Scientific Note

Rapid detection of *Phaeocytostroma sacchari* in sugarcane using conventional polymerase chain reaction[1]

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ABSTRACT

Diseases caused by fungi associated with adverse weather conditions are one of the main causes of decreases in sugarcane production. This study aimed to develop a protocol for a fast identification of *Phaeocytostroma sacchari*, which is the causal agent of bark rot in sugarcane. The reference sequences of three DNA regions of *P. sacchari*, namely internal transcribed spacer, ribosomal large subunit and translation elongation factor 1-alpha (TEF1-α), were analyzed with specific primers design. The specific primers generated that aligned in their entirety with *P. sacchari* were selected and synthesized. Polymerase chain reaction (PCR) assays were performed to confirm the primer specificity, using *P. sacchari* isolates and 10 species of other genera. Two sets of primers that amplify the TEF-1α region (PsF1/Psf1 and PsF2/PsR2) showed a high specificity and sensitivity in detecting *P. sacchari* using conventional PCR, what will allow large-scale surveys of this pathogen in sugarcane crops.

KEYWORDS: *Saccharum officinarum* L., sugarcane bark rot, translation elongation factor 1-alpha.

RESUMO

Detecção rápida de *Phaeocytostroma sacchari* em cana-de-açúcar por reação em cadeia de polimerase convencional

Doenças causadas por fungos associadas a condições climáticas adversas são uma das principais causas da queda na produção de cana-de-açúcar. Objetivou-se desenvolver um protocolo para identificação rápida de *Phaeocytostroma sacchari*, agente causal da podridão da casca de cana-de-açúcar. Sequências de referência de três regiões de DNA de *P. sacchari*, sendo região espaçadora transcrita interna, grande subunidade ribossômica e fator de elongação da tradução 1-alfa (TEF1-α), foram analisadas por meio de desenhos de *primers* específicos. Os *primers* gerados que se alinharam em sua totalidade com *P. sacchari* foram selecionados e sintetizados. Ensaios baseados na reação em cadeia de polimerase (PCR) foram realizados para verificar a especificidade do *primer*, utilizando-se isolados de *P. sacchari* e 10 espécies de outros gêneros. Dois conjuntos de *primers* que amplificam a região TEF-1α (PsF1/Psf1 e PsF2/PsR2) demonstraram alta especificidade e sensibilidade na detecção de *P. sacchari* com PCR convencional, o que permitirá o levantamento em larga escala do patógeno na cultura de cana-de-açúcar.

PALAVRAS-CHAVE: *Saccharum officinarum* L., podridão da casca da cana-de-açúcar, fator de elongação da tradução 1-alfa.

The sugarcane (*Saccharum officinarum* L.) global production has increased rapidly in the last decade due to the growing demand for sugar, and Brazil has been the largest producer worldwide (Bordonal et al. 2018).

Several factors may affect the production of sugarcane, such as insect pests and plant pathogens that interfere with its development. Among the pathogens, fungi are notable due to the recent great increases in the incidence of these microorganisms in sugarcane crops (Nechet et al. 2016).

Phaeocytostroma sacchari causes bark rot in sugarcane crops, and more than 50 countries have reported its presence, including Brazil (Abbott et al. 1964, Viswanathan et al. 2003, Carabez et al. 2014, Melo et al. 2023). In general, the symptoms are associated with unfavorable growth conditions, such as drought and excess of ripening, and the fungi then takes advantage of plants under stress and colonize them (Abbott et al. 1964). *P. sacchari* belongs to the Ascomycota phylum and the Diaporthales order (Sutton 1964). It shows a rapid mycelial growth and forms pycnidia that internally house conidiogenic cells (Muthumary 2019).

Rot symptoms are characterized by bark discoloration, varying from green to yellow. In addition, a reddish color in the nodal region may extend to leaves (Viswanathan et al. 2003). Different

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varieties of sugarcane have shown up to 100 % of susceptibility to this pathogen, evidencing that, under favorable environmental conditions, it has the potential to be highly aggressive, causing significant losses in the final yield (Viswanathan et al. 2003).

