REP-PCR PATTERNS AND SEQUENCE ANALYSIS OF THE INTERNAL TRANSCRIBED SPACER OF rDNA OF Stemphylium solani.

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INTRODUCTION
Leaf blight of tomato (Lycopersicon esculentum) caused by Stemphylium solani is of economic importance to Brazil. Recently, a new leaf blight of cotton caused by S. solani was reported in Brazil, causing heavy yield losses especially in susceptible cultivars (Mehta, 1998). A genetic study of S. solani isolates conducted earlier using RFLP of the ITS region showed identical patterns for all the strains (Mehta, 1999). In the present study, a more detailed analysis of the ITS region by sequencing was performed to understand the phylogenetic relationship between some of the S. solani isolates from tomato and cotton.

Characterization of plant pathogenic bacteria especially at the pathovar level, is being done by Repetitive Extragenic Palindromic (REP) elements, Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences, and BOX elements (collectively known as rep-PCR). Recently, REP and ERIC-PCR fingerprinting is also being used for plant pathogenic fungi (Edel et al., 1995). Besides cloning and sequencing of the ITS region, in the present study, we also performed ERIC and REP-PCR fingerprinting.

MATERIALS AND METHODS
Fungal isolates. Thirty-three monosporic isolates of S. solani (28 from cotton, 2 from tomato from the State of São Paulo and 3 from tomato from the State of Goiás) were analysed by ERIC and REP-PCR fingerprinting. An isolate of A. macrospora from cotton was included for comparison, since it produces somewhat similar symptoms to those produced by S. solani.

DNA extraction. Total DNA was extracted and the amount of DNA was quantified by a DyNa Quant 200 Fluorometer, manufactured by Pharmacia, and was confirmed by gel electrophoresis. DNA samples were diluted in TE buffer to give a concentration of 20 ng µL⁻¹ and stored at −21°C. Samples which showed degradation of DNA in gel electrophoresis quantification were discarded and DNA extraction was repeated. RNA was eliminated by RNAase (10 µg/ml).

ERIC and REP-fingerprinting. Sequences of the primers used to amplify DNA of all the 34 fungal isolates were ERIC1R-5'ATGTAAGCTCCTGGGGTTCAC3'; ERIC2-5'AAGTAAGTGACTGGGGTGAGCG3'; REP1R-15'IIICGICGICATCIGGC3'; REP2-I-5'ICGICCTATCIGGCCTAC3'. PCR reactions were performed in 25 µl volumes containing, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2 mM MgCl₂, 200 µM of each dATP, dCTP, dGTP and dTTP, 1.3 µl of BSA, 50 picomoles of each primer, 100 ng of genomic DNA, and 1 unit of Taq polymerase (Pharmacia, USA). Negative controls, without DNA template were maintained in all the reactions. Amplification was performed in a Thermal Cycler (MJ Research, Inc. Watertown, MA, USA: for ERIC: 7 min at 94°C, followed by 30 cycles of denaturation of 1 min at 94°C, annealing of 1 min at 52°C, 2 min at 72°C and a final extension of 16 min at 65°C. and for REP: 6 min at 95°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing of 1 min at 40°C, 8 min at 65°C, and a final extension of 16 min at 65°C. Amplification products (25 µl) were electrophoresed in 2% agarose gels with TBE running buffer, stained with ethidium bromide and were scanned into a computer imaging file using a Kodak EDAS 120 digital camera. ERIC and REP-PCR analyses were repeated once to confirm the results. Data were analysed considering the presence or the absence of bands. A distance matrix was analysed by the unweighted pair group method (UPGMA in the NTSYS-pc, version 1.8), using the Jaccard coefficient.

Amplification of the ITS region. The ITS region of four isolates of S. solani was amplified using the primer pairs ITS4/ITS5. The sequences for the ITS4 and ITS5 primers were 5’TCCTCCGCTTATTGATATGC3’ and 5’GGAAGTAAAAGTCGTAACAAGG3’, respectively.

Amplification conditions were: 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C and a final extension of 5 min at 72°C. Amplifications were performed in a total volume of 50 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2 mM MgCl₂, 200 µM of each

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dATP, dCTP, dGTP, dTTP, 25 picomoles of each primer, 100 ng of genomic DNA, and 1 U of Taq polymerase. Aliquots of 5 µl of amplification products were electrophoresed in 1.4% agarose gels and stained with ethidium bromide to verify the fragment size.

