African Journal of Biotechnology Vol. 11(86), pp. 15251-15257, 25 October, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1079
ISSN 1684-5315 ©2012 Academic Journals

Full Length Research Paper

Early detection of gray mold in grape using conventional and molecular methods

Hala Abdel Wahab[1] and Rania A. A. Younis[2]*

[1]Department of Plant Pathology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.
[2]Department of Genetics, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Accepted 5 September, 2012

Botrytis cinerea **affects grape quality and yield, and can be difficult to manage due in part to non-symptomatic, quiescent infection in berry development. The aim of this study was to develop a dual system for the detection, isolation and quantification of *B. cinerea*. After three days of samples replication on the modified selective medium (mKERS), the results showed a significant infection effect on the majority of inflorescence samples, especially on the small berries which demonstrated *Botrytis* infection in all tested plants and appeared to be highly susceptible to *Botrytis* infection prior to harvest. Moreover, infection variation was determined in almost all inflorescence samples taken from different plants. The real-time PCR assay was used to determine the DNA quantity of *B. cinerea* in each sample tested. A linear relationship was found in these two systems, conventional and molecular assays, to demonstrate the infection of different samples with *B. cinerea*. Although, the real-time PCR assay was highly expensive, it appeared to be more rapid and sensitive than the conventional selective medium assay, allowing both detection and quantification of *B. cinerea* within 3 h. However, conventional assay has an advantage of both detection and isolation of viable cells of *B. cinerea*, which resulted in making a wide collection of different isolates. Furthermore, this conventional assay is cheaper than molecular test, especially when we carry out a routine work. This dual method proved to be selective and sensitive assays and should be used to monitor *Botrytis* infection in the field.**

Key words: *Botrytis cinerea*, inflorescence infection, latent/quiescent infection, real time polymerase chain reaction/real-time quantitative PCR (PCR/qPCR).

INTRODUCTION

Botrytis cinerea is a broad-host-range necrotrophic fungus, responsible for economic losses in fruit, vegetables and flowers, causing soft-rotting symptoms (Jarvis, 1980; Elad et al., 2007). Infection of grape (*Vitis vinifera*) often occurs at bloom time, followed by a period of latency, during which the pathogen is present (quiescent or latent) inside the berry without causing disease symptoms, generally until grape berries begin to ripen. Gray mold causes financial losses for the growers, reducing not only yield but also grape quality. *Botrytis* bunch can be difficult to manage due in part to non-symptomatic, quiescent infection in berry development. For early infection, individual conidia of *B.cinerea* are deposited onto inflorescences (McClellan and Hewitt, 1973; Coertze et al., 2001).

Existing technologies for pathogen detection, although useful for some applications of studying pathogen biology and informing grower decisions, have their limitations including insufficient sensitivity or the inability to distinguish quiescent from active infections (Meyer et al., 2000; Holz et al., 2003; Dewey and Meyer, 2004; Gindro et al., 2005). Furthermore, there is much literature on the detection of microorganisms using PCR (Taylor et al., 2001; Peters et al., 2004; Schena et al., 2004; Okubara et al., 2005; Chilvers et al., 2007). The conventional PCR assays do not allow precise quantification and, hence could not be used to determine if pathogen inoculum is above action thresholds. Recently, the real-time

*Corresponding author. E-mail: raniayounis25@gmail.com.

quantitative PCR (qPCR) applications was developed in the second half of the 1990s (Heid et al., 1996). This new technique has offered the ability of simultaneous detection and quantification of DNA based on nucleic acid sequences and concentrations (Million et al., 2006; Zeng et al., 2006). The qPCR technology has many advantages: quantitative properties, high sensitivity and specificity, which make this technique suitable for routine usage and disease management decisions (Bustin, 2004; West et al., 2008; Postollec et al., 2011). Cadle-Davidson (2008) reported a qPCR method based on Taqman chemistry for monitoring *B. cinerea* infection. However, this protocol uses a long freezing assay protocol and does not include internal control. Celik et al. (2009) also developed a quantitative analysis of *Botrytis* by qPCR but only on artificially contaminated table grapes. Recently, another qPCR method was developed to detect and quantify *Botrytis* in the vineyard (Diguta et al., 2010).

The purpose of this study was to use a dual system for detecting, isolating and quantifying *B. cinerea* in inflorescence samples which have quiescent infection. This would demonstrate the strengths and weaknesses of qPCR in comparison with the selective medium method developed in this study.