A major obstacle to the diagnosis of this fungus, considering host symptoms, is the similarity with other diseases. To overcome this issue, it is necessary to use reliable techniques, such as molecular detection. The identification of fungal pathogens using polymerase chain reaction (PCR) and specific primers has been widely used (Ni et al. 2012). It is an accurate, specific and cost-effective method easily adaptable for plant disease detection (Kang et al. 2021). Therefore, this study aimed to develop a protocol for identifying *P. sacchari* using conventional PCR.

The design of specific primers considered reference sequences of three regions of *P. sacchari*, namely internal transcribed spacer (ITS), ribosomal large subunit (LSU) and translation elongation factor 1-alpha (TEF1-α) (FR748047, FR748105 and FR748079, respectively) (Lamprecht et al. 2011).

The primers were obtained from the GenBank (Benson et al. 2012) of the National Center for Biotechnology Information - NCBI (Bethesda, MD, USA) and the DNA sequences of each region were processed for the generation of specific primers using automated software packages, including the Primer3 Plus (Hung & Weng 2016) and Primer-BLAST (Ye et al. 2012). Conserved and variable regions were searched and selected to design high-specificity primers for the detection of *P. sacchari*.

All generated primers were subjected to BLAST analysis and compared to the NCBI database (Kumar & Chordia 2015). The BLAST search was carried out in order to comparing *P. sacchari* with the nucleotide sequences of other fungi available in the database. Only specific primers that aligned uniquely with the *P. sacchari* sequence in its entirety (100 %) were considered. For each pair of primers, an analysis of ideal parameters was carried out, namely primer length, melting temperature, guanine and cytosine percentage and annealing temperature, among others. The possibility of formation of secondary structures was also evaluated (staples, autodimer and cross-dimer) (Kumar & Chordia 2015). The best set of primers for detecting the species was selected and synthesized for subsequent biological assays, in order to test their effectiveness using conventional PCR.

Genomic DNA was extracted from mycelium fragments cultivated in potato-dextrose-agar medium for 7 days, according to a protocol adapted from Dellaporta (1983), to test primer efficiency. After the DNA extraction, a temperature gradient was created in a thermocycler to identify the ideal annealing temperature, using a PCR reaction volume containing 1.3 µL of sterile water, 2.5 µL of 10X buffer, forward and reverse primers, concentration of 2.5 pmol L^{-1}, in an amount of 0.1 µL each, and 1.0 µL of DNA product at the concentration of 50 ng μL^{-1}. Electrophoresis in 1.2 % (w/v) agarose gel was performed using 1X TBE buffer and GelRed™ staining, and photographed after a period under ultraviolet light. The analysis was based on amplification, and only the bands consistently reproducible in agarose gel were considered for subsequent tests and identification of ideal annealing temperature.

The primer efficiency was verified through PCR reaction composed of 1X PCR Master Mix 2X produced by Ludwig Biotechnology®, forward and reverse primers, and DNA from *P. sacchari* isolates from the fungal collection of the Núcleo de Pesquisa em Fitopatologia (Universidade Federal de Goiás, Goiânia, Goiás state, Brazil). The thermal cycler used was the Mastercycle Nexus (Eppendorf, Germany).

The parameters were stipulated based on primer designs (Table 1). The gradient identified the ideal annealing temperature. In this test, negative controls containing sterile water were included to replace the DNA volume.

To test the primer specificity, PCR reactions were performed on *P. sacchari* isolates and on ten other genera of phytopathogenic fungi, namely *Nigrospora sphaerica*, *Lasiodiplodia theobromae*, *Corynespora cassiicola*, *Pseudofusicoccum stromaticum*, *Pestalotiopsis microspore*, *Alternaria alternata*, *Diaphorte phaseolorum*, *Colletotrichum falcatum*, *Fusarium solani* and *Macrophomina phaseolina*.

