

Pathogenicity, Molecular Characterization, and Cercosporin Content of Brazilian Isolates of *Cercospora kikuchii*

Álvaro M. R. Almeida¹, Fernanda F. Piuga⁵, Silvana R. R. Marin¹, Eliseu Binneck¹, Fábio Sartori²,
Leila M. Costamilan³, Maria R. O. Teixeira⁴ & Marcelo Lopes⁶

¹Embrapa Soja, Cx. Postal 231, CEP 86001-970, Londrina, PR, Brazil, e-mail: amra@cnpso.embrapa.br; ²Milenia Biotecnologia & Genética Ltda, Rua Pedro Antônio de Souza, 405, CEP 86031-610, Londrina, PR, Brazil, ³Embrapa Trigo, Cx. Postal 451, CEP 99001-970, Passo Fundo, RS, Brazil, ⁴Embrapa Agropecuária Oeste, Cx. Postal 661, CEP 79804-970 Dourados, MS; ⁵UNOPAR, Av. Paris, 675, CEP 86041-140, Londrina, PR, Brazil; ⁶Departamento de Ciências Agrárias, Universidade de Cruz Alta, UNICRUZ, CEP 98025-810, Cruz Alta, RS, Brazil

(Accepted for publication 05/10/2005)

Corresponding author: Álvaro M. R. Almeida

ALMEIDA, A.M.R., PIUGA, F.F., MARIN, S.R.R., BINNECK, E., SARTORI, F., COSTAMILAN, L.M., TEIXEIRA, M.R.O. & LOPES, M. Pathogenicity, molecular characterization, and cercosporin content of Brazilian isolates of *Cercospora kikuchii*. Fitopatologia Brasileira 30:594-602. 2005.

ABSTRACT

Cercospora kikuchii, involved with the defoliation of soybean (*Glycine max*) plants, is normally associated with *Septoria glycines* in late season. Seventy-two isolates from different regions in Brazil, obtained mainly from purple stained seeds, showed phenotypic variation. Cercosporin content and rate of colony growth was highly variable among isolates. A strong correlation between cercosporin content and virulence was identified. Genetic variation among and within populations was evaluated based on 86 RAPD loci. The RAPD analysis clustered all isolates into seven groups. No relationship was observed between RAPD groups and geographic origin or cercosporin content. The sequence of the internal spacer regions (ITS1-5.8S-ITS2) from 13 isolates chosen according to the previous RAPD and clustering analysis showed high similarity (97%-100%) to the GenBank sequences of *C. kikuchii* (AY266160, AY266161, AY152577 and AF291708). It is clear from this work that Brazilian isolates of *C. kikuchii* from different geographic regions, are variable in relation to virulence, RAPD profiles and cercosporin content. Cercosporin content could be a good parameter for choosing an adequate isolate for screening resistant or tolerant cultivars. Considering that this pathogen is easily seed-borne, findings are expected to show the same haplotypes in different regions. Migration could be favoured by infected seeds as demonstrated by RAPD analysis.

Additional keywords: genotypic diversity, PCR-RFLP, RAPD, ITS sequence, virulence.

RESUMO

Patogenicidade, caracterização molecular e teor de cercosporina de isolados brasileiros de *Cercospora kikuchii*

Cercospora kikuchii está envolvido na desfolha da soja (*Glycine max*), normalmente em associação com *Septoria glycines*, no final do ciclo da cultura. Setenta e dois isolados, obtidos principalmente de sementes com mancha púrpura e oriundas de diferentes regiões do Brasil, mostraram variabilidade fenotípica. O teor de cercosporina e a velocidade de crescimento de colônias foram bastante variáveis entre os isolados. Uma forte correlação foi identificada entre o teor de cercosporina e virulência. Diferenças genéticas, entre e dentro da população analisada, foram observadas por RAPD com a análise de 86 loci. As análises de RAPD permitiram agrupar os isolados em sete grupos. Nenhuma relação foi identificada entre os grupos de RAPD e a origem geográfica ou teor de cercosporina. A sequência da região espaçadora do DNA ribossomal (ITS1-5,8S-ITS2) foi determinada em 13 isolados escolhidos nos diferentes agrupamentos. A similaridade das sequências obtidas comparadas às sequências de *C. kikuchii* depositadas no GenBank (AY266160, AY266262, AY152577 e AF291708) variou de 97 a 100%. Este trabalho demonstrou que os isolados brasileiros de *C. kikuchii* de diferentes origens são variáveis quanto à virulência, aos padrões de RAPD e ao teor de cercosporina. O teor de cercosporina pode ser um bom parâmetro na escolha de um isolado adequado para seleção de cultivares tolerantes ou resistentes a esse patógeno. Considerando que ele é facilmente transmitido por sementes não é surpresa encontrar os mesmos haplotipos em diferentes regiões. A migração poderia ser favorecida por sementes infetadas como demonstrado na análise de RAPD.

Palavras-chave adicionais: diversidade genética, PCR-RFLP, RAPD, sequência ITS, virulência.

INTRODUCTION

Cercospora leaf blight in soybean [*Glycine max* (L.) Merrill] is caused by *Cercospora kikuchii* (Matsumoto & Tomoyasu) M.W. Garner. It was first described in Korea then

three years later in the USA (Gardner, 1925). Originally, its importance was restricted to the purple seed stain; however, Lehman (1950) observed that inoculated soybean plants in the greenhouse developed symptoms on hypocotyls, stems, leaves and petioles. Walters (1978) described defoliation in

soybean plants caused by *C. kikuchii* and called the disease Cercospora leaf blight. In the same report, he observed different reactions among soybean cultivars, reporting that cultivars such as Davis and Tracy were less affected than others. Currently *C. kikuchii* is associated with three symptoms on soybeans: purple seed stain, seedling death and leaf blight (Walters, 1978; Schuh, 1991).

