

BAITING BIOASSAYS AND DNA-PCR BASED METHODS FOR DETECTION OF PHYTOPHTHORA SPECIES ASSOCIATED WITH CHESTNUT INK DISEASE FROM SOIL, ROOT TISSUES AND WATER

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Introduction

Chestnut ink disease (CID) is a destructive and a lethal disease of sweet chestnut (*Castanea sativa* Mill.) that is associated with different soil borne *Phytophthora* species. CID is very difficult to control because cultural and chemical measures provide limited effects and didn't prevent disease spread.

The aim of this study was to develop and validate different methodologies used for *Phytophthora* detection from natural soil, symptomatic vegetal tissues and water.



Fig. 1-4. Chestnut ink disease symptoms on *Castanea sativa*

Material and Methods

Baiting techniques bioassays and DNA-PCR assays based on the multicopy ITS region was compared for method validation. Efficiency, specificity and time response span were the parameters evaluated and compared to validate proposed objectives.

Biological bait

Soil samples were used for the isolation of *Phytophthora*.

From each sample, 20 ml of soil was taken and placed in a 120 mm diameter Petri dish. In each plate, 8 ml of distilled water was added and five 0.5 cm diameter discs of chestnut leaves were placed.

The plates were placed at room temperature and after 72 hours the chestnut leaf discs were removed and placed in PDA (Potato Dextrose Agar, Difco) culture medium. The presence of *Phytophthora* was confirmed by microscopic observation of the hyphae.



Fig. 5. Baiting technics bioassays

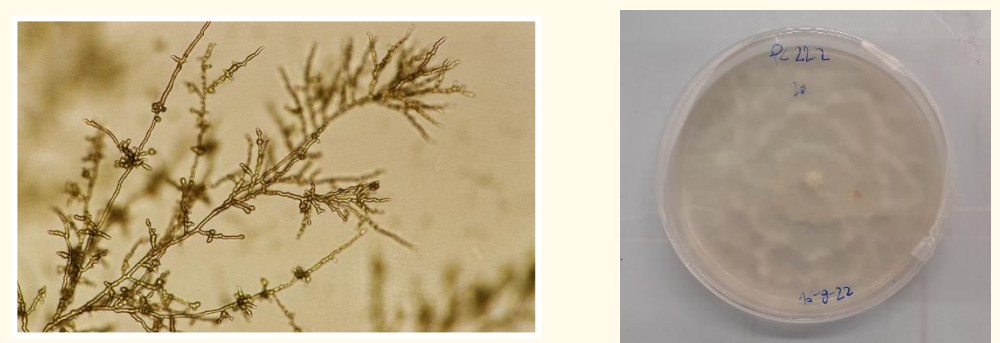


Fig. 6. Mycelium and colony morphology of *Phytophthora* sp. on PDA

Phytophthora detection

Phytophthora sp. identification was carried out by amplification and sequencing of the ITS (ITS4, 5.8S, ITS6), ribosomal DNA (rDNA) region using the universal primers ITS4 and ITS6.



Fig. 7. Thermocycler PCR

Resultados e Conclusões

Biological bait

Table 1. Positive isolates of *Phytophthora* sp. on PDA medium obtained from chestnut leaf discs.

Samples	Repetition 1	Repetition 2	Repetition 3
1	1/5	0/5	0/5
2	2/5	3/5	3/5
3	0/5	0/5	0/5
4	3/5	1/5	0/5
5	3/5	5/5	3/5

Phytophthora spp. was detected in all soil samples except sample 3.

The effectiveness of the method, i.e. the positive isolation of *Phytophthora* species varied, however, from 20 to 100 % depending on the sample.

This method allows the processing of a high number of samples, which enables its use in phytopathological diagnostic laboratories.

