Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 1, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

COMPARISON OF CLASSICAL VERSUS qPCR METHODS IN THE DETECTION OF *Botrytis cinerea* IN RASPBERRIES

Quthama AL ZAIDI, Florentina BADEA, Camelia Filofteia DIGUȚĂ, Florentina MATEI

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: alzaidy2004.n3@gmail.com

Abstract

Botrytis cinerea, is a necrotrophic fungus that causes gray mold on fruits and vegetables. This mold ranked second in the list of the top ten fungal pathogens. The aim of this study was to develop a system for rapid and accurate identification and quantification of B. cinerea on fresh raspberry by quantitative real-time PCR (qPCR) and a comparison was made between traditional methods (cell culture) and molecular tool. Raspberry samples were purchased from the local supermarkets. Raspberry diluted samples were cultivated on Dichloran Rose Bengal Chloramphenicol agar selective medium used for the growth of mold, then DNA was extracted from the same samples and qPCR was done. In classical methods (cell culture) the result was negative (no fungal colony growth). By the qPCR technique, B. cinerea was detected in two out of the three samples. In qPCR technology, the test proved to be selective, rapid and sensitive and can be used for the diagnosis of B. cinerea.

Key words*: raspberries, Botrytis cinerea, qPCR*

INTRODUCTION

The fungal genus *Botrytis* contains many species that vary in their biology, ecology, physical characteristics, and host range. About 30 species have been characterized as a result of advancements in molecular genetics, particularly the creation of pertinent phylogenetic markers. Relevant losses in a variety of commercially significant horticultural and floral crops are caused by *Botrytis species* (Poveda et al., 2020). The most often researched polyphagia fungus is *Botrytis cinerea* Pers.: Fr. The *Botrytis* community decided to use *Botrytis cinerea* as the generic name in 2013 at the *Botrytis* Symposium in Bari, Italy, despite the fact that *B. cinerea* is the name of the asexual stage (anamorph) and *Botryotinia fuckeliana* is the name of the sexual stage (Fillinger et al., 2016), sclerotic grow within degenerating host tissues as part of this fungus's life cycle, serving as a crucial survival mechanism. In temperate areas, sclerotic begins to grow in the early spring to produce conidiophores and multinucleate conidia. This fungus reproduces sexually by spermatizing sclerotic, which results in the development of apothecia and asci. Eight binucleate ascosporic are the main source of inoculum inside a crop (Holz et al., 2007). The steps of the *B. cinerea* infection process are typically listed as follows: penetration of the host surface, death of the host tissue/primary lesion formation, lesion expansion/tissue maceration, and sporulation (Choquer el at., 2007). The symptoms caused by this necrotrophic fungus are extremely varied and difficult to generalize to plant organs and tissues. The most common symptoms of soft rots on leaves and soft fruits are collapse, water infiltration of the parenchyma tissues, and the quick emergence of grey masses of conidia. When it comes to many fruits and vegetables, the illness frequently starts on adjacent senescent flowers before spreading as a soft rot and harming nearby developing fruit (blossom-end rot), including raspberries, strawberries and apples. More than 50 hosts have been reported being infected by seed-borne diseases, which frequently start by rotting the herbaceous stems at ground level and also cause additional soft-rot lesions to emerge on leaves and pods (Williamson et al., 2007). In this sense *B. cinerea* is an intriguing model system for necrotrophic diseases, although studying it can be challenging due to the frequent changes in karyotypes among naturally strains (Fillinger et

