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Research Article

Integrated Microbial and Molecular Analysis for the Identification of Pathogenic Fungus Species from Soil Samples of Agricultural Fields

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Abstract

Alternaria species are common soil fungi, important both as saprophytes on plant debris and as serious plant pathogens and aeroallergens. In this work, soil samples from agricultural fields around Bhopal were used to isolate filamentous fungi, with a special focus on Alternaria. Fungi were isolated on Potato Dextrose Agar (PDA) using serial dilutions, purified and examined based on colony characters and microscopic morphology using Lactophenol Cotton Blue (LPCB) staining. Among the isolates, one dominant dark, fast-growing fungus showed olive-brown colonies and typical Alternaria-type conidia: pigmented, multi-septate spores often with a tapering beak and formed in chains. These features matched published descriptions of Alternariaalternata and allowed identification at species level by morphology alone. The study demonstrates that a simple PDA culture combined with LPCB staining is still a useful and low-cost workflow for isolation and preliminary identification of Alternariaalternata from soil, and confirms this species as a frequent culturable component of local agricultural soils.

Keywords:

Alternariaalternata; soil fungi; PDA isolation; Lactophenol Cotton Blue; morphological identification1. Introduction

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Key Words

Alternaria, Mycotoxins, PCR Amplification, Sanger Sequencing ,ITS Region

Introduction

Fungal pathogens are a big challenge in agricultural and natural ecosystems because they reduce crop yield, contaminate food with mycotoxins, and sometimes even infect animals and humans. Soil is the main reservoir of these fungi. It contain saprotrophs, beneficial symbionts and also many plant-pathogenic and opportunistic human-pathogenic species (Iwen et al., 2002; Akhtar et al., 2022). Correct and fast identification of these fungi is therefore very important if we want to understand disease risk and design control measures. Classical identification of soil fungi is mostly based on colony morphology, microscopic features and spore characters on media such as Potato Dextrose Agar (PDA) or Malt Extract Agar. While these methods are still useful, they are often too slow and sometimes not accurate, especially for species that look similar under the microscope or do not sporulate well in culture (Martin &Rygiewicz, 2005; Bernreiter, 2017). Many soil fungi also grow slowly or have specific nutritional

requirements, so they may be missed entirely by culture- based approaches (Beemrote *et al*., 2024).Because of these limitations; molecular methods have become central in modern mycology. Among all markers, the internal transcribed spacer (ITS) region of the ribosomal DNA is now widely accepted as the "universal DNA barcode" for fungi (Bellemain et al., 2010; Nilsson et al., 2014). The ITS region (ITS1-5.8S-ITS2) is flanked by conserved rRNA genes but itself is variable enough to separate many species, which makes it ideal for PCR amplification and sequencing from unknown isolates (White et al., 1990; Iwen et al., 2002). Sanger sequencing of PCR-amplified ITS fragments, followed by comparison against curated databases, is still one of the most common methods for routine fungal identification because it is reasonably cheap and technically simple (Langsiri et al., 2023).However, Sanger sequencing is not perfect. Mixed templates, such as co-infected cultures or environmental DNA, can produce overlapping chromatograms that are hard to interpret. In addition, closely related species complexes (for example some Fusarium and Aspergillus groups) sometimes show too little variation in ITS alone, and require additional loci (Nilsson et al., 2014; Samson et al., 2006). Newer methods like Illumina or nanopore, long- read sequencing can provide deeper community profiles and resolve mixed infections, but they are more expensive and not always available in small laboratories (Lindahl et al., 2013; Langsiri et al., 2023).In agriculture, soil-borne fungi such as Fusarium, Rhizoctonia, Alternaria and Aspergillus are responsible for root rots, wilts, damping-off and foliar diseases that seriously impact crop yields (Summerell et al., 2010; Rotem, 1994). Many of these pathogens also produce mycotoxins, including trichothecenes, fumonisins and aflatoxins, which pose chronic health risks through contaminated food and feed (Bhat et al., 2020; De Hoog et al., 2000). With global movement of plant material and climate change, there is evidence that ranges of plant pathogens are shifting and new disease problems appear in previously unaffected regions (Harrison et al., 2020).The present work was designed as a small-scale study to isolate fungi from agricultural field soils around Bhopal, to extract their genomic DNA, and to identify selected isolates by PCR amplification and Sanger sequencing of the ITS region. By doing so, we try to link simple microbiology with more advanced molecular tools and to show how even a basic laboratory can perform reliable fungal identification using this integrated pipeline.

2. Materials and method

Soil samples were collected from different agricultural fields around Bhopal district (Madhya Pradesh, India). At each site, loose plant debris and stones were removed from the surface, and soil was taken from approximately 2–10 cm depth using a clean stainless-steel spatula. About 200–300 g of soil per site was placed into sterile zip-lock bags, properly labelled with date and location, and transported to the laboratory. Samples were stored at 4 °C until further processing. Before isolation, soil was air-dried at room temperature and passed through a 2 mm sieve to remove big particles. For fungal isolation, 1 g of sieved soil was added to 9 mL sterile distilled water in a test tube and vortexed well to make a 10^{-1} suspension. Serial dilutions were prepared up to 10^{-5}. From the final dilution, 0.1–1.0 mL was pipetted and spread onto Potato Dextrose Agar (PDA) plates. PDA medium was prepared according to standard composition (potato infusion, dextrose, agar; pH around 5.6), sterilised by autoclaving at 121 °C for 15–20 minutes and poured into sterile Petri plates. In some plates, chloramphenicol (12 mg/100 mL) was added after cooling to reduce bacterial growth.Plateswere incubated at 28–30 °C for 5–7 days. Emerging fungal colonies were observed daily. Colonies with distinct morphology (colour, margin, elevation, growth rate) were picked with a sterile needle and sub-cultured onto fresh PDA to obtain pure cultures. These pure isolates were maintained on PDA slants at 4 °C for short-term storage.Microscopic identification of the isolates was done using

