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Analyzing Microbes

Manual of Molecular Biology Techniques

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Chapter 6

Molecular Identification of Microbes: I. *Macrophomina phaseolina*

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Abstract

This chapter will help us in the isolation of *Macrophomina phaseolina* from soil and infected plants and examination of morphological and physiological features for identification by using microscopic and cultural characters. In the later part, we will learn recent research findings to identify this fungus using PCR-based molecular techniques.

6.1 Introduction

Macrophomina phaseolina (Tassi) Goid., a soil-borne fungus, causes charcoal rot [1]. The fungus can infect the root and lower stem of over 500 plant species and is widely distributed all over the world. The pathogen causes wide range of diseases in the arid and semi-arid regions of the world. *M. phaseolina* persists in soil as sclerotia formed in infected host tissue and later released in the soil during decaying process. As a root inhabitant, the fungus is wide-spread in warmer area, invades immature, damaged, or senescent tissues; plants are generally attacked at seedling and flowering, when conditions are hot and dry. Infection develops from sclerotia, which can survive for a few years in roots. *M. phaseolina* is widely distributed among areas with variable soil types and annual rainfall, indicating that this fungus can persist under highly diverse environmental conditions. In case of soil-borne phase, the pathogen remains either on the dead organic debris or on the root stubbles, which are left over after the crop harvest. High soil temperature (40 °C), low soil pH (5.4–6), low soil moisture (8–16 %), and high humidity (90 %) favor infection and disease development. Long periods of drought and hot temperatures interspersed with rain showers create ideal conditions for the fungal pathogenesis.

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Table 6.1
List of PCR primers

Primer name	Sequence	PCR product size
Universal primers		
ITS-1	5′ TCCGTAGGTGAACCTGCGG 3′	~650
ITS-4	5′ TCCTCCGCTTATTGA TATGC 3′	
Specific primers		
MPKF1	5′ CCGCCAGAGGACTATCAAAC 3′	~350
MPKR1	5′ CGTCCGAAGCGAGGTGTATT 3′	

6.2 Materials

- Diseased field soil
- Mesh-2 mm, 45 μm
- Sodium hypochlorite
- Acidified Potato Dextrose agar plates (pH 5.6)
- *M. phaseolina* culture
- Potato Dextrose broth
- Lysis buffer—(50 mM Tris–HCl, pH 7.8, 50 mM Na2-EDTA, 3 % SDS), 1 % 2-mercaptoethanol should be added freshly)
- Primers (Table 6.1)
- Thermal cycler
- *Taq* DNA polymerase
- 10× PCR buffer
- Milli Q Water
- 50× TAE buffer

6.3 Methods

6.3.1. Isolation

1. Sieve the air-dried soil through mesh.
2. Dissolve 5 g of soil in 0.525 % sodium hypochlorite and allow standing for 10–20 min.
3. Wash the deposit in sterile distilled water over a sieve with a 45 μm mesh.

4. Introduce the deposit into a 250-ml flask and incorporate in to 100 ml of PDA.
5. Pour into the petriplates and incubate at 32–34 °C for 3–4 days.
6. Colony morphology: On PDA colonies range in color from white to brown or gray and darken with age.

6.3.2. Microscopy

1. Hyphal branches generally form at right angles to parent hyphae, but branching is also common at acute angles.
2. Pycnida: 100–200 μm in diameter; dark to grayish, becoming black with age; globose or flattened globose; membranous to subcarbonaceous with an inconspicuous or definite truncate ostiole.
3. The pycnida bear simple, rod-shaped conidiophores, 10–15 μm long.
4. Conidia: 14–33 × 6–12 μm, single celled, hyaline, and elliptic or oval.
5. Microsclerotia: jet black in color and appear smooth and round to oblong or irregular.

6.3.3. DNA Extraction

See Chap. 1 (Fungal DNA isolation).

