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Prevalence of *Botrytis cinerea* at different phenological stages of table grapes grown in the northern region of South Africa

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ABSTRACT

Botrytis cinerea, is one of the major causal agents of postharvest decay in table grapes, contributing to 20% losses world-wide. The aim of this study was to determine the prevalence of *B. cinerea* at different phenological growth stage (full bloom, pea size and fully mature berries) and the impact of agro-climatic sites on pathogen incidence. Droplet digital polymerase chain reaction (ddPCR) was used simultaneously with culture based methods to detect and quantify *B. cinerea*. *Botrytis cinerea* was detected at different phenological stages on asymptomatic grape samples. Prevalence and level of *B. cinerea* differed between phenological stages and sites. Full bloom stage of site B showed the highest prevalence of *B. cinerea* (82.9%) compared to site A (33.3%). For the latter phenological stages (pea size and mature stage), site A had the highest prevalence (100% for both), compared to 35.2% and 44.4% observed at site B, respectively. Furthermore, the *B. cinerea* concentration varied between stages within the two sites. The concentration of *B. cinerea* at site A showed a threefold increase from pea size (2.67 copies μL^{-1} of DNA) to mature berry stage (9.16 copies μL^{-1} of DNA), compared with the decline noted for similar growth stages at site B. *Botrytis cinerea* inoculum build up on asymptomatic grapes could be effectively monitored as the berry develops at critical phenological stages. Preharvest monitoring of the pathogen can help growers improve well-established cultural and management practices, hence limit the risk of postharvest decay.

1. Introduction

Botrytis cinerea, Pers.: Fr, a fungal pathogen that causes gray mold, is regarded as one of the most economically important postharvest pathogens of table grapes (Gubler et al., 2013), blueberries (Rivera et al., 2013), strawberries (Feliziani and Romanazzi, 2016) and other fresh produce (Carisse and Van Der Heyden, 2015). The pathogen, *B. cinerea*, was placed second in a list of the world's top fungal plant pathogens, due to its importance scientifically and economically (Dean et al., 2012). McClellan and Hewitt (1973) described the different infection pathways of *Botrytis*, as one of the infections occurring at full bloom and, through the floral parts (petals, styles, stigmas or stamen). Established fungal hyphae of *B. cinerea* remains dormant during pre-harvest phenological stages until conducive environmental conditions such as fruit injuries and high sugar content enable pathogen proliferation (Romanazzi et al., 2016). Infections of *B. cinerea* that are not detected at harvest, during packing and/or transportation may lead to the growth and subsequent spread of the disease in table grapes postharvestly even when stored at low temperature (−0.5 °C), thereby, reducing the market value of the product (Crisosto et al., 2002; Celik et al., 2009). Abundant sporulation may arise from a single infected berry and contaminate the entire batch of grapes (Romanazzi et al., 2016) leading to the manifestation of the fungus later in the supply chain. As a result, the pathogen is responsible for severe economic damage accounting for 20% postharvest loses world-wide, valued between 10 and 100 billion Euros per year (Anonymous, 2015).

To reduce such losses, several technologies are available for the control of gray mold including the use of fungicides (Feliziani et al., 2014) and sulfur dioxide (Gándara-Ledezma et al., 2015). Despite pressure to develop alternative applications, some registered active ingredients are still used to control gray mold on different crops, including table grapes. However, several conventional fungicides have been banned across the world especially in Europe (Romanazzi et al., 2016), a market accessed by the South African table grape industry. In this context early, rapid, and accurate detection of *B. cinerea* in table grapes is essential for, amongst others, developing disease prediction models and alternative control applications.

Among the many culture independent approaches recently used (Saito et al., 2013; Jongman et al., 2017), a highly sensitive detection tool, the droplet digital Polymerase Chain Reaction (ddPCR) which does not require calibration standards (Flatschart et al., 2015; Koppel and Bucher, 2015; Pinheiro et al., 2012) has since been introduced. This

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recent technology was commercialised to allow precise quantification and detection of the target nucleic acid in a sample. It determines absolute concentrations of nucleic acid copies in discrete, volumetrically defined, water-in-oil droplet partitions of a sample into 20,000 smaller segregated reactions. Then using a standard PCR reaction method, the target is amplified in each partition and individually counted as positive or negative by the associated target dependent florescence signal. It further offers a simple workflow capable of generating highly stable partitioning of DNA molecules (Pinheiro et al., 2012).

Most studies using ddPCR have mainly focused on clinical research areas such as cancer (Albano et al., 2015; Beltrame et al., 2015; Combaret et al., 2015) and human saliva (Bahn et al., 2015). Other applications of the ddPCR include quantification of potato bacterial pathogens (Dreo et al., 2014), *Phytophthora nicotianae* (Timbo et al., 2004), bovine viral diarrhea viruses (Flatschart et al., 2015), and analysis of genetically modified organisms (Koppel and Bucher, 2015). However, to our knowledge its application in plant pathology is still new and more specifically in table grape or other fruit types in pre-harvest environments is limited.

In our study, the ddPCR, was used to detect and quantify *B. cinerea* in asymptomatic table grape tissues. The aim of the study was to determine the prevalence of *B. cinerea* using the ddPCR absolute quantification method at different berry developmental stages. In addition, the effect of different agro-climatic sites on prevalence and incidence of the pathogen was determined.