al., 2016). One of the most thoroughly researched necrotrophic fungal diseases, *B. cinerea*, causes gray mold rot in more than 500 plant species (Williamson et al., 2007; Mercier et al., 2019). Raspberry, grape, strawberry, and tomato are just a few of the commercially significant crops which this disease has a terrible effect on (Dean et al., 2012) and can exist in the stems, leaves, flowers, fruits, and seeds of several plant species. It may manifest overt illness symptoms prior to harvest or after harvest (Fillinger and Elad, 2016). In addition, one of the most significant post-harvest infections in fresh fruits and vegetables has been identified as being induced by *B. cinerea* (Zhang et al., 2014). *B. cinerea* causes annual economic losses that easily surpass $10 billion in the world (Weiberg et al., 2013). *B. cinerea* has been ranked as the second major plant pathogen due to its scientific and economic value (Dean et al., 2012). Because *B. cinerea* has a wide host range, a variety of assault methods, and both asexual and sexual phases that can live in either favorable or unfavorable conditions, it is challenging to control (Fillinger and Elad, 2016). Conidia, the asexual spores of *B. cinerea*, are easily disseminated by wind or water, and sclerotic, the sexual spores of *B. cinerea*, are necessary for survival in adverse environments (Brandhoff et al., 2017). The raspberry is a third-generation fruit with an improved flavor, excellent nutritional and therapeutic properties. It is a non-climacteric variant (McDougall et al., 2017; Xing et al., 2018). But raspberries also have strong respiration rates, delicate fruit tissues, and are vulnerable to mechanical harm when being harvested. This results in softer tissues that are more vulnerable to infection by pathogens after harvesting, which makes shelf life and storage difficult (Tezotto-Uliana et al., 2013; Guerreiro et al., 2015). Researchers have conducted significant research regarding the identification and occurrence of postharvest raspberries diseases, in order to solve the issues of postharvest storage and the management of raspberries fresh supply of (Carisse et al., 2018; López et al., 2016).

The production of organic fruit has steadily expanded over the past few decades, as has its market share in the global food industry. The growth of strawberries, blueberries, and raspberries fruits that are significant exports from Central Europe is driven in large part by rising consumer demand for organic fruit production. Fungi-related infections, which affect plants and fruits from the time of sowing to the point of market sale and result in unanticipated product degradation, are the main worries of food producers. Identifying the causal agents of soil-borne diseases has been accomplished using morphological methods of identification. These conventional techniques are time consuming, are prone to mistakes, and may lead to false results. Due to these drawbacks, more effective approaches are increasingly being adopted, such as using molecular-level analytical techniques. Polymerase chain reaction (PCR)-based techniques enable the rapid amplification of specific DNA fragments (Malarczyk et al., 2019). Experts investigating morphological characteristics of microorganisms, such as colony appearance and the formation of asexual structures on microbiological media or on the host, are classic techniques of identifying fungal pathogens. Samples that have been isolated on suitable agar media can be examined under a light microscope to detect even the smallest features. Colonies are evaluated and counted may be assessed using this procedure (classic techniques), which takes a lot of time. On occasion, colonies must fulfil specific requirements in order to produce conidia, which can impede laboratory work-flow (Frąc et al., 2015). For identification, selective media have been suggested and deployed. Although most species are not host specific and many fungi may live on plants, the identification of external infection symptoms caused by fungus on their hosts can also be utilized to confirm the pathogen. A correct identification based solely on the morphology of the colonies is highly challenging, because to the lack of carrier specificity and symptom changes between plant populations at different latitudes. Interpretations of the pathogen's morphology are also arbitrary and heavily dependent on personal experience. The human aspect could result in erroneous pathogen detection and misguided plant protection measures (Malarczyk et al., 2019). The aim of this study was to compare the classical traditional methods for the detection of *B. cinerea* in raspberry fruits and compare them with qPCR technique, show the pros and cons

and which are better and find the best and fastest methods for the detection of *B. cinerea* in raspberry.

MATERIALS AND METHODS

Raspberry samples were purchased from different food stores of different international origins, taking into account the most popular markets. The samples codes and origin are presented below:
T1: Mega Image - origin: Morocco
T2: Shop & Co. - origin: Portugal
T3: Lidl- origin: Spain
Sample preparation: 5 g of each sample were placed in a sterile vial homogenized with 45 ml of distilled water. They were cultured on Dichloran Rose Bengal Chloramphenicol agar. The total DNA was extracted from the same samples to be used for the qPCR technique.

