Research Note

MONITORING PHAKOPSORA *RUST IN PUERTO RICO*[1,2]

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Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* was first detected in the continental United States in Louisiana in 2004. The threat of the introduction of ASR throughout the Caribbean after its report on soybean [*Glycine max* L. (Merr.)] in Louisiana in 2004 motivated the establishment of a soybean rust surveillance in Puerto Rico (Scheneider et al., 2005). Our original hypothesis was that the Caribbean would be a pathway for ASR entering North America because of the proximity to South American countries where this pathogen was present. In South America, *P. pachyrhizi* has been reported in Argentina (Rossi, 2003), Bolivia, Brazil, Uruguay (Yorinori et al., 2005), Colombia (Godoy, 2006), and Ecuador (Linzan, 2005. National Agricultural Research Institute, Ecuador, *pers. comm*.). *Phakopsora pachyrhizi* can reduce yields in areas where the pathogen commonly occurs; yield losses can reach up to 80% (Kuchler et al., 1984; Miles et al., 2003). Soybean is a valuable crop in Puerto Rico; in fact, seed companies established on the island since the 1970s cultivate approximately 500 ha of soybeans, with a market value of $30 million per year (Pérez, 2006. Monsanto Caribe, *pers. comm*.).

The American soybean rust (AmSR) caused by *Phakopsora meibomiae* is endemic to Puerto Rico on numerous legumes; it is restricted to the mountainous areas (Estévez de Jensen et al., 2006). In 1976 an AmSR outbreak occurred in soybeans, dry beans and *Phaseolus coccineus*, thus indicating that the rust was a major concern in Puerto Rico (Bromfield, 1984). In May 2004, a survey of ASR rust was conducted in Puerto Rico, and *Phakopsora meibomiae* was identified in *Lablab purpureus* (hyacinth bean) and *Teramnus uncinatus* (Hernández, 2004). The objectives of this study were to conduct a survey in different areas of Puerto Rico to identify the presence of *Phakopsora* spp. in cultivated and wild legumes and to search for the presence/absence of the ASR, by using sentinel plots. *Phakopsora* spp. identification was conducted with conventional PCR and ELISA techniques.

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Phakopsora rust monitoring and identification

Sentinel soybean plots were planted at three locations: Adjuntas, Isabela, and Juana Díaz at the Agricultural Experiment Stations (Table 1). Each sentinel plot was sown on three planting dates (Table 1). Plots were sown with soybeans and dry beans in a complete randomized block design that was replicated four times. Plots consisted of ten 0.25- × 0.63-m rows.

Plots were monitored weekly for early symptoms, which include chlorosis (yellowing or flecking) and pustules (blisters or lesions) mainly on the undersides of the lower leaves. Weekly evaluations included the presence of signs and symptoms of soybean rust. Symptomatic and asymptomatic soybean and dry bean leaves were collected and placed in double plastic bags to prevent the release of spores, and were stored on ice prior to examination at the University of Puerto Rico Plant Diagnostic Clinic at Juana Díaz Agricultural Experiment Station. Seven evaluations in each trial were conducted at Isabela and Juana Díaz to monitor diseases and pests. The location, description of the symptoms, and scouting dates were recorded for each location. After storage for less than 24 h at 4° C, samples were removed from the bags and symptoms were described. Leaves were placed in a humid chamber for 12 h and then examined under the stereoscope. Digital images of lesions were also taken with either a dissecting or compound microscope and submitted to the Digital Diagnostic Identification System (DDIS) (Xin et al., 2001). Because it can be difficult to morphologically differentiate between *P. pachyrhizi* and *P. meibomiae* (Ono and Hennen, 1992), which has been reported in Puerto Rico since 1976, an ELISA test was conducted with an Envirologix Qualiplate Kit TM[6] (Envirologix, Portland, ME) (Wayne et al., 2007). Samples were also evaluated by using conventional Polymerase Chain Reaction (PCR) in a thermocycler (Perkin Elmer 480). The PCR assay was performed in a total volume of 20 µl containing 10 µl of master mix, 3 µl of each primer forward and reverse, and 4 µl genomic DNA. Oligonucleotide primers used were specific to *P. pachyrhizi* (5' TAA GAT CTT TGG GCA ATG GT 3' "forward" / 5' GCA ACA CTC AAA ATC CAA CAA CAA T 3' "reverse") or to *P. meibomiae* (5' GAA GTT TTT GGGCAA ATC AC-3' "forward" / 5' GCA CTC AAA ATC CAA CAT GC 3' "reverse") (Frederick et al., 2002). Polymerase Chain Reaction was performed with the following cycling conditions: 94° C denaturation for 2 min, 35 cycles of 94° C for 30 s, 65° C for 30 s and 72° C for 30 s, followed by an extension of 72° C for 6 min. Negative controls were tested using the same reaction mixture and amplification conditions without DNA template. Polymerase Chain Reaction products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (Wayne et al., 2007).

