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Full Length Research Paper

Development of a sensitive nested-polymerase chain reaction (PCR) assay for the detection of *Ustilago scitaminea*

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A species-specific polymerase chain reaction (PCR) assay was developed for rapid and accurate detection of *Ustilago scitaminea*, the causal agent of sugarcane smut disease. Based on nucleotide differences in the internal transcribed spacer (ITS) sequences of *U. scitaminea*, a pair of species-specific primers, SL1 (5`-CAGTGCACGAAAGTACCTGTGG-3`) and SR2 (5`-CTAGGGCGGTGTTCAGAAGCAC-3`) was designed by using a panel of fungal and bacterial species as controls. The primers SL1/SR2 specifically amplified a unique PCR product about 530 bp in length from *U. scitaminea* strains with a detecting sensitivity at 200 fg of the fungal genomic DNA in a 25 µl reaction solution. To increase sensitivity, a nested-PCR protocol was further established, which used ITS4/ITS5 as the first-round primers followed by the primer pair SL1/SR2. This protocol increased the detection sensitivity by 10,000-fold compared to the PCR method and could detect the fungal DNA as low as 20 ag. The nested-PCR detected *U. scitaminea* from young sugarcane leaves with no visible smut disease symptoms. The findings from this study provide a sensitive and reliable technique for the early detection of *U. scitaminea,* which would be useful for sugarcane quarantine and production of germ-free seedcanes.

Key words: Sugarcane, *Ustilago scitaminea*, nested-polymerase chain reaction (PCR), molecular detection.

INTRODUCTION

Sugarcane (*Saccharum* hybrids spp.), a major industrial crop, is widely cultivated in tropical and subtropical regions for sugar production. Smut disease of sugarcane, caused by the fungus *Ustilago scitaminea* Syd., leads to considerable yield losses and reduction in cane quality (Ferreira and Comstock, 1989). Sugarcane smut was first reported from Natal in South Africa in 1877 (McMartin, 1945), and numerous outbreaks were noted in Africa and Asia in the following decades. Smut remained confined to the Eastern hemisphere until it was found in Argentina in 1940. In China, smut was reported in 1932 for the first time (Antoine, 1961; Presley, 1978), and it has caused serious problems in sugarcane production (Shen, 2004). Now, the disease occurs in all sugarcane-producing countries except Papua New Guinea (Presley, 1978; Singh et al., 2004).

Early and accurate diagnosis of plant diseases is a crucial component of any crop-management systems. Plant diseases can be managed most effectively if control measures are introduced at an early stage of disease development. Reliance on symptoms often is not adequate since the disease may be well established before symptoms appear, and symptom severity can be highly variable.

The polymerase chain reaction (PCR) techniques, including PCR and nested-PCR etc., have been widely used as rapid, specific and sensitive tools for the detection and evaluation of plant diseases (Kamel et al.,

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Abbreviations: PCR, Polymerase chain reaction; ITS, internal transcribed spacer.

Table 1. The fungal and bacterial strains used in this study.

Code	Species	Host	Geographic origin
U1	*U. scitaminea* (MAT-1)	Sugarcane (F134)	Guangzhou, China
U2	*U. scitaminea* (MAT-2)	Sugarcane (F134)	Guangzhou, China
U3	*U. scitaminea* (MAT-1)	Sugarcane (ROC16)	Zhanjiang, China
U4	*U. scitaminea* (MAT-2)	Sugarcane (ROC16)	Zhanjiang, China
U5	*U. scitaminea* (MAT-1)	Sugarcane (ROC22)	Wenyuan, China
U6	*U. scitaminea* (MAT-2)	Sugarcane (ROC22)	Wenyuan, China
S1	*Gibberella fujikuroi* (Sawada) wollenw	Sugarcane	Guangzhou, China
S2	*Colletotrichum fuleatum* Went.	Sugarcane	Guangzhou, China
S3	*Mycovellosiella koepkei* (Kruger) Deighton.	Sugarcane	Guangzhou, China
S4	*Bipolaris sacchari* (Butler.) Shoemaker	Sugarcane	Zhanjiang, China
S5	*Bipolaris stenospila* (Drechs.) Shoemaker	Sugarcane	Guangzhou, China
M1	*U. maydis* (MAT-1)	Maize	Guangzhou, China
M2	*U. maydis* (MAT-2)	Maize	Guangzhou, China
Lxx	*Leifsonia xyli* spp.*xyli*	Sugarcane	Zhanjiang, China
Xa	*Xanthomonas albilineans*	Sugarcane	Guangzhou, China

