Rapid diagnosis of strawberry crown rot pathogens and fungicide resistance for improved disease control

Nan-Yi Wang, Adrian I. Zuniga, Andre Bueno Gama and Natalia A. Peres

Summary

Rapid and accurate disease diagnosis and detection of fungicide-resistant isolates are important for effective disease management programs in strawberry production. In Florida, *Macrophomina phaseolina*, *Phytophthora cactorum*, *Colletotrichum gloeosporioides*, and *C. acutatum* are the primary organisms causing strawberry crown rot. Even though the disease can be caused by different pathogens, symptoms are indistinguishable, and the impact of crown rot can be equally devastating. To inform strawberry growers of disease diagnostic results in time for effective deployment of chemical control practices, we developed a multiplex high-resolution melting (HRM) assay to detect above-mentioned strawberry crown rot pathogens. Furthermore, we successfully employed this HRM assay in the plant diagnostic clinic of UF-GCREC and examined over 500 plant samples in the 2018-2019 strawberry season. The newly designed primers for detection of resistance to QoI fungicides (i.e. Abound, Cabrio) in *C. acutatum* using HRM did not perform as expected, indicating different primer design strategies are needed for improvement. Identification of the *sdh*B mutations H272R, H272Y, and P225F that confer resistance to the SDHI fungicides (FRAC 7, i.e. Fontelis, Merivon, Luna, Kenja) in *Botrytis cinerea* isolates was successful using HRM with two sets of designed primers.

Methods

Development and validation of a molecular diagnostic tool for the rapid and accurate detection of the strawberry crown rot pathogens

To develop a molecular tool for rapid and accurate detection of the crown rot pathogens of strawberry, we first designed primers targeting the conserved region of the ribosomal DNA internal transcribed spacer (ITS) sequences of several fungal species. This list included *C. acutatum*, *C. gloeosporioides*, *M. phaseolina*, *P. cactorum, Pestalotiopsis* spp., and *Fusarium oxysporum*. These primers were tested under the previously validated HRM conditions using a LightCycler 480 system. In addition, we adopted and tested primers published in other detection systems such as real-time PCR and recombinase polymerase amplification (RPA) for the application in HRM.

Implementation of a high-throughput HRM assay in the UF-GCREC plant diagnostic clinic

For implementation of a high-throughput HRM assay for diagnosis of strawberry crown rot diseases, symptomatic strawberry plants sent to the plant diagnostic clinic of UF-GCREC during the 2018-2019 season were processed for crude DNA extraction according to the previously described protocol and tested by the HRM assay developed in this study. Additionally, conventional culture isolation was performed for validation and comparison to the HRM results.

Development of a high-throughput detection assay to quickly determine the QoI fungicide resistance profile of *Colletotrichum* species

Primer-template complementarity is crucial for PCR-based detection methods as mismatches can adversely affect priming efficiency. To develop a rapid assay for determining whether *Colletotrichum* species were resistant to QoI fungicides, we took advantage of this concept and designed primers with a perfect match to the expected target but with a 3' end mismatch to its counterpart, i.e., primers for QoI-

sensitive isolates were expected to have lower priming efficiency on QoI-resistant isolates and vice versa. Priming efficiency was determined at various annealing temperatures from 60 to 68°C using HRM.

Development of a HRM assay to determine the SDHI fungicide resistance profile of *B. cinerea* isolates

During the 2017-2018 strawberry season, 25 *B. cinerea* isolates were collected from two commercial farms in Florida. After isolation, isolates were incubated on HA medium for 7 days at 23˚C. After incubation, conidial suspentions of 10^6 conidia/ml were prepared and 1µl was used for HRM analysis. Two sets of HRM markers were designed based on the *sdh*B gene of *B. cinerea* from strawberry to detect either the H272R/Y or the P225F mutations. Priming efficiency was determined at 58°C annealing temperature using HRM. The 25 *B. cinerea* isolates were sequenced to confirm HRM results.

Results

Development and validation of a molecular diagnostic tool for the rapid and accurate detection of the strawberry crown rot pathogens

Six primer combinations were tested, among which the primer pair ITS-F3 and ITS-R1 produced distinctive melting peaks with good amplification levels (**Figure 1**) for *C. acutatum*, *C. gloeosporioides*, *M. phaseolina*, and *Pestalotiopsis* spp. However, we observed two overlapping melting peaks for *C. acutatum* and *F. oxysporum* (**Figure 1**). This makes the primers unsuitable for accurate detection of strawberry crown rot pathogens such as *F. oxysporum,* a common soilborne fungus with a broad distribution.

Instead of designing primers targeting various fungal pathogens simultaneously, we adopted primers used in other detection systems for *M. phaseolina* and *Phytophthora* species and tested their application in HRM. The results showed that primer pairs Mps_TaqMan_F/ Mps_TaqMan_R and TrnM-F/TrnM-R amplified specific melting peaks for *Phytophthora* species and *M. phaseolina*, respectively (**Figure 2**). We further tested the multiplexing capability of these primers with AcG1-F1/AcG1-R1 (for *Colletotrichum* species) and found that the HRM assay in the presence of individual DNA showed excellent specificity, with each primer pair generating the specific melting peak corresponding to the target pathogen and without any primer interactions (**Figure 2**). However, amplification of different melting peaks was observed in the presence of mixed DNA samples and were concentration- and target DNA-dependent (data not shown).

