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Differentiation of *Stemphylium solani* isolates using random amplified polymorphic DNA markers

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***Stemphylium solani* isolates infect two varieties of eggplant, *Solanum aethiopicum* and *Solanum melongena* grown in Senegal (West Africa) and are morphologically quite similar. Using the random amplified polymorphic DNA (RAPD) procedure with arbitrary 10-mer primers, we were able to differentiate these *S. solani* isolates into two groups directly related to their plant host origin. A RAPD product of approximately 480 bases pairs obtained with OPF-20 primer were polymorphic between the two groups. Four new primers (F20F1, F20F2, F20R1 and F20R2) based on nucleotide sequence analysis of this 480 bases pairs RAPD fragment were developed. Such primers used pairwise amplified a single fragment from the DNA of *S. solani* isolates whatever their host origin. However, DNA extracted from *Fusarium oxysporum* (f. sp. *vasinfectum*, f. sp. *elaeidis*), *Verticillium dahliae* and *Phyllosticta* sp. isolates did not amplify using these primers. Our results indicate that these primers sets were good tools for specific identification of these two eggplants *S. solani* isolates by polymerase chain reaction (PCR).**

Key words: *Stemphylium solani* isolates, *Solanum aethiopicum*, *Solanum melongena*, random amplified polymorphic DNA (RAPD) markers, identification.

INTRODUCTION

The genus *Stemphylium* Wallr. was established in 1833 (Wallroth, 1833) with *Stemphylium botryosum* Wallr. as the type species. There were 33 published taxa of *Stemphylium* in 2002 (Câmara et al., 2002). Since then, many new species have been described (Simmons, 2004; Pei et al., 2009, 2011; Wang et al., 2009). Species of *Stemphylium* genus are dematiacious hyphomycetes, many of them are economically important pathogens on a

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Table 1. Geographic and host origin of *Stemphylium* isolates used in this study.

Isolates code	Localities	Hosts origin
S0	Kourounar	*Solanum aethiopicum*
PJ1	Pikine	*Solanum aethiopicum*
PJ2	Pikine	*Solanum aethiopicum*
S2	Thiaroye	*Solanum aethiopicum*
S2'	Thiaroye	*Solanum aethiopicum*
S3	Thiaroye	*Solanum aethiopicum*
C2	Kolda	*Solanum aethiopicum*
C3	Kolda	*Solanum aethiopicum*
C23	Ziguinchor	*Solanum aethiopicum*
C31	Ziguinchor	*Solanum aethiopicum*
C4	Kolda	*Solanum melongena*
C5	Kolda	*Solanum melongena*
C8	Kolda	*Solanum melongena*
C13	Kolda	*Solanum melongena*
C15	Kolda	*Solanum melongena*
C16	Kolda	*Solanum melongena*
C21	Ziguinchor	*Solanum melongena*
C25	Ziguinchor	*Solanum melongena*
C27	Ziguinchor	*Solanum melongena*
C28	Ziguinchor	*Solanum melongena*

wide range of plants (Mehta, 2001; Câmara et al., 2002; Tomioka and Sato, 2011). Until the emergence of molecular phylogenetic analysis, the taxonomy of *Stemphylium* species was based primarily on conidial morphology, including variation in conidial shape, size, length/width ratio, color, septation and ornamentation (Simmons, 1985, 2001). However, many of these characters overlap among species, making species determination difficult. The emergence of polymerase chain reaction (PCR) techniques has enhanced tradetional approaches to fungal taxonomic investigations. DNA sequence data are now being commonly used to verify morphological concept and other taxonomic hypothesis (Hsiang and Goodwin, 2001; Hunter et al., 2006; Pei et al. 2011). The ITS-rDNA sequence is being widely used to identify phylogenetic relationships among fungal taxa, especially at the species level (Callac and Guinberteau, 2005; Sotome et al., 2009). In a previous study, ITS-rDNA sequence analysis confirmed the identification of 45 fungal isolates as *Stemphylium solani* (Ndir et al., 2008), but was not able to reveal polymorphism among them. Random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams et al., 1990) has been used successfully to detect genetic variability in many groups of plants fungi (Assigbetse et al., 1994; Alfonso et al., 2000; Macdonald et al., 2000; Collopy et al., 2001). In the present paper, we used RAPD procedure on a sample of twenty isolates to find polymorphism between 10 *S. solani* isolates from *Solanum aethiopicum* and 10 others from *Solanum melongena* and we used the sequence information of some RAPD marker to develop a PCR-specific assay for these *S. solani* isolates identification.

