RAPID AND ACCURATE PCR-BASED AND BOILING DNA ISOLATION METHODOLOGY FOR SPECIFIC DETECTION OF *Sclerotium cepivorum* IN GARLIC (*Allium sativum*) CLOVES

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SUMMARY

Allium *white rot caused by the pathogenic fungus* Sclerotium cepivorum *is a devastating disease of garlic worldwide. In this study, we developed and evaluated a molecular assay for the detection of* S. cepivorum*in mycelia and infected garlic cloves, through PCR, using a specific primer set combined with a rapid boiling DNA extraction. The PCR products were amplified in all the samples infected with* S. cepivorum*, but not in other samples including phylogenetically closely related fungi. This methodology is specific and has a shorter execution time compared to standard methods.*

Introduction

Allium white rot is prevalent in many regions where garlic is cultivated worldwide and can reduce crop yields drastically (Davis *et al*., 2007). Garlic (*Allium sativum* L) is an economically important crop in Mexico. However, this crop is affected by the fungal disease known as *Allium* white rot, caused by *Sclerotium cepivorum* Berk (Couch and Kohn, 2000), which is considered the main fungus disease in several states of the country (Velásquez-Valle and Reveles-Hernández, 2016) In the State of Guanajuato the losses may reach up to 100% (Pérez-Moreno *et al*., 1998). In the absence of an *Allium* host, *S. cepivorum*persists in soil as sclerotia that can survive for more than 20 years and which can germinate in response to sulfides of *Allium* species, producing an infective mycelium (Coley-Smith *et al*., 1990). Many approaches have been used to control *Allium* white rot, including application of fungicides, soil fumigants and soil solarization, biological control agents (Miñambres *et al*., 2010) and composted onion waste (Coventry *et al*., 2002). However, the efficacy of these methods depends on the assurance that the pathogen is *S. cepivorum*and on the stage of development of the disease, which is not evident

KEYWORDS / *Allium* White Rot / Garlic / ITS Sequence / PCR Detection / *Sclerotium cepivorum* /

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UNA METODOLOGÍA RÁPIDA Y SEGURA, BASADA EN PCR Y AISLAMIENTO DE DNA POR HERVIDO, PARA LA DETECCIÓN ESPECÍFICA DE *Sclerotium cepivorum* EN DIENTES DE AJO (*Allium sativum*)

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RESUMEN

La pudrición blanca en Allium *causada por el hongo patógeno* Sclerotium cepivorum *es una enfermedad devastadora del ajo en todo el mundo. En este estudio desarrollamos y evaluamos un ensayo molecular para la detección de* S. cepivorum*en micelios y en dientes de ajo infectados, mediante PCR, usando un set de primers específicos, combinado con una rápida extracción de ADN basada en ebullición. Los productos de PCR fueron amplificados en todas las muestras infectadas con* S. cepivorum*, pero no en otras muestras, incluyendo hongos filogenéticamente cercanos. Esta metodología es específica y tiene un tiempo de ejecución más corto en comparación con los métodos estándar.*

UMA METODOLOGIA RÁPIDA E SEGURA, BASEADA EM PCR E ISOLAMENTO DE DNA POR FERVIDO PARA A DETECÇÃO ESPECÍFICA DE *Sclerotium cepivorum* EM DENTES DE ALHO (*Allium sativum*)

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RESUMO

A podridão branca do Allium *causada pelo fungo patogénico* Sclerotium cepivorum *é uma doença devastadora do alho em todo o mundo. Neste estudo, desenvolvemos e avaliamos um ensaio molecular para a detecção de* S. cepivorum*em micélios e em dentes de alho infectados, por PCR, utilizando um conjunto de primers específicos, combinado com uma rápida extração de DNA em ebulição. Os produtos de PCR foram amplificados em todas as amostras infectadas com* S. cepivorum*, mas não em outras amostras, incluindo fungos filogeneticamente próximos. Essa metodologia é específica e possui um tempo de execução menor em comparação aos métodos padrão.*

until the infection is well advanced (Ulacio-Osorio *et al*., 2006; Xu *et al*., 2010).