PCR detection

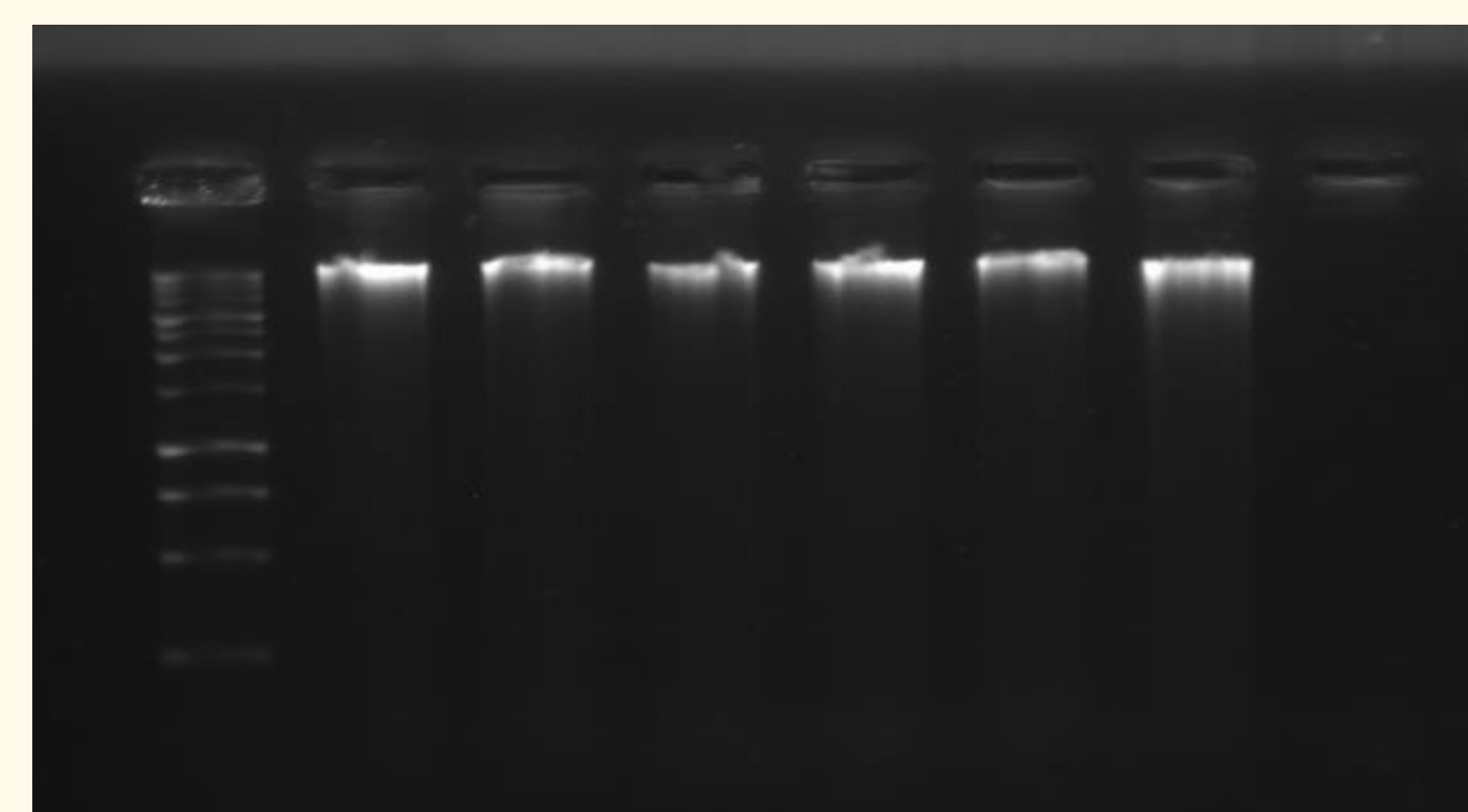


Fig. 8. Genomic DNA extracted from mycelium of *Phytophthora* isolates. Column 1 - 1kb DNA Ladder (Promega) Column (2-7) - *Phytophthora* isolates.