2003; Farid et al., 2006; Kawther, 2008; Li et al., 2009; Liu et al., 2009). For detection of *U. scitaminea,* so far only one PCR-based method was reported, which was based on the *b*E mating-type gene sequence of *Ustilago maydis* (Albert and Schenck, 1996). Comparing with the PCR-based methods, nested-PCR detection offers higher sensitivity and has been adapted in detection of plant diseases (Li et al., 2009; Liu et al., 2009).

The objective of this study was to develop a sensitive and accurate nested-PCR protocol for the detection of *U. scitaminea* to provide technical support for sugarcane quarantine, breeding of resistant varieties and production of disease-free sugarcane seedlings.

MATERIALS AND METHODS

Fungal and bacterial samples

A total of 15 microbial pathogens collected in China including 13 fungal strains and two bacterial strains were used in this study (Table 1).

Plant materials

The youngest fully expanded leaves above growth point were collected from sugarcane varieties ROC 22 and YT 97-639, which are susceptible to smut disease. The plants were grown in a sugarcane plantation, Guangdong, China.

DNA extraction

Monosporidial mating-type cultures of *U. scitaminea* and *U. maydis* were prepared following the method of Moosawi-Jorf and Mahin (2007). Sporidial DNA and bacterial DNA were extracted by cetyltrimethyl ammonium bromide (CTAB) method as described by Shen et al. (2006). Mycelial DNA of other fungal species was isolated as described by Brigon et al. (1993). Sugarcane genomic DNA was extracted from young leaves by the CTAB method as stated by Zhang et al. (2006).

Amplification of internal transcribed spacer (ITS) regions

The ITS region and the 5.8S rRNA gene were amplified from *U. scitaminea* strains using the universal fungal primers ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5 (5`-GGAAGTAAAAGTCGTAACAAGG-3`) (White et al., 1990). Each PCR reaction mixture contained 1 µl of genomic DNA (10 to 20 ng), 0.2 µl of rTaq DNA polymerase (5 U/µl, TaKaRa Biotechnology (Dalian) CO., LTD.), 2.5 µl of 10 × PCR reaction buffer (with Mg^{2+}), 2 µl of 2.5 mM dNTP mixture, 1 µl of 5 µM primers ITS4/ITS5, and ddH_2O 17.3 µl in a total volume of 25 µl. The PCR thermal cycling reaction was started by denaturationat 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 56°C for 40 s, and 72°C for 1 min, and then a final extension at 72°C for 10 min using a BIO-RAD DNAEngine Peltier Thermal Cycler. Products were cooled and kept at 4°C for further analysis.

Sequencing of ITS regions

All the PCR products were purified by using TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 and cloned into the plasmid vector PMD18-T, and then transformed into competent cells of *Escherichia coli*. Positive colonies were selected using the white-blue-colony method (Sambrook et al., 1989). The colonies were then cultured in liquid Luria Broth (LB) culture medium. The PCR products were sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.

Sequence analysis and primer design

The ITS sequences of *U. Scitaminea* were aligned and analyzed using DNAStar (5.01) Megalign program. The specific primer pair SL1 and SR2 was then designed based on conserved regions.

Specificity of PCR amplification

The specificity of the primer pair SL1 and SR2 in detection of *U.*

scitaminea was tested using a range of fungal and bacterial species shown in Table 1 as controls in PCR reactions.

Nested–PCR amplification

The nested-PCR amplification was performed in two rounds. The first round PCR amplification used primers ITS4 and ITS5 with the same reaction conditions as those for amplification of ribosomal DNA (rDNA) ITS regions. Then, 1 µl PCR products were used as templates for the second round of PCR amplification with the primers SL1 and SR2 using the same reaction conditions as the first round.

Determination of the sensitivity of the PCR and nested-PCR protocols

By preparing 10-fold serial dilutions, the genomic DNA of *U. scitaminea* strain U1 were diluted with ddH_2O to 200 ng, 20 ng, 2 ng, 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, 2 fg, 200 ag, 20 ag and 2 ag per microliter, respectively. These DNA samples were then used as templates for PCR and nested-PCR amplification using the same conditions previously described. An aliquot (10 µl) of each PCR products was analyzed by electrophoresis and visualized by ethidium bromide staining.