Morphological characterization

A sample of 1 ml was taken from each sample and diluted 4 times serial dilution (1, 2, 3 and 4) were worked out, then cultured on the selective medium (Dichloran Rose Bengal Chloramphenicol Agar) used for mold growth and placed in the incubator for 72 hours at a temperature of 37°C.

Standard curve for *B. cinerea*

Microbial DNA was extracted from raspberry samples from each sample (T1, T2 and T3). 1 ml was taken from each sample, vortexed and centrifuged for 5 minutes at 10,000 rpm, then *B. cinerea* DNA was extracted and diluted. DNA was quantified using QuickDrop, then DNA extraction was performed with kit Quick DNA Fungal/Bacterial Miniprep kit (Zymo Research Germany).

Real-time PCR amplification

Specific *B. cinerea* primers targeting the ribosomal region between 28S and 18S genes (intergenic spacer) were used: Bc3F (5'–GCTGTAATTTCAATGTGCAGAATCC-3') and Bc3R (5'-GGAGCAACAATTAATCGCATTTC-3'). The DNA sample (5 mL) was mixed in a final volume of 25 mL with *B. cinerea* primer mixture. The program was used: 3 min at 95ºC, followed by 40 cycles of 15 s at 95ºC and 30 s at 62ºC. A melting curve was established by decreasing the temperature from 90ºC by 0.5ºC every 10 s. All reactions were performed in triplicate. The cycle threshold (C_t), or the PCR cycle where fluorescence first occurred, was determined automatically using BIO-RAD software after setting the baseline to 100. The efficiency (E) of the PCR assay was calculated using the formula, $E = [10^{-1/slope} -1] \times 100$, where the slope was extracted from the curve $C_t = f(\log Q0)$ and Q0 is the initial DNA or cell population in the assay. E was expressed as percentage.

Statistics

The mean SD is used to express all values. The statistical program SIGMASTAT 3.0 from statistic was used to analyses the data. Inc. One-way ANOVA was used to analyses group differences. As indicated by Zar, post hoc comparisons were made using the Holm-Sidak comparison test (1996). Statistical significance was defined as a P value of 0.001 or 0.05.

RESULTS AND DISCUSSIONS

Culture-based techniques are sometimes difficult to use because they are laborious and time-consuming and may not be able to detect pathogens in the viable but non-culturable state (VBNC) produced by stress, when they are present in the food (Foddai et al., 2020). The qPCR reaction has a high efficiency, specificity and sensitivity, and it is able to amplify the lowest concentration of DNA. (Si Ammour et al., 2019). Agar with Dichloran Rose Bengal Chloramphenicol has been used to grow *B. cinerea* spores from samples for the detection of fungi. Then qPCR was used to detect the possible presence of *B. cinerea* in the raspberries samples using primers Bc3F and Bc3R. The material was used according to the description of the study (Diguta et al., 2010), (Tanovic et al., 2014).

Culture-based method to detect *B. cinerea*

After plating raspberry samples on Dichloran Rose Bengal Chloramphenicol agar, no mycelium or any indication of *B. cinerea* was detected. Figure 1 shows plates with Dichloran Rose-Bengal Chloramphenicol (DRBC) Agar media without any *B. cinerea* growth. Two hypothesis can be approached: no *B. cinerea*

was present on the sample, or the detection method is not suitable when the contamination level is low.