2. Materials and methods

2.1. Site description and sample collection

Samples were collected from vineyards located in the northern table grape production region of South Africa during the 2015/16 season. Two commercial farms (site A and B) were selected for this study. These two sites differ with respect to geographic location and climatic conditions, as described in Carmichael et al (2017). Site A is at a lower altitude (899 m above sea level) than site B (1123 m above sea level). Annually, site B receives higher average rainfall (350–700 mm) than site A (150–350 mm). The cultivar used was 'Crimson Seedless' grafted on R110 rootstock. Other crops cultivated at both sites included citrus and water melon.

A split plot design was applied, with six replicates per site and three subplots per replicate. Three experimental vines were randomly selected in each subplot. A total of 263 samples comprising of full bloom (stage 23) ($n = 47$), pea size (stage 31) ($n = 108$) and mature stages (stage 38) ($n = 108$) according to the modified E-L scale (Combe, 1995) were collected. Sampling was done at three points around the vine, representing East, West and inside canopy to harmonise the effect of micro-climate per sample. Following the same strategy, bioaerosol samples were collected by direct impaction using an air sampler (SAS Super 100, Cherwell laboratories, England). All samples were collected aseptically with disposable sterile gloves, placed in sterile brown paper bags and were immediately transported to the laboratory in a cooler box with ice packs. Analyses were initiated within 24 h. Weather data to define the differences for the two sites at the time of sampling was obtained from the South African Weather Services.

2.2. Measurement of physiological maturity parameters at harvest

Berry mass (g), firmness (N), and total soluble solids (TSS) (%) were measured on five individually selected berries of each sample at commercial harvest stage. Berries were carefully detached by cutting the pedicels. For berry mass, individual berries were weighed on a calibrated scale (Mettler instrumente AG CH-8606 Greifensee - Zürich, Switzerland). Firmness was determined using a penetrometer. The berry diameter was determined using a tape measure along the transversal section of each berry. Total soluble solids (%) were measured from extracted juice using a hand digital refractometer (PR-32, Atago, TSS 0–32 %, Palette, Tokyo, Japan).

2.3. Isolation of Botrytis cinerea from table grape flowers and berries

Methods described by Diguta et al. (2010) were followed with modifications. Table grape samples (flowers and berries), 25 g, were placed in a beaker containing 225 mL sterile peptone buffered water (PBW) (Merck, Johannesburg, South Africa) supplemented with 0.025% (v/v) Tween 80 (Associated Chemical Enterprises, Johannesburg). This was then partially submerged in an ultrasonic water bath (Labotec, Johannesburg) sonicated for 5 min. at 25 °C to dislodge microbes. A 1 ml of the washing was used in a standard dilution series in PBW up to 10^4. Subsequently 0.1 ml washing was plated on 90 mm plates with Malt Extract Agar (Merck, Johannesburg) (detection limit, 1 $CFUg^{-1}$). All plates were incubated at 25 °C for four days, after which fungal colonies were counted and grouped according to visual appearance and representatives of each group were isolated and purified. All isolated fungi were maintained in sterile water kept at room temperature. The remaining microbiota washing (224 ml) was concentrated by filtration through a 0.45 µm pore size nitro-cellulose membrane (Sartorius Stedim Biotec, Goettingen, Germany). The filter was then stored at 4 °C before DNA extraction.

2.4. DNA extraction

The DNA was extracted directly from the filter paper using the fungal / bacterial Zymo Research kit (ZymoReseach, USA), as per manufacture's specifications. The eluted DNA was quantified using Qubit® Fluorometer (dsDNA HS (High Sensitivity Assay Kit)) (Lifescience Technology, Johannesburg). Isolated DNA was stored at −20 °C for further experimental procedures.

2.5. Controls and method optimization

To test the sensitivity of the primers, DNA from *Alternaria alternata* (Fr.) Keissl. (PPRI 10993, Biosystematics, Agricultural Research Council, South Africa) isolated from *Solanum lycopersicum* was used as a negative template control. Template DNA of *B. cinerea* isolated from 'Thompson Seedless' table grapes was used as a positive control and identified by the diagnostic centre of the Forestry and Agricultural Biotechnology Institute, University of Pretoria. In order to obtain a clear separation between the negative and positive droplets from the ddPCR output, optimization of the positive control to the best amplitude was necessary. Three dilutions, (1:10, 1:100 and 1:1000) of the positive control with sterile sabex water were tested. The 1:10 diluted positive template had the best amplitude (results not shown), and was used for the ddPCR analysis.

2.6. Droplet digital PCR assays

The DNA from the filter papers was used to detect and quantify *Botrytis cinerea* using the QX100™ Droplet Digital™ PCR system (Bio-Rad, Pleasanton, CA, USA) (detection limit, 1 DNA copy μL^{-1}). The reaction mix for ddPCR consisted of 1 µL DNA, 10 µL Supermix (2 × Q × 200 EvaGreen Mix) (Bio-Rad), 8.6 µL sterile H_2O and 0.2 µL of each primers. Primers specific to *B. cinerea* (Bc3_F: 5′-GCTGTAATTTCAATGTGCAGA ATCC-3′; Bc3_R: 5′-GGAGCAACAATTAATCGCA TTTC-3′) (GenBank: AM233400.1) (Suarez et al., 2005) were used. All samples were analysed in duplicates. After droplet generation, 40 µL of the generated droplet emulsion was loaded into a 96 well PCR plate. PCR was performed in a T100™ thermal cycler (Bio-Rad). The amplification conditions were 5 min. at 95 °C DNA polymerase activation, followed by 39 cycles of 30 s at 95 °C for denaturing and 60 s at 60 °C for annealing and extension, followed by a final hold of 5 min. at 90 °C for droplet stabilisation and cooling to 4 °C. After thermal cycling, the plate was