Detection of the fungal pathogen from sugarcane plants

Sugarcane smut is one of the easily recognizable sugarcane diseases because of its obvious whip-like sorus symptoms. In addition to the characteristic whip-like sorus, which appears at the late stage of disease development, early symptoms include stiff and erected leaves and the lengthened upper internodes (Comstock et al., 1983). Eight young sugarcane leaves showing early symptoms of smut disease were collected and total DNA samples were prepared for PCR and nested-PCR amplifications.

To test the feasibility of early detection from the *U. scitaminea* contaminated farm land, 13 sugarcane plants with no visible smut disease symptoms were randomly selected and labeled. A young leaf was collected from each selected plant for the total genomic DNA extraction and detection by PCR and the nested-PCR.

RESULTS

ITS sequence alignment and primers design

ITS fragments were amplified from the genomic DNA of six different *U. scitaminea* strains with the fungal universal primers ITS4/ITS5. The PCR products from various strains were cloned separately and sequenced. Sequence alignment revealed that the ITS sequences of the samples were identical and shared about 99% homology with the published ITS sequence of *U. scitaminea* (GenBank accession Nos.:EF185080, EF185076, EU427308, AY345007, FM179316, AF135433, DQ004830 and AM999337). To design species specific primers, we compared the ITS sequence of *U. scitaminea* with those of related fungal pathogens, including *Sporisorium erythraeense* (Syd. and P. Syd.) Vánky. *Sporisorium holwayi* (G.P. Clinton and Zundel) Vánky, *U. maydis* (DC.) Corda, *Sporisorium occidentale* (Seym. ex G. P. Clinton) Vánky and Snets, *Sporisorium scitamineum* (Syd.) M. Piepenbr., M. toll and Oberw..(synonym of *U. scitaminea*), *Sporisorium spinulosum* S.H. He and L. Guo, and *Ustilago trichophora* (Link) Körn.

These ITS sequences shared over 83% of homology containing both highly conserved and variable regions. By targeting the variable regions, a pair of primers was designed and named as SL1 (5`-CAGTGCACGAAAGTACCTGTGG-3`) and SR2 (5`-CTAGGGCGGTGTTCAGAAGCAC-3`) (Figure 1).

Specificity of the designed primers

A total of 13 fungal and two bacterial DNA samples were subjected to PCR amplification with primers SL1 and SR2, and the results show that the primers were highly specific for *U. scitaminea*. A 530 bp unique band was obtained from each of the six *U. scitaminea* strains collected from various areas in China (Figure 2A), but not from other fungal and bacterial species (Figure 2A). As a parallel control, the same fungal DNA samples were amplified using the universal primers ITS4 and ITS5, and a distinct PCR band were detected from all the fungal genomic DNA samples (Figure 2B).

Sensitivity of the PCR detection and the nested-PCR detection

The results show that the minimum amount of the fungal DNA could be detected by the PCR using the primer combination SL1 and SR2 of about 200 fg (Figure 3), while nested-PCR could detect as low as 20 ag of the fungal genomic DNA (Figure 4), which indicates at least 10,000-fold higher sensitivity than the PCR method.

Detection of the pathogen from the symptomatic sugarcane plants

Using PCR, the fungal DNA molecules were detected from seven out of the eight leaves (Figure 5A). In contrast, the nested-PCR could amplify the PCR band from all the eight leaves showing early stage symptoms of smut disease (Figure 5B).

As a control, a healthy young leave was also collected from the sugarcane growing in the greenhouse without *U. scitaminea* contamination. As expected, negative results were shown in both PCR and nested-PCR amplification (Figures 5A and B).