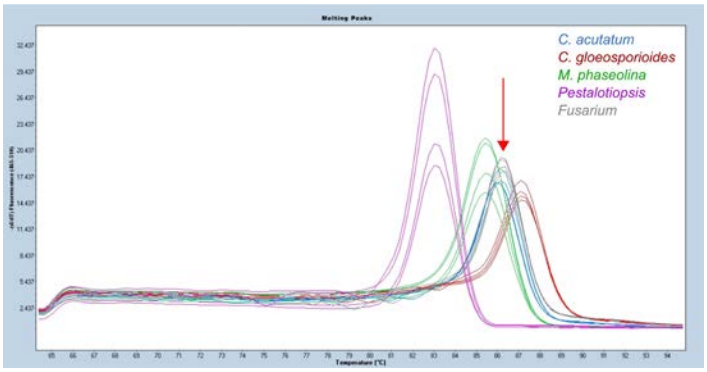

Figure 1. High-resolution melting (HRM) analysis for detection of strawberry crown rot pathogens and a common soilborne fungus. The red arrow indicates two overlapping melting peaks representing *Colletotrichum acutatum* (blue line) and *Fusarium oxysporum* (gray line).

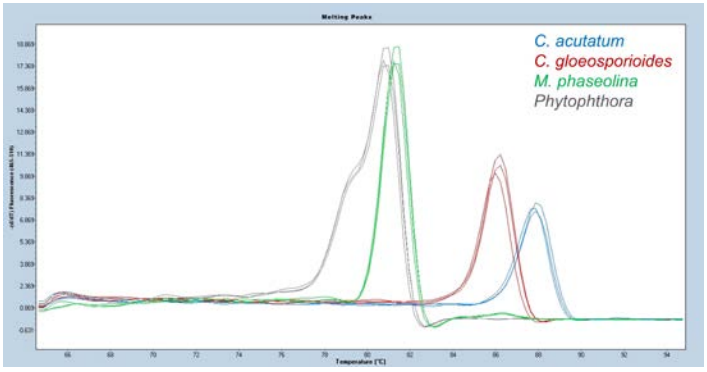

Figure 2. High-resolution melting (HRM) analysis for detection of strawberry crown rot pathogens using mixed primers. The primer mix contained three sets of primers, for *Colletotrichum acutatum* (blue line) and *C. gloeosporioides* (red line), for *Phytophthora* species (green line), and for *Macrophomina phaseolina* (gray line).

Implementation of a high-throughput HRM assay in the UF-GCREC plant diagnostic clinic

During the 2018-2019 season, we processed and examined 529 plant samples (equal to 131 clinic samples) using the HRM assay, among which 54% (285/529) tested positive and 23% (121/529) negative by both HRM and culture isolation methods (**Table 1**). In addition, 22% (116/529) of plant samples tested positive by HRM but negative by culture isolation, indicating that HRM is more sensitive in detecting strawberry crown rot pathogens than

culture isolation (**Table 1**). One percent (7/529) of plant samples were positive by culture isolation but negative by HRM. This is likely due to the limited amount of symptomatic crown tissue or the use of different parts of the necrotic crown tissue for diagnosis. Of the HRM-positive plant samples, 5% tested positive for *C. acutatum*, 35% for *C. gloeosporioides*, 37% for *Phytophthora* species, and 23% for *M. phaseolina* (**Figure 3**). Statistical analysis using Fisher's exact test also suggested a significant relationship in pathogen detection between HRM and culture isolation ($P < 0.0001$).

Table 1. Summary of diagnostic results for strawberry samples processed in the plant diagnostic clinic of UF-GCREC in the 2018-2019 season.

HRM	Culture isolation		
	Positive	Negative	Total
Positive	285	116	401
Negative	7	121	128
Total	292	237	529

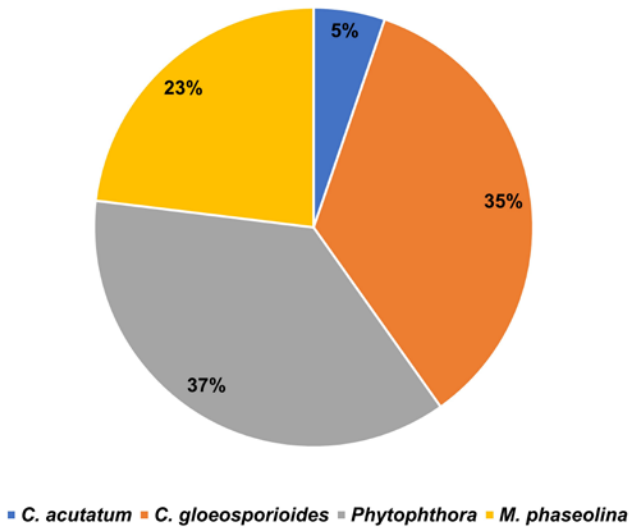

Figure 3. Summary of diagnostic results for strawberry samples testing positive for at least one species using high-resolution melting (HRM) analysis in the 2018-2019 season.

