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Phenotypic and Genotypic Variability in *Cercospora kikuchii* Isolates from Santa Fe Province, Argentina

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1. Introduction

More than 100 fungal diseases affecting soybean (*Glycine max* (L.) Merr.) culture have been found worldwide, though only 35 of them are of great economic concern since they cause a 10 to 15% yield decrease (Ivancovich & Botta, 2003; Yeh & Sinclair, 1980).

In Argentina, "Late-cycle diseases" (LCD) constitute a complex of infections affecting the culture of this leguminous, particularly in the reproductive stages. The first symptoms appear from the onset of carpel formation, thus producing premature plant maturation, a reduction in yield (5 to 8%) and seed quality loss. Due to the climate conditions of the region, leaf blight and purple seed stain, is one of the LCD prevailing in the central-northern region of Santa Fe Province (Formento, 2005; Ivancovich & Botta, 2003).

The causal agent of the disease is the fungus *Cercospora kikuchii* (T. Matsumoto & Tomoyasu) M.W. Gardner, which produces irregular injuries on the leaves, forming reddish-purple necrotic areas, pale pink to dark purple stains on the seeds, together with cracks on the outer coat (Formento, 2005). *C. kikuchii* belongs to the *Cercospora* taxonomic complex, which is assumed to be host-specific (Crous & Braun, 2003), and therefore its species are normally identified from the phenotypic characteristics they show when grown on their natural substrate, being much more difficult to be characterized from artificial media (Almeida et al., 2005; Gams et al., 2007).

One of the most important factors determining the pathogenicity of this fungus is the production of cercosporin, a red exotoxin (Kuyama & Tamura, 1957; Upchurch et al.,1991). Another aspect to consider is the genetic variability found in some fungi, which has determined the description of different races; that is why the use of some methodology to detect inter- and intraspecific variations between isolates is recommended (Kuyama & Tamura, 1957). In this sense, the Random Amplified Polymorphic DNA (RAPD) technique allows the differentiation between strains since amplification focuses only on the whole genome (Tigano et al., 2003; Williams et al., 1990).

Although some Brazilian research groups have been studying aspects related with this phytopathogen (Almeida et al., 2003) only a few reports have been found in Argentina and, in particular in Santa Fe Province, about epidemiology and population structure of *C. kikuchii.*

The aim of the present work was to determine the occurrence of phenotypic and genotypic variability between isolates of *C. kikuchii* in various regions of Santa Fe Province.

2. Methods

2.1 Fungal isolation

Nineteen samples with visible symptoms of leaf blight obtained from soybean lots not less than 0.5 ha in size, were processed. Soybean samples were collected from several places like Emilia, Margarita, Esperanza, Gobernador Crespo and San Justo, in Santa Fe Province. Sampling was carried out between January and May (2005-2006 campaign) under similar climatic conditions (Table 1).

Meteorological Station	Average			
	Tmax ºC	Tmin ºC	Tmed ºC	RH %
1	26.8	14.4	22.8	67
2	28.8	15.0	22.5	73
3	28.1	15.0	22.9	70

Table 1. Climatic conditions corresponding to different meteorological stations in Santa Fe Province, Argentina. 2005-2006 Campaign. 1: Esperanza; 2: Emilia, Gobernador Crespo and San Justo; 3: Margarita; Tmax: maximum temperature; Tmin: minimum temperature; Tmed: medium temperature; RH: relative humidity.

Fig. 1. Tissue pieces being disinfected by immersion.

Fig. 2. Tissue pieces in moist chambers.

Tissue pieces were disinfected by immersion in 3% w/v sodium hypochlorite for 3 min, rinsed with sterile distilled water (Fig.1) and incubated in moist chambers at 26 ± 0.5 ºC, under alternate light cycles (16 h cold light and 8 h in the dark) (Salvador & Garrido, 1990). (Fig. 2).

Observation with stereoscopic magnifying lens (BOECO Germany, BTB 3-C) was performed from the 3rd day of incubation onwards. Once the conidial structure growth was verified, conidia were taken with a sterile needle and suspended in 1 mL sterile water so as to obtain a homogeneous suspension. To produce monosporic cultures, an aliquot (100 µL) was spread over Potato Dextrose Agar (PDA) in a Petri dish and incubated as described above (Dunkle & Levy, 2000). Isolates were labeled with letter C and correlative numbers (Table 2), stored and subsequently included in the strain collection.