Positive controls included DNA samples from soybean infected with *P. pachyrhizi* (University of Florida) and DNA samples from *Lablab purpureus* infected with *P. meibomiae* from Adjuntas, Puerto Rico. Five samples of *Lablab purpureus* DNA were sent to the University of Florida (UF/IFAS) for confirmation and identification using Real Time PCR.

Survey of Phakopsora spp. from legume hosts

From 2005 to 2007, a detailed survey of wild and cultivated legumes was conducted in 26 municipalities of Puerto Rico (Table 2). Approximately 21 plants reported as hosts or that had developed symptoms after inoculation with *Phakopsora pachyrhizi* (USDA/

[6]Trade names in this publication are used only to provide specific information. Mention of a trade name does not constitute a warranty of equipment or materials by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

TABLE 1.—*Location, altitude, date of planting, average air temperatures and precipitation in Adjuntas, Isabela and Juana Díaz.*

	Location	Altitude (m)	Date of Planting	Average Air Temperatures °C		Total Annual Precipitation mm
				Max	Min	
Adjuntas	N 18°10´ W 066°47'	594	Feb. 28, 2006 May 26, 2006 July 25, 2006	23	16	1,340
Isabela	N 18°27 W 067°03'	128	Nov. 22, 2005 Feb. 17, 2006 May 2, 2006	27	22	1,200
Juana Díaz	N 18°10´ W 066°47'	28	Nov. 16, 2005 Dec. 15, 2006 Feb. 14, 2006	27	21	1,143

TABLE 2.—*List of legumes collected during the survey.*

Legume	Common name
Cajanus cajan	pigeon pea
Canavalia ensiformis	canavalia
Clitoria ternatea	butterfly pea
Crotalaria pallida	smooth rattlebox
Desmodium heterophyllum	variable-leaf ticktrefoil
Glycine max	soybean
Glycine wrightii	glycine
Lablab purpureus	bonavit bean, chícharo
Macroptilium atropurpureum	purple bean
Macroptilium lathyroides	pea bean, phasey bean, wild bean
Mimosa ceratonia	mimosa
Mucuna pruriens	velvet bean
Pachyrrhizus erosus	wild yam bean
Phaseolus lunatus	butter bean, lima bean
Phaseolus vulgaris	dry bean, green bean
Pueraria phaseoloides	tropical kudzu
Teramnus uncinatus	frijolillo
Vigna luteola	harypod cowpea
Vigna unguiculata	cowpea, black-eyed pea

APHIS, 2002) were collected. Disease severity was evaluated on five leaves by using the modified scale of Vakili (Vakili, 1981): 1 = immune reaction; 2 = <5% leaf area infected; 3 = infection between 5 and 25% leaf area infected; 4 = severity between 26 and 50% leaf area infected; and 5 = >51% leaf area infected.

Symptoms under search were chlorosis (yellowing or flecking) on the upper side of the leaves, and pustules (blisters or lesions) on the undersides of the leaves. Samples collected were placed in a double plastic bag with a dry paper towel and were transported in a cooler to the laboratory for analysis. We collected 21 plants that were reported as hosts or that had developed symptoms after inoculation with *P. pachyrhizi* (USDA/ APHIS, 2002). Plants listed in Tables 2, 3, 4 and 5 show the legumes collected and the location in the survey. A positive result for PCR indicated that a 330 bp amplicon was observed with UV illumination after staining with ethidium bromide.