The importance of this disease has increased in all countries where soybean is grown, especially in tropical areas (Wrather *et al.*, 1997). In Brazil, *C. kikuchii* has been associated with purple seed stain (Miyasaka, 1958) for a long time. A study published by Sedyama *et al.* (1971) did not associate stained seeds with reduced germination; however, losses have been detected due to defoliation resulting from the development of large necrotic areas. This symptom is more prevalent in late season and is usually associated with brown spot, caused by *Septoria glycines* Hemmi (Almeida *et al.*, 1997).

Although control of this disease may be achieved through genetic resistance, very little information is available about the genetic variability of the pathogen. Ideally, newly bred soybean genotypes should be screened for Cercospora leaf blight prior to being released to the farmers, thus requiring that plant breeders understand the pathogen variability.

Evidence that variability occurs among isolates of *C. kikuchii* came from visual analysis of cultural and morphological characteristics on potato-dextrose-agar (PDA). Colonies originating from different regions in Brazil showed considerable phenotypic variation. The color of the mycelium among isolates ranged from white to dark olive and black. Some isolates even showed dense mycelia with slow growth in contrast to others that exhibited faster development.

In this work, the variability among *C. kikuchii* isolates from different areas in Brazil was analysed by RAPD, sequencing of the ITS regions of the rDNA, cercosporin content, virulence on soybean cultivars and cultural characteristics. A previous report has been published (Almeida *et al.*, 2004).

MATERIAL AND METHODS

Fungal cultures

Seventy-two isolates of *C. kikuchii* from different regions of Brazil were obtained from either infected soybean seeds or leaves (Table 1). Purple stained seeds from different geographic areas were separated from soybean lots and surface sterilized in 1% sodium hypochloride for 1 min, followed by a sterile distilled H₂O wash. Blotter testing was carried out according to Neergaard (1979) for two-four days at 25 °C. A small piece of the tip of the mycelium was transferred to Petri dishes containing PDA plus 1% streptomycin sulphate and incubated at 25 °C for ten days. From the border of each uniform colony a small piece of mycelium was transferred to a new PDA dish and incubated

as mentioned before. For DNA extraction, small plugs from the stock culture of each isolate were grown in 200 ml potato-dextrose broth at 26 °C for two weeks under 18 h light.

Mycelial growth

One 8 mm diameter plug from each isolate was transferred to the center of Petri dishes with PDA, kept at 26 °C with 18 h light. The colony diameter was measured after eight, 12 and 16 days. Three plates per isolate were used.

Cercosporin production assay

Cercosporin concentration was determined spectrophotometrically according to Jenness *et al.* (1989). Isolates were grown on PDA and incubated at 25 °C for six days under 18 h light. One mycelium plug (7 mm diameter) was cut from the border of the colonies, transferred to tubes containing 8 ml of 5 N KOH and kept in the dark for 4 h. Three tubes per isolate were used. After centrifugation at 10,000 rpm the samples were evaluated for cercosporin concentration in a spectrophotometer at A₄₈₀ nm using a molar extinction coefficient of 23,300 (Jenness *et al.*, 1989). The average volume for one plug was 0.2 ml. The experiment was repeated twice.

Virulence analysis

Inoculation was performed according to Callahan *et al.* (1999) with modifications: 10 g of mycelium scraped from a 14 day-old-colony was washed in sterilized water and homogenized in a blender with 100 ml of sterile 0.2% water agar for 40 sec. Mycelial suspension was sprayed onto both sides of leaves from 25 day-old plants using 4 Kg/cm² pressure. After inoculation the plants were covered with plastic bags for 48 h and kept at a greenhouse with temperature ranging from 28-33 °C. The experiment was conducted in a completely randomized block design with 20 treatments consisting of five soybean cultivars ('Davis', 'Conquista', 'Sambaíba', 'IAS-2' and 'Paraná') inoculated with four isolates (169, 179, 301 and 444). Each replication consisted of one pot with three plants per pot, and there were three replications per treatment. Plants were rated at two and three weeks after inoculation based on diagrammatic scale adapted from James (1971). The experiment was conducted twice. The same inoculum was used to inoculate pods of the same cultivars using a syringe with 25 x 7 needle.

DNA Extraction

Mycelia of each of the 72 isolates were washed and centrifuged at 3,000 x g for 10 min. The resulting pellets were washed in sterilized water, lightly squeezed in filter paper and stored at -80° C. DNA was extracted according to the method of Almeida *et al.* (2001b) and maintained at -80 °C.

RAPD analysis

Seventeen random oligonucleotide (10-mer) primers (Operon Technologies, Inc., Alameda, CA, USA) were used.

TABLE 1 - Soybean (*Glycine max*) isolates of *Cercospora kikuchii* obtained from purple stained seeds from different cultivars and origins in Brazil