Isolate	Location	Lot	Cultivar/varietal
C14	Emilia	A01	A5409
C15	Emilia	A01	A5409
C16	Emilia	A01	A5409
C17	Emilia	A01	A5409
C18	Emilia	A01	A5409
C19	Emilia	A01	A5409
C20	Margarita	A04	TJ2070
C21	Margarita	A04	TJ2070
C22	Margarita	A04	TJ2070
C23	San Justo	A02	A4910
C24	Margarita	A04	TJ2070
C25	G. Crespo	A04	A8000
C26	Emilia	B01	A5409
C27	G. Crespo	A04	A8000
C28	Esperanza	Lote Rural	A7636
C29	Esperanza	Lote Rural	A7636
C30	Esperanza	Lote Rural	A7636
C31	Esperanza	Lote Rural	A7636
C32	Esperanza	Lote Rural	A7636

Table 2. Origin and designation of *Cercospora kikuchii* regional isolates. G. Crespo: Gobernador Crespo.

2.2 Phenotypic characterization

Each fungus was inoculated with a single touch in the center of a Petri dish containing PDA and incubated under the conditions previously described. Macro and microscopic observations were conducted after 15 days, taking into account color and diameter of the colony, red pigment production (cercosporin) and the presence or absence of typical elements of reproduction. For the latter case, plates were discarded only after 25 days of incubation.

Cercosporin production was confirmed following Jenns et al. methodology (Jenns et al, 1989) with slight modifications as described by González et al. (González et al., 2008).

2.3 Genotypic characterization

Every isolate was streak-plated over PDA and incubated for 4 days under the conditions described above. Following incubation, 5 mL sterile water was added and the surface of the colony was rubbed with a wire loop in order to free the fungal elements. The resulting suspension was added to 100 mL of culture medium for *Colletotrichum* (Martinez Culebras et al., 2000), and incubated 48 h in the dark at 28-30 ºC in orbital motion (Orbital Shaker, Forma Scientific, Inc.) at 180 rpm. The mycelium was placed on a nylon filter (200 µm pore diameter) and dried with absorbent paper until all moisture was removed. Once dry, it was extended forming a layer as thin as possible and was dried in stove at 37 $\pm$ 0.2 ºC until constant weight. Then it was ground to thin powder in a mortar.

The same procedure was applied to two strains belonging to the NITE Biological Resource Center (Japan) collection: *C. kikuchii* NBRC 6711 and *C. sojina* NBRC 6715.

In order to extract total DNA, Di Conza et al. (2007) protocol was followed. DNA was quantified through absorbance reading in a spectrophotometer, and its quality determined by means of electrophoresis in 0.8% w/v agarose gel (Sambrook et al., 1989). RAPDs were carried out following Williams et al. (1990), using 20 oligonucleotides (FAGOS/Ruralex, Argentina) (Table 3).

The reaction mixture was prepared for a 50 µL total volume with these components: 2.5 mM magnesium chloride, 125 µM of each dNTPs (INBIOHIGHWAY), 1 µM oligonucleotide, 5 U *taq* DNA polymerase (INBIOHIGHWAY) and 20 ng DNA per reaction. Amplification was carried out using a MJ Research Thermal Cycler under the following conditions: 1 cycle of 5 min at 95ºC, 40 cycles comprising 75 s at 94ºC, 90 s at 36ºC and 150 s at 72ºC, and 1 final cycle of 10 min at 72ºC. Amplification products were separated by electrophoresis in 1.5% w/v agarose gel with 0.5X TBE 0.089M Tris-borate, 0.002 M EDTA. The run time was 180 min, with a 100V constant voltage. The gel was stained with ethidium bromide, and the molecular weight marker was 100-bp DNA Ladder (Promega). Band profiles obtained were photographed and analyzed with Gel Doc XR System (BIORAD-Life Science Cat. # 170-8170) using the Quantity One Software.

All RAPD reactions were done in duplicate.