MATERIALS AND METHODS

In order to detect and isolate *B. cinerea* from inflorescence samples of grape (Var, Flame Seedless), six inflorescence samples were taken from different plants. Infection detection test was done on axis, peduncles, pedicels and small berries (Table 1) by both conventional and molecular test

Preparation of mKERS medium

The modified selective medium, mKERS, has been developed based on previous published medium, KERS medium (Kritzman and Netzer 1978; Kerssies, 1990). The composition of this medium was: glucose, 20 g; $NaNO_3$, 1 g; KH_2PO_4, 1.2 g; $MgSO_4.7H_2O$, 0.2 g; KCl, 0.15 g; chloramphenicol, 0.05 g; pentachloronitrobenzene 0.02 g; tannic acid, 5 g; $CuSO_4$, 2.2 g; Cabrio Top (fungicide), 0.1 g; agar, 25 g. This medium, with an unadjusted pH of approximately 5.4, was autoclaved at 121°C for 20 min. Various fungal species (*Aspergillus* sp., *Rhizopus* sp. and *Penicillium* sp.), associated with gray mold infection, were obtained from Plant Pathology Department, Faculty of Agriculture, Ain Shams University and grown on PDA medium as a control. For each fungus, mycelial plug-inoculated plates were incubated at 23°C until mycelia had reached the edge of the plates. These different fungi were then transferred to mKERS medium. The mycelium growth and the brown halo formation of either *B. cinerea* or other fungal species were also noted.

Detection of *B. cinerea* on mKERS medium

Inflorescence samples were tested for *B. cinerea* detection using mKERS medium. As there is no need to decontaminate the samples before replication using such selective medium, all samples were only dipped separately in sterile water for 5 min, dried on paper towels and then replicated on mKERS medium.

First, preliminary test was done to detect *Botrytis* infection in all inflorescence samples in order to evaluate the clarity of brown halo formation on such developed mKERS medium. For statistical analysis, 15 pieces of each inflorescence sample (axis, peduncles, pedicels and small berries) were plated onto mKERS medium/plate, and then replicated in eight plates (about 120 pieces in total for each sample type). All replicates were incubated at 23°C for 3 to 28 days, during which the samples were examined daily for development of *B. cinerea*. Cultures were transferred to potato dextrose agar (PDA; Difco) and then identified morphologically by the light microscope. All *B. cinerea* isolates were preserved in paraffin oil at 4°C.

Statistical analysis

Statistical analysis of data was determined by analysis of variance. Two ways of analysis were done: the average number of infected inflorescence samples either within each plant or among different plants using the General Linear Model (GLM) procedure of SAS user's Guide (SAS Institute, 1996). The level of significance was set at $p<0.05$.

DNA extraction

DNA extraction from inflorescence samples was performed using AxyPrep Multisource Genomic DNA Miniprep Kit, cat. No. AP-MN-MS-GDNA-50, according to the manufacture manual.

Real-time PCR amplification

Specific *B. cinerea* primers targeting the ribosomal region between 28S and 18S genes (intergenic spacer) reported by Rigotti et al. (2002) were used: Bc424f: 5'-GCT TCC CCC GTA TCG AAG A-3'; Bc491r: 5'-CGA ACG GCC AGG TCA TCT-3', and targeting actin CrActin-F: 5'-GGC TGG ATT TGC TGG AGA TGA T-3' CtActin-R 5'-TAG ATC CTC CGA TCC AGA CAC TG-3' (Yuling et al., 2007). All primers were purchased from Metabion, Germany. The 5 µl from DNA sample (10 ng/µl) was mixed in a final volume of 25 µl with 2X Maxima SYBR Green supermix (Fermentas, Lithuania), water and primer mixture at 0.56 µM to detect intergenic spacer (IGS) regions of the nuclear ribosomal of *B. cinerea* or to detect actin gene in grape (*V. vinifera*). Reactions were performed in an Agilent-stratagene real-time PCR Mx3000P QPCR apparatus. We used a program of: 10 min at 95°C, followed by 40 cycles of three-steps amplification run at 95°C for 30 s, 55°C for 45 s and 72°C for 45 s for amplifying IGS region, and 10 min at 95°C, followed by 40 cycles of three-step amplification run at 95°C for 30 s, 45°C for 45 s, and 72°C for 45 s for amplifying actin gene. All reactions were performed in triplicate. The cycle threshold (C_t) was determined automatically using MxPro QPCR software after setting the baseline at 100.

