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Use of AFLP markers to estimate molecular diversity of *Phakopsora pachyrhizi*

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ABSTRACT

Background: Asian soybean rust (SBR) caused by *Phakopsora pachyrhizi* Syd. & Syd., is one of the main diseases affecting soybean and has been reported as one of the most economically important fungal pathogens worldwide. Knowledge of the genetic diversity of this fungus should be considered when developing resistance breeding strategies. We aimed to analyze the genetic diversity of *P. pachyrhizi* combining simple sampling with a powerful and reproducible molecular technique.
Results: We employed Amplified Fragment Length Polymorphism (AFLP) technique for the amplification of *P. pachyrhizi* DNA extracted from naturally SBR-infected plants from 23 production fields. From a total of 1919 markers obtained, 77% were polymorphic. The high percentage of polymorphism and the Nei's genetic diversity coefficient (0.22) indicated high pathogen diversity. Analysis of molecular variance showed higher genetic variation within countries than among them. Temporal analysis showed a higher genetic variation within a year than between years. Cluster, phylogenetic and principal co-ordinate analysis showed that samples group by year of collection and then by country sampled.
Conclusions: The study proposed combining a simple collection of urediniospore with a subsequent analysis by AFLP was useful to examine the molecular polymorphism of samples of *P. pachyrhizi* collected and might have a significant contribution to the knowledge of its genetic diversity. Also, AFLP analysis is an important and potent molecular tool for the study of genetic diversity and could be useful to carry out wider genetic diversity studies.

1. Introduction

Asian soybean rust (SBR), caused by the biotrophic fungus *Phakopsora pachyrhizi* Syd. & Syd, is one of the main diseases affecting soybean [1]. In South America SBR was first reported in Paraguay and Brazil in 2001 causing important yield losses up to 75% [2]. Six SBR resistance loci, *Rpp1-6*, have been mapped in soybean [3]. However, resistance conferred by these alleles is not always durable since it can become ineffective after only a few years. This evidence suggests rapid changes in virulence probably based on the high genetic diversity in *P. pachyrhizi*. For that reason it is important to estimate the genetic diversity of this pathogen in order to develop efficient disease management approaches as well as breeding strategies to obtain new long-term resistant cultivars. Early genetics diversity studies of *P. pachyrhizi* were based on comparisons of the nucleotide sequence in conserved regions [4,5] and as it was expected, they resulted in a low polymorphism. Later, Twizeyimana et al. [6] found high molecular genetic diversity using variable simple sequence repeat (SSR) markers. On the other hand, Amplified Fragment Length Polymorphism (AFLP) markers [7] have been used successfully to characterize

genetic diversity of several pathogens, including fungal rust of other crops [8,9]. These markers that allow detecting a large number of polymorphic bands simultaneously, have not yet been tested in *P. pachyrhizi*.

Another important aspect that should be considered in diversity studies of *P. pachyrhizi*, due to its biotrophic nature, is the sampling procedure. Several studies involved urediniospores collection by scraping or by using infected leaves segments [4,5] with the potential risk of contaminations in the analysis. In a recent study Twizeyimana et al. [6] cultivated urediniospores on detached leaves from a very susceptible cultivar through several cycles of infection and collection to establish purified cultures; however it could be time consuming.

In the present study an approach combining urediniospore collection and subsequent genetic analysis by AFLP was established and used to examine the molecular polymorphism of isolates of *P. pachyrhizi* collected from various "agro-ecological" locations in Argentina, Brazil, Paraguay and Uruguay.

2. Material and methods

2.1. P. pachyrhizi sampling

Bulk urediniospores were collected from 20 to 40 leaves of 10 to 20 naturally SBR-infected plants chosen randomly from commercial production fields. Each sample pool represents more than one rust genotype. Four samples were collected in Argentina and five in Paraguay during 2008; while in 2009 three samples were collected in Brazil, seven in Paraguay and four in Uruguay, accounting a total of 23 samples (Fig. 1). A variable number of samples were collected at each country and year depending on the presence of the disease. Numerous samples were collected in Paraguay since the pathogen was spread all over the producing area, whereas in Argentina, a very large soybean producer, *P. pachyrhizi* only caused phytosanitary problems in limited regions at the North of the country. Samples from Brazil were collected from geographically dispersed locations, while samples from Uruguay were taken at the single producing area at that moment. Thus, the sampling approach by using countries (not individual localities) as the level of hierarchical sub-division in the diversity study included a minimum of three samples in each country.