lactophenol cotton blue (LPCB) staining. A small portion of mycelium and spores from the pure culture was picked with a sterile needle and placed on a clean glass slide. One drop of LPCB stain was added and gently mixed with the fungal material. A coverslip was placed carefully to avoid air bubbles. The slide was then examined under a compound microscope at 10×, 40× and, when required, 100× magnification. Features like hyphal septation, conidiophore structure, shape, septation and arrangement of spores were recorded and compared with standard mycological keys and atlases (De Hoog et al., 2000; Rotem, 1994). Particular attention was given to Alternaria- type conidia, which are typically dark, multi- septate and formed in chains.

Results and Discussion

PDA plates inoculated with soil dilutions showed rich fungal growth after a few days of incubation. Colonies of different colours (white, grey, olive, dark brown) and textures (cottony, velvety, powdery) appeared, indicating that the agricultural soils harboured a diverse community of filamentous fungi. By repeated sub-culturing, single-colony isolates were obtained. Among these, one dominant morphotype with dark, fast-growing colonies was consistently recovered from several soil samples.On PDA, this fungus formed olive-brown to dark brown colonies with a slightly cottony to floccose surface. The colony margin was irregular, and with age the reverse of the plate became almost blackish. Such cultural features are in line with descriptions of Alternaria species commonly found in agricultural soils and on plant residues (Rotem, 1994; Summerell et al., 2010).Microscopic examination of this isolate using LPCB staining revealed septate, pigmented hyphae and characteristic conidiophores bearing chains of dark, obclavate to ellipsoid conidia. The conidia were multi-septate, with both transverse and sometimes longitudinal septa, often with a tapering beak at one end. These morphological characters fit well with Alternariaalternata, a widely distributed saprophyte and plant pathogen known to cause leaf spots and related diseases on many crops, as well as acting as an aeroallergen in humans (Rotem, 1994; De Hoog et al., 2000).

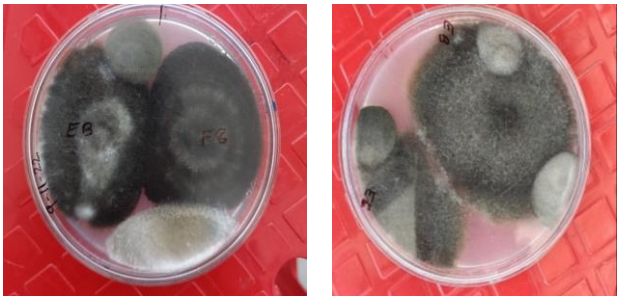

Figure 1.Mixed culture of pathogenic fungus on PDA media.

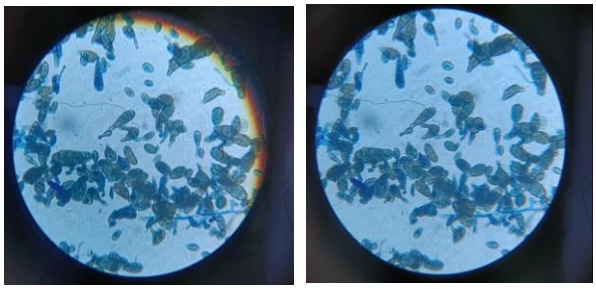

Figure 2.Figure showing the Alternaria alternate spores after lactophenol cotton blue staining.

The repeated isolation of Alternariaalternata from different field soils in the Bhopal area suggests that this species is well adapted to local agro-ecosystems, surviving on plant debris and soil organic matter. This is in agreement with other studies which report A. alternata as a common soil inhabitant and important causal agent of foliar diseases and post-harvest spoilage (Rotem, 1994; Akhtar et al., 2022). Its presence in these soils may therefore represent a potential inoculum source for Alternaria leaf spot epidemics on nearby crops, especially under favourable conditions of humidity and temperature. Because this work focused on simple culture and microscopy, other fungi present in the samples were not characterised in detail.

However, the PDA isolation combined with LPCB staining proved sufficient to recognise A. alternata at least to species level, showing that even without molecular tools, careful observation and comparison with standard keys can still give meaningful taxonomic information. At the same time, it should be remembered that PDA favours only a subset of culturable fungi, and that many non-sporulating or slow-growing species may remain undetected (Bernreiter, 2017; Cale et al., 2021).

Conclusion

In summary, the study showed that soil from local agricultural fields contains Alternaria alternata as a frequent culturable fungus. Considering its known roles as a plant pathogen and allergen, this finding underlines the need for regular monitoring of soil-borne inoculum in crop fields and for further work to connect these soil isolates with actual disease symptoms observed in plants..

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