MATERIALS AND METHODS

Fungal materials

Out of 45, a set of twenty *S. solani* isolates collected from naturally infected hosts: *S. aethiopicum* and *S. melongena* in different vegetables growing areas throughout the Senegal were used as listed in Table 1. Using morphological characters and molecular analysis of the ITS regions including 5.8S rDNA (RFLP and sequencing), we identified these 45 isolates as closely related to members of *S. solani* species by comparing our ITS sequence data with reference isolates in GenBank (data not shown). Isolate *May S*3 was therefore chosen from our collection and deposited in GenBank database under accession number AF 426739. Four *Fusarium* and three *Verticillium* strains were obtained from IRD-Montpellier, one strain from INRA-Montfavet was identified as *Phyllosticta* sp. All cultures were single-spored and stored on potato dextrose agar slants (PDA, Difco) at 4°C until further processing.

Fungal culture and DNA extraction

Isolates were grown in 200 ml of potato-dextrose-broth (PDB, Difco) for 7 days at 25°C. The mycelium was harvested by filtration, frozen 48 h at -80°C and lyophilized for 24 h. Total DNA was extracted following the method of Lee et al. (1988) with modifications and was performed by phenol-chloroform-isoamyl alcohol (25:24:1) procedure. The DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA) to a final concentration of 5 ng μl^{-1}.

DNA amplification

RAPD primers

The primers used are listed in Table 2 and were obtained from kit F, Operon Technologies (Alameda, CA, USA).

Amplification conditions

RAPD reactions were performed in a total volume of 25 µl containing 2.5 mM $MgCl_2$ (1.5 µl), 10 mM dNTPs (0.25 µl), 20 p.mol. of primer (4 µl), 25 ng of genomic DNA, 1 U of *Taq* DNA polymerase (Promega Charbonnières, France) and 2.5 µl of 10X *Taq* polymerase buffer. Negative controls without DNA were included in all reactions. The amplifications were conducted as follows: 4 min at 94°C, 30 cycles (1 min at 94°C, 1 min at 36°C and 1 min 30 s at 72°C), 5 min at 72°C. PCR reactions using sequence-specific primers were performed in the same conditions using 5 Units of *Taq* DNA polymerase, with an initial denaturation step at 94°C for 4 min followed by 24 cycles (30 s at 94°C, 1 min at 56 to 60°C and 1 min at 72°C), 5 min at 72°C. Amplification products were separated by electrophoresis on 1.4 % agarose gels stained with ethidium bromide and visualized under UV light. A 1 kb molecular weight ladder (Eurogentec, France) was used as size standard.

DNA templates

RAPD amplification reactions were conducted with each primer on

Table 2. Codes and sequences of the 6 primers tested for RAPD analysis of *S. solani* isolates, with total number of amplified DNA fragments and number of polymorphic DNA fragments obtained with each primer.

Primer code	Sequences 5' to 3'	Amplified fragment	Polymorphic fragment
OPF-02	GAGGATCCCT	7	2
OPF-04	GGTGATCAGG	5	2
OPF-10	GGAAGCTTGG	10	1
OPF-13	GGCTGCAGAA	8	2
OPF-14	TGCTGCAGGT	5	1
OPF-20	GGTCTAGAGG	10	1

the DNA of 10 *S. solani* isolates from *S. aethiopicum* and 10 others from *S. melongena.* PCR assays using specific primers were conducted with the DNA from *Stemphylium*, *Fusarium*, *Verticillium* and *Phyllosticta* isolates. All amplification reactions were repeated in separate experiments. RAPD data were analyzed considering the presence or the absence of bands.

Specific primers selection

An OPF-20 RAPD fragment of about 480 bp obtained from the amplification was extracted and purified from a low melting point 1% agarose gel by using QIA quick Gel Extraction Kit Protocol (QIAGEN) and ligated into the *Eco*RI site of pGEM-T Easy Vector (Promega). After transformation of *Escherichia coli* (TM 109-High Efficiency Competent Cells), white colonies were selected. Plasmid DNA was prepared and after restriction digest, clones containing the expected size insert were sequenced (Genome Express, France). From the sequence obtained, two sets of primers were defined and used to amplify DNA from different fungal species.

RESULTS

RAPD analysis

No banding pattern was observed in negative controls without DNA (data not shown). RAPD patterns were established for the 20 isolates of *S. solani* with the six primers listed in Table 2. These primers were chosen from the 16 tested because of the clear amplification pattern they produced. The profiles were reproductible from one experiment to another. The size of amplified DNA fragments generated with the 6 primers ranged from 500 to 2500 bases pairs.