Key to the control is the ability to detect the pathogen in seed cloves. The standard method to detect the pathogen is to use infected tissue or soil to collect sclerotia (Bakonyi *et al*., 2011). However, isolation onto agar media and morphological identification is required to confirm the presence of *S. cepivorum,* since sclerotia of similar size can be produced by other species (Xu *et al*., 2010). This approach is time-consuming, as isolation requires several days of incubation, and accurate identification of the resulting colonies based on their morphology requires specialist expertise.

Polymerase chain reaction (PCR) analysis has been used to detect several plant fungal pathogens. Its main advantages are: i) time effective, ii) versatile, and iii) sensitive in identifying specific products. Standard protocols for the genetic detection of plant pathogens are based on NaOH-SDS, CTAB or commercial kits. However, alternative strategies such as boiling methods have never been developed and tested for the detection of *S. cepivorum*in garlic cloves.

In this study we developed and tested a PCR-based method to detect *S. cepivorum*in both mycelia and infected garlic cloves (*Allium sativum*) using the boiling DNA extraction method.

Materials and Methods

Experimental materials

The experimental work was carried out on *Sclerotium cepivorum* strain C2 isolated from Cortazar, Guanajuato, México; *S. cepivorum*K1 isolated from Zacatecas, Zacatecas, México; *Sclerotinia sclerotiorum*, *Fusarium oxysporum* and *Phytophthora capsici* isolates, and *Allium sativum* Tacátzcuaro cloves, provided by the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP-Bajío). *S. cepivorum*was maintained on agar plates with Czapeck media at 16ºC; *S. sclerotiorum* and *F. oxysporum* were maintained on agar plates with PDA medium at 25º; and *P. capsici* was maintained on agar plates with V8 media at 25ºC. Liquid media were used to obtain individual mycelia, which were harvested by centrifugation after 5 days and frozen at -80ºC.

Allium sativum *Tacátzcuaro infection*

Garlic cloves were germinated individually in 50ml glass flasks containing 20ml double-distilled water and incubated for 72h at 16ºC, then one square centimeter of 7 days old *S. cepivorum* mycelia grown in Czapeck media was added to infect germinated garlic cloves and incubated for 72h at 16ºC. Garlic cloves were harvested using sterile forks and frozen at -80ºC.

DNA isolation

Frozen mycelia and garlic cloves were used to extract genomic DNA by the boiling method (Holmes and Quigley, 1981); 2-4mg of mycelium or 80-120mg of non-infected garlic tissue were transferred into an Eppendorf tube containing 200mg of glass beads of 500µm in diameter and 150µl TE (Tris-Base 50mM, EDTA 2mM), vortexed for 2min, boiled for 5min and vortexed for 1min. To each tube 150µl of phenol: chloroform (5:1 v:v) was added, vortexed for 1min and centrifuged at 12000rpm for 5min. Each supernatant was transferred to a sterile Eppendorf tube, adding 100µl chloroform, vortexed for 1min and centrifuged at 12000rpm for 5min. DNA was re-suspended in 50ul H_2O. The Dellaporta method was used as a standard DNA extraction method (Dellaporta *et al*., 1983).

Primer design and PCR

In order to synthesize amplicons around of 200bp, two sets of primers, ITSKF/ITSKR and ITSLF/ITSLR, were de-

signed from the ITS sequence with the OligoPerfect Designer program (Invitrogen) with reference to the ITS sequences of *S. cepivorum* (Z99683.1, Z99682.1, Z99681.1 and JX442 200.1). The PCR reaction was performed using as template approximately 50ng or 100ng of DNA extracted by the boiling and Dellaporta methods, respectively. The PCR reaction was carried out as follows: 4min at 94ºC, 30 cycles of 1min at 94ºC, 30s at 58ºC or 68ºC, 30s at 72ºC and a final extension of 4min at 72ºC. The PCR products were separated on 1.2% agarose gel (Fermentas) stained with GelRed (Botium), using the GeneRuler 100pb DNA Ladder (Life Technologies) as size marker.