Isolate/Origin	State	Cultivar	Isolate/Origin	State	Cultivar
1-Londrina	PR	Embrapa 25	37 -Anápolis	GO	Unknown
2-Londrina	PR	Embrapa 133	38-Anápolis	GO	Unknown
3-Londrina	PR	Embrapa 136	39-Anápolis	GO	Unknown
4-São Carlos do Ivaí	PR	BR-37	40-Bagé	RS	Unknown
5-São Carlos do Ivaí	PR	BR-37	41-Bagé	RS	Unknown
6-Terra -Boa	PR	Ft-Abyara	42-Bagé	RS	Unknown
7-Terra -Boa	PR	BR-16	43-Passo-Fundo	RS	Unknown
8-Terra -Boa	PR	BR-16	44-Passo-Fundo	RS	Unknown
9-Santa Cecília do Pavão	PR	BR-37	45-Cruz-Alta	RS	Unknown
10-Santa Cecília do Pavão	PR	BR-37	46-Cruz-Alta	RS	Unknown
11-Santa Cecília do Pavão	PR	BR-37	47-Cruz-Alta	RS	Unknown
12-NovaCantu	PR	Ft-Abyara	48-Ijuí	RS	Unknown
13-Nova Cantu	PR	Unknown	49-Ijuí	RS	Unknown
14-Nova Cantu	PR	BR-16	50-Londrina	PR	Clark
15-Peabiru	PR	BR-16	51-Londrina	PR	Clark
16-Rondonópolis	MT	Unknown	52-Londrina	PR	Clark
17-Rondonópolis	MT	Unknown	53-Londrina	PR	Cometa
18-Rondonópolis	MT	Unknown	54-Londrina	PR	Cometa
19-Chapadão do Céu	MT	Unknown	55-Londrina	PR	Cometa
20-Chapadão do Céu	MT	Unknown	56-Imperatriz	MA	Tracajá
21-Chapadão do Céu	MT	Unknown	57-Imperatriz	MA	Tracajá
22-Luiziana	GO	Unknown	58-Imperatriz	MA	Tracajá
23-Luiziana	GO	Unknown	59-Imperatriz	MA	Sambaíba
24-Luiziana	GO	Unknown	60-Imperatriz	MA	Sambaíba
25-Mineiros	GO	Unknown	61-Imperatriz	MA	Sambaíba
26-Mineiros	GO	Unknown	62-Imperatriz	MA	Seridó
27-Mineiros	GO	Unknown	63-Imperatriz	MA	Seridó
28-Goiatuba	GO	Unknown	64-Imperatriz	MA	Seridó
29-Goiatuba	GO	Unknown	65-Francisco Beltrão	PR	FT-2000
30-Goiatuba	GO	Unknown	66-Francisco Beltrão	PR	FT-2000
31-Rio-Verde	GO	Unknown	67-Francisco Beltrão	PR	FT-2000
32-Rio-Verde	GO	Unknown	68-Laranjeira do Sul	PR	BR-16
33-Rio-Verde	GO	Unknown	69-Barreiras	BA	Unknown*
34-Senador Carneiro	GO	Unknown	70-Londrina	PR	Unknown*
35-Senador Carneiro	GO	Unknown	71-Sta. Cruz-Bolivia		Unknown
36-Senador Carneiro	GO	Unknown	72-Sta. Cruz-Bolivia		Unknown

*Isolated from infected leaves with typical symptoms of *Cercospora* leaf blight.

Reaction mixtures were prepared for a final volume of 25 μ l of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 5% Triton-X100, 10 mM of each of the four deoxyribonucleotide triphosphates, 0.4 μ M primer, 25 U/ml Taq polymerase and 20 ng of genomic DNA. Amplification was performed in a Perkin Elmer PCR system 9600 programmed for 45 cycles. Each cycle consisted of a denaturation step at 94 °C for 1 min, a primer annealing step at 36 °C for 1 min, and a primer extension step at 72 °C for 2 min. Following amplification, the products were electrophoresed on 1% agarose gel in 0.5x Tris-borate-EDTA (TBE) buffer pH 8.0 for 15 V/cm, stained with ethidium bromide, and visualized by UV fluorescence.

ITS RFLP analysis and sequencing

The ITS region from 12 selected isolates, chosen according to a previous clustering analysis, was amplified with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')

and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.* (1990). Amplification reactions were performed in 50 μ l -volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 200 μ M each deoxynucleoside triphosphate, 0.5 μ M each primer, 10 ng of genomic DNA and 2.5 U Taq DNA polymerase. Temperature parameters were 94 °C for DNA denaturation, 3 min for the first cycle and 1 min for the remaining cycles, 45 °C for 1 min for primer annealing, and 72 °C for 2 min for primer extension with a total of 35 cycles. Amplified products were analyzed by electrophoresis in 1.2% agarose gel and visualized after staining with ethidium bromide. A sample of 20 μ l of each PCR product was digested with *Rsa*I, *Eco*RV, *Dra*I, *Eco*RI, *Hae*III, *Mse*I, *Pst*I, *Taq*I, according to the manufacturer's instructions. Digested DNA was run on 2% agarose gels. The gels were stained with ethidium bromide (0.5 μ g/ml), and DNA was visualized under UV light.

Sequencing was performed by the chain-termination

method using the ABI Big Dye Terminator Cycle sequencing kit v 2.0 (Applied Biosystems Inc., Foster City, CA, USA) on an ABI PRISM model 3100 DNA sequencer.

Analysis of genetic similarity

The RAPD bands and restriction fragments were scored as present (1) or absent (0). Data were analyzed using the numerical taxonomy package NTSYSpc version 2.02.j. A similarity matrix was produced with the SIMQUAL program using the Nei & Li (1979) distance coefficient which measures the proportion of band mismatches between pairs of isolates. Cluster analysis was performed with the cluster program SAHN using the unweighted pair-group method with arithmetic averages (UPGMA). Principal coordinate analysis was obtained using the eigen values and eigen vectors for real symmetric matrices inside the ordination subroutine. Bootstrap analysis was obtained by 1000 replications with the program WINBOOT (Yap & Nelson, 1996).

Analysis of molecular variance

Analysis of molecular variance (AMOVA) was performed using WINAMOVA (Excoffier *et al.* 1992) on *C. kikuchii* isolates to partition the total variance into that attributable to differences within and among populations components and among populations within groups (states). Isolates from one specific state constituted a population. For this analysis a total of 19 populations were considered (Londrina, São Carlos do Ivaí, Francisco Beltrão, Terra Boa, Santa Cecília do Pavão, Nova Cantú, Rondonópolis, Chapadão do Sul, Luiziana, Mineiros, Goiatuba, Rio Verde, Senador Carneiro, Anápolis, Bagé, Passo Fundo, Cruz Alta, Ijuí and Imperatriz) and together with 70 isolates from the states of PR, MT, GO, RS and MA. Two isolates from Bolivia were not included because of the low number. The variance among populations and the F statistic were tested by nonparametric randomization analysis. Estimation for gene flow (N_m) among populations and among states was performed using POPGENE, version 1.31; (<http://www.ualberta.ca/~fyeh/>).