6.3.4. Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a technique used to amplify a part of DNA that lies between two regions of known sequences. Two oligonucleotides are used as primers for a series of synthetic reactions that are catalyzed by DNA polymerase. These oligonucleotides specifically anneal to the target sequences on opposite strands and flank the region that is to be amplified.

6.3.4.1. Amplification of 5.8S rDNA Gene and ITS Regions

Prepare the PCR master mix following Table 6.2. Redistribute 44 μl of master mix into PCR tube and add 4–6 μl genomic DNA.

6.3.4.2. PCR Program for ITS Primers

Lid heating—Enabled	
Step 1 = 95 °C for 5 min	(Initial denaturation)
Step 2 = 95 °C for 1 min	(Denaturation)
Step 3 = 50 °C for 30 s	(Primer annealing)
Step 4 = 72 °C for 1 min 20 s	(Elongation)
Step 5 = 34 Cycle	(Repeat steps 2–3)
Step 6 = 72 °C for 10 min	(Final elongation)
Hold at 4 °C for 10 min	

Table 6.2
Preparation of PCR master mixture for single reaction

Reaction mixture	Primer sets and reagents concentration	
	ITS1 and ITS4	MPKF1 and MPKR1
Genomic DNA	20–40 ng	10–25 ng
Forward primer	50 pmol	5 pmol
Reverse primer	50 pmol	5 pmol
dNTPs mix	0.2 mM	0.2 mM
10× PCR buffer	5 μl	2 μl
Taq DNA polymerase	1 U	0.4 U
Milli Q Water	Make up to 50 μl	Make up to 20 μl

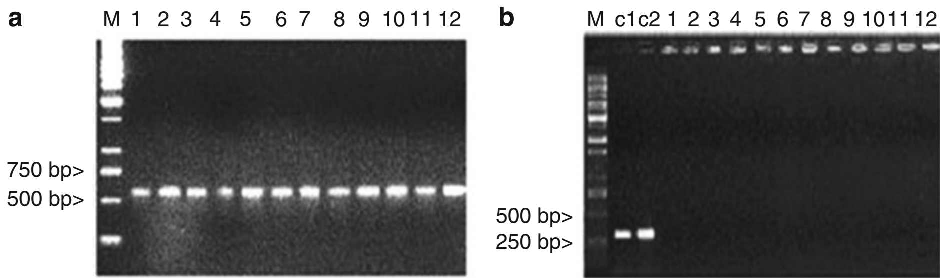

Fig. 6.1 PCR Amplification of rDNA gene cluster: (**a**) Primers ITS1 and ITS4 were used for amplification of nearly 650 bp fragment. *Lanes 1–10* showing amplified products of *M. phaseolina* isolates. (**b**) Amplification of *M. phaseolina* with specific primers (*MpKF1 and MpKR1*) produced 350 bp amplicon in lanes c1 and c2. *Lanes 1–12* showing no amplified product with different test microbes. M- 1 kb molecular ladder.

6.3.4.3. PCR Program for ***M. phaseolina*** *Specific Primers [1]*

Lid heating—Enabled	
Step 1 = 95 °C for 5 min	(Initial denaturation)
Step 2 = 95 °C for 30 s	(Denaturation)
Step 3 = 56 °C for 1 min	(Primer annealing)
Step 4 = 72 °C for 2 min	(Elongation)
Step 5 = 25 Cycle	(Repeat steps 1–3)
Step 6 = 72 °C for 10 min	(Final elongation)
Hold at 4 °C for 10 min	

6.3.5. Gel Electrophoresis

PCR amplified products together with marker (1 kb Fermentas, USA) were resolved by gel electrophoresis (4 V cm^{-1}) on 1.4 % agarose gels in 1× TAE buffer containing 0.5 mg ml^{-1} Et–Br and visualized under UV transilluminator (Fig. 6.1).

Reference

1. Babu KB, Srivastava AK, Saxena AK, Arora DK (2007) Identification and detection of *Macrophomina phaseolina* by using species-specific oligonucleotide primers and probe. Mycologia 99:733–739