Cloning and sequencing of amplified DNA. The amplified ITS region of the isolates, two each from cotton and tomato, were cloned using the p-GEM T-easy cloning kit (Promega, Madison, USA). Plasmid DNA was extracted by the Nucleospin Nucleic Acid Purification Kit (Clontech) and used in the sequencing reactions. The sequencing reactions were performed in a total volume of 10 µl containing 300-500 ng of DNA, 5 picomoles of primer (M13 forward or M13 reverse), 3 µl of buffer (20 mM TrisHCl, pH 9.0; 5 mM MgCl₂) and 1 µl of big dye terminator cycle sequencing ready reaction kit (Perkin Elmer). The reactions were conducted with an initial denaturation of 5 min at 95ºC, followed by 25 cycles with a denaturation of 1 min at 95ºC, annealing of 10 s at 55ºC and extension of 4 min at 65ºC. The sequencing was performed in an automatic sequencer (ABI PRISM™ 377, Perkin Elmer) and repeated twice for all the clones.

Phylogenetic analysis. Twenty five ITS sequences, available in the Genbank, showing homology to the ITS sequence of S. solani were chosen to construct a phylogenetic tree. The sequences were aligned using the ClustalW program. The evolutionary distance was calculated using the Jukes-Cantor distance and the phylogenetic tree was constructed by the Neighbour-Joining method using the MEGA package. Bootstrap analysis with 500 replications was performed to provide support for the branches of the tree. Gaps were treated as missing information.

RESULTS & DISCUSSION
ERIC and REP analysis revealed a total of 31 PCR products out of which, 21 were polymorphic. Data obtained with both primers were combined to construct a dendrogram which revealed a low level of polymorphism among the cotton isolates. However, clear differences were noticed between the tomato and the cotton isolates as well as between the tomato isolates originated from different geographic regions. A. macrospora fell into a completely separate group (Fig. 1, 2A).

![Fig. 1. A.B-Sample gels of the REP-PCR profiles respectively, of some isolates of S. solani. Lane 1-2 are the tomato isolates from the State of São Paulo, 9 through 28 are the cotton isolates, 31-33 are the tomato isolates from Goiás and No. 34 is A. macrospora.](image)

The sequencing of the ITS region of the four isolates of S. solani revealed sequences of 605 bp. The two strains from cotton and one strain from tomato originated from the State of Goiás showed identical sequences, whereas the strain from tomato originated from the State of São Paulo showed differences in 9 bases. The high level of similarity of the ITS region observed (100-98.5%), indicates that the ITS region is highly conserved in these isolates. The ITS sequences of the S. solani isolates Nos. 1, 21 and 31 were deposited in the Genbank under accession numbers AF203451,AF203448,AF203449,AF203450, respectively. A phylogenetic tree was constructed using the ITS sequences of the four S. solani isolates and 25 ITS sequences of fungal pathogens showing homology to the ITS of S. solani. Setosphaeria rostrata was used as an outgroup. The sequence sizes of the ITS region of different fungal isolates tested in this study varied considerably, and hence, the sequences were edited after alignment in order to obtain the sequences with approximately the same length. The phylogenetic tree showed that S. solani is closely related to S. botryosum, S. alfalfae, S. herbarum, P. herbarum and P. gracilariæ (Fig. 2B). The Alternaria spp. formed a phylogenetically distinct group, but was closely related to S. solani. Chlochiiobolus and Pyrenophora species presented a high homology score in the Genbank query, however, they formed distinct groups and were distantly related to S. solani.

The separation of the cotton isolates from the tomato isolates by PCR amplification using the primers ERIC and REP gives evidence that they belong to distinct pathotypes of S. solani. Distinction was also observed between the isolates from tomato originated from the State of Goiás and São Paulo. The isolates of
tomato originated from the State of Goiás showed to be more similar to the cotton isolates than did the tomato isolates originated from São Paulo. Similar results were obtained by Mehta (1999) using RAPD analysis. The similarity between the isolates of cotton and those of tomato originated from the State of Goiás can also be observed in the analysis of the ITS region. The ITS sequences of the *S. solani* isolates showed a high level of similarity and could not distinguish the isolates from cotton and tomato originated from the State of Goiás. However, a distinct sequence for the isolate of tomato from São Paulo was revealed. The variation between the tomato isolates from Goiás and São Paulo, might be due to the occurrence of two different species of *Stemphylium*. The phylogenetic tree showed that *Stemphylium* and *Pleospora* form one group which is more related to *Alternaria* spp. ERIC and REP-PCR fingerprinting gave consistent results which were comparable with the sequencing and RAPD (Mehta, 1999), and hence, can also be used to assess intra-specific diversity of the plant pathogenic fungi.

In the present study, we have reported for the first time the ITS sequence of *S. solani* and inferred the phylogenetic relationships of *S. solani* with other fungal species.

**Fig. 2 A**

**Fig. 2 B**

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**REFERENCES**