RESULTS

Cultural characteristics, cercosporin content and mycelial growth

Seventy-two *C. kikuchii* isolates from the most diverse soybean growing regions were observed for variation in the color of the mycelium and their morphology. Most colonies (51) had a purple tinge when observed from the underside of the Petri dish. Some colonies (21), however, were white to light gray or black. The mycelia on top varied greatly between white or dark gray to light violet. Since isolates exhibited different growth rates on PDA, seven randomly chosen isolates (1, 28, 31, 47, 63, 68 and, 75) were followed during a 16 day-period to estimate daily growth rate. Isolates 31 and 28 exhibited the fastest growth (0.71 cm/day) and slowest growth rate (0.29 cm/day), respectively.

The concentration of cercosporin was variable among isolates and among replications ranging from 0.3 to 41 nmol/ml for isolates 37 and 47, respectively.

Virulence analysis

Virulence analysis performed with four isolates with different capacities for cercosporin synthesis showed that all isolates were able to infect soybean leaves, and that there was a correlation coefficient of 83% between cercosporin content and disease severity (Table 2). Isolates that showed purple to red halos in PDA media were also those that produced more cercosporin and were the most virulent. Lesions had a central necrotic area with a yellow halo visible one to two weeks after inoculation. Symptoms began to appear four to seven days after inoculation as irregular spots. Yellow halos developed three to seven days after the formation of the necrotic lesions. Isolates that produced low levels of cercosporin caused very small number of tiny necrotic foliar lesions. When inoculated into pods they were also able to induce purple seed stain (data not shown).

Analysis of genetic similarity

Of the 17 primers screened, 14 produced reproducible RAPD fragments in two trials. A total of 86 selected polymorphic amplicons were detected and generated a matrix of genetic distance. A dendrogram with seven clusters was obtained through the UPGMA (Figure 1). The matrix of similarities generated with all 72 isolates showed genetic distances ranging from 33% to 99%. The first cluster was comprised of 13 isolates with similarities ranging from 73% to 79%, with an average of 77%. The second cluster contained the majority of the isolates (51) for similarities ranging from 73% to 88%, for an average of 85%. The third cluster was formed by two isolates with similarities of 76%. The fourth cluster contained three isolates with similarities of 72% to 84% and an average of 79%. The fifth, sixth and seventh clusters contained only one isolate each. The geographic origin and virulence of the isolates did not correlate with RAPD groups. No marker was specific to an isolate's ability to produce cercosporin.

Bootstrap analysis of the 1,000 interactions showed values ranging from 53% to 100%. Principal coordinates analysis confirmed the clustering of isolates into seven groups with 79% of total variance explained by the two vectors used (Figure 2).

Analysis of molecular variance

Genetic differentiation among and within populations was observed based on 86 RAPD loci. The AMOVA analysis within population and the coefficient of genetic differentiation among populations are shown in Table 3. The "among States" effect explained 2.64% of the total variance. Within populations and among populations represented 75.09% and 22.27% of the total variance, respectively (Table 3), suggesting that the majority of genetic diversity was found within populations. Gene flow (N_m) among

TABLE 2 - Virulence analysis of *Cercospora kikuchii* isolates showing different cercosporin concentrations in soybean (*Glycine max*) cultivar

Soybean Cultivar	Isolate (Cercosporin content)				Average
	12 (6.1*)	3 (12.5)	20 (16.1)	63 (33.0)	
IAS-2	1.00 B**	1.67 B**	2.00 B**	2.57 B**	1.83 B**
Davis	1.67 AB	3.33 A	3.00 A	4.67 A	3.16 A
Sambaiba	1.67 AB	3.67 A	3.67 A	5.33 A	3.58 A
Paraná	1.67 AB	2.67 A	3.00 A	5.00 A	3.08 A
Conquista	2.00 A	3.00 A	3.67 A	5.33 A	3.50 A
Average	1.60 c	2.87 b	3.07 b	4.60 a	

* Cercosporin content (nmol/ml) measured according to Jens *et al.*, 1989.

**1= 0%-1% infected leaf area; 2=2%-5% infected leaf area; 3= 6%-10% infected leaf area; 4=11%-25% infected leaf area; 5= 26%-50% infected leaf area; 6=51% -75% infected leaf area; 7=>75% infected leaf area (adapted from James, 1971). Values followed by the same capital letter within a column and small letters in a line are not significantly different at P<0.05 according to Tukey's test. No. of sampled leaves =8.

