pa = Polygonum arenastrum*, *Pl = Plectranthus logotubus*, *Pr = Pterocarpa rhoifolia*, *Ps = Pisum sativum*, *Psp = Polygonum sp*., *Pss = Panax schin-seng*, *Pv = Phaseolus vulgaris*, *Qs = Quercus serrata*, *Rc = Rumex crispus*, *Rj = Rhamnus japonica*, *Sb = Staphylea bumalda*, *Ss = Sambucus sieboldiana*, *Ssp = Syringa spp*., *Sv = Syringa vulgaris*, *Tp = Trifolium pratense*, *Va = Vicia amoena*, *Vo = Viburnum opulus*, *Wh = Weigela hortensis*.



specimens of Sinaloa branch next to a sequence described as *Erysiphe sp*. also associated with common bean. This sequence corresponds to a Brazilian specimen described by Almeida *et al*. (2008). This specimen showed characteristics of *E. polygoni s. lat*., as also described in the present work. However, there is a large number of *Erysiphe* species on legumes (Braun *et al*. 2010), including the common and widespread species *E. pisi* and *E. trifoliorum*. Unfortunately anamorphs of *Erysiphe* spp. infecting Fabaceae are morphologically not sufficiently distinguishable to allow species identification. However, phylogenetic analysis revealed that our specimens branched together with specimens of *Oidium* sp. and *E. diffusa* from soybean and wild soybean (Takamatsu *et al*. 2002, Almeida *et al*. 2008) and are genetically distant to specimens of *E. polygoni* (Almeida *et al*. 2008). Although we could find the teleomorphic stage of the fungus, our molecular studies suggest that the specimens from Sinaloa, Mexico were the *Oidium* stage of *E. diffusa*.

The powdery mildews from Sinaloa are closely related to *Erysiphe* sp., a powdery mildew of common bean from Brazil. Also it seems plausible that the causal agents of the disease in Sinaloa, Mexico and in Brazil (Almeida *et al*. 2008) are different strains of *E. diffusa*. But they differ from typical *E. diffusa* from soybean and wild beans by their symptomatic traits. A more detailed study is needed including a higher number of specimens and the use of additional molecular techniques. Additionally, efforts should be focused on finding the teleomorph. Furthermore, the role of wild bean and other legume species should be investigated as potential sources of *E. diffusa* on commercial bean varieties in Sinaloa and other bean producing areas.

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