Phakopsora spp. rust monitoring and identification

No symptoms of rust were visible on soybeans or dry beans during the seven evaluations conducted from each sentinel plot at each location. Leaf samples placed in humid chamber conditions did not show presence of rust uredospores. Moreover, DNA amplification of asymptomatic leaf tissue of selected samples of soybean and dry bean from sentinel plots showed neither *Phakopsora pachyrhizi* nor *P. meibomiae*. The location of the sentinel plots exhibited variable climatic conditions. On the north coast at Isabela, average temperatures ranged from about 24° C in January to 27° C in July. Temperatures on the south coast averaged roughly 1° C higher. For every rise in elevation of 300 m, temperatures fell about 2.2° C. In Juana Díaz average low air temperatures ranged from 21 to 24° C and average high air temperatures ranged from 21 to 27° C. The coolest areas on the Island are in Adjuntas, where ideal mean temperatures for rust development (24° C) occur during the months of December, January and February. However, rainfall is limited

TABLE 3.—*Distribution of* Phakopsora meibomiae *in cultivated and wild hosts in the Municipality of Adjuntas, Puerto Rico.*

Host	Altitude (meters)	GPS	Disease severity 1- 5	PCR[2] Ppa[3]	PCR[2] Pme[4]	ELISA
Lablab purpureus	453	N 18°11.897' W 066°43.729'	3	—	+	—
Lablab purpureus	560	N 18°11.162' W 066°45.411'	3	—	+	+
Glycine spp.	453	N 18°11.897' W 066°43.729'	1	—	—	—
Pueraria phaseoloides	453	N 18°11.897' W 066°43.729'	1	—	—	—
Phaseolus vulgaris	594	N 18°10' 633", W 066°47'520"	1	—	—	—
Macroptillium lathyroides	593	N 18°10'454", W 066°47'301"	1	—	—	—
Vigna luteola	453	N 18°11'554", W 066°30'946"	2	—	+	—
Clitoria ternatea	454	na[1]	2	—	+	—
Glycine max	454	N 18°10'474", W 066°47'961"	1	—	—	—
Phaseolus vulgaris	454	N 18°10'474", W 066°47'961"	1	—	—	—

[1]not assessed.
[2]PCR = Polymerase Chain Reaction.
[3]Ppa = *Phakopsora pachyrhizi.*
[4]Pme = *Phakopsora meibomiae.*

TABLE 4.—*Distribution of* Phakopsora meibomiae *in cultivated and wild hosts in the Municipality of Isabela, Puerto Rico.*

Host	Altitude (meters)	Location	Disease severity 1-5	PCR[1]		ELISA
				Ppa[2]	Pme[3]	
M. pruriens	128	N 18°28.012' W 067°03.289'	1	—	—	—
L. purpureus	128	N 18°28.012' W 067°03.289'	1	—	—	+
Cajanus cajan	128	N 18°28.012' W 067°03.289'	3	—	+	—
Pachyrhizus erosus	128	N 18°28.012' W 067°03.289'	1	—	—	—
Cannavalia ensiformis.	128	N 18°28.012' W 067°03.289'	2	—	+	—
Desmodium heterophyllum	128	N 18°28.012' W 067°03.289'	1	—	—	—
Crotalaria pallida	128	N 18°28.012' W 067°03.289'	1	—	—	—
L. purpureus	128	N 18°28.012' W 067°03.289'	1	—	—	—

[1]PCR = Polymerase Chain Reaction.
[2]Ppa = *Phakopsora pachyrhizi.*
[3]Pme = *Phakopsora meibomiae.*

TABLE 5.—*Distribution of* Phakopsora meibomiae *in cultivated and wild hosts in the Municipality of Juana Díaz, Puerto Rico.*

Host	Altitude (m)	Location	Disease severity 1-5	PCR[1]		ELISA
				Ppa[2]	Pme[3]	
Glycine max	28	N 18°01.558' W 066°31.529'	1	—	—	—
Lablab purpureus	28	N 18°01.558' W 066°31.529'	1	—	—	—
Phaseolus vulgaris	28	N 18°01.558' W 066°31.529'	1	—	—	—
Vigna unguiculata	28	N 18°01.558' W 066°31.529'	1	na	na	—
Macroptillium lathyroides	28	N 18°01.558' W 066°31.529'	1	na	na	—

[1]PCR = Polymerase Chain Reaction.
[2]Ppa = *Phakopsora pachyrhizi.*
[3]Pme = *Phakopsora meibomiae.*

FIGURE 1. Symptoms of *Phakopsora meibomiae* in legumes collected in Adjuntas, P.R. A) *Lablab purpureus*; B) *Canavalia* sp.; C) *Pachyrrhizus erosus.*

during these months, all of which is not conducive to soybean rust infection. Humid conditions and temperatures ranged from 15 to 28° C, optimal for soybean rust infection, as well as for soybean growth (Melching et al., 1989). Uredospore infection requires 6 to 12 h of moisture, and the spread of the disease is enhanced by moist conditions (Marchetti et al., 1976; Melching et al., 1989).