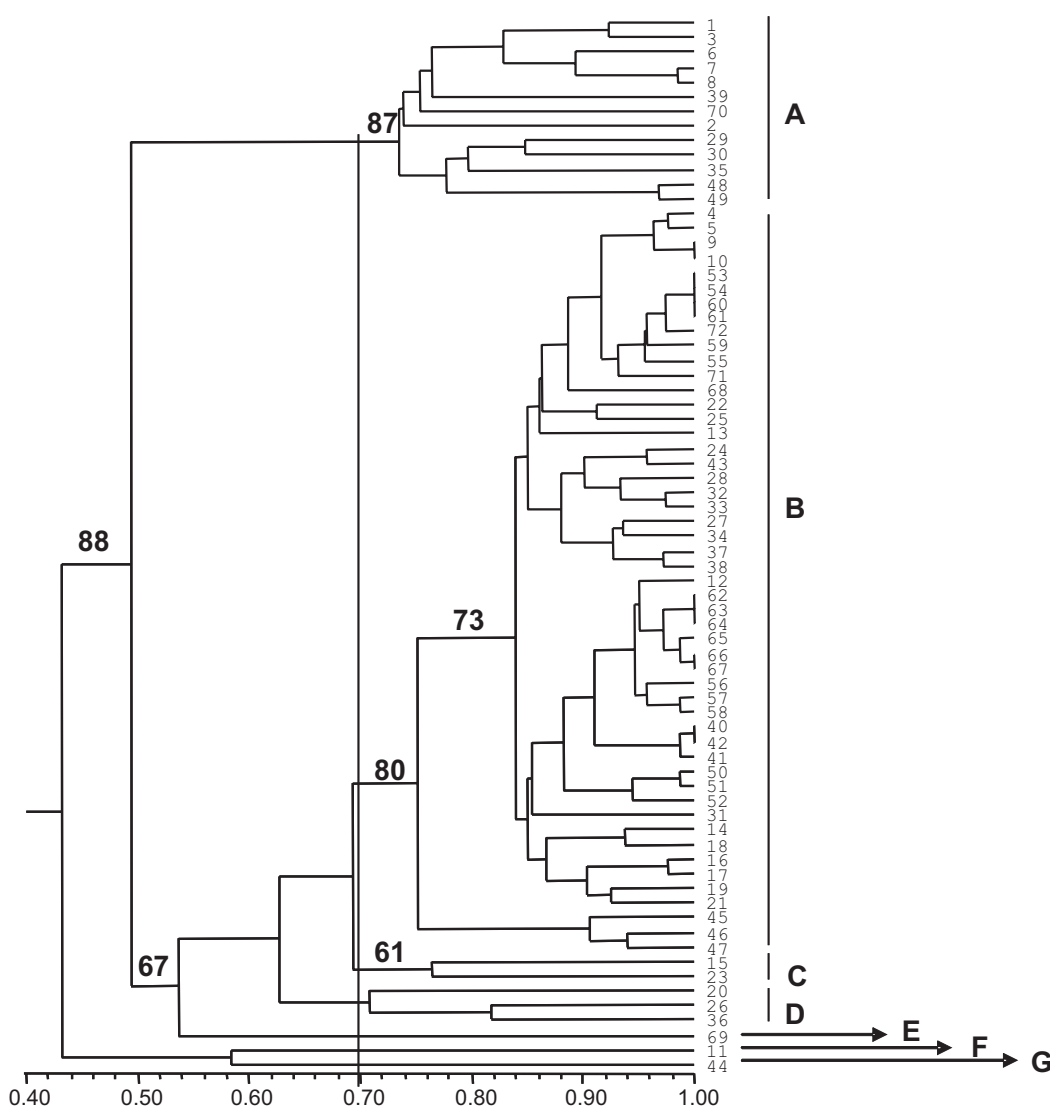


FIG. 1 - Genetic similarity among 72 *Cercospora kikuchii* isolates sampled from different regions in Brazil. Phenogram was estimated by clustering a distance matrix (Dice coefficient) of binary random amplified polymorphic DNA data using the unweighted pair group method of arithmetic means in the NTSYS program. Numbers at the major branches indicate the percentage occurrence of the cluster to the right of the branch in 1,000 bootstrapped data sets. Only branches occurring in 50% or more of the bootstrapped phenograms are labeled. A to G indicate the seven clusters of isolates found.

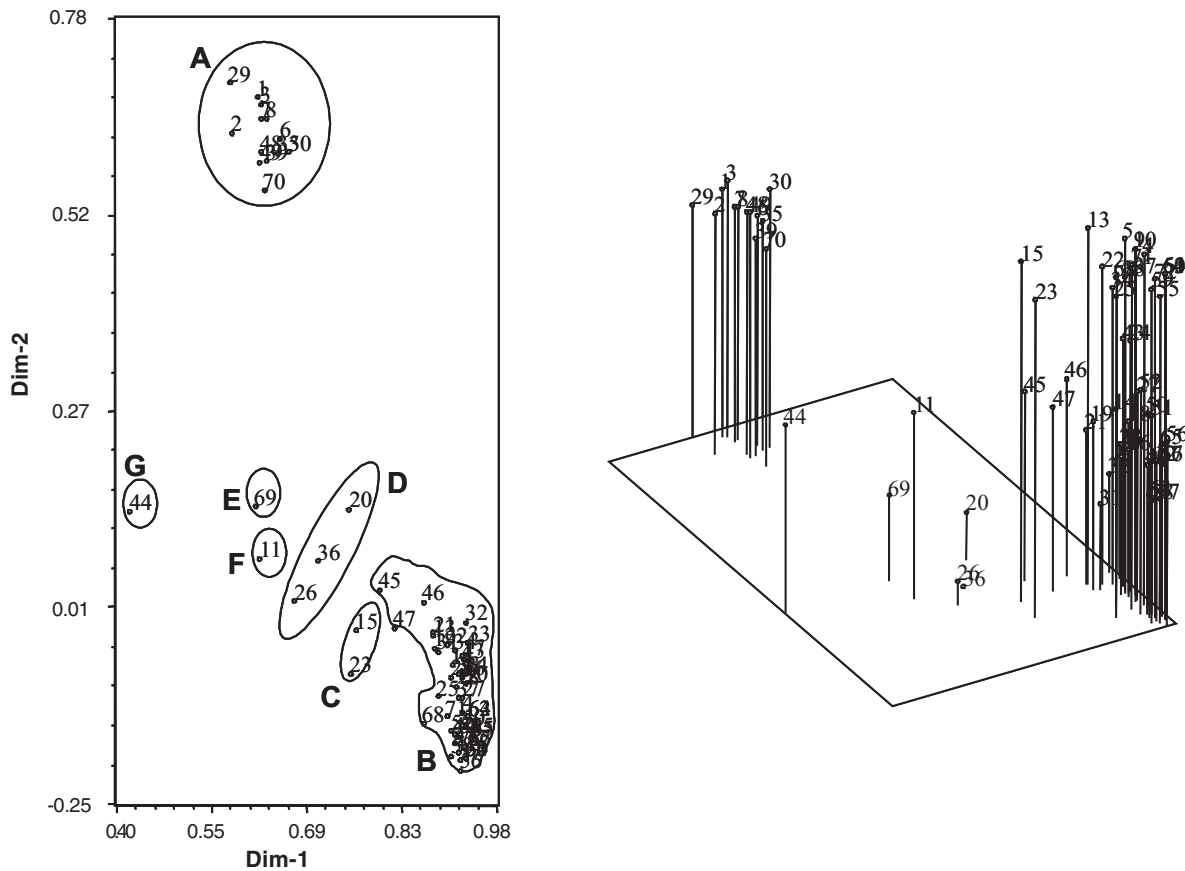


FIG. 2 - Two and three dimensional principal coordinate analysis of 72 *Cercospora kikuchii* isolates grouped in seven clusters (A through G). X and Y-axes account for 79% of total variation observed. X, Y and Z-axes account for 82.5% of total variance observed.

populations over all loci (0.45) was smaller than among States that showed N_m of 1.33.