A matrix with 0 and 1 corresponding to absence and presence of band, respectively, for each one of the images resultant from each oligonucleotide used, was constructed, giving a total of 18 binary matrices with order mx21, where 18 represents the amount of nucleotides considered, m the bands obtained and 21 the total fungi analyzed. From these matrices, distance matrices (21x21) were obtained with the Jaccard coefficient, designed for asymmetric binary variables (Everitt & Hothorn, 2009; Johnson & Wichern, 1998) (Table 4).

Then, an average distance matrix (21x21), essential to apply the cluster technique, was calculated with the 18 distance matrices (Abonyi & Feil, 2007; Bolshakova & Azuaje, 2003; Peña, 2002).

The choice of clustering algorithm depended on the type of data and the purpose of using the technique. It was considered convenient to apply more than one clustering algorithm for a database and then compare the agreement between the results.

Therefore, a partition (FANNY) and a hierarchical (AGNES) algorithm were selected. FANNY computes "diffuse" clusters, giving each item a degree of belonging to the cluster. The algorithm combines the optimum choice of the amount of clusters (k) and the parameter r, linked to the degree of diffusivity or *membership exponent*.

AGNES makes agglomerative hierarchical clusters, the distance between clusters (k) being analyzed by Ward method.
Silhouette Coefficient (SC) and Agglomeration Coefficient (AC) were used for FANNY and AGNES, respectively, to evaluate cluster quality (Kaufman & Rousseeuw, 1990).
Data were processed with R version 2.10.1 Software (Torgo, 2003).

Oligonucleotide	Sequence
OPA-01	5′-CAGGCCCTTC-3′
OPA-02	5′-TGCCGAGCTG-3′
OPA-03	5′-AGTCAGCCAC-3′
OPA-04	5′-AATCGGGCTG-3′
OPA-05	5′-AGGGGTCTTG-3′
OPA-06	5′-GGTCCCTGAC-3′
OPA-07	5′-GAAACGGGTG-3′
OPA-08	5′-GTGACGTAGG-3′
OPA-09	5′-GGGTAACGCC-3′
OPA-10	5′-GTGATCGCAG-3′
OPA-11	5′-CAATCGCCGT-3′
OPA-12	5′-TCGGCGATAG-3′
OPA-13	5′-CAGCACCCAC-3′
OPA-14	5′-TCTGTGCTGG-3′
OPA-15	5′-TTCCGAACCC-3′
OPA-16	5′-AGCCAGCGAA-3′
OPA-17	5′-GACCGCTTGT-3′
OPA-18	5′-AGGTGACCGT-3′
OPA-19	5′-CAAACGTCGG-3′
OPA-20	5′-GTTGCGATCC-3′

Table 3. Oligonucleotides used in RAPD reaction

		Fungi j		Total
		1	**0**	
Fungi i	**1**	a	b	a+b
	0	c	d	c+d
Total		a+c	b+d	a+b+c+d

Table 4. Contingency table summarizing the coincidences in the presence/absence of bands between pairs of fungi. Letters i and j: correspond to the same fungus or to different fungi; 1: presence of bands; 0: absence of bands; a, b, c and d are the frequencies[1].

[1]coefficient of distance is given by:

$J = \frac{b+c}{a+b+c}$. No 0-0 matches in numerator or denominator.

3. Results

3.1 Phenotypic characterization

When considering PDA cultures, the colonies, with sizes between 26-50 mm diameter, appeared as white, green-greyish and pink colored, depending on the isolate. The reverse side showed green, brown or red-brown color. All of them presented a reddish touch on their edge and abundant colorless exudate which became amber to ochre with the passing of time. The pigment diffused to the medium in some of them. Fructification was observed after 15 days of incubation, though sporulation was scarce and unevenly distributed over the colony surface.