During three years of plot evaluations at Juana Díaz, Isabela and Adjuntas, endemic diseases were observed in dry beans, fungi such as *Alternaria* spp. causing leaf spot, and *Uromyces appendiculatus* causing bean rust (only in Isabela). In soybeans the most prevalent foliar diseases were frog eye leaf spot caused by *Cercospora* spp., and bacterial pustule caused by *Xanthomonas* spp. In Juana Díaz, *Phomopsis* spp. was isolated from soybean leaf spots, and during 2006 and 2007 an outbreak of *Peronospora* sp. occurred in Isabela at R3 plant stage. Bacterial pustule, a common disease occurring in Isabela, showed symptoms similar to those of soybean rust. However, bacterial pustules had the clear yellow halo associated with the disease.

No incidence of *P. pachyrhizi* rust on *Lablab purpureus* or any other legume species evaluated was observed at the three locations, possibly because of lack of precipitation. Rainfall favors rust infection in Puerto Rico (Vakili, 1976; Vakili, 1979). At locations where environmental conditions were not ideal for rust development, such as Juana Díaz, the absence of soybean rust may favor the expansion of soybean winter nurseries on the Island.

Phakopsora spp. rust detection survey of legume hosts

Soybean rust infects over 95 species of plants belonging to 42 genera (Bromford, 1980). The northern and southern central mountainous areas of Puerto Rico have a diversity of legumes growing in the wild. Rust symptoms in *Lablab* occurred as angular lesions on the upper leaf surface, or small yellow flecks or specks. Lesions were situated near the leaf veins and when developed were cone-shaped. Initially the pustules are small, irregular with reddish brown color. When pustules are mature they produce large numbers of light-colored, powdery spores (urediospores). The rust in *Lablab purpureus* was identified as *Phakopsora meibomiae*; none of the samples were positive for *P. pachyrhizi*. During 2005, 2006 and 2007, *Lablab purpureus* samples were collected in 27 municipalities of Puerto Rico (Tables 3, 4 and 5). At the locations visited, severity of rust infection varied, depending on the location and leaf position in the plant. Most of the infected leaves were located at the lower part of the plant in close proximity with the ground and under the shade. Disease severity measured on a scale of 1 to 5 varied from 2 to 4 measured in percentage leaf area covered (Figure 1). Mature tan colored rust pustules located on the adaxial leaf surface erupted with uredinia containing beige to tan-colored urediniospores (Figure 1). Urediniospores were ornamented, ellipsoid, and were

TABLE 6.— *Distribution of* Phakopsora meibomiae *in cultivated and wild hosts in different Municipalities, Puerto Rico.*

Site of Collection	Hosts	Location	Disease severity 1-5	PCR[2]		ELISA
				Ppa[3]	Pme[4]	
Corozal	*Lablab purpureus*	N 18°16.908' W 066°18.224'	3	—	—	—
Corozal	*Mimosa ceratonia*	na[1]	-	na	na	—
Cayey	*L. purpureus*	na	3	—	+	—
Cayey	*Teramnus uncinatus*	na	2	—	+	—
San Sebastián	*L. purpureus*	na	3	—	+	—
San Sebastián	*Phaseolus lunatus*	na	2	—	+	—
San Sebastián	*Vigna spp.*	na	1	—	—	—
San Sebastián	*M. atropurpureum*	na	1	—	—	—
Yahuecas	*L. purpureus*	N 18°12.812 W 066°37.823	3	—	+	—
Yahuecas	*Crotalaria pallida*	"	1	—	—	—
Yahuecas	*Macroptillium lathyroides*	"	1	—	—	—
Yahuecas	*Vigna sp.*	"	1	—	+	—
Castañer	*L. purpureus*	na	3	—	+	—
Castañer	*M. lathyroides*	na	1	—	—	—
Castañer	*Vigna luteola*	na	1	—	—	—
Jayuya	*L. purpureus*	N 18°13.040' W 066°36.072'	3	—	+	—
Jayuya	*Phaseolus lunatus*	na	na	—	—	—
Guaynabo	*L. purpureus*	na	na	—	—	—

[1]not assessed.
[2]PCR = Polymerase Chain Reaction.
[3]Ppa = *Phakopsora pachyrhizi.*
[4]Pme = *Phakopsora meibomiae.*

TABLE 6.—*(Continued) Distribution of* Phakopsora meibomiae *in cultivated and wild hosts in different Municipalities, Puerto Rico.*