Internal control for DNA extraction and amplification

An internal control was included in the assay by detecting actin gene (Accession number: XM_002282480) in grape (*V. vinifera*). The detection of actin gene was done to ensure that controls for DNA preparation and PCR amplification were available and there were no inhibitors.

Melting curve analysis

For qPCR with SYBR-Green I, melting curves were programmed in

Table 1. Determination of the average number of inflorescence samples infected with *B. cinerea*.

Plant	Inflorescence sample (Average number)				
	Axis	Pedicel	Peduncle	Small berry	F value
1	0.058^{Aa}	0.000^{Cb*}	0.000^{Bb*}	0.043^{Ba}	0.053
2	0.145^{Aa}	0.000^{Cb*}	0.000^{Bb*}	0.019^{Bb}	0.0005
3	0.000^{Ab*}	0.059^{Bb}	0.464^{Aa}	0.033^{Bb}	0.035
4	0.000^{Ab*}	0.091^{Bb}	0.180^{Ba}	0.034^{Bb}	0.036
5	0.108^{Aa}	0.158^{Aa}	0.000^{Ba*}	0.021^{Ba}	0.315
6	0.000^{Ab*}	0.096^{Bab}	0.127^{Bab}	0.233^{Aa}	0.126
F value	0.153	0.002	0.004	0.0001	

A, B, C, Means with different letters within the same column are significantly different ($p<0.05$); a, b, means with different letters within the same row are significantly different ($p<0.05$). * 0.00 = no detected infection with *B. cinerea*.

order to check the expected amplification product. The thermal protocol for dissociation was defined as 15 s at 95 °C, 20 s at 60°C and 20 min slow ramp between 60 and 95°C, after the PCR program. The data for dissociation curve was captured during this slow ramp. The melting curve was visualized with the software in the dissociation window using the corresponding MxPro QPCR software procedure (Agilent).

Quantification assays

Standard curves were calculated for quantification purposes using ten-fold serial dilutions of purified DNA from *B. cinerea* in sterile distilled water covering the range of 10 to 1×10^{-6} ng per reaction. DNA concentration was determined fluorometrically using the Fluorescent DNA quantitative kit (Bio-Rad, California, USA) and a SmartSpec-Plus Spectrophotometer (Bio-Rad, California, USA). PCR amplification reactions were carried out in triplicate in three independent experiments. Standard curves were generated by plotting the genomic DNA from *B. cinerea* against the C_t values exported from the Agilent-stratagene real-time PCR Mx3000P QPCR apparatus for each plate. The C_t values for unknown samples were extrapolated from standard curves.

RESULTS

Determination of infected samples on mKERS medium

Preliminary study have demonstrated the appearance of brown halo formation surrounding the infected inflorescence samples after 3 days of incubation on mKERS medium, while the other fungal genera used as negative controls did not show any brown halo formation during the same period. Table 1 showed statistical analysis of the average number of infected samples of inflorescence within the same plant and among different plants. Regarding the F values of the average number of each infected inflorescence sample (axis; peduncles; pedicels or small berries) among different plants (1-6), statistical analysis indicated that *Botrytis* infection affected pedicels, peduncles and small berries significantly in all tested plants as they showed significant differences ($p<0.05$) between them. In contrast, they showed insignificant differences ($p > 0.05$) for axis indicating that there was no effect of *Botrytis* infection on axis in all tested plants (Table 1). Moreover, the results showed that *Botrytis* infection mostly occurred in pedicels, peduncles and small berries taken from plants 5, 3 and 6, respectively. The results indicate that there was no *Botrytis* infection in axis taken from plants 3, 4 and 6, pedicels and peduncles taken from plants 1 and 2, peduncles taken from plants 1, 2 and 5 while, the small berries of all these plants showed infection with *B. cinerea* (Table 1). Regarding the F values of the average number of all infected inflorescence samples (axis, peduncles, pedicels and small berries) within each tested plant, data analysis exhibited significant differences of infected inflorescences among plants 1, 2, 3 and 4 ($p<0.05$). The plants 1 and 2 demonstrated the highest *Botrytis* infection in axis, while they did not show any infection in pedicels and peduncles (Table 1). In addition, the plants 3 and 4 showed the highest *Botrytis* infection in peduncles, whereas they did not show any infection in axis (Table 1). Moreover, the plants 5 and 6 revealed insignificant differences among different inflorescence samples for *Botrytis* infection (Table 1).