Detection of the pathogen from the asymptomatic sugarcane plants

The PCR results showed that only one sample was positive with a positive rate at 7.7% (1/13) (Figure 6A). In

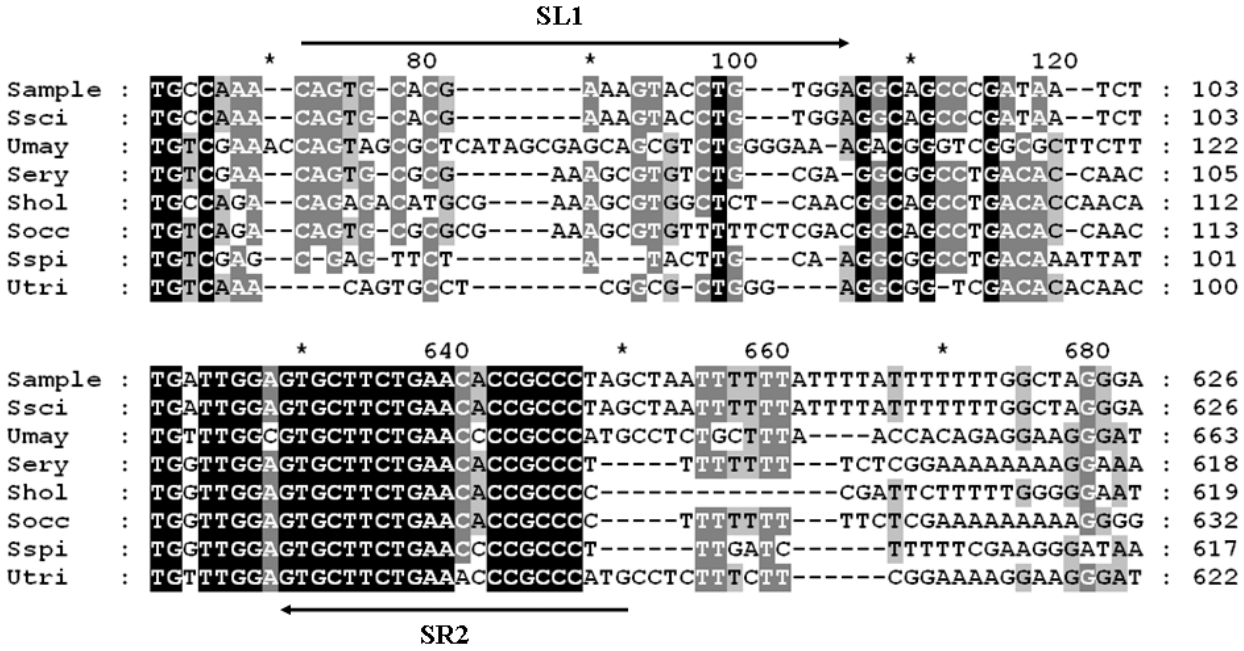

Figure 1. Partial alignment of the ITS sequences of *U. scitaminea* (Sample) and other fungal pathogens. Sery, *S. erythraeense* (NCBI Accession No., AY740049); Umay, *U. maydis* (NCBI Accession No., FJ167353); Shol, *S. holwayi* (NCBI Accession No., AY344980); Socc, *S. occidentale* (NCBI Accession No., AY344985); Ssci, *S. scitamineum* (NCBI Accession No., AY345007); Sspi, *S. spinulosum* (NCBI Accession No., GU139172); Utri, *U. trichophora* (NCBI Accession No., AY345009). The identical and highly conserved residues were highlighted in black and grey, respectively. The arrows indicate the locations of the primers SL1 and SR2 designed in this study.