Site of Collection	Hosts	Location	Disease severity 1-5	PCR[2]		ELISA
				Ppa[3]	Pme[4]	
Fajardo	*L. purpureus*	na	na	—	—	—
Coamo	*L. purpureus*	na	3	—	+	—
Utuado	*L. purpureus*	na	3	—	+	+
Utuado	*Mucuna pruriens*	na	2	—	+	—
Lajas	*L. purpureus*	na	1	—	—	—
Aibonito	*L. purpureus*	na	3	—	+	—
Yauco	*L. purpureus*	na	3	—	+	—

[1]not assessed.
[2]PCR = Polymerase Chain Reaction.
[3]Ppa = *Phakopsora pachyrhizi.*
[4]Pme = *Phakopsora meibomiae.*

FIGURE 2. Agarose Gel of Polymerase Chain Reaction of *Phakopsora* spp. in *Lablab purpureus*. DNA amplification of ITS region of rDNA produced a 330 bp fragment observed with UV illumination after staining with ethidium bromide. Lane 1 = 100 bp ladder; lane 2 = Positive control for *Phakopsora pachyrhizi* (Ppa); lane 3 = *Lablab* Aibonito 1:10 Ppa; lane 4 = *Lablab* Aibonito 1:10 *Phakopsora meibomiae* (Pme); lane 5 = *Lablab* Aibonito 1:100 Ppa; lane 6 = *Lablab* Aibonito 1:100 Pme; lane 7 = *Lablab* Aibonito 1:1000 PPa; lane 8 = *Lablab* Aibonito 1:1000 Pme; lane 9 = *Lablab* Corozal 1:10 Ppa; lane 10= *Lablab* Corozal 1:10 Pme; lane 11 = *Lablab* Corozal 1:100 Ppa; lane 12 = *Lablab* Corozal 1:100 Pme; lane 13 = *Lablab* Corozal 1:1000 Ppa; lane 14 = *Lablab* Corozal 1:1000 Pme.

DNA dilutions: 1:10, 1:100 and 1:1000

slightly yellowish-brown when observed under a compound microscope (Marchetti et al., 1975). No teliospores were observed on leaf tissue. The DNA extracted from symptomatic leaves did not amplify for *P. pachyrhizi* even though ELISA tests for this species were positive (Tables 3, 4, 5 and 6). Three of those samples were also analyzed by USDA/APHIS in Beltsville by using Real Time PCR, confirming negative results for *P. pachyrhizi*.

Pachyrhizus erosus planted at Isabela in 2006 was infected with rust. Disease severity was moderated; symptoms included small red-brown lesions and a few pustules on the underside of the leaves. Pustules were scattered and urediniospores were ornamented, ellipsoid, hyaline and yellowish-brown when viewed with a compound microscope, similar to pustules of *P. meibomiae* from *Lablab purpureus* (Figure 2). Polymerase Chain Reaction tests from leaf tissue infected with rust were positive for *P. meibomiae* but not for *P. pachyrhizi*. Interestingly, ELISA test was positive for *P. pachyrhizi*, thus indicating a false positive.

In 2007, *Cannavalia ensiformis* was found to be infected with rust in Isabela. Symptoms included tan lesions and abundant pustules on the underside of the leaf containing numerous urediniospores. Lesions were expanded and were visible on the upper leaf surface. Mature rust pustules erupted with volcano-like uredinia containing beige- to tan-colored urediniospores. Polymerase Chain Reaction tests of DNA extractions from pustules developed on the underside of the leaf were positive for *P. meibomiae*

(Figure 2). At the location of Yahuecas in Adjuntas (Table 6), *Vigna* spp. showed pustules only on a few older leaves. Pustules were visible and widely spread on the abaxial leaf surface and were widely spread and more numerous towards the tip of the leaf. Pustules were hard and showed very few uredinospores (Figure 1). Under the stereoscope rust pustules had conspicuous uredinia and a few hyaline urediniospores.

This survey covered a wide variety of legume species and ecological niches. Of all samples tested, only *Phakopsora meibomiae* was identified on *Lablab purpureus, Cannavalia ensiformis, Pachyrhizus erosus, Mucuna pruriens* and *Vigna* sp. as previously reported (Vakili and Bromfield, 1976; Rytter et al., 1984). We sampled at the same location in Adjuntas, where in 1976 an outbreak of *Phakopsora* rust had occurred on *Phaseolus vulgaris, Phaseolus coccineus* and *Glycine max* (Vakili, 1981). However, *Phakopsora pachyrhizi* was not detected; the only species prevalent in the legumes was *P. meibomiae*. In Puerto Rico neither of the rust species has been confirmed on soybeans at this time.

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