As the statistical analysis demonstrated the highest significant variation ($p = 0.0001$) among the small berries in different plants (Table 1), these samples were subjected to test for quantifying the DNA of *B. cinerea* residing in the tissues of small berries in all tested plants (A1-A6) (Table 2) by qPCR. Since the plant 6 showed insignificant variation among different inflorescence samples, but exhibited the highest *Botrytis* infection in both small berries and peduncles (Table 1), qPCR assay was also tested on peduncles (A7) (Table 2).

Calibration curves

Genomic DNA obtained from *B. cinerea* was used as a template for qPCR with primers Bc424f and Bc491r. As expected, the PCR product melting temperature was 87 ± 1.00°C as shown in Figure 1. The standard curve generated with the Bc424f/Bc491r pair in the conditions

Table 2. Determination of DNA concentration of *B. cinerea* in inflorescence samples using qPCR.

Sample number	C_t (dR)	Concentration (pg)
A1	26.24	10
A2	26.54	10
A3	18.29	3410
A4	21.1	490
A5	17.65	5310
A6	20.79	610
A7	19.52	1460
NTC	38.49	0

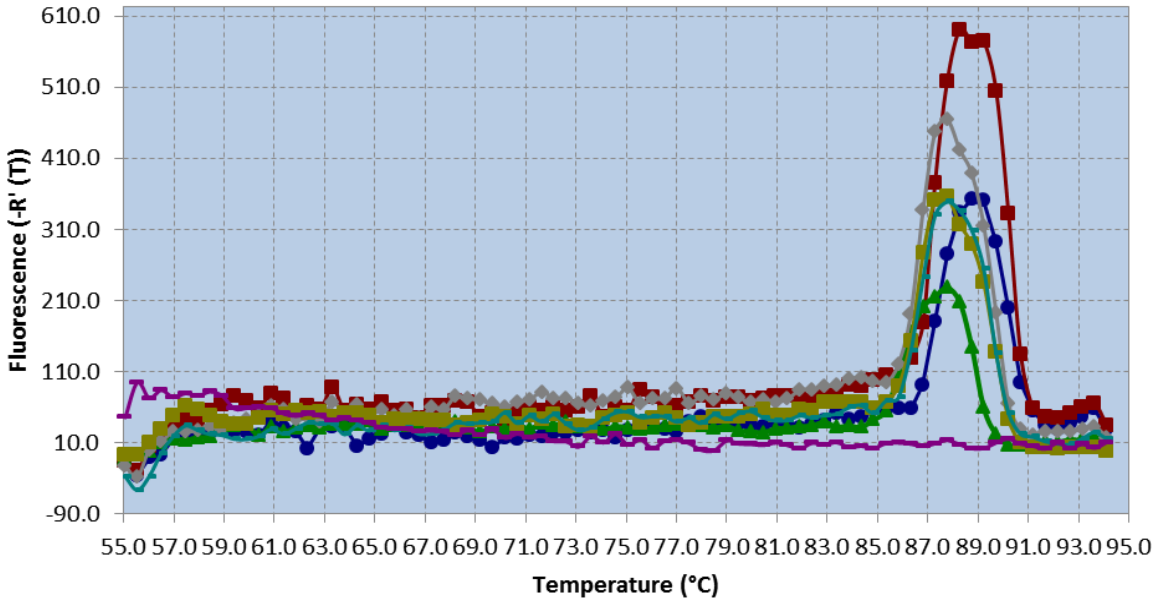

Figure 1. Dissociation curve obtained from genomic DNA of *B. cinerea* with SYBR Green I.

described previously was shown in Figure 2. The standard curve for *B. cinerea* was generated by plotting the log of DNA (pg) against the C_t value determined by qPCR. Linearity was observed across the whole range used and the very high correlation coefficient (R^2 = 1) indicated very low inter assay variability. The slope of the standard curve was -3.32, which corresponded to an amplification efficiency of 99%. The limit of detection was defined as the lowest pathogen quantity that could be detected using SYBR Green qPCR method. Under conditions that include SYBR Green, the maximum C_t value that could be used was 38, which corresponded to a DNA concentration of 2.3 pg.