Cloning and sequence analysis

The PCR product was purified from the agarose gel and cloned into the pCR4-TOPO vector according to the manufacturer (Invitrogen). Chemically competent cells of *Escherichia coli* TOP10 (Invitrogen) were transformed and grown on LB agar plates containing kanamycin at a final concentration of 50µg·ml^{-1}. Transformed colonies were transferred into Falcon tubes (Corning) containing 3ml of LB broth with kanamycin and ampicillin added at a final concentration of 50µg·ml^{-1} for plasmid DNA extraction. Each plasmid DNA was digested with the enzyme *Eco*RI to verify the presence of the insert.

Plasmid DNA containing the insert was sent to LANGEBIO (National Laboratory of Genomics for Biodiversity) for sequencing. The sequences were compared to the database of the NCBI (National Center for Biotechnology Information).

Results

Design of new primers to produce amplicons of 200bp by PCR

In this study, two sets of nested primers ITSKF (19-mer)/ITSKR (20-mer) and ITSLF (22-mer)/ITSLR (23-mer) (Table I) were identified as highly specific and efficient primers. DNA isolated by the boiling method from Tacátzcuaro cloves inoculated with *Sclerotium cepivorum* strain C2 was used as template to amplify the *S. cepivorum*ITS region by PCR; the results are shown in Figure 1. The specific DNA fragments were approximately 200bp (Lanes 3 and 4). The ITS1SCF/ITS2SCR primers (Haq *et al.*, 2003) were used as positive control (Lane 2) and as negative control DNA was isolated by the boiling method from Tacátzcuaro cloves not inoculated with *S. cepivorum*strain C2 and new primers ITSLF/ITSLR (Lane 5); this result confirms that the designed new primers specifically allow the amplification of a ITS region of 200bp by PCR.

Identification and comparison of Sclerotim cepivorum *strain C2 ITS sequence*

The 200bp fragments obtained by PCR were cloned into the vector pCR4-TOPO®-TA, which was used to transform chemically competent *Escherichia coli* cells. Plasmid DNA was purified to obtain its sequence, which is shown in Figure 2. The sequence obtained was aligned with the NCBI database using the BLASTN algorithm (Altschul *et al.*, 1990). The specific DNA fragment shared 100% identity with *S. cepivorum* ITS sequences Z99683.1, Z99682.1, Z99681.1 and JX442200.1, confirming the identification of *S. cepivorum* (Table II).

Specificity of the new primer set for Sclerotium cepivorum *ITS amplification*

To demonstrate the specificity of the new designed primer ITSLF/ITSLR for *S. cepivorum* identification, PCR was performed using as template DNA isolated by the boiling method from several plant pathogens: *Sclerotinia sclerotiorum*, *Fusarium oxysporum* and *Phytophthora capsici*. The results are shown in Figure 3; no cross reaction was observed in PCR with any of the non-target species (Lanes 4, 5 and 6). PCR

TABLE I
NUCLEOTIDE SEQUENCES OF THE PRIMERS USED TO AMPLIFY THE INTERNAL TRANSCRIBED SPACER REGION (ITS) OF *Sclerotium cepivorum*

Primer name	Sequence (5' — 3')	Tm (ºC)	Reference
ITSKF	GCTGCCTTTGGGGCCTCGT	58	This study
ITSKR	AGGGGCGCAATGTGCGTTCA	58	This study
ITSLF	GGGGCCTCGTATGCTCGCCAGA	68	This study
ITSLR	CCCGGAATACCAAGGGGCGCAAT	68	This study
ITS1SCF	AGCTGCCTTTGGGGCCTC	54	Haq *et al.*, 2003
ITS2SCR	TTAGGTTTTGACAGAAGCACAT	54	Haq *et al.*, 2003

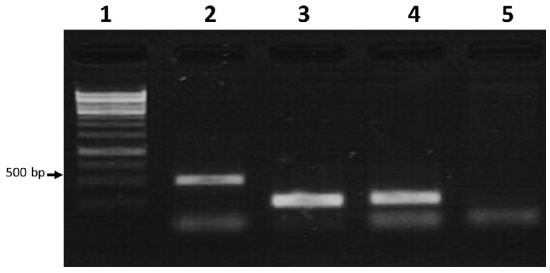

Figure 1. Internal transcribed spacer sequence (ITS) from *Sclerotium cepivorum* amplified by PCR using new primers and a boiling DNA method from infected garlic as template. Lanes 1: 1kb DNA Ladder, 2: ITS1SCF/ITS2SCR, 3: ITSKF/ITSKR, 4: ITSLF/ITSLR, and 5: ITSLF/ITSLR DNA from non-infected garlic as template.