ITS-RFLP and sequencing

PCR with primers ITS1 and ITS4 produced a DNA fragment of approximately 560 bp for the 12 isolates tested. No size variation was found among the amplified ITS regions. Of eight restriction enzymes used only three (*Nde* II, *Hinf* I and *Alu* I) cut the fragment. The observed band sizes were 310 and 200 bp for *Nde* II, 300 and 210 bp for *Hinf* I and, 380 and 140 bp for *Alu* I. No polymorphism was detected

among the samples. *Cercospora sojina* Hara was used as an out group and it exhibited a band of approximately 600 bp with the same number of restriction sites as *C. kikuchii* but producing fragments of different sizes (for example 400 bp and 150 bp with *R*s).

The PCR-amplified and cloned sequences of the 5.8 rDNA gene and its ITS flanking regions from all the 12 isolates were aligned. Sequences demonstrated identity of 97% to 100% with four sequences of *C. kikuchii* (GenBank accession no. AY266160, AY266161, AY152577 and AF291708). The comparison of Brazilian sequences (12) with

TABLE 3 - Analysis of molecular variance at three-levels for populations of *Cercospora kikuchii*, using random amplified polymorphic DNA phenotypes*

Source	df	Variance	Total variance (%)	Φ statistic	p
Among States	5	0.3066	2.64		
Within populations	51	8.7133	75.09	0.249	0.001
Among populations and within States	14	2.5846	22.27		

*Probability of having an equal or more extreme variance component statistic than the observed values by chance alone. Tested by nonparametric randomization analysis, using 1,000 repetitions.

these four *C. kikuchii* sequences from the GenBank showed the values ranging from 0 to 6 nts differences. The overall mean number of differences among Brazilian isolates was 1.2 nts over all 12 pair-wise comparisons. Most of the differences were inside the ITS2. The numbers of transitions within each of the 12 sequences from Brazilian isolates ranged from 0 to 3 while no transversions were observed. In another comparison, using the same 12 sequences with an isolate of *C. sojina* (GenBank AY266158) the values ranged from 2 to 5 nts (Table 4).

DISCUSSION

The objective of this work was to investigate phenotypic and genotypic variation among isolates of *C. kikuchii* in order to provide information for the soybean breeding program at Embrapa Soja. Data from this study revealed a considerable degree of variation in the population of *C. kikuchii* confirming previous results obtained by Almeida *et al.* (2001a) who had observed differences in morphological types and levels of pigmentation of the media where the isolates were grown.

Red pigmentation around colonies was caused by cercosporin, a non-host-specific phytotoxin isolated in 1957 (Kuyama & Tamura, 1957) from *C. kikuchii*. The role of cercosporin in the pathogenicity of *C. kikuchii* was first demonstrated by Upchurch *et al.* (1991) who considered it crucial for the infection of soybean plants. They observed that spontaneous and UV-induced mutants that did not produce cercosporin were not able to cause infection when inoculated on soybean leaves. In our study the differences in virulence among the isolates were associated with cercosporin content, and constitute unprecedented information for this pathosystem. Cercosporin when excited by light produces

hydroxyl radicals and singlet oxygen. Singlet oxygen catalyses peroxidation of lipid membrane, disrupting cellular integrity, inducing leakage of cytoplasmic contents and causing cell death (Daub & Ehrenshaft, 2000).

The observation of red color around the colony appeared valueless in predicting virulence, since several isolates with no reddish color were virulent. However, all isolates with pigmented colonies were the most virulent. Isolates that produced more cercosporin were also more virulent.

It was not clear in our studies if the lack of color in the extract for colorimetric assay observed in some isolates could be due to the sensitivity of the method used since isolates with very low amounts of cercosporin were also able to cause lesions when inoculated on soybean leaves or purple stained seeds when inoculated into pods. Cercosporin content varied considerably among replications from the same colony and from different colonies.

The RAPD has been successfully used to assess the genetic variability of *Cercospora* species (Inglis *et al.*, 2001; Weiland *et al.*, 2001). The genetic diversity observed among isolates through RAPD analysis was also an important result from this work. Seven RAPD groups were present among 72 isolates studied; however, the groups could not be correlated with cercosporin content, virulence or geographic origin of the isolates.

Similarly to other studies (Goodwin *et al.*, 2001; Almeida *et al.*, 2001b) molecular techniques such as RAPD provided strong evidence of genetic diversity among fungi. Under our conditions and for the proposed objectives these proved reliable and of low cost in relation to other methods.