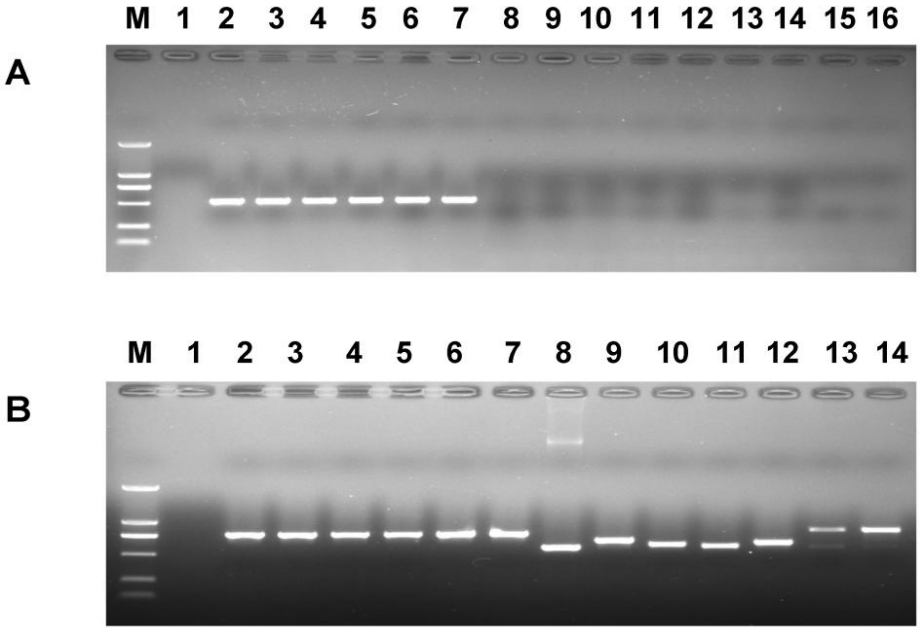

Figure 2. A. Electrophoresis of PCR-amplified products with specific primers SL1/SR2. Lane M, DNA marker DL2000; lane 1, ddH_2O; lanes 2 to 16, U1, U2, U3, U4, U5, U6, S1, S2, S3, S4, S5, M1, M2, Lxx and Xa, respectively (strain codes; see Table 1). **B.** Electrophoresis of PCR-amplified products with the fungal universal primers ITS4 and ITS5. Lane M, DNA marker; lane 1, ddH_2O; lanes 2 to 14, U1, U2, U3, U4, U5, U6, S1, S2, S3, S4, S5, M1 and M2, respectively (strain codes; Table 1). The DNA marker used in the experiments was DL2000 from Guangzhou Geneshun Biotech Ltd. The bands from upper to bottom are 2000, 1000, 750, 500, 250 and 100 bp, respectively.

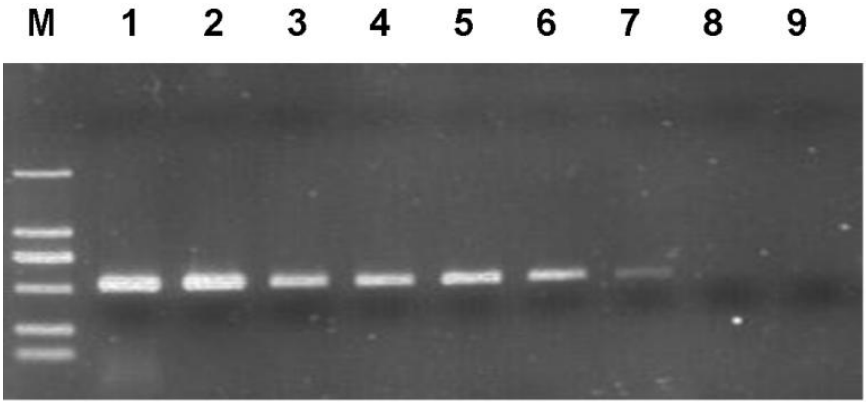

Figure 3. Sensitivity of PCR for the detection of *U. scitaminea* with primers SL1/SR2 using different quantities of genomic DNA. Lane M, DNA marker DL2000; lanes 1 to 8, PCR products using DNA template at quantity of 200 ng, 20 ng, 2 ng, 200 pg, 20 pg, 2 pg, 200 fg and 20 fg in a 25 µl PCR reaction system, respectively; lane 9, ddH_2O.

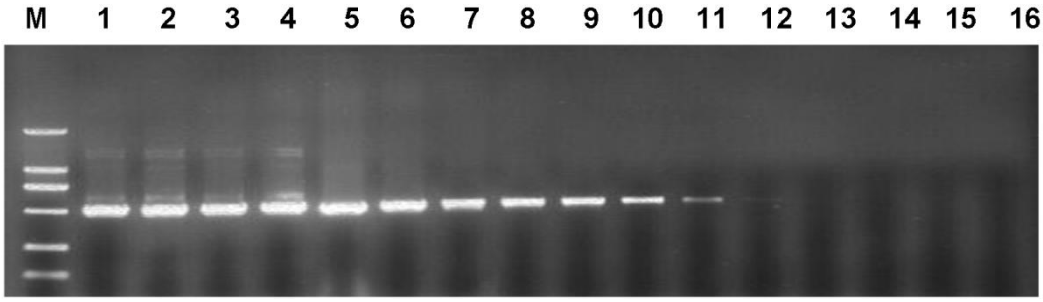

Figure 4. Sensitivity of nested-PCR for the detection of *U. scitaminea*. Lane M, DNA marker DL2000; lanes 1 to 12, nested-PCR products with DNA template at quantity of 200 ng, 20 ng, 2 ng, 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, 2 fg, 200 ag, 20 ag and 2 ag in a 25 µl PCR reaction system, respectively; lane 13, ddH_2O; lanes 14 to 16, negative control using the genomic DNA of healthy seedcane as template.