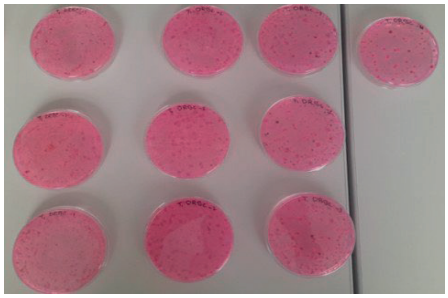

Figure 1. Aspects of total cells charge of raspberries fruits. No *B. cinerea* was detected on DRBC medium on plate

PCR-based method to detect *B. cinerea*

The amplification plots e generated with the Bc3F/Bc3R primer pair is presented in Figure 2.

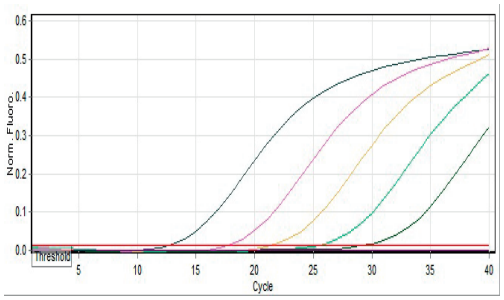

Figure 2. The amplification plots of *B. cinerea*

The standard curve for *B. cinerea* was generated by plotting the Ct value determined by qPCR and the logarithm of the DNA concentration (fg). Linearity was observed throughout the range used and a correlation coefficient (R^2 = 0.9929) indicated very low inter-assay variability. The slope of the standard curve was -3.737, which corresponded to an amplification efficiency of 85.18%. Under the conditions described, the maximum C_t value that could be used was 32, which corresponds to a DNA concentration of 9.8 fg as shown in Figure 3.

The concentration of *B. cinerea* DNA present in each raspberry sample is shown in the Figure 4. In the present experiment, *B. cinerea* was detected in samples 1 and 2. In sample 3, *B. cinerea* was not detected by qPCR (being lower than the established detection limit of 9.8 fg *B. cinerea* DNA).

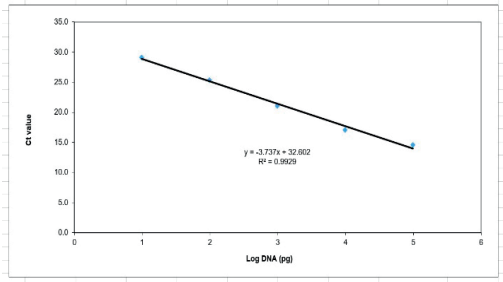

Figure 3. Standard curve generated from the amplification of *B. cinerea* DNA

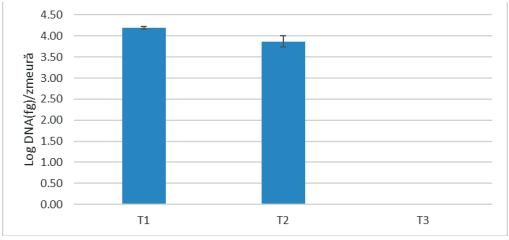

Figure 4. The concentration of *B. cinerea* DNA detected on raspberries by qPCR

The current results were compared with previous studies, where it was compared with (Malarczyk et al., 2019), who diagnosed plant pathogenic fungi that affect berry crops, as well as (Si Ammour et al., 2019), who detected *Botrytis cinerea* in Grapevine Bunch Trash by qPCR. And it was demonstrated that the qPCR reaction has a high efficiency of up to 96%, which indicates excellent sensitivity, and that it is able to amplify the lowest concentration of DNA.

CONCLUSIONS

In this article has been compared a specific and sensitive qPCR protocol with conventional methods for the detection and quantification of *B. cinerea* in raspberries. Conventional methods are useful methods, but they are somewhat inaccurate and require more time and effort compared to qPCR.

Conventional methods require cell counting, as well as the rapid growth of some types of bacteria, which leads to concealment of the main cause, while the qPCR method is characterized by accuracy. It can detect the least amount of *B. cinerea* presented in the sample, obtained accurate results. A commercially available kit was used to isolate fungal DNA, which is an

effective and straightforward technique that enables the routine examination of more samples per day.

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