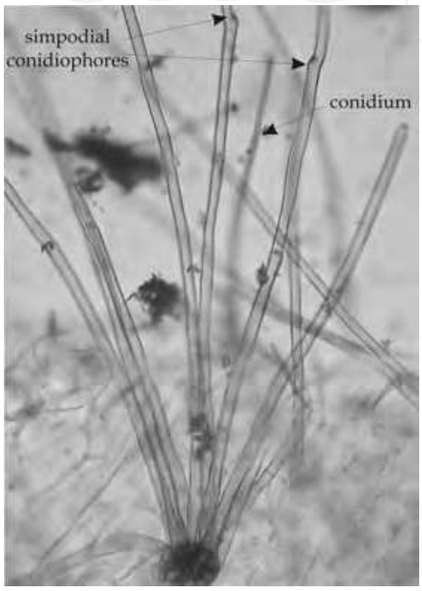

Fig. 3. Growth of *Cercospora kikuchii* on natural substratum (400x)

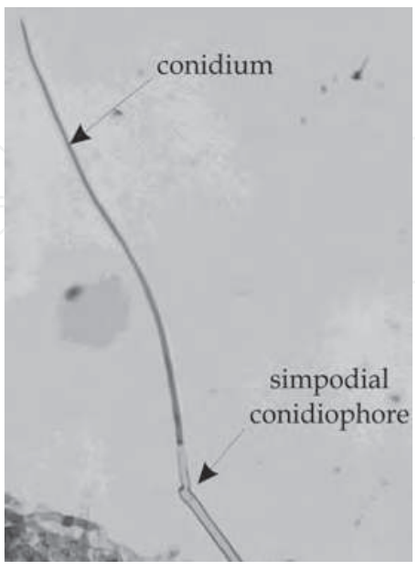

Fig. 4. *Cercospora kikuchii*. Single conidium (400x)

Similar morphologic structures were observed in the 19 isolates. The direct examination of the injury showed brown pigmented, fasciculated and septate conidiophores (200-300 µm long per 4-5 µm wide), with simpodial growth and conidiogenous cells integrated, terminal or intercalary, with thickened and darkened conidiogenous loci (Figure 3). Single, long, acicular, hyaline, pluriseptate (12 to 26 septa) conidia with truncate bases and subacute apices (170 to 190 µm long per 3 µm wide at the base) were observed (Figure 4) as described by Ellis (1971), Crous & Braun (2003) and Solheim (1929).
Conidia formation process involves internal and external walls of the conidiogenous cells, so when the conidio arises, a scar appears in its origin, on the conidiogenous cell (Cai, 2004; Fernández et al., 1991).

3.2 Genotypic characterization

A total of 90 bands were obtained, 100% of them were polymorphic. Amplification size ranged between 107 bp and 2750 bp, an average of 51 amplified fragments/oligonucleotide being produced. OPA-01 was the oligonucleotide which produced the least amount of bands (1) and OPA 12 the most (23) amplified fragments (Table 5). Amplifications with OPA-02 and OPA-06 were not satisfactory.
Figure 5 shows band profiles obtained with oligonucleotide OPA-14.

Oligonucleotide	Maximum number of bands	Minimum number of bands	Total bands	Number of polymorphic bands	Pair of bases (bp)
OPA-01	19	1	49	49	1846-168
OPA-03	17	9	49	49	1437-202
OPA-04	16	6	46	46	1451-156
OPA-05	19	7	48	48	1485-107
OPA-07	14	5	49	49	1599-197
OPA-08	16	3	58	58	2009-253
OPA-09	14	7	53	53	1561-134
OPA-10	18	4	56	56	1797-114
OPA-11	13	5	45	45	1422-212
OPA-12	23	7	55	55	1883-200
OPA-13	17	8	51	51	1495-156
OPA-14	15	6	44	44	1999-309
OPA-15	16	7	53	53	2730-241
OPA-16	18	8	50	50	2252-215
OPA-17	16	4	60	60	2673-213
OPA-18	18	9	51	51	1655-229
OPA-19	18	9	58	58	2750-208
OPA-20	16	7	51	51	1572-242
Total			926	926	

Table 5. Total number of bands and polymorphic bands obtained with the oligonucleotides selected for RAPD

A FANNY cluster analysis with k= 6 and r =1.2 (optimum combination) was carried out. Few distances near 0.2 were observed (Fig 6). C21 and C22 (isolated from Margarita and

same cultivar) were strongly related, as well as C30 and C31, both of them isolated from Esperanza (same cultivar). Great genetic distances for the remaining fungi were detected (Fig.7).