contrast, the nested-PCR assay revealed four positive samples, and the positive rate was 30.8% (4/13) (Figure 6B). Subsequent observation confirmed that smut disease symptoms appeared in the four positive sugarcane plants (data not shown). The results are consistent with the findings from the sensitivity analyses on PCR and nested-PCR.

DISCUSSION

The PCR is one of the most powerful molecular techniques for pathogen detection and disease diagnosis. Ward (1995) suggested that PCR amplification can be a more specific test than DNA hybridization, since PCR primers are dependent on a high degree of similarity in two separate regions of 15 to 25 bp. Thus, the accuracy and efficiency of PCR assay mainly depend on the characteristics of primers. For species-specific detection, PCR reactions targeting the rDNA genes have been commonly used as these genes possess the characteristics suitable for the detection of pathogens at the species level (Wu et al., 2005; Liu et al., 2009). These rDNA molecules are highly stable and exhibit a mosaic of conserved and variable regions within the genome (Hibbett, 1992). Based on a similar thinking, we designed the primers SL1 and SR2 by targeting the variable regions of the conserved ITS sequence for detection of sugarcane smut disease pathogen *U. scitaminea*. The specificity of the primer pair SL1/SR2 was demonstrated by using the genomic DNA samples from 13 fungal and two bacterial species, and the results show that the positive PCR band could only be detected from *U. scitaminea* isolates but not from other microorganisms.

Comparing with PCR, nested-PCR can greatly improve the detection sensitivity. Our nested-PCR method showed a high sensitivity with the detection limit at 20 ag of *U. scitaminea* genomic DNA, which is at least 10,000-fold more sensitive than PCR method used in this study. Our results are similar to a previous study on detection of

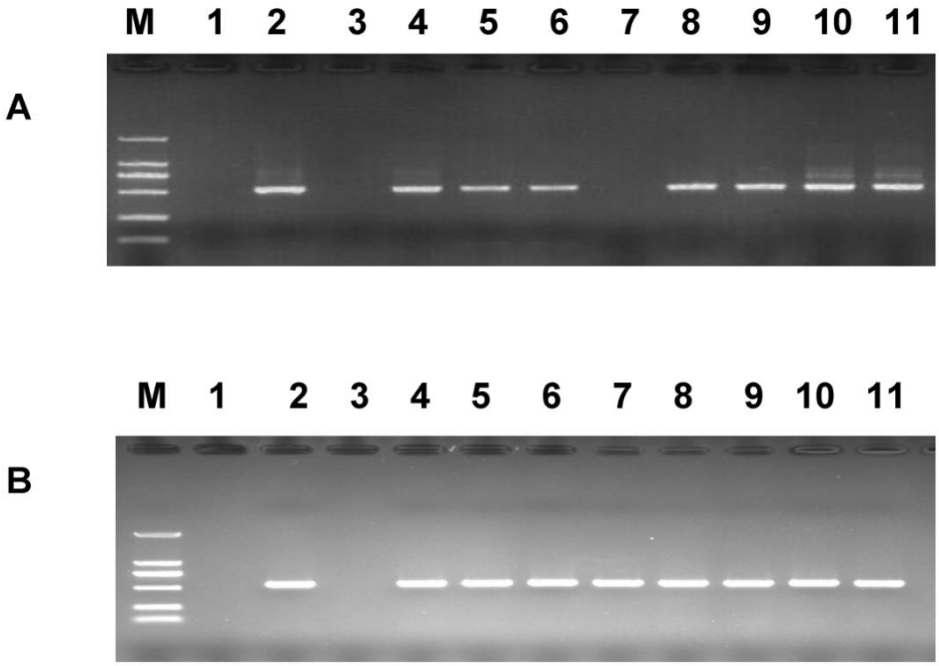

Figure 5. Detection of the pathogen from sugarcane young leaves in plants with visible early stage symptoms of smut disease by PCR (A) and nested-PCR (B). M, DNA marker DL2000; lane 1, ddH_2O; lane 2, positive control (*U. scitaminea* genomic DNA); lane 3, negative control (genomic DNA of healthy seedcane); lanes 4 to 11, sugarcane young leaves from the plants with visible early stage symptoms.