After designing the primers for each gene region using the Primer3 Plus software (Hung & Weng 2016) and submitting to BLAST analysis (Ye et al. 2012), only the primers designed from the TEF-1α region aligned in its entirety (100 %) to *P. sacchari* DNA sequences (Figure 1). These data corroborate reports that the TEF-1α region, even if considered a conserved region, undergoes faster mutations in relation to other gene regions and leads to an increase in the phylogenetic signal, enabling differentiation among species (Irimia & Roy 2008).

Table 1. Primers based on the TEF1-α gene region for detecting *Phaeocytostroma sacchari* isolates.

Primer sequence (Forward/reverse)	Gene	Primer	Tm (ºC)	G + C (%)	Product size (bp)
CAGAGCAGCACGCATTTGTC	TEF1-α	PsF1	60.5	55	500
GGGGCGTCTGTAGTCAGTTT		PsR1	59.7	55	
TTCAATCCCAGAGCAGCACG	TEF1-α	PsF2	60.7	55	506
GGCGTCTGTAGTCAGTTTGTT		PsR2	58.5	47.6	

Tm: melting temperature; G + C: percentage of guanine and cytosine.

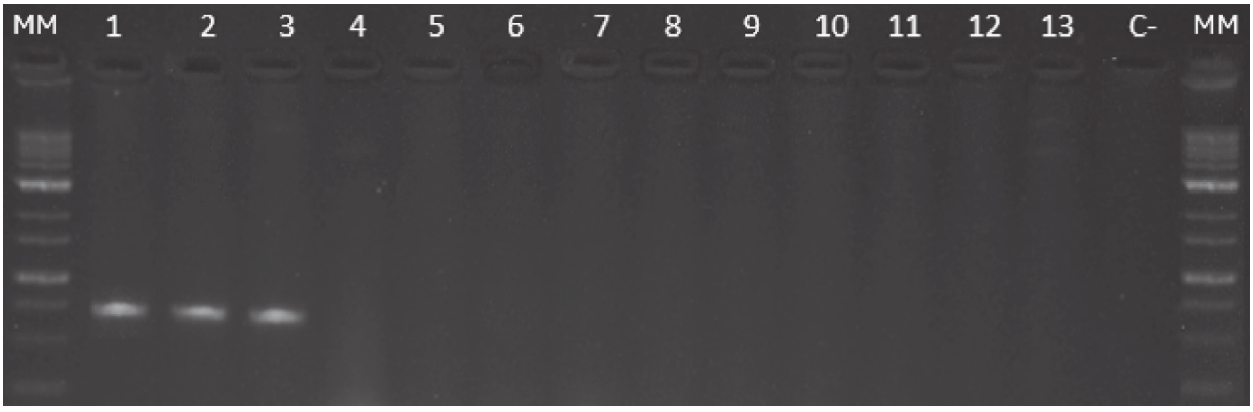

Figure 1. Agarose gel electrophoresis (1.2 %) of the primer specificity test. MM: molecular maker (1kb); 1: *Phaeocytostroma sacchari* - VPA1; 2: *Phaeocytostroma sacchari* - VPA3; 3: *Phaeocytostroma sacchari* - VPA4; 4: *Nigrospora sphaerica*; 5: *Lasiodiplodia theobromae*; 6: *Corynespora cassiicola*; 7: *Pseudofusicoccum stromaticum*; 8: *Pestalotiopsis microspora*; 9: *Alternaria alternata*; 10: *Diaphorte phaseolorum*; 11: *Colletotrichum falcatum*; 12: *Fusarium solani*; 13: *Macrophomina phaseolina*; C-: negative control with sterile water.

Santos et al. (2020) reported that the design of a specific primer from the TEF-1α region is effective for diagnosing different species of *Macrophomina*. After comparison with the database, five pairs of primers were designed based on the elongation factor of the gene region (TEF1-α/FR748079; NCBI) that generated different fragment sizes. After submitting to a gradient test to identify the temperature of ideal annealing, only two pairs of primers amplified the *P. sacchari* isolate (Table 1).

