

## Molecular revolution in plant health: Rapid assays for fungal wilt pathogens of subtropical fruits

Early generation molecular tools, such as PCR-RFLP, Bio-PCR, and colony PCR, allowed direct pathogen identification but faced challenges of cross-reactivity. Second-generation assays, including multiplex PCR and LAMP-PCR, improved detection efficiency and field applicability. Next-generation diagnostics have leveraged genomic and transcriptomic insights, resulting in highly sensitive qRT-PCR, multiplex nested RT-PCR, and comparative genomics-based assays. Notably, unique *Ceratocystis fimbriata*-specific genes (Cf-PME and Cf-XYL) were identified and developed into a semi-quantitative colony PCR assay, enabling early and precise detection of the mango wilt pathogen directly from infected tissues.

**Keywords:** Fusarium detection, Mango wilt, Molecular diagnostics, Next-generation diagnostics PCR-based assays

INDIA ranks second in fruit production, yet the productivity of subtropical fruits such as mango, guava, banana, and papaya has steadily declined over the last few years due to climate change, high summer temperatures, and pest and disease outbreaks. Fungal diseases pose a serious problem in mango, guava, and banana, while viral diseases are more severe in papaya and banana. Diagnostics is the first and foremost step in disease management, as confirmation of the presence and load of the pathogen in the soil and plant determines the extent of the management modules to be implemented. Moreover, efficient, rapid detection assays for specific pathogens that are user-friendly and ready to use in the farmer's field are essential for complete disease control. Early detection of viral pathogens is relatively easy using immunodiagnostic and molecular assays, whereas detection of fungal pathogens is more challenging. Traditional diagnostic methods for fungal pathogens, such as symptom observation, culture-based identification, and morphological or biochemical analysis, though considered the "gold standard," are often time-consuming and unreliable, particularly during the early stages of infection. Several molecular diagnostic assays have been developed and validated for the early detection of fungal pathogens that cause mango wilt, mango malformation, mango anthracnose, guava wilt, and banana wilt.

### Early generation molecular diagnostics

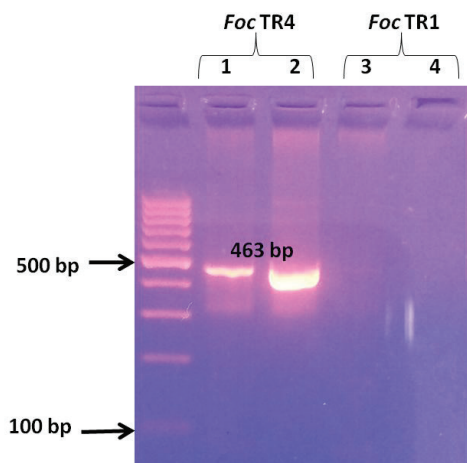
The nuclear ribosomal DNA (rDNA) operon is the most commonly used target for bacteria and fungi for several reasons. Although rDNA is the main target of many nucleic acid-based analyses, other targets for

detecting fungi include  $\beta$ -tubulin, actin, elongation factor 1 $\alpha$ , and mating-type genes. PCR-RFLP is a variant of the restriction fragment length polymorphism of specific amplicons. Another common molecular diagnostic assay is Bio-PCR, a direct colony PCR assay for pathogen detection that does not involve DNA extraction; instead, it uses the colony/mycelial mat for PCR reactions, mixed with standard Tris-EDTA buffer. A colony PCR assay was optimized with a DNA template prepared from 10 mg of fungal mycelium of *Fusarium* dissolved in 50  $\mu$ L of Tris-EDTA buffer using ITS1-ITS4 primers and species-specific primers. These techniques had issues with cross-species amplification, which led to the detection of other *Fusarium* species in larger samples.