GCTGCCTTTGGGGCCTCGTATGCTCGCCAGAGAATATCAAAACTCTTTTTATTAATGT

CGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA

TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA

TCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGG

Figure 2. Partial internal transcribed spacer sequence (ITS) of *Sclerotium cepivorum* strain C2, using new ITSKF/ITSKR (underline) and ITSLF/ITSLR (double underline) primers (accession number KR108327.1).

TABLE II
SEQUENCES PRODUCED SIGNIFICANT ALIGNMENTS IN GENBANK OF THE NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION

Description	Identity (%)	Accession number
Sclerotim cepivorum ribosomal RNAs and internal transcribed spacers	100	Z99683.1
Sclerotium cepivorum ribosomal RNAs and internal transcribed spacers	100	Z99682.1
Sclerotium cepivorum ribosomal RNAs and internal transcribed spacers	100	Z99681.1
Sclerotium cepivorum strain 12, internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene	100	JX442200.1

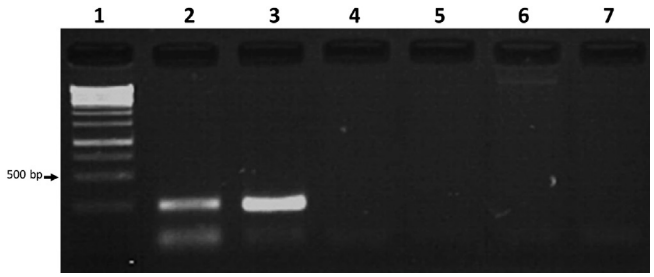

Figure 3. Internal transcribed spacer sequence (ITS) from *Sclerotium cepivorum* amplified by PCR using ITSLF/ITSLR nested primers. Lanes 1: 1kb DNA Ladder, 2: DNA from *Sclerotium cepivorum* strain C2, 3: DNA from *Sclerotium cepivorum* strain K1, 4: DNA from *Sclerotinia sclerotiorum*, 5: DNA from *Fusarium oxysporum*, 6: DNA from *Phythopthora capsica*, and 7: DNA from non-infected garlic.

only achieved the synthesis of approximately 200pb amplicons for *S. cepivorum*strain C2 and *S. cepivorum*strain K1 (Lanes 2 and 3), confirming the high specificity of the PCR assay to detect the fungus causing white rot disease.

Discussion

Several tools have been used to differentiate fungi, including microbiological and phenotipic analysis (Bakonyi *et al*., 2013; Divya and Chouhan, 2014) and PCR (Haq *et al*., 2003; Xu *et al*., 2010). Detection of fungal pathogens by PCR is rapid and sensitive compared to other techniques. In this study we developed and tested a PCR-based assay for specific identification of *Sclerotium cepivorum* in both mycelia and infected garlic cloves, using rapid DNA extraction by the boiling method and new specific primers ITSKF/ITSKR and ITSLF/ITSLR. The boiling method for DNA extraction has not been used previously to identify *S. cepivorum*in garlic cloves. The boiling method is performed in ~30min, whereas the standard Dellaporta method, highly recommended for DNA extraction from plant tissue, requires about 2 to 10h (if overnight incubation is included) to be completed.

The amplicons obtained were approximately 200bp (Figure 1), which were confirmed by sequencing (Figure 2) and correspond to *S. cepivorum*C2. No amplification was obtained of DNA extracted from an non-infected garlic clove by the boiling method and using ITSKF/ITSKR or ITSLF/ITS LR primers (Figures 1 and 3), confirming the specificity of these primers to detect the presence of *S. cepivorum*.