There was no association between the groups of isolates, based on RAPD markers and cercosporin content. Isolates that produced high contents of cercosporin were

TABLE 4 - Pair-wise comparison between number of nucleotide differences of internal transcribed spacer regions of the ribosomal genes from Brazilian *Cercospora kikuchii* isolates (6 through 17) and *C. sojina* (AY266158) and *C. kikuchii* (AY266160, AY266161, AY152577 and AF291708) from GenBank

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]
[1] AF291708																	
[2] AY152577	4																
[3] AY266161	6	2															
[4] AY266160	4	0	2														
[5] AY266158	8	4	4	4													
[6] AY6 33835	5	1	1	1	5												
[7] AY633836	6	2	2	2	4	3											
[8] AY633837	5	1	1	1	5	0	3										
[9] AY633838	5	0	2	0	4	1	2	1									
[10] AY633839	5	1	1	1	5	0	3	0	1								
[11] AY633840	5	1	1	1	3	0	3	0	1	0							
[12] AY633844	5	1	1	1	5	0	3	0	1	0	0						
[13] AY633841	5	1	3	1	5	2	3	2	1	2	2	2					
[14] AY633842	4	0	2	0	4	1	2	1	0	1	1	1	1				
[15] AY739106	5	1	1	1	2	2	1	2	1	2	2	2	2	1			
[16] AY633843	4	0	2	0	4	1	2	1	0	1	1	1	1	0	1		
[17] AY739107	5	1	1	1	3	2	1	2	1	2	2	2	2	1	0	1	1

clustered together with low cercosporin producers. For example, isolates 3 (12.5 nmol/ml) and 63 (33.0 nmol/ml) were high producers of cercosporin and were separated in clusters A and B, respectively. Isolates 27 (11.4 nmol/ml) and 37 (0.3 nmol/ml) were clustered together but cercosporin content was significantly higher in isolate 27.

The effect of plant genotype on the genetic diversity of the isolates used was not consistent, probably due to the low number of isolates from each cultivar. This theory is currently under investigation. Most of the isolates, despite being isolated from different soybean genotypes, clustered together.

The occurrence of different haplotypes (RAPD profiles) in the same region suggests that the pathogen populations are not genetically uniform across the area, and this work may indicate how rapidly this pathogen evolved in different environments, since isolates collected from seeds were produced in both tropical and sub-tropical Brazilian regions.

Isolates that formed a single cluster (E, F and G) as well as other isolates from clusters A and B were selected for ITS sequence analysis. The results showed high similarity (97%-100%) with published sequences of *C. kikuchii* (GenBank AY266160, AY266161, AY152577 and AF291708). The number of nucleotide differences of ITS of the rDNA among all 12 sequences observed in this work ranged from 0 to 3, with an average of 1.2 nts. However, Goodwin *et al.* (2001), in a previous publication, mentioned that the mean number of nucleotide differences between three isolates of *C. kikuchii* ranged from 2 to 7 with an average of 4.7. These differences may be due to different sets of sequences (TREEBASE) used by Goodwin *et al.* (2001) for comparing their isolates.

An additional comparison of Brazilian isolates (12) with an isolate of *C. sojae* (GenBank AY266158) showed values ranging from 2 to 5 nts, different from the values of Goodwin *et al.* (2001) that found larger values ranging from 4 to 9 nts.

The variability found in this work was confirmed through additional statistical analysis. The estimator $\hat{\Theta}_{st}$ is highly different from zero ($P > 0.001$), indicating large genetic differentiation among populations from different regions (Hartl & Clark, 1997). The majority part of genetic variance was found within populations. The AMOVA analysis showed that the smallest fraction of variance was observed among populations within states that demonstrated substantial gene flow among states. When all isolates were analysed without clustering into states, a low estimated of genetic flow ($Nm=0.45$) was observed, suggesting that the variability among states is smaller than among collecting sites. This fact could be explained by the flux of seeds from traditional areas in the South to new areas in the Central and northern regions of Brazil. Traditional areas where soybean was cultivated for more than 30 years could have induced more diversity than new areas especially because of the genetic background of cultivars used over the years.

Populations of *C. kikuchii* are pathogenically, genotypically and geographically variable. Considering that this pathogen is easily transmitted by seeds it is not surprising to find the same haplotypes in different regions. Migration could be favoured by infected seeds as demonstrated by the clustering analysis.

In Brazil, there has been a rapid increase in soybean producing area since 1970; therefore, the traffic of seeds from traditional areas to new areas could be responsible for the geographical variability since *C. kikuchii* is a seed borne pathogen. However, this fact alone cannot explain all the genetic diversity observed. To what extent the soybean genotype may favor the development of new genetically distinct isolates is unknown. Unfortunately, an insufficient number of isolates was obtained from each area to permit the evaluation of gene flow among populations more precisely.

For a pathogen without known sexual reproduction, the observed diversity can be explained by single mutations and chromosomal aberrations like deletions, transpositions and chromosomal losses (Kistler & Miao, 1992). According to Kempken & Kuck (1998) transposons may increase variability in fungi. Also, fusion between vegetative cells of fungi may form heterokaryons (Carlile, 1986).

Control of Cercospora leaf blight may be possible through resistant cultivars although no resistant gene has been identified so far. However, different levels of susceptibility as observed by Walters (1978) were found in this study. These differences occurred with all isolates tested. Identification of a resistant gene against *C. kikuchii* would be a great contribution for breeding programs. However, the pathogen's variability must be considered in order to avoid drawbacks with the release of new cultivars. For countries with large soybean areas like Brazil it is very important to know in advance the variability of the pathogen, in order to avoid resistant released cultivars becoming susceptible when sown in different areas. The level of variability among isolates as identified in this work may help to define the breeding method for effective resistance.

ACKNOWLEDGMENTS

We thank Dr. E.S. Calvo and Dr. A.L. Nepomuceno for providing conditions for sequencing; L.C. Benato, M.C. Pinto and N. Valentin for helping in several steps of this work; M. Meyer and M.F. Gastal for providing infected seeds and, Dr. John Rupe (Univ. of Arkansas, USA), Dr. Marisa A. S. V. Ferreira (UnB), Dr. M. C. Bassoi and J.F. Ferraz de Toledo for discussions and suggestions on the manuscript.

Approved by the Head of Research and Development of Embrapa Soja as manuscript 141/2003.