Given the optimum combination obtained with Fuzzy analysis (FANNY), the AGNES algorithm was applied considering the number of clusters found (k=6).

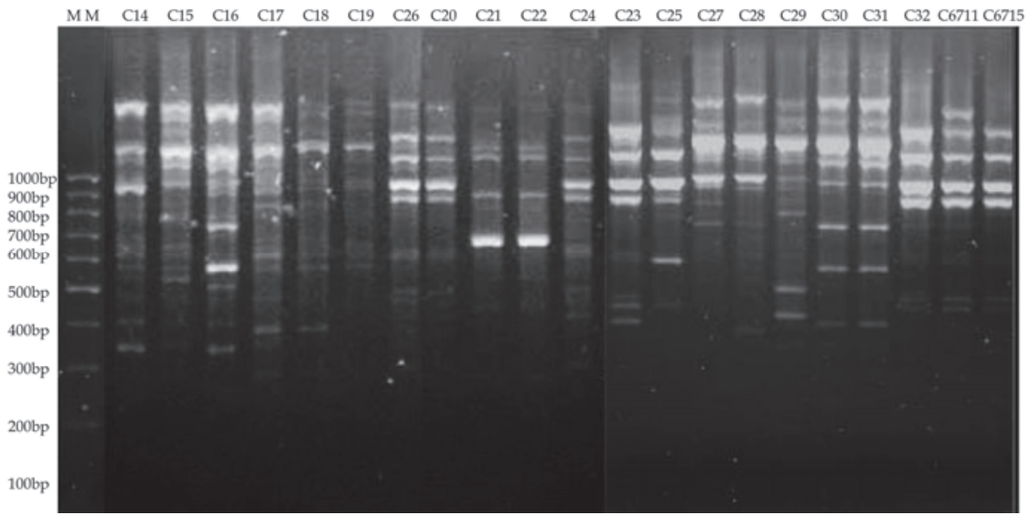

Fig. 5. Sample gel with patterns produced by *Cercospora kikuchii* isolates and NBRC strains using OPA-14. MM: molecular marker 100 bp; C14, C15, C16, C17, C18, C19, C26, C20, C21, C22, C24, C23, C25, C27, C28, C29, C30, C31, C32: regional isolates; C6711: *Cercospora kikuchii* NBRC 6711; C6715: *Cercospora sojina* NBRC 6715.

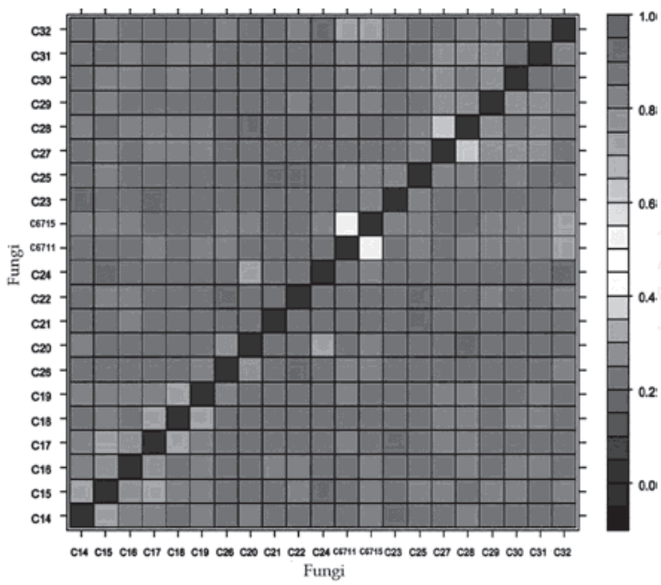

Fig. 6. Average Distance Matrix. C32, C31, C30, C29, C28, C27, C25, C23, C24, C22, C21, C20, C26, C19, C18, C17, C16, C15, C14: regional isolates; C6711: *Cercospora kikuchii* NBRC 6711; C6715: *Cercospora sojina* NBRC 6715.