The PCR condition for new primers, for which the Tm are 58 and 68ºC, and annealing and synthesis times are 30sec, reduced significantly the total PCR-program time from 4h, used for primers ITS1SCF/ITS2SCR (Figure 1, Lane 2), to 2.5h using our primers. The PCR assay described here is highly specific for *S. cepivorum*(Figure 3, Lanes 2 and 3), because no PCR products were amplified for other plant pathogens such as *Fusarium oxysporum* and *Phytophthora capsici*, including a phylogenetically closely related fungus *Sclerotinia sclerotiorum* (Holst-Jensen *et al*., 1998; Xu *et al*., 2010).

Conclusion

The methodology proposal in this study has two main major advantages over standard methods; i) it is highly specific because no other closely related fungi were falsely detected, thus avoiding problems associated with misidentification of samples contaminated with other fungi and ii) the total time required to establish the diagnosis was significantly reduced to ~3h compared to other methods that range from 6 to 14h. However, further studies with a larger sample are necessary to assess the efficacy of this method. The shorter execution time and accuracy of this strategy are very critical, especially in pathogens with a high potential of dispersion.

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REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J. Mol. Biol. 215*: 403-410.

Bakonyi J, Vajna L, Szeredi A, Tímár E, Kovács GM, Csősz M, Varga A (2011) First report of *Sclerotium cepivorum* causing white rot of garlic in Hungary. *New dis. rep. 23*: 5.

Coley-Smith JR, Mitchell CM, Sansford CE (1990) Long-term survival of sclerotia of *Sclerotium cepivorum* and *Stromatinia gladioli*. *Plant Pathol. 39*: 58-69.

Couch BC, Kohn LM (2000) Clonal spread of *Sclerotium cepivorum* in onion production with evidence of past recombination events. *Phytopathology 90*: 514-521.

Coventry E, Noble R, Mead A, Whipps JM (2002) Control of Allium White Rot (*Sclerotium cepivorum*) with composted onion waste. *Soil Biol. Biochem. 34*: 1037-1045.

Davis RM, Hao JJ, Romberg MK, Nunz JJ, Smith RF (2007) Efficacy of germination stimulants of sclerotia of *Sclerotium cepivorum* for management of White Rot of garlic. *Plant Dis. 91*: 204-208.

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation version II. *Plant Mol. Biol. Rep. 1(4)*: 19-21.

Divya KS, Chouhan JB (2014) Study of fungal diversity with reference to physical and chemical parameters. *Int. J. Environ. Sci. 5*: 401-406.

Haq MA, Collin HA, Tomsett AB, Jones MG (2003) Detection of *Sclerotium cepivorum* within onion plants using PCR primers. *Physiol. Mol. Plant Pathol. 62*: 185-189.

Holmes D, Quigley M (1981) A rapid boiling method for the preparation of bacterial plasmids. *Analyt. Biochem. 114*: 193-197.

Holst-Jensen A, Vaage M, Schumacher T (1998) An approximation to the phylogeny of *Sclerotinia* and related genera. *Nord. J. Bot. 18*: 705-719.

Miñambres GG, Conles MY, Lucini EI, Verdelli RA, Meriles JM, Zygadlo JJ (2010) Application of thymol and iprodine to control garlic White Rot (*Sclerotium cepivorum*) and its effect on soil microbial communities. *World J. Microbiol. Biotechnol. 26*: 161-170.

Pérez-Moreno L, Olalde-Portugal V, Salinas-González JG, Sánchez-Pale JR, Entwistle AR (1998) Current known distribution of white rot *Sclerotium cepivorum* Berk. in Mexico. In: Entwistle, A.R., L. Pérez-Moreno (eds.) *Proceedings of the 6th International Workshop on Allium white rot*. Irapuato, Guanajuato, Mexico. pp. 6-9.

Ulacio-Osorio D, Zavaleta-Mejía E, Martínez-Garza A, Pedroza-Sandoval A (2006) *Strategies for management of Sclerotium cepivorum* Berk in garlic. J. *Plant Pathol. 88*: 253-261

Velásquez-Valle R, Reveles-Hernández M (2016) Effect of agents of alternative management on the garlic white rot development. *Revista Mexicana de Micologia 44*: 41-47.

Xu Z, Harrington TC, Gleason ML, Batzer JC (2010) Phylogenetic placement of plant pathogenic *Sclerotium* species among teleomorph genera. *Mycologia 102*: 337-346.