The detection and quantification of *B. cinerea* infected inflorescence samples

To obtain an accurate estimate of the target DNA molecules (*B. cinerea* DNA) in the inflorescence samples, different controls were needed: controls to test the efficiency of the PCR itself (PCR positive control) and controls to demonstrate the effect of the grape matrix, which includes natural inhibitory compounds, on the recovery of DNA from the *B. cinerea*, as well as its effect on PCR (PCR internal control). To achieve these goals, the actin gene was used as an internal control and the standard curve was created as shown in Figure 3. From the standard curve of IGS of *B. cinerea* obtained previously, we used C_t value to quantify the DNA concentration of *B. cinerea* by pg in inflorescence samples as shown in Table 2.

The absolute quantification qPCR method was used to assess the level of *B. cinerea* infection in each small berries sample as shown in Figure 4. The DNA concentration of *B. cinerea* residing in each small berries sample (A1-A6) and peduncles sample (A7) was given in Table 2. The lowest DNA concentration appeared in

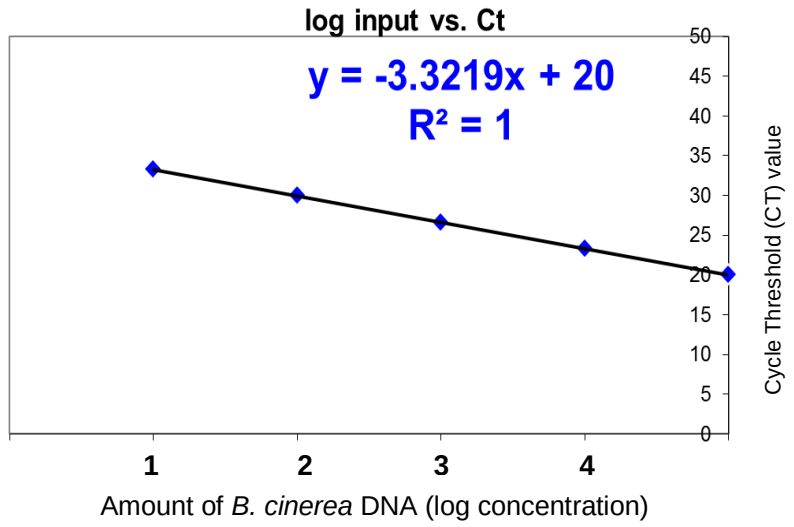

Figure 2. Standard curve generated from the amplification of 10-fold dilutions of target genomic DNA of *B. cinerea*. This curve revealed a good linear relationship ($R^2 = 1$) between the $\log_{10}$ value of the starting DNA concentration and the threshold cycle.

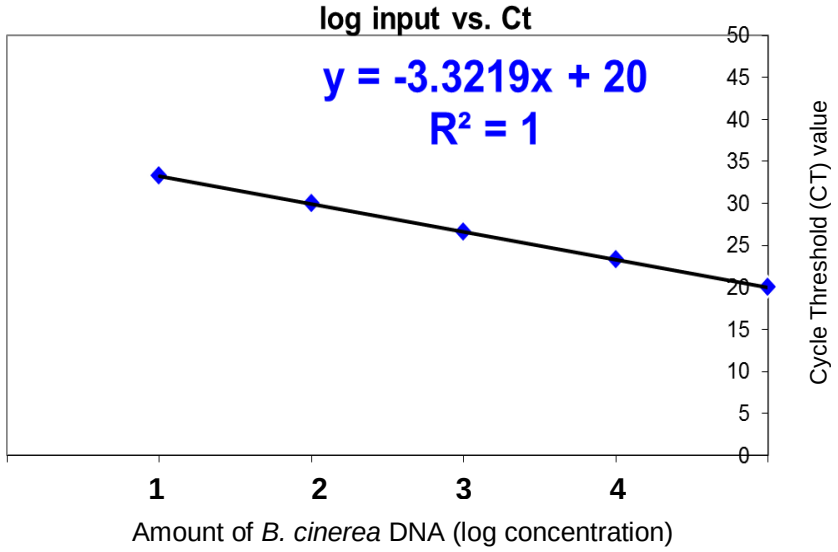

Figure 3. Standard curve generated from the amplification of 10-fold dilutions of internal control (actin). This curve revealed a good linear relationship ($R^2 = 1$) between the $\log_{10}$ value of the starting cell concentration and the threshold.

samples A1 and A2 which showed low level of *B. cinerea* (10 pg DNA). In contrast, the highest DNA concentration revealed in samples A3 and A5 (3410 and 5310 pg respectively) indicated a high level of *B. cinerea* residing in these samples. Moreover, the DNA concentration of B.cinerea residing in peduncles sample (A7) appeared to be higher than that obtained from the small berries sample (A6) taken from the same plant (6).