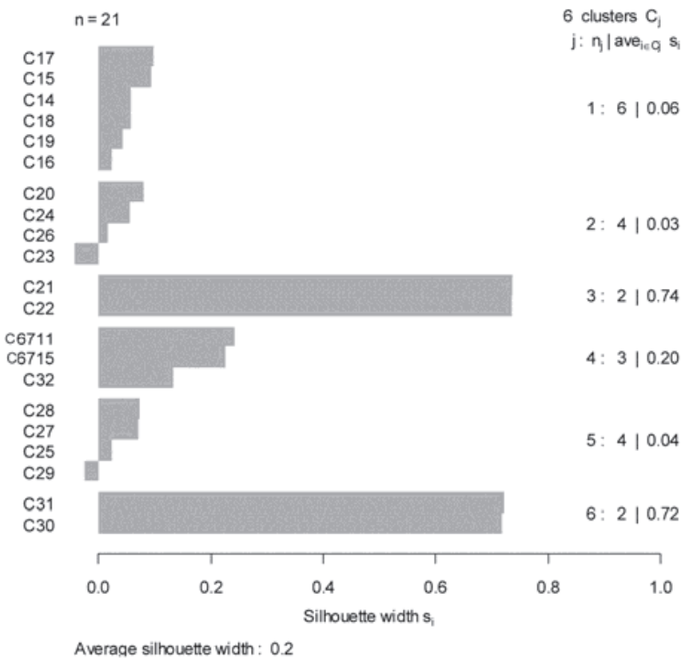

Fig. 7. Silhoutte plot of FANNY (x= average distance; k=6; membership exponent (r) = 1.2). C17, C15, C14, C18, C19, C16, C20, C24, C26, C23, C21, C22, C32, C28, C27, C25, C29, C31, C30: regional isolates; C6711: *Cercospora kikuchii* NBRC 6711; C6715: *Cercospora sojina* NBRC 6715.

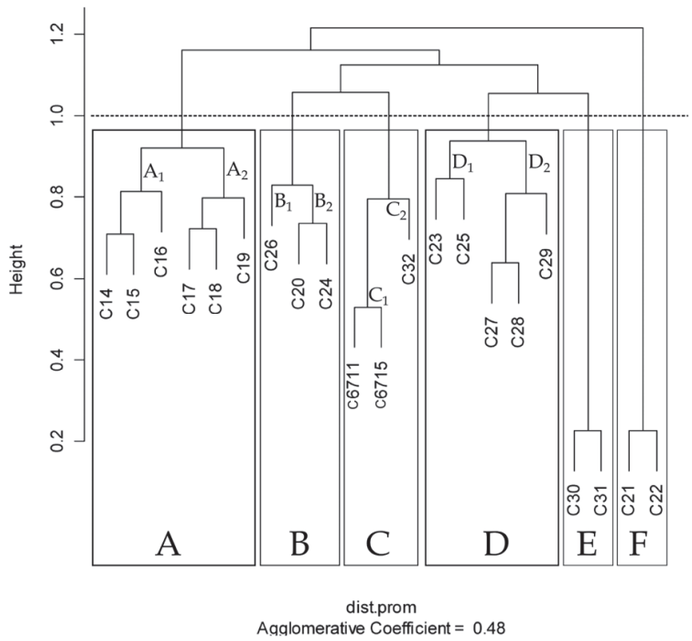

Fig. 8. Dendrogram of AGNES (x= average distance, method = Ward). C14, C15, C16, C17, C18, C19, C26, C20, C24, C32, C23, C25, C27, C28, C29, C30, C31, C21, C22: regional isolates; C6711: *Cercospora kikuchii* NBRC 6711; C6715: *Cercospora sojina* NBRC 6715.

The AGNES cluster analysis resulted in a dendrogram that showed great genetic distances between different fungi (Figure 8). Cluster A included six isolates, C14, C15 and C16 in cluster A_1 and C17, C18 and C19 in A_2, all of them from the same origin (Emilia, Table 2). Cluster B included three fungi, which were grouped in two subclusters. B_1 included C26 which, although isolated from Emilia, belonged to another lot, and B_2 included C20 and C24, both isolated from the same region (Margarita) and cultivar (Table 2).
Cluster C included both strains NBRC (C6711 and C6715) (subcluster C_1) and C32 isolated from Esperanza (subcluster C_2). Cluster D included 5 fungi, C23 and C25 from different origin and cultivar (subcluster D_1) and C27 (same origin than C25), C28 and C29 isolated from Esperanza (subcluster D_2) (Table 2). Finally, clusters E and F included C30 and C31, (isolated from Esperanza) and C21 and C22 (from Margarita), respectively.