Samples were obtained by harvesting urediniospores from pustules using a stereo microscope and a micropipette with a Tween 20 (2% v/v) solution. An additional exhaustive microscopic inspection was carried out to assure the clean composition of urediniospores in each sample.

2.2. DNA extraction

Total genomic DNA was extracted from 10 mg of urediniospores using a cetyltrimethyl ammonium bromide (CTAB) protocol from Villavicencio et al. [10] with minor modifications. DNA concentration was determined spectrophotometrically according to Sambrook et al. [11]. Frozen ground mycelium of pure cultures maintained in our laboratory of *Fusarium* sp., *Macrophomina phaseolina* and *Colletotrichum* sp. was employed for DNA extraction using the same procedure, to include them in the diversity study as outgroup controls.

2.3. PCR identification

To confirm the identity of the collected samples, PCR-amplification with *P. pachyrhizi*-specific primers Ppa1 (5′-TAAGATCTTTGGGCAATG GT-3′) and Ppa2 (5′-GCAACACTCAAAATCCAACAAT-3′) was performed [12]. As a positive control, DNA from an infected sample with *P.*
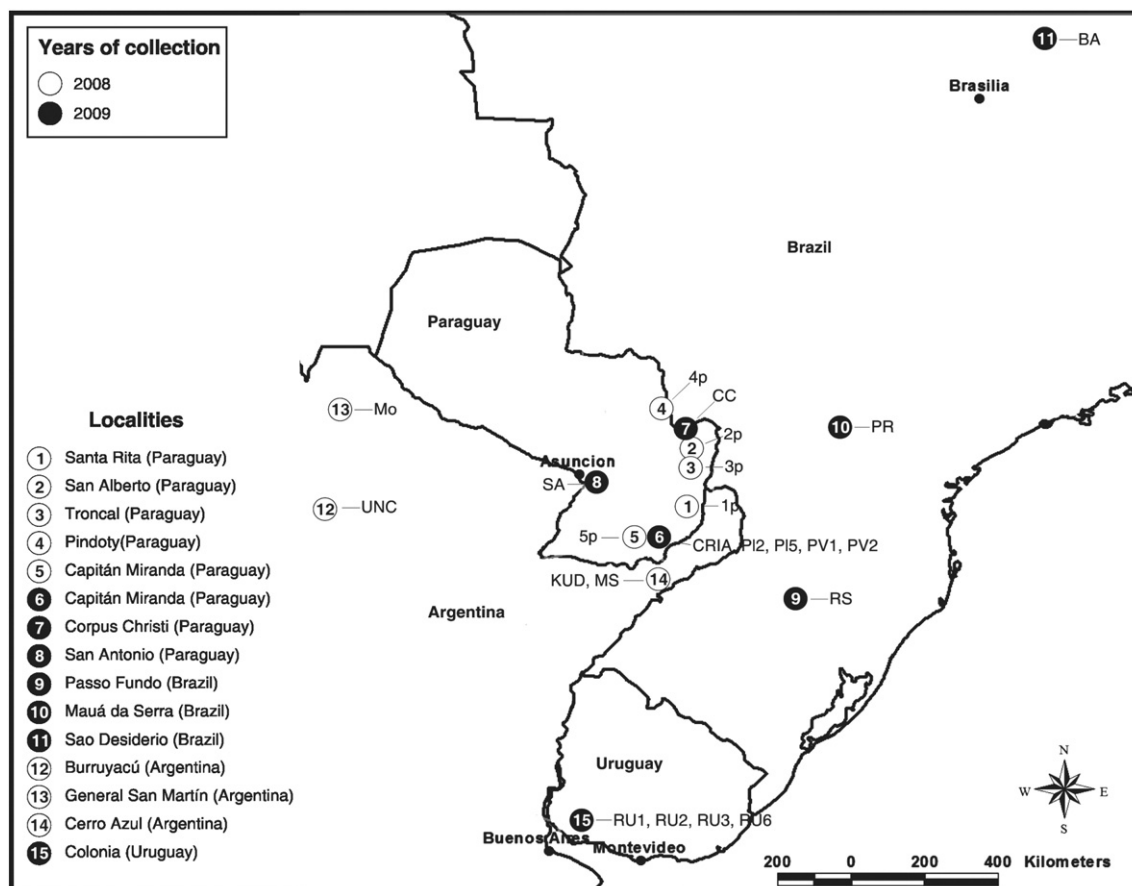

Fig. 1. Geographic locations of the different sampling points where *P. pachyrhizi* was collected between 2008 and 2009 seasons. Names in the map belong to the 23 samples collected.

pachyrhizi previously collected at a location in Northwest Argentina was used, whereas soybean DNA was used as a negative control.