DISCUSSION

In this study, a dual method was used as a simple and reliable tool for the detection, isolation and quantification of *B. cinerea*. To indicate relative disease pressure in each inflorescence sample, the use of conventional mKERS assay allowed the calculation of the average number of infected inflorescence samples and isolation of

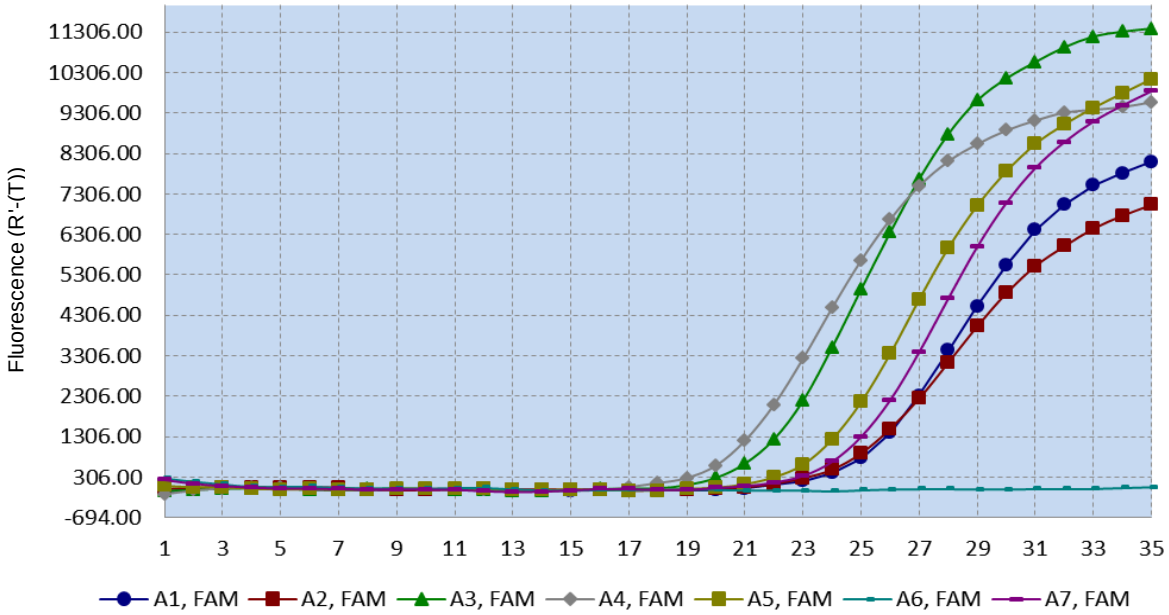

Figure 4. Amplification plots obtained from genomic DNA of inflorescences with SYBR-Green I.

the pathogen. Variation of the average number of infected inflorescence samples with *B. cinerea* was investigated depending on plant and inflorescence part as the plants 3 and 4 showed the highest *Botrytis* infection in peduncles which did not show any *Botrytis* infection in both plants 1 and 2. No effect of *Botrytis* infection was shown in axis in 50% of the tested plants. Moreover, all tested plants demonstrated infection in small berries with high significant differences ($p = 0.0001$) indicating an increased susceptibility of berry's tissues to *Botrytis* infection. This means that sample type had a clear impact on *B. cinerea* infection which presents latently on inflorescence organ. In all cases, each inflorescence part could be an important source of *Botrytis* infection resulting in gray mold disease. Furthermore, qPCR assay demonstrated compatible results with the previous conventional method in some cases. This was indicated as the lowest DNA concentration (10 pg DNA) of *B. cinerea* which appeared to be in the small berries taken from plant 2 which previously demonstrated the lowest number of infected samples on mKERS medium. In contrast, the DNA concentration of *B. cinerea* was highest (5310 pg) in the small berries taken from plant 5 which proportionally demonstrated a low number of infected samples using mKERS medium. This was due to the existence of numerous non viable cells of *B. cinerea* (in berry's tissues) which were unable to grow on the medium. Although, the molecular test is more sensitive and rapid for the detection and quantification of pathogen, the conventional method is also necessary for the detection and isolation of the viable pathogen cells leading to isolates collection that would be used for further analysis. Nevertheless, the qPCR assay could be useful to detect and quantify fungal DNA, regardless its viability. Indeed, these two assays, conventional and molecular assays, have a complementary or dual advantage for studying latent infection. Furthermore, qPCR test should serve as a decision-making tool in vineyards as it could monitor the evolution of *B. cinerea* attack prior to harvest and consequently optimize the concentration and the number of sprays of fungicides.