### Second generation molecular diagnostics

Second-generation molecular diagnostic assays were multiplexed and more efficient than first-generation assays. Multiplex PCR is a technique that uses several pairs of primers annealing to different target sequences, permitting simultaneous analysis of multiple targets in a single sample. A multiplex PCR-based assay using gene-specific primers for the pectin lyase b gene and glutamine synthetase was developed for the detection of *Colletotrichum gloeosporioides*, the causal agent of anthracnose disease in mango, with higher sensitivity and accuracy. Similarly, a simple multiplex PCR assay using three primer pairs – ITS, chitin synthase, and species-specific – was developed and validated for the detection of *Fusarium oxysporum* f.sp. *psidii*, which causes wilt disease of guava. Secreted-in-xylem (SIX) gene-specific primers and FocTR4-specific primers have been successfully used

for the molecular identification of *Fusarium oxysporum* f. sp. *cubense* (Foc) tropical race 1 and tropical race 4, which cause wilt disease in banana. Loop-mediated isothermal amplification–polymerase chain reaction (LAMP-PCR) is a robust, flexible tool that represents a significant advance in point-of-care (POC) diagnostics. Despite their high sensitivity, these methods are limited by cross-species genomic similarities that compromise specificity, the need for costly instruments, and limited field applicability.



FocTR4 specific primer for molecular detection of wilt pathogen race TR4 in banana

### Next generation molecular diagnostics (NGMD)

The next generation of molecular diagnostics involves whole-genome sequencing, metagenomic analysis, and quantitative real-time PCR (qRT-PCR) assays. In general, qRT-PCR assays have been successfully used to detect numerous viruses, such as papaya ring spot virus and banana bunchy top virus, which exhibit  $10^3$ – $10^4$ -fold greater sensitivity than immunoassays. They have also been used to quantify fungal pathogen load and confirm the presence of spore-forming fungi through real-time quantification using specific genes. This assay can detect even very small amounts. A mango malformation-inducing agent was identified as a *Fusarium* complex, as demonstrated by real-time quantification of polyketide synthase gene expression. This gene plays a key role in the biosynthesis of fumonisins, a major toxin produced by a specific group of *Fusarium* species.

Another variant assay, multiplex nested RT-PCR, simultaneously and sensitively detects 3–4 pathogens in a compartmentalized tube using a combination of specific primers. This newly developed method combines the advantages of multiplex RT-PCR with the sensitivity and reliability of nested RT-PCR, all in a single closed tube. This technique is now being used for virus detection in

other horticultural crops, such as citrus and papaya.

Comparative genome analysis of *Ceratocystis fimbriata*, *C. manginecans*, and *C. albifundus* identified unique genes, Cf-PME and Cf-XYL, encoding pectin methyl esterase and xylanase, which discriminate *Ceratocystis fimbriata* from other species. These genes were used to develop a semi-quantitative colony PCR assay for the early detection of the mango wilt pathogen, validated in wilt-affected soil samples from different geographic locations.

### Salient Features of NGMD:

- Enables rapid detection using a standardized colony PCR assay.
- Applicable for both laboratory and field-sample diagnostics.
- Reduces diagnostic time from days to a few hours.

### Advantages of NGMD:

- High specificity and sensitivity compared to morphological methods.
- Reduces chances of misdiagnosis and ensures timely management actions.
- Compatible with standard PCR platforms available in diagnostic laboratories.

### Applications of NGMD:

- Monitoring and surveillance of orchards for pathogen presence.
- Useful for quarantine, breeding, and disease management programs.

### CONCLUSION

Early diagnosis of fungal and viral pathogens is essential for the development of effective prophylactic strategies in disease management. Molecular detection systems should be optimized through automated extraction and purification of nucleic acids from pathogens, and through advanced genotyping systems using robotics. Advancements in microarrays, real-time, and next-generation sequencing platforms have reduced cost-related drawbacks, making molecular diagnostic systems ready for field-level use. Presently, molecular assays such as qPCR (quantitative PCR), multiplex quantitative PCR, and next-generation diagnostics are widely used for early detection of fungal and viral pathogens. These rapid, sensitive, cost-effective, and field-deployable diagnostics enable effective disease management and surveillance in nurseries, ensuring the supply of healthy planting material.

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