2.4. AFLP protocol, electrophoresis and gel staining

The AFLP protocol was performed according to Vos et al. [7] with some modifications. Amplification reactions with 34 primer combinations having one or three selective nucleotides were performed. Each combination was repeated at least twice by an independent AFLP procedure to confirm band patterns. Final amplification products were separated on 6% denaturing polyacrylamide gels (Amersham Pharmacia Biotech, UK) using a Sequi-Gen GT System (Bio-Rad, CA). A molecular weight marker was included in all gels (size range 250–1500 bp). Gels were stained with silver nitrate according to manufacturer instructions for the Silver Staining System (Promega Corporation, WI).

2.5. Data analysis

Percentage of polymorphism and Nei's genetic diversity [13] were calculated to measure the genetic variability among samples from different countries and years of collection, using Info-Gen software (Software for statistical analysis of genetic data, version 2014). An analysis of molecular variance (AMOVA) was performed also using Info-Gen software. AMOVA produces estimates of variance components and F-statistic analogues, designated as phi-statistics (Phi$_{ST}$) that reflect genetic diversity at different levels of hierarchical subdivision. The two hierarchical levels analyzed were: regional (by countries) and temporal (by years) but only considering samples from Paraguay, since they were collected in 2008 and 2009.

Each band (monomorphic or polymorphic) was scored in a dominant manner and transformed into either a 0 (absent) or 1 (present) matrix. Only bands that could be scored unequivocally were included in the analysis. The range between 80 and 1000 bp of the band size was considered for the analysis. Genetic similarity was calculated by using Jaccard index (Sj) [14]. Cluster analyses were carried out using McQuitty similarity analysis (Unweighted Pair Group Method with Arithmetic Mean, UPGMA) to determine the genetic diversity of the samples. All calculations were conducted by using Info-Gen software. To evaluate the robustness of the groups formed, the binary data set was subjected to bootstrapping using the WINBOOT program [15]. The dendrogram was reconstructed 1000 times and the frequency which a particular group was formed was considered to reflect the strength of the group. The limit of 50% was considered to indicate statistical support for the topology at a node in the bootstrap consensus tree [16].

In addition to evaluate the molecular evolution of the *P. pachyrhizi* collected samples, a minimum evolution tree (neighbor-joining tree) was constructed using the software Mega 6 [17] based on the distance matrix from Info-Gen.

A Principal Co-ordinate Analysis (PCoA) was performed using Info-Gen software, which uses spectral decomposition to approximate a matrix of distances/dissimilarities by the distances between a set of points in few dimensions. The points were used to visualize grouping of the samples.

3. Results

The presence of *P. pachyrhizi* was confirmed in all samples collected exhibiting typical SBR symptoms, since only a fragment of expected size (332 bp) was amplified with species-specific PCR-primers.

The AFLP banding pattern was highly reproducible for the 23 samples of *P. pachyrhizi* using 34 pairs of AFLP primers (Supplementary Fig. 1). Out of 1919 total markers obtained, 77% were polymorphic. The average percentage of polymorphic loci was 68% and the Nei's genetic diversity coefficient was 0.22 (Table 1). The

Table 1
Summary of statistics for AFLP markers from *P. pachyrhizi* samples collected from Argentina, Brazil, Paraguay and Uruguay in 2008 and 2009.

Countries	Year of sampling	No. samples	Polymorphic loci (%)	Nei's genetic diversity
Argentina	2008	4	33	0.15
Brazil	2009	3	17	0.09
Paraguay	2008	5	47	0.2
Paraguay	2009	7	59	0.24
Uruguay	2009	4	9	0.04
Total		23	68	0.22

highest genetic diversity was found in Paraguay in 2009 while the lowest was detected in Uruguay, the same year.