LITERATURE CITED

ALMEIDA, A.M.R., MARIN, S.R.R., BINNECK, E., PIUGA, F.F., SARTORI, F., COSTAMILAN, L.M., TEIXEIRA, M.R. & LOPES,

- M. Pathogenicity, molecular analysis and cercosporin content of Brazilian isolates of *Cercospora kikuchii*. Abstracts of the VII World Soybean Research Conference, Foz do Iguaçu. 2004. pp.69-70.
- ALMEIDA, A.M.R., SARTORI, F., CALVO, E.S., MARIN, S.R.R. & FUKUJI, T.S. Diferenciação morfo-bio-molecular de isolados de *Cercospora kikuchii* obtidos de sementes de soja, no Brasil. *Fitopatologia Brasileira* 26:328. 2001a.
- ALMEIDA, A.M.R., ABDELNOOR, R.V., CALVO, E.S., TESSMAN, D. & YORINORI, J.T. Genotypic diversity among brazilian isolates of *Sclerotium rolfsii*. *Journal of Phytopathology* 149:493-502. 2001b.
- ALMEIDA, A.M.R., FERREIRA, L.P., HENNING, A.A., VELOSO, J.F.S. & YORINORI, J.T. Doenças da Soja. In: Kimati, H., Amorin, L., Bergamin Filho, A., Camargo, L.E.A. & Rezende, J.A.M. (Eds.). *Manual de Fitopatologia*. São Paulo. Ceres. 1997. pp.642-664.
- CALLAHAN, T.M., ROSE, M.S., MEADE, M.J., EHRENSHAFT, M. & UPCHURCH, G.G. CFP, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. *Molecular Plant-Microbe Interactions* 12:901-910. 1999.
- CARLILE, M.J. Genetic Exchange and Gene Flow: Their Promotion and Prevention. In: Rayner, A.D.M. & Moore, D. (Eds.). *Evolutionary Biological of the Fungi*. Cambridge. Cambridge University Press. 1986. pp.203-214.
- DAUB, M.E. & EHRENSHAFT, M. The photoactivated *Cercospora* toxin cercosporin: contribution to plant diseases and fundamental biology. *Annual Review of Phytopathology* 38:461-490. 2000.
- EXCOFFIER, L., SMOUSE, P.E. & QUATTRO, J.M. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131:479-491. 1992.
- GARDNER, M.W. Indiana plant diseases. *Proceedings of the Indiana Academy of Science*. 35:237-257. 1925.
- GOODWIN, S.B., DUNKLE, L.D. & ZISMANN, V.L. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. *Phytopathology* 91:648-658. 2001.
- HARTL, D.L. & CLARK, A.G. *Principles of Population Genetics*. 3.ed. Sunderland (MA): Sinauer Associates. 1997. pp.111-122.
- INGLIS, P.W., TEIXEIRA, E.A., RIBEIRO, D.M., VALADARES-INGLIS, M.C., TIGANO, M.S. & MELLO, S.C.M. Molecular markers for the characterization of brazilian *Cercospora caricis* isolates. *Current Microbiology* 42:194-198. 2001.
- JAMES, W.C. *A manual of disease assessment keys for plant diseases*. Canadian Department of Agriculture. Publication No. 1458. 1971.
- JENNS, A.E., DAUB, M.E. & UPCHURCH, R.G. Regulation of cercosporin accumulation in culture by medium and temperature manipulation. *Phytopathology* 79:213-219. 1989.
- KEMPKEN, F. & KUCK, U. Transposons in filamentous fungi: facts and perspectives. *BioEssays* 20:652-659. 1998.
- KISTLER, H.C. & MIAO, V.P.W. New modes of genetic change in filamentous fungi. *Annual Review of Phytopathology* 30:131-152. 1992.
- KUYAMA, S. & TAMURA, I. Cercosporin. A pigment of *Cercospora kikuchii* Matsumoto et Tomoyasu. II. Cultivation of fungus, isolation and purification of pigment. *Journal of the American Chemistry Society* 79:5725-5726. 1957.
- LEHMAN, S.G. Purple stain of soybean seeds. *North Carolina Agriculture Experimental Station Bulletin* 369:1-11. 1950.
- MIYASAKA, S. Contribuição para o Melhoramento da Soja no Estado de São Paulo. (Tese de mestrado) Piracicaba, ESALQ. 1958.
- NEEGAARD, P. *Seed Pathology*. London. MacMillan. 1979.
- NEI, M. & LI, H. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings National Academy of Science USA* 76:5269-5273. 1979.
- SCHUH, W. Influence of temperature and leaf wetness period on conidial germination in vitro and infection of *Cercospora kikuchii* on soybean. *Phytopathology* 81:1315-1318. 1991.
- SEDIYAMA, T., CARDOSO, A.A. & ALMEIDA, A.M.R. Estudo do comportamento de variedades e linhagens de soja. *Seiva* 31:257-266. 1971.
- UPCHURCH, R.G., WALKER, J.A., ROLLINS, J.A., EHRENSHAFT, M. & DAUB, M.E. Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. *Applied and Environmental Microbiology* 57:2940-2945. 1991.
- WALTERS, H.J. *Cercospora* leaf blight of soybeans. *Phytopathology News* 12:165-166. 1978.
- WEILAND, J., EIDE, J., RIVERA-VARAS, V. & SECOR, G. Genetic diversity of *Cercospora beticola* in the U.S. and association of molecular markers with tolerance to the fungicide triphenylin hydroxide. *Phytopathology* 91:S94. 2001. (Abstract)
- WHITE, T.J., BRUNS, T., LEE, S. & TAYLOR, J. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis, M.A., Gelfand, D.H. Sninsky, White, T.J. (Eds.). *PCR Protocol, a guide to methods and applications*. San Diego, CA. Academic Press. 1990. pp.315-322.
- WRATHER, J.A., ANDERSON, T.R., ARSYAD, D.M., GAI, J., PLOPER, L.D., PORTA-PUGLIA, A. & RAM, H.H. Soybean disease loss estimates for the top 10 soybean producing countries in 1994. *Plant Disease* 81:107-110. 1997.
- YAP, I. & NELSON, R.J. Winboot: a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. *International Rice Research Institute, Manila, Philippines. International Rice Research Institute Research Paper No. 14*. 1996.