Table 2 showed the total number of amplified products and the number of polymorphic fragments produced with each primer. Amplification with primer OPF-14 resulted in a single marker band (OPF14-1500 bp), which was specific for *S. solani* isolates from *S. melongena*. Of the 10 amplified products generated with primer OPF-20, one of approximately 480 bp was amplified strongly with *S. solani* isolates only from *S. melongena*. Figure 1 shows amplification products generated with primers OPF-14 (A) and OPF-20 (B).

All six primers generated two distinct amplitypes within *S. solani* isolates. These primers revealed polymorphism useful to classify the isolates. Using RAPD approach, we were able to identify two groups according to their plant host origin. The RAPD-480 bp polymorphic between the two groups was chosen for its specificity, its strong intensity and its size, which would facilitate its cloning.

Characterization of the 480 bp RAPD fragment

The RAPD fragment amplified with OPF-20 primer from only *S. melongena* isolates revealed a 502 bp sequence length. Using the BLAST search program (National Center for Biotechnology Information), there were no obvious similarities with any of the sequences available in the GenBank database.

Using this sequence information (Figure 2), two sets of specific primers were synthesized (F20F1-F20R1 and F20F2-F20R2) (Table 3). These primers were designed without any percent of the primer OPF-20 sequence (ten first nucleotides).

PCR identification of *Stemphylium solani* isolates

Amplification of entire genome of all *S. solani* isolates tested using these new primers resulted in a single PCR product for all whatever their plant host origin. The length of the single amplicon produced was of 400, 406, 485 and 492 bp, with the new primers in the following association: F20F1-F20R1, F20F2-F20R1, F20F1-F20R2 and F20F2-F20R2 (Table 4). The Figure 3 showed a DNA fragment of 400 bp yielded with one of these sets of primers for all *S. solani* DNA samples (F20F1-F20R1). To confirm the PCR identification of the isolates collected in our vegetables growing areas, one *S. solani* isolate from the two previous groups were associated with DNA samples from three other fungal species in a second amplification test using the same set of primers. As shown in Figure 4, a DNA fragment of expected size (400 bp) was amplified for *S. solani* isolates. However, DNA from *Verticillium dahliae*, *Fusarium oxysporum* and *Phyllosticta* sp. isolates did not amplify with F20F1-F20R1 primer pair.

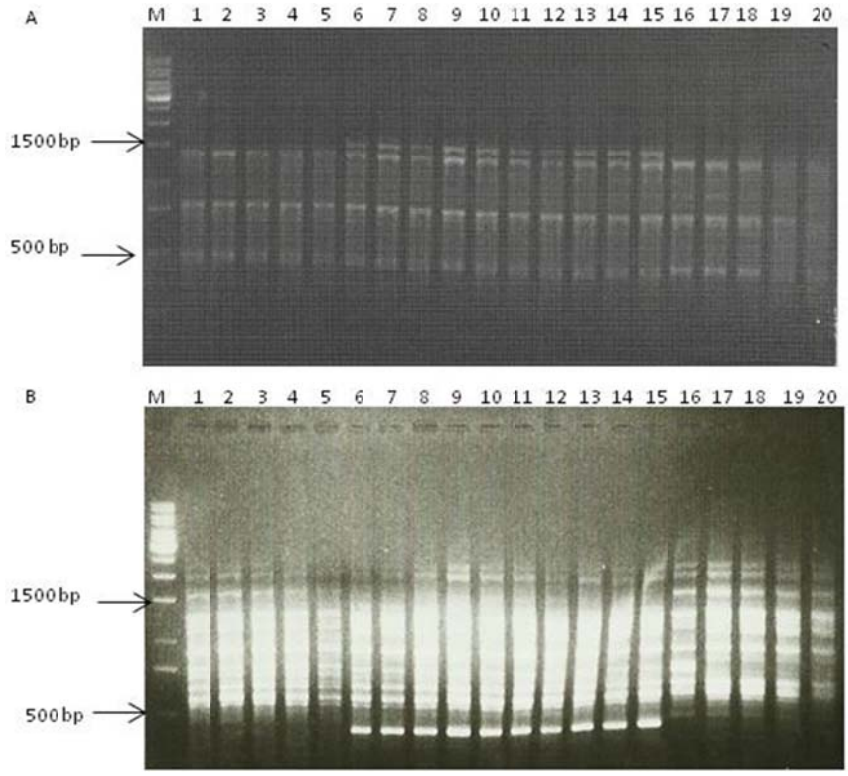

Figure 1. Random amplified polymorphic DNA (RAPD) patterns obtained for 20 isolates of *S. solani* with: A) primer OPF-14 and B) primer OPF-20. Lanes 1-5, 16-20 are isolates from *Solanum aethiopicum*, and lanes 6-15 are isolates from *Solanum melongena*. Lane M shows the molecular weight marker (1kb DNA ladder).