In light of recent studies, numerous quantitative assays using real-time PCR have been developed to specifically detect microbial targets in many types of samples, including, but not limited to, molds (Alaei et al., 2009; Carisse et al., 2009; Luo et al., 2010). The qPCR technique had the advantage of the detection of *B. cinerea* over classic culture-based methods as it enhanced specificity and reduced processing time (2 to 3 h), leading to quicker results. In addition, previous studies had showed that the use of immunoassays lacks sensitivity to detect small quantities of pathogen (Meyer et al., 2000; Dewey and Meyer, 2004) and the use of conventional PCR lacks the precision for DNA quantification (Gindro et al., 2005). Nevertheless, molecular technique is always more expensive than conventional assay. Although classical method, using selective medium, is too slow (Martinez et al., 2010), as shown here in this study (results obtained after three days), this technique would not be useless as we need a low cost method for a routine work in order to detect the pathogen viability and make isolates collection necessary for further studies. Indeed, we should use mKERS medium combined with qPCR assays for detection, isolation and quantification of *B.cinerea*. Thus, such dual method could be suitable for the risk assessment of *Botrytis* gray mold at the field level.

ACKNOWLEDGEMENTS

This project was supported financially by the Science and Technology Development Fund (STDF), Egypt, Grant No 2131. We are grateful to Dr. Sherif Edris and Dr. Mohamed Mostafa for their assistance in this paper.

REFERENCES

Alaei H, Baeven S, Maes M, H¨ofte M, Heungens K (2009). Molecular detection of *Puccinia horiana* in *Chrysanthemum_morifolium* through conventional and realtime PCR. J. Microbiol. Meth. 76:136-145.

Bustin SA ed. (2004). A-Z of Quantitative PCR. IUL Biotechnology Series, La Jolla, CA Series 5.

Cadle-Davidson L (2008). Monitoring pathogenesis of natural *Botrytis cinerea* infections in developing grape berries. Am. J. Enol. Viticult. 59:387-395.

Carisse O, Tremblay DM, L´evesque CA, Gindro K, Ward P, Houde A (2009). Development of a TaqMan real-time PCR assay for quantification of airborne conidia of *Botrytis squamosa* and management of Botrytis leaf blight of onion. Phytopathol. 99:1273-1280.

Celik M, Kalpulov T, Zutahy Y, Ish-shalom S, Lurie S, Lichter A (2009). Quantitative and qualitative analysis of Botrytis inoculated on table grapes by qPCR and antibodies. Postharvest Biol. Tec. 52:235-239.

Chilvers MI, du Toit LJ, Akamatsu H, Peever TL (2007). A real-time, quantitative PCR seed assay for *Botrytis* spp. that cause neck rot of onion. Plant Dis. 91:599-608.

Coertze S, Holz G, Sadie A (2001). Germination and establishment of infection on grape berries by single airborne conidia of *Botrytis cinerea*. Plant Dis. 85:668-677.

Diguta CF, Rousseaux S, Weidmann S, Bretin N, Vincent B, Guilloux-Benatier M, Alexandre H (2010). Development of a qPCR assay for specific quantification of *Botrytis cinerea* on grapes. FEMS Microbiol. Lett. 313:81-87.

Dewey FM, Meyer U (2004). Rapid, quantitative tube immunoassays for on-site detection of *Botrytis*, *Aspergillus* and *Penicillium* antigens in grape juice. Analytica Chimica. Acta. 513:11-19.

Elad Y, Williamson B, Tudzynski P, Delen N (2007). *Botrytis* spp. and diseases they cause in agricultural systems - An introduction. *Botrytis:* Biology, Pathology and Control. Netherlands, Springer. pp. 1-8.