4. Discussion

Soybean is one of the most important crops in Argentina, and it has been characterized by an incredible rate of adoption and growth. Twenty-one percent of the cultivated lands of Argentina are in Santa Fe Province, whose main crop, soybean, makes it the main national producer (Aizen et al., 2009; Penna & Lema, 2002). However, soybean crops are affected by several diseases which decrease the total production.
This study aims at contributing to the knowledge of one of the most frequent soybean phytopathogens. Phenotypic and genotypic variations among isolates of *C. kikuchii* from infected soybean corresponding to the centre-northern region in Santa Fe Province, geographically separated but with similar climatic conditions, were studied.
When grown on PDA, and considering micromorphology, the isolated fungi showed colonies with different macroscopic aspects. These results would be in agreement with those reported by Almeida et al. (2005) on Brazilian isolates of *C. kikuchii*. The red pigment observed around some of the colonies was caused by the presence of cercosporin, a pathogenicity factor of *C. kikuchii*, as described by many authors (Assante et al., 1997; Fajola, 1978; Upchurch et al., 1991).
In general, fungi belonging to genus *Cercospora*, as well as other similar genera, exhibit great difficulty for sporulating on artificial culture media (Avila de la Calle et al., 2004; Cadwell, 1994; Yeh & Sinclair, 1980). This limitation was also proved in this study.
Information about which is the optimum medium for studying *Cercospora* sporulation is diverse and confuse, there being no uniform criteria between the different authors. This particular fact evidences the great "inter" and "intra" variability among species belonging to this genus (Brunelli, 2004; Cai, 2004; Chen et al., 1979; Jenns et al., 1989; Salvador & Garrido, 1990).
Given that genus *Cercospora* belongs to the group of Dematiaceous (dark-colored) fungi, it shows septated and olive-brown pigmented hyphae. It is worth mentioning that the number of conidia, its size and number of septa are affected both by the environment and the culture media used (Cai, 2004). Yeh & Sinclair (1980) reported that conidiophore and conidia size differed between isolates even when incubated under the same conditions.
Since typing is a necessary first step in knowing pathogens (Redondo et al., 2009), techniques based on DNA polymorphisms are especially valuable to enhance epidemiological studies.

Genetic variability could be found both between isolates from the same region and between those from different regions, thus confirming previous results (González et al., 2008; Lurá et al., 2007), as reported by Almeida et al. and Cai for *C. kikuchii* and other species (Almeida et al., 2003, 2005; Cai, 2004). No isolates turned out to be 100 % similar. These findings are not easy to be explained. However, it is essential to consider that this fungus is transmitted through the soybean seeds and, being necrotrophic, it can survive in the stubble. As a great increase in the soybean producing area has occurred in Argentina in the last decade, the turnover of seeds from traditional to new production areas has increased accordingly (Secretaría de Agricultura, Ganadería, Pesca & Alimentos [SAGPyA], 2003). The survival of the fungus in the stubble, on the other hand, makes it the main source of primary inoculum for the re-infection in the next campaign (Sillón, 2007).

According to Pujol Vieira dos Santos et al. (2002) and Stenglein & Ballati (2006), many factors could have been affecting polymorphism analysis, e.g. the intraspecific variants of a pathogen, the number of samples selected for analysis, genetic flow between populations, environmental adaptation and selective pressure and migration.

Other factors to be considered, which could explain the genetic variability detected in the study, would be the changes in DNA within the populations of filamentous fungi. As reported by MacDonald (1997), these changes are the consequence of mutations, deletions, pairing systems or gene migration or flow, and population selection, since sexual reproduction is not known in *C. kikuchii* (Almeida et al., 2005). Daboussi & Capy (2003) and Kempken & Kück (1998), on the other hand, state that changes mediated by transposable elements, together with transposition and recombination, provide a wide range of genetic variation, which is useful for the natural self-adaptation of the population to the changing environment and the interaction with another organisms.