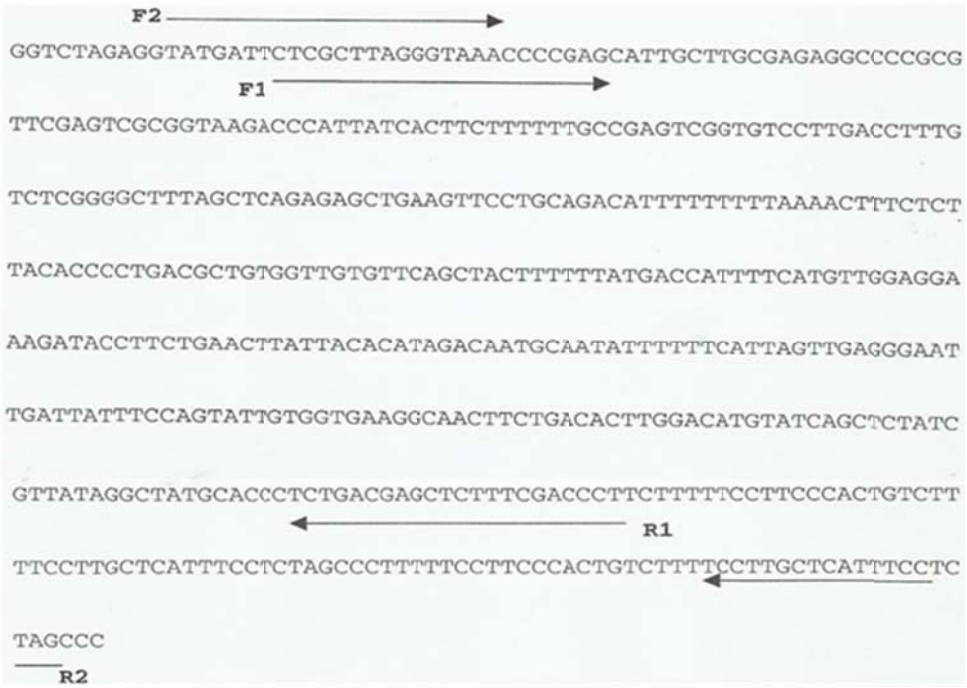

Figure 2. Nucleotide sequence of OPF20-RAPD fragment. The new primers for specific amplification used in this study are indicated by arrows.

Table 3. Nucleotide sequences of the four primers developed from the 502 bp RAPD fragment and their annealing Temperature (°C).

Primer	Sequence	Annealing temperature (°C)
F20F1	5' CTCGCTTAGGGTAAACCCCGAGC 3'	64
F20R1	5' GAAGGGTCGAAAGAGCTCGTCAG 3'	62
F20F2	5' TATGATTCTCGCTTAGGGTAAACC 3'	57
F20R2	5' GCTAGAGGAAATGAGCAAGG 3'	56

Table 4. Length of PCR fragments amplificated with the new primers.

Primer	Fragment length (bp)	
	F20F1	F20F2
F20R1	400	406
F20R2	485	492

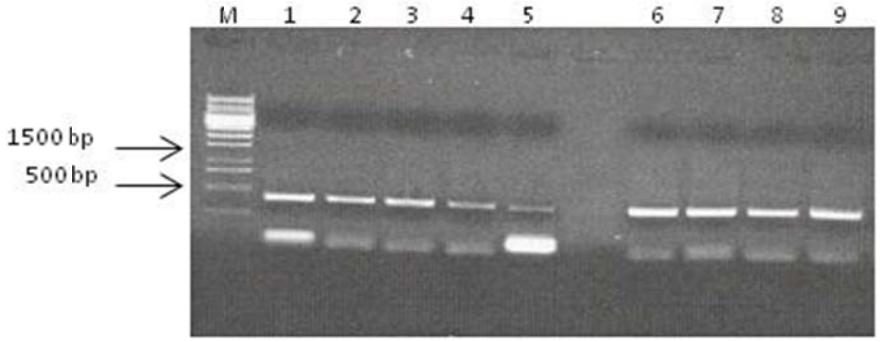

Figure 3. Specific PCR amplification of *S. solani* isolates with primers set F20F1-F20R1. Lane M: 1-kb marker; lanes 1, 2, 3, 4, 5 show amplification producted from *Solanum aethiopicum* isolates (S3, C2, C3, C31, C32), lanes 6, 7, 8, 9 isolates from *S. melongena* (C4, C5, C8, C13).