Development of a high-throughput detection assay to quickly determine the QoI fungicide resistance profile of *Colletotrichum* species

The HRM assays showed that the relative fluorescence intensity decreased with an increase in annealing temperatures (**Figure 4**). Among primers tested, R-NF2/NR1 primers for QoI-resistant isolates of *C. acutatum* exhibited a stronger mismatch effect on priming efficiency across the tested annealing temperatures, compared to S-NF2/NR1 primers for QoI-resistant isolates (**Figure 4**). Although no fluorescence was observed when primer-template mismatches occurred at 68°C, this temperature severely affected the priming efficiency, thus making it inapplicable to the HRM assay (**Figure 4**).

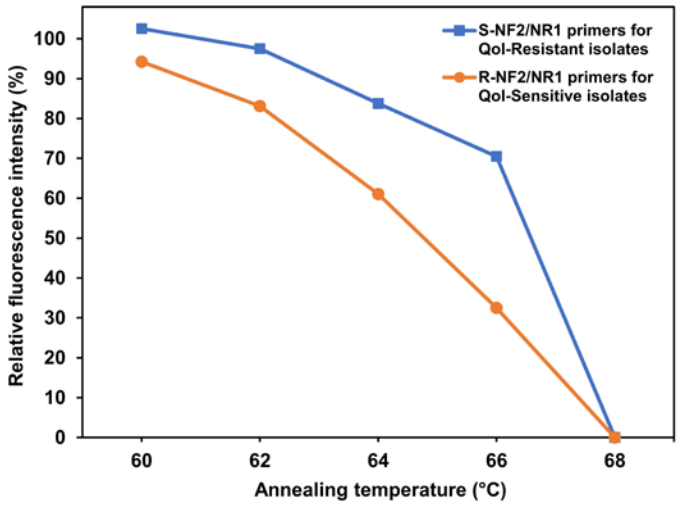

Figure 4. Effect of annealing temperature on primer-template mismatch. S-NR2/NR1 primers were designed for a perfect match to the G143 allele of *Colletotrichum acutatum* QoI-sensitive isolates and R-NR2/NR1 primers to the A143 allele of *C. acutatum* QoI-resistant isolates.

Development of a HRM assay to determine the SDHI fungicide resistance profile of *B. cinerea*

The HRM and Sanger sequencing assays confirmed the presence of the H272R and H272Y mutations (**Figure 5**) in four and two isolates, respectively, whereas the P225F (**Figure 6**) was only detected in one isolate. Our study provides a fast and cost-effective method to detect frequent (H272R/Y) and powerful (P225F) *sdh*B mutations. Furthermore, HRM analysis could be implemented in plant diagnostic clinics to monitor predominant genotypes in strawberry fields and inform growers about SDHI fungicides with high risk for selection of resistance that would limit BFR control.

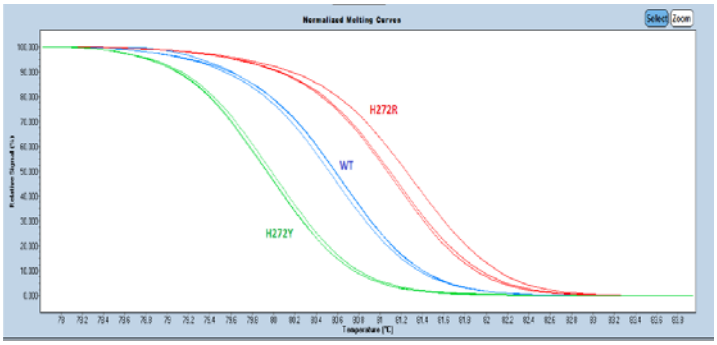

Figure 5. High-resolution melting (HRM) analysis for detection of *sdh*B mutations in *Botrtytis cinerea* isolates from strawberry. The primers F4/R4 identified the mutations H272R (red lines) and H272Y (green line).

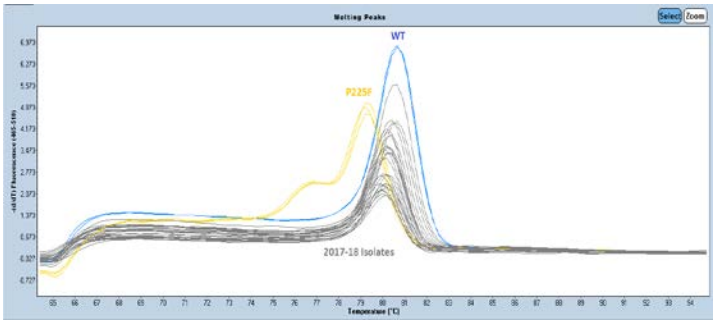

Figure 6. High-resolution melting (HRM) analysis for detection of *sdh*B mutations in *Botrtytis cinerea* isolates from strawberry. The primers F1/R1 identified the mutation P225F (yellow lines).

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Contact

Dr. Natalia A. Peres
UF/IFAS Gulf Coast Research and Education Center
P: 813.419.6605
E: nperes@ufl.edu