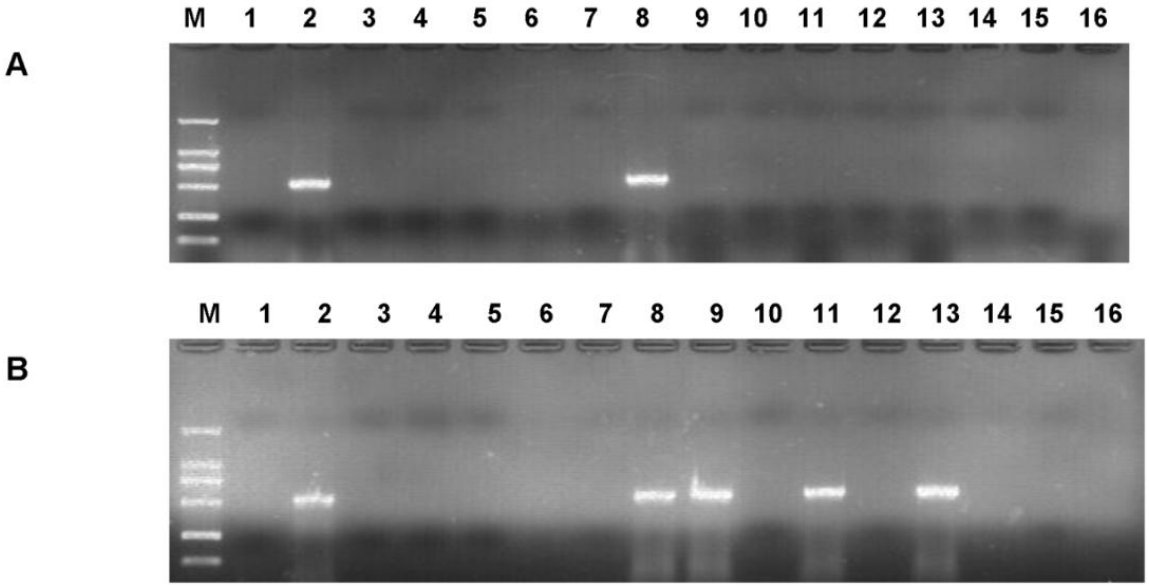

Figure 6. Detection of *U. scitaminea* from sugarcane young leaves with no visible smut disease symptoms. **A.** Detection by PCR. **B.** detection by the nested-PCR; M, DNA marker; lane 1, ddH_2O; lane 2, positive control (*U. scitaminea* genomic DNA); lane 3, negative control (genomic DNA of healthy seedcane); lanes 4 to 16; sugarcane young leaves from the plants with no visible smut disease symptoms.

a root rot pathogen of *Camellia oleiferra,* which showed that the nested-PCR increased the detection sensitivity by about 10,000-fold, comparing with PCR amplification (Li et al., 2009), except for that their nested-PCR

detection limit was at 100 ag genomic DNA. Significantly, in contrast to the previously reported PCR method for detection of sugarcane smut pathogen, which showed a detection limit at 50 pg of the fungal DNA, the minimum detection limit of the PCR and the nested-PCR developed in this study were at 200 fg and 20 ag of *U. scitaminea* genomic DNA, respectively, representing about 250- and 2,500,000-fold increase in detection sensitivity, respectively. The usability of the nested-PCR protocol was verified by demonstration of its capacity to detect the fungal DNA from the *U. scitaminea*-contaminated sugarcane plants with unapparent smut disease symptoms.

Conclusion

This study has developed a nested-PCR method for the detection of *U. scitaminea* with higher sensitivity and specificity. The method would be a useful tool for various applications, such as sugarcane quarantine, production of germ-free sugarcane seedlings, early disease diagnostics and evaluation of smut disease resistance in breeding programs.