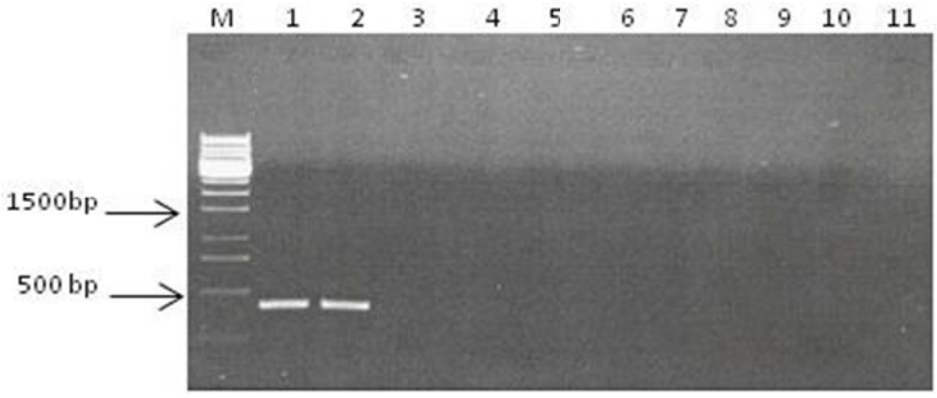

Figure 4. Specific PCR assay on selected fungal species using primers set F20F1-F20R1. Lane M: 1 kb marker; lane 1 isolate S3 (*S. solani* from *S. aethiopicum*), lane 2 isolate C4 (*S. solani* from *S.melongena*), lane 3-5 (V6, V14, V7) (*Verticillium dahliae*), lanes 6-7 (455R23, US1) (*Fusarium o*.f.sp. *vasinfectum*) lanes 8-9 (6F, PB32) (*Fusarium o*.f.sp. *elaeidis*) lane 10: T5 (*Phyllosticta* sp.), lane 11: negative control.

DISCUSSION

RAPD analyses used in this study appear to be extremely powerful and can separate individuals having intra specific variability. It gives more comprehensive information regarding the genetic variability among the fungal populations as it is based on the entire genome of an organism (Zimand et al., 1994; Achenbach et al., 1997; Mehta, 2001). In a previous study, RAPD data were also used to separate cotton and tomato *S. solani* isolates in Brazil (Mehta, 2001). Similar RAPD analyses have been successfully applied to characterize genomic variability in numerous fungal pathogens (Andebrhan and Furtek, 1994; Alfonso et al., 2000; Pollastro et al., 2000). In many phytopathogenic fungi, RAPD analyses have proved useful for detecting genomic polymorphisms directly related to host specialization (Hamelin et al., 1993; Assigbetse, 1994). Data presented here may form the basis for further studies using larger samples to assess host specialization among *S. solani* isolates.

As a result of this study, RAPD markers generated with *S. solani* isolates from *S. melongena* but never observed with *S. aethiopicum* isolates were identified. Such markers were exploited in an attempt to set up a new diagnostic technique based on PCR. Using the RAPD OPF-20 fragment sequence information, we synthesized two sets of specific oligonucleotide primers that could identify *S. solani* isolates from two senegalese eggplants among other fungal species. The PCR primers we designed with these data were successfully used to identify these *S. solani* isolates, however a step of fungal culture still remains necessary (Tooley et al., 1997; Le Cam et al., 2001; Pollastro et al., 2000; Zimand et al., 1994). In order to use these specific primers in the detection of this organism, further development is needed to detect the fungus from infected host plant as recommended by Le Cam et al., (2001). DNA-based techniques recently developed, enabled many uses in mycology and provided tools for both discrimination of closely related fungi and also identification of fungal species. The RAPD analyses have proved useful for distinguishing the isolates of *S. solani* used in this study. Additionally, the specific primers and PCR assay we have described may be used as a quick and reliable protocol for Senegalese *S. solani* isolates identification. Further studies need to be conducted to test these primers on other *Stemphylium* species and related fungi in order to assess the extent and limits of their specificity.

Conflict of interests

The authors did not declare any conflict of interest.

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