Gindro K, Pezet R, Viret O, Richter H (2005). Development of a rapid and highly sensitive direct-PCR assay to detect a single conidium of *Botrytis cinerea* Pers.: Fr *in vitro* and quiescent forms in planta. Vitis 44:139-142.

Heid CA, Stevens J, Livak KJ, Williams PM (1996). Real time quantitative PCR. Genome Res. 6:986-994.

Holz G, Gutschow M, Coertze S, Calitz FJ (2003). Occurrence of *Botrytis cinerea* and subsequent disease expression at different positions on leaves and bunches of grape. Plant Dis. 87:351-358.

Jarvis WR (1980). Epidemiology. In: Coley-Smith JR, Verhoeff K, Jarvis WR,eds. The Biology of *Botrytis.* London: Academic Press. pp. 219-250.

Kerssies A (1990). A selective medium for *Botrytis cinerea* to be used in a spore-trap. Netherlands J. Plant Pathol. 96:247-250.

Kritzman G, Netzer D (1978). A selective medium for isolation and identification of *Botrytis* spp. from soil and onion seed. Phytoparasitica 6:3-7.

Luo Y, Gao W, Doster M, Michailides TJ (2010). Quantification of conidial density of *Aspergillus flavus* and *A. parasiticus* in soil from almond orchards using real-time PCR. J. Appl. Microbiol. 106:1649-1660.

Martinez J, Simon V, Gonzalez B, Conget P (2010). A real-time PCR-based strategy for the detection of Paenibacillus larvae vegetative cells and spores to improve the diagnosis and the screening of American foulbrood. Lett. Appl. Microbiol. 50:603-610.

McClellan WD, Hewitt B (1973). Early botrytis rot of grapes: Time of infection and latency of *Botrytis cinerea* Pers. in *Vitis vinifera* L. Phytopathology 63:1151-1157.

Meyer UM, Spotts RA, Dewey FM (2000). Detection and quantification of *Botrytis cinerea* by ELISA in pear stems during cold storage. Plant Dis. 84:1099-1103.

Million L, Reboux G, Bellanger P, Roussel S, Sornin S, Martin C, Deconinck E, Dalphin JC, Piarroux R (2006). Quantification de *Stachybotrys chartarum* par PCR en temps réel dans l'environnement domestique, hospitalier, et agricole. J. Mycol. Med. 16:183-188.

Okubara PA, Schroeder KL, Paulitz TC (2005). Real-time polymerase chain reaction: Applications to studies on soil borne pathogens. Can. J. Plant Pathol. 27:300-313.

Peters IR, Helps CR, Hall EJ, Day MJ (2004). Real-time RT-PCR: Considerations for efficient and sensitive assay design. J. Immunol. Methods 286:203-217.

Postollec F, Falentin H, Pavan S, Combrisson J, Sohier D (2011). Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiol. 28:848-861.

Rigotti S, Gindro K, Richter H, Viret O (2002). Characterization of molecular markers for specific and sensitive detection of Botrytis cinerea Pers.: Fr. in strawberry (Fragaria x ananassa Duch.) using PCR. FEMS Microbiol. Lett. 209:169-174.

SAS Institute (1996). SAS/STAT Users Guide: Statistics. SAS Institute, Inc., Cary, NC., USA p. 7.

Schena L, Nigro F, Ippolito A, Gallitelli D (2004). Real-time quantitative PCR: A new technology to detect and study phytopathogenic and antagonistic fungi. Eur. J. Plant Pathol. 110:893-908.

Taylor E, Bates J, Kenyon D, Maccaferri M, Thomas J (2001). Modern molecular methods for characterisation and diagnosis of seed-borne fungal pathogens. J. Plant Pathol. 83:75-81.

West JS, Atkins SD, Emberlin J, Fitt BDL (2008). PCR to predict risk of airborne disease. Trends Microbiol. 16:380-387.

Yuling Y, Shimano F, Ashihara H (2007). Involvement of rapid nucleotide synthesis in recovery from phosphate starvation of *Catharanthus roseus* cells. J. Exp. Bot. 58:1025-1033.

Zeng QY, Westermark SO, Rasmuson-Lestander Å, Wang XR (2006). Detection and quantification of *Cladosporium* in aerosols by real-time PCR. J. Environ. Monit. 8:153-160.