REFERENCES

Albert HH, Schenck S (1996). PCR amplification from a homolog of the *b*E mating-type gene as a sensitive assay for the presence of *Ustilago scitaminea* DNA. Plant Dis., 10: 1189-1192.

Antoine R (1961). Smut. In: Sugarcane Disease of the World, eds. Martin JP, Abbott EV, Hughes CG. Elsevier Publishing Company, Amsterdam, Neatherland, pp. 327-354.

Brigon P, Gigot C, Chaubet N (1993). *In vivo* footprinting by LMPCR procedure. In: Plant Molecular Biology Manual, eds. Gelvin SB, Schilperoort RA , Verma DPS. Kluwer Academic Publishers, Belgium, B18: 1-34.

Comstock JC, Ferreira SA, Tew TL (1983). Hawaii`s approach to control sugarcane smut. Plant Dis., 67(4): 453-457.

Farid B, Maria JF, Aziz M (2006). A rapid PCR based method to distinguish between *Enterococcus* species by using degenerate and species-specific *sod*A gene primers. Afr. J. Biotechnol., 5(9): 697-702.

Ferreira SA, Comstock JC (1989). Smut. In: Disease of Sugarcane, eds. Ricaud C, Egan BT, Gillaspie Jr AG, Hughes CG. Elsevier Press, Amsterdam, Neatherland, pp. 211-229.

Hibbett DS (1992). Ribosomal RNA and fungal systematics. Trans. Mycol. Soc., 33: 533-556.

Kamel AA, Ibrahim NA, Mohmed AA, Mohmed SK, Joseph AV (2003). PCR identification of *Fusarium* genus based on nuclear ribosomal DNA sequence data. Afr. J. Biotechnol., 2(4): 82-85.

Kawther S (2008). Genotyping of bovine viral diarrhea virus using multiples PCR with and without RNA extraction. Afr. J. Microbiol. Res., 2: 316-318.

Li H, Song GT, He MJ, Hao Y, Zhou GY (2009). Detection of *Camellia oleifera* root rot pathogen with nested-PCR. J. Zhejiang For. Coll., 26(6): 849-853.

Liu JN, Li H, Zhou GY (2009). Specific and rapid detection of *Camellia oleifera* anthracnose pathogen by nested-PCR. Afr. J. Biotechol., 8(6): 1056-1061.

McMartin A (1945). Sugarcane smut: Reappearance in Natal. Southern Afr. Sugar J., 29: 55-57.

Moosawi-Jorf SA, Mahin BI (2007). *In vitro* detection of yeast-like and mycelial colonies of *Ustilago scitaminea* in tissue-cultured plantlets of sugarcane using polymerase chain reaction. J. Appl. Sci., 7(23): 3768-3773.

Presley J (1978). The culmicolous smut of sugarcane. Sugar y Azucar, 73(10): 34-39.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual, 2nd edn Cold Spring Harbor Laboratory, Cold Spring Harbor Press, New York, USA.

Shen WK (2004). Sugarcane diseases in Guangdong sugarcane regions in recent years and integrated controlling methods. Sugarcane and Canesugar, 1: 1-5.

Shen WK, Zhou GH, Deng HH, Zhou LY (2006). Detection of sugarcane ratoon stunting disease pathogen with PCR and nucleotide sequence analysis. Chin. Agric. Sci. Bull., 22(12): 413-416.

Singh N, Somai BM, Pillay D (2004). Smut disease assessment by PCR and microscopy in inoculated tissue cultured sugarcane cultivars. Plant Sci., 167: 987-994.

Ward BB (1995). Diversity of culturable denitrifying bacterial. Arch. Microbiol., 163(3): 167-175.

White TJ, Bruns T, Lee S, Taylor JW (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications, eds. Innis MA, Gelfand DH, Sninsky JJ, White TJ. Academic Press, Inc., New York, pp. 315-322.

Wu Q, Chen ZN, Fan HZ, Jin XZ (2005). Identification of corn bacterial wilt pathogen by nested-PCR based on 16S rDNA. Acta Phytopathol. Sin., 35(5): 420-427.

Zhang HY, Liu XZ, Li TS, Yang YM (2006). Genetic diversity among flue-cured tobacco (*Nicotiana tabacum* L.) revealed by amplified fragment length polymorphism. Bot. Stud., 47: 223-229.