With reference to the strains *C. kikuchii* NBRC 6711 and *C. sojina* NBRC 6715, from the Culture Collection of the National Institute of Technology and Evaluation (NITE), Japan, no data were found concerning either their isolation source, location or country of origin. The low similarity they showed, as compared to the regional isolates, could be attributed to the fact that they come from regions naturally different from those selected for this work.

As regards the two oligonucleotides that gave unsatisfactory results to amplification, very few bands were detected with OPA-06, which could be accounted for considering that efficiently amplified DNA regions must be located between two sites complementary to the primers and separated by a distance of a few kb (Williams et al., 1990). In the *C. kikuchii* under study, the sites for this primer could be less frequent, which made the amplification of fragments technically impossible. OPA-02, on the other hand, showed a pattern with numerous bands, its reading and interpretation becoming thus very difficult.

The 6 groups identified by the clustering techniques allowed to distinguish both genetic variability among them and similarity among the fungi belonging to the same group. After comparing the results of both clustering methods, a high genetic homogeneity could be observed in two out of the six ones (third and sixth FANNY groups with the corresponding F and E AGNES groups) since Silhouette coefficient associated to them turned out to be higher than 0.70 with FANNY algorithm and agglomerative coefficient in AGNES technique was close to zero for the same two clusters. Besides, clusters A and C (AGNES) corresponded with the 1st and 4th FANNY clusters, respectively. As regards the rest of the

fungi, little homogeneity was confirmed between the isolates comprising clusters B and D generated by AGNES and clusters 2 and 5 obtained with FANNY.

It was shown that *C. kikuchii* isolates from the same geographic region appeared in different groups. Molecular analyses showed intraspecific variability within *C. kikuchii* isolates from soybean collected in different regions, so it was difficult to establish a relationship between this variability and that of the soybean cultivars from which *C. kikuchii* isolates were obtained. Similar results had been previously reported by González et al. (2008) and Almeida et al. (2005). According to the results here obtained, Argentinian populations of *C. kikuchii* are phenotypically, genotypically and geographically variable. In agreement with Almeida et al. (2005), who consider that this pathogen is easily transmitted by seeds, it is not surprising to find the same haplotypes in different regions.

In Argentina, and in this region in particular, there has been a rapid increase in the soybean producing area since 1970 (Aizen et al., 2009); therefore, the traffic of seeds from traditional to new areas could be responsible for the geographical variability since *C. kikuchii* is a seed borne pathogen. Unfortunately, an insufficient number of isolates was obtained from each area to permit the evaluation of gene flow among populations more accurately.

For countries like Argentina, with large soybean areas it is very important to know the variability of the pathogen in advance in order to prevent resistant cultivars when sown in different areas.

Few studies have been reported concerning the molecular characterization of genus *Cercospora*. Therefore, widening the scope of knowledge about this pathogen biology, and developing strategies to control the cultures intended for human and/or animal use in this region, therefore, would be a great contribution of this work.

5. Conclusions

Results revealed a considerable degree of phenotypic and genotypic variation in the population of *C. kikuchii* infecting soybean crops from the centre-northern region of Santa Fe Province, Argentina.

From the phenotypic- in vitro- viewpoint, isolates were differentiated by color and size of the colonies, as well as by sporulation capacity. As far as genotypic aspect is concerned, differences in the genome of fungi from the different regions were detected, aside from differences among isolates from the same population.

The two statistical techniques applied proved to be adequate since not only genetic variants could be detected among the isolates under study but also similar clusters were obtained in both of them, thereby giving validity to the results. The detection of genetically similar isolates, on the other hand, would make decision-making easier so as to intervene in health issues such as the prevention of diseases produced by these phytopathogenic fungi.

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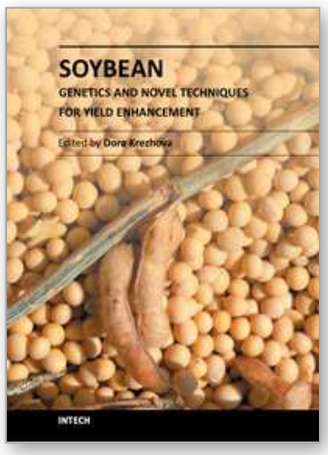

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