The AMOVA results for the regional analysis by year are shown in Table 2. Among samples collected in 2008, a high genetic variation was observed within countries (93% of total variation). The Phi$_{ST}$ value was 0.07 representing a low genetic divergence among samples from different countries. The same trend was observed for samples collected in 2009. The AMOVA analysis by year for samples collected in Paraguay revealed a high genetic variation within a year (87%). The Phi$_{ST}$ value obtained was 0.13, indicating a moderate degree of variance between the two years of sampling.

To estimate the genetic similarity between the samples collected, Jaccard's distances were calculated and a dendrogram was constructed (Fig. 2). The cluster analysis of *P. pachyrhizi* revealed that samples from Uruguay group separated at a 0.94 distance from the main cluster. At a distance value of 0.87 two clusters according to year of collection were differentiated. In addition, within each of the two clusters all samples from the same country were sub-clustered. Outgroup controls of *Fusarium* sp., *M. phaseolina* and *Colletotrichum* sp. had a dissimilitude value of 0.97. The reliability of the dendrogram tested by bootstrap analysis with 1000 replications showed that the majority of the clusters exhibited very high bootstrap values.

In the phylogenetic analysis (Fig. 3), at least five groups could be clearly distinguished: i) samples from Argentina and Paraguay collected in 2008; while the rest of the groups corresponding to samples collected in 2009 were: ii) and iii) from Paraguay, iv) from Brazil and v) from Uruguay. As in the diversity analysis, samples grouped mainly by the year of collection and samples from Uruguay were the most distant.

In the PCoA, the two first principal co-ordinate axes accounted for 24% of the total variance (Fig. 4). Groups generated by PCoA support the results obtained by the dendrogram analysis. Samples from Argentina and Paraguay grouped at the left on the ax 1 (CP 1), indicating a higher molecular similarity between them, while samples from Brazil and Uruguay grouped together at the right on this ax. No clear differences were found between samples from Paraguay in different years on the CP 1, but when analyzing CP 2, two groups are clearly differentiated, according to the year of sampling.

4. Discussion

Previous studies using molecular techniques conducted to estimate the molecular diversity of *P. pachyrhizi* showed low levels of

Table 2
AMOVA by country for 23 samples of *P. pachyrhizi*.

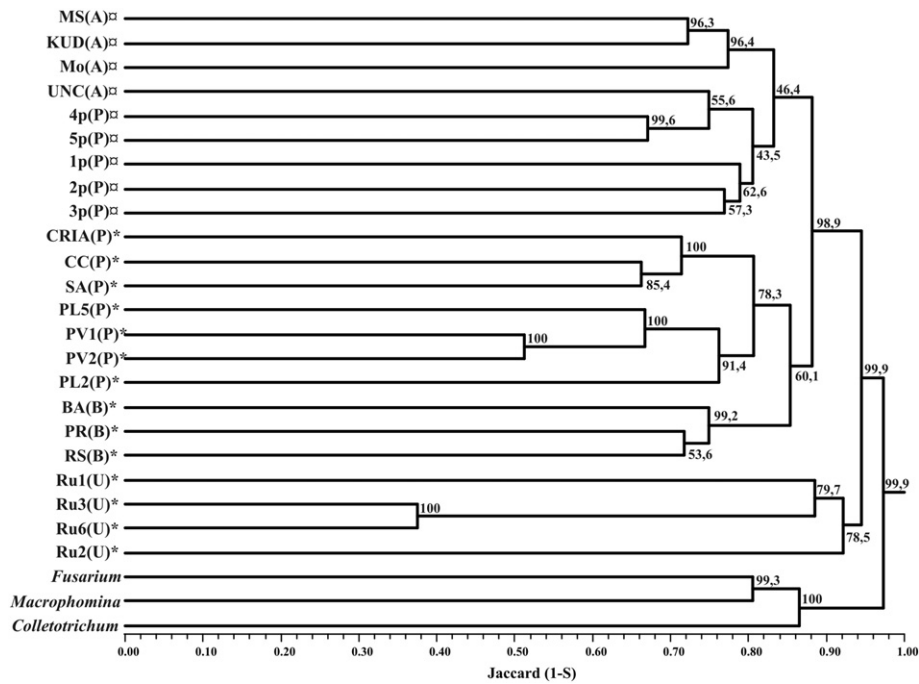| Source of variation	2008		2009	
	D.f.[a]	Variation (%)	D.f.[a]	Variation (%)
Among countries	1	7.1	2	8
Within countries	7	92.9	6	92
Total	8	100	8	100
Phi$_{ST}$	0.07		0.08	

[a] Degree of freedom.