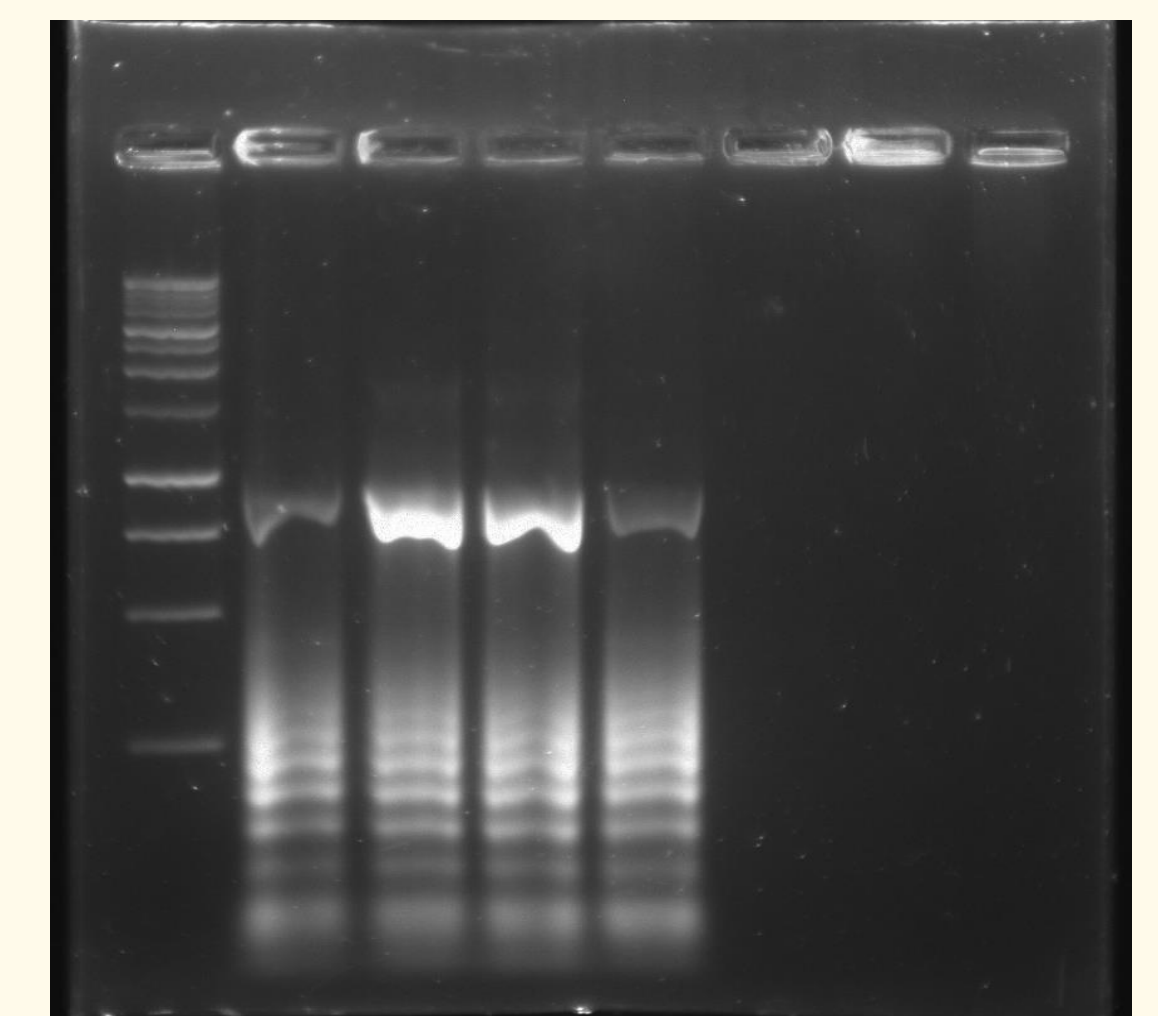


Fig. 9. PCR amplification fragment using genomic DNA from *Phytophthora* isolates with primers ITS4-ITS6. Column 1 - Molecular marker (1 kb DNA Ladder, Promega); Column (2-5) - *Phytophthora* isolates.

Molecular identification methods based on ITS1, 5.8S and ITS2 region provided the identification of *Phytophthora* spp. in an objective and reliable way that are valuable and basic requisites for integrated management programs for evaluating the applied controls measures to minimize impacts and control the disease.

Molecular methods for species detection and identification as Real-time PCR and the relatively new detection (Loop-Mediated Isothermal Amplification) by its specific sensitive are currently being developed for *Phytophthora* identification and diagnosis of ink disease of chestnut.

Acknowledgment: Project BioChestnut - PDR2020-101-030959 and Programa Experimental para o Tratamento Biológico do Cancro do Castanheiro com Base em Estirpes Hipovirulentas de *Cryphonectria parasitica* (CIMO-IPB).