After performing the gradient test and following the manufacturer's recommendations of the Master Mix produced by Ludwig Biotechnology®, the efficient cycle for the PsF1/PsR1 primer consisted of three steps. The first step was initial denaturation at 94 ºC for 5 min; the second consisted of 35 cycles, with three stages per cycle, denaturation at 94 ºC for 30 s, annealing at 52 ºC for 30 s, and extension at 72 ºC for 30 s; and the third consisted of a final extension at 72 ºC for 10 min. For the PsF2/PsR2 primer, the efficient cycle consisted of initial denaturation at 94 ºC for 5 min, the second stage consisted of 35 cycles, with three stages per cycle, denaturation at 94 ºC for 30 s, annealing at 55 ºC for 30 s, and extension at 72 ºC for 30 s. The third stage consisted of a final extension at 72 ºC for 10 min.

The detection efficiency test revealed that the PsF1/PsR1 primer, designed based on the TEF1-α region, amplified different *P. sacchari* isolates by amplifying a band with approximately 500 bp. Also, the PsF2/PsR2 primer showed DNA amplification in different *P. sacchari* isolates.

The specificity test used ten fungus genera. The primer pairs PsF1/PsR1 and PsF2/PsR2 could detect *P. sacchari* exclusively. Both primers designed in this study did not amplify other phytopathogenic fungi, proving their specificity to *P. sacchari* (Table 2).

All tests were replicated four times to analyze the reproducibility of results and did not lead to different results. All results proved the efficiency of the specific primers PsF1/PsR1 and PsF2/PsR2 in detecting *P. sacchari* using conventional PCR. The TEF1-α region separates species. Although it is a conserved region, this genic region presents a greater phylogenetic signal, when compared to the other genomic regions. It provides a precise and consistent

Table 2. Amplification results using *Phaeocytostroma sacchari*-specific primers.

Isolates[a]	Amplification results[b]	
	PsF1/PSR1[c]	PsF2/PsR2[c]
P. sacchari (VPA1)	+	+
P. sacchari (VPA3)	+	+
P. sacchari (VPA4)	+	+
Nigrospora sphaerica	-	-
Lasiodiplodia theobromae	-	-
Corynespora cassiicola	-	-
Pseudofusicoccum stromaticum	-	-
Pestalotiopsis microspora	-	-
Alternaria alternata	-	-
Diaphorte phaseolorum	-	-
Colletotrichum falcatum	-	-
Fusarium solani	-	-
Macrophomina phaseolina	-	-

[a] Culture collection of phytopathogenic fungi from the Núcleo de Pesquisa em Fitopatologia (Universidade Federal de Goiás, Goiânia, Goiás state, Brazil); [b] presence (+) and absence (-) of evident band with expected size; [c] Specific primers for *P. sacchari* (PsF1/PsR1; PsF2/PsR2) from the region translation elongation, factor 1-alpha.

species identification, enabling distinguishing among species in a same genus (Irimia & Roy 2008).

Lazarotto et al. (2016) addressed the importance of using primers to amplify the TEF1-α region. Together with database comparison and phylogenetic analysis, it allowed identifying *Fusarium solani* associated with *Pinus* spp. The authors also addressed the importance of using PCR for specific identification of phytopathogens and development of methods to inhibit pathogenic microorganisms.

This gene region is useful not only for detecting phytopathogens, but also for establishing phylogenetic relationships (Laraba et al. 2022), monitoring and developing methods to inhibit the pathogenic microorganism (Lazarotto et al. 2016).

The *P. sacchari* specific primers designed here contribute to other studies by allowing evaluation of the geographic distribution of this disease. This is the first study in which specific primers were developed for detecting *P. sacchari* and reveals that both the PsF1/PsR1 and PsF2/PsR2 primer sets can be used for detection of the causal agent of sugarcane bark rot. This facilitates large-scale surveys aiming to monitor the distribution of the pathogen in sugarcane crops.

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