Fig. 2. Dendrogram based on Jaccard's similarity coefficient of 23 samples of *P. pachyrhizi* collected in this study in 2008 (¤) and 2009 (*), including samples from Argentina (A), Brazil (B), Paraguay (P) and Uruguay (U), presented as distance (1-S, S: similarity). Bootstrap values for 1000 replications are indicated at the corresponding node for each cluster. Samples of *Colletotrichum* sp., *Fusarium* sp., *M. phaseolina* were included as outgroup controls.

polymorphism [4,5] since conserved regions within species as beta-tubulin gene and ITS were analyzed. In a recent study, Twizeyimana et al. [6] found a higher level of genetic diversity in *P. pachyrhizi* by using SSR markers in the genetic analysis. These molecular markers can be very informative, multi-allelic and reproducible [7], however, they target specific regions of the genome.

Fig. 3. Neighbor-joining tree obtained from SJ similarity coefficient generated from AFLP fingerprint data of 23 *P. pachyrhizi* samples collected in 2008 (¤) and 2009 (*).

In that sense, AFLP markers are a relevant method, characterized by its versatility, cost-effectiveness, and independence of prior sequence information and broad applicability [18]. This technique permits the simultaneous screening of many different regions distributed randomly throughout a genome [19], revealing more polymorphic loci, even considering the loss of information due to their dominant nature. Furthermore, no previous report on the use of these markers for studying genetic diversity in *P. pachyrhizi* was found. Results of the present work clearly showed that this is a powerful molecular tool to study the genetic variability of this pathogen since a great number of markers and percentage of polymorphism were obtained, even though a relatively small number of samples were characterized. It must be pointed out that the level of genetic diversity found in this work by using AFLP markers, was comparable to that previously reported [6] using SSR markers (77% vs. 87%, respectively). This result highlights the ability of ALFP to accurately estimate genetic diversity, especially when considering that SSR markers are designed to specifically amplify hyper variable genome regions and could thereby over estimate diversity.

It must be noted that although the aim of the present study was to establish a methodology for genetic diversity assessment, it was useful to carry out a preliminary analysis of the *P. pachyrhizi* samples collected. Results from clustering method, phylogenetic and PCoA analysis grouped samples mainly by the year of collection and then by each country. These results suggest potential genetic changes between subsequent years within the same region, as it has been previously proposed by Ray et al. [20] and Paul et al. [21], who observed changes in phenotypic reactions to rust inoculation in different years. It is plausible that an annually recurrent introduction of new pathogen genotypes could occur from the African continent since global circulation patterns dictate that aerial currents blow in an east to west direction from Africa over the Atlantic (Trade Winds) and reach the east coast of South America, thereby facilitating aero-biota transport [22].

All the samples collected in Uruguay clustered separately from the rest of the samples. The soybean genotypes used in this country are
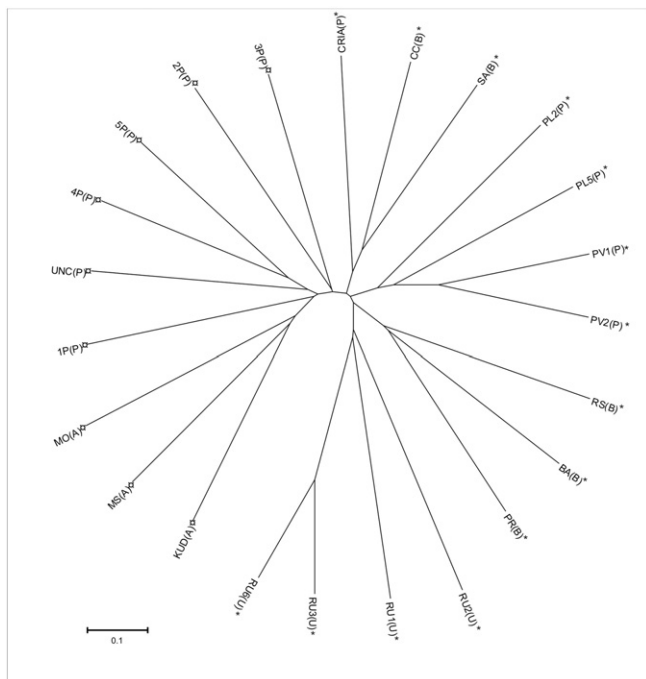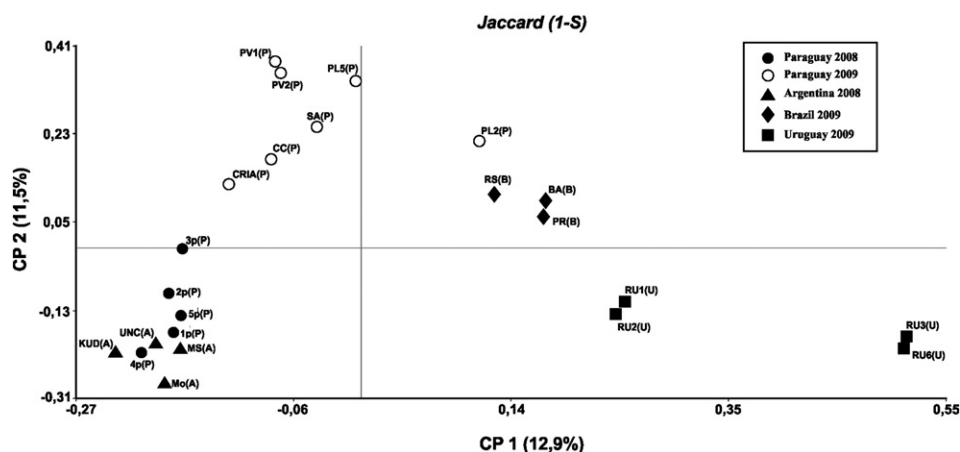
Fig. 4. Principal coordinate analysis plot of *P. pachyrhizi* samples analyzed in this study. CP 1 and CP 2 are first and second principal co-ordinates estimated with 1919 AFLP markers.

adapted to temperate climates and belong to different maturity group compared to those cultivated in Northern Argentina, Paraguay and Brazil with a subtropical climate. These differences could favor different plant/pathogen interactions that could result in the proliferation of different pathogen genotypes among regions. It must also be pointed out that the lowest genetic diversity was detected in the samples from Uruguay; which could be probably explained by samples collection was in a single production area of the country at that time and/or that the disease attack took place by the end of the crop cycle giving less time for pathogen multiplication cycles limiting the possibility for genetic changes to occur.

Despite the groups obtained by clustering and PCoA analysis, the low phi-statistics revealed low genetic divergence in samples collected from locations as distance as Bahia (Brazil) (Fig. 1: locality 11) and Colonia (Uruguay) (Fig. 1: locality 15). The low genetic differentiation observed among samples from different countries could be explained by the spreading of *P. pachyrhizi* over long distances helped by air currents, as previously documented by Isard et al. [23].

The high genetic diversity found for *P. pachyrhizi* could seem surprising for a species that appears to be maintained mainly asexually (the sexual stage in field has not yet been reported). However, there are many plant pathogens considered to be strictly asexual that are characterized by unusually high levels of genetic diversity, such as *Puccinia triticina* [24,25]. For imperfect fungi, such levels of genetic diversity could be explained by parasexuality and heterokaryosis [26]. In this sense parasexual nuclear recombination occurred between different isolates *P. pachyrhizi* after germ tube fusion or hyphal anastomosis which may explain the genetic diversity found [27]. Additionally an unexpected large genome above 850 Mb size with expanded multigene families and very large amount of transpos-able elements (>45%) was reported [28]. In summary, the sampling by collecting urediniospores together with the molecular approach proposed, allowed for detection the genetic diversity in *P. pachyrhizi* sampled in four countries of South America. While encouraging results have been reported here that suggest high genetic diversity of the pathogen, further research should be conducted increasing number of samples to investigate regional population structures of *P. pachyrhizi*. Our results give an interesting insight into the genetic diversity of *P. pachyrhizi*, which could explain the great ability of this pathogen to overcome single resistance genes. In accordance with this, new breeding strategies to increase the duration of resistance need to be considered in future soybean improvement. One approach could be pyramiding major resistance genes, as described by Lemos et al. [29] who introduced three soybean rust resistance genes in a single cultivar. Another possible alternative strategy could be based on the identification and introduction of quantitative resistance, conferred by many genes with additive effects that generate less selective pressure on the pathogen, and may therefore provide a more durable resistance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejbt.2015.06.007.

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