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Detection transposable elements in *Botrytis cinerea* in latent infection stage from symptomless apples

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PEER REVIEW

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Comments
Authors of this manuscript have presented an interesting work on PCR based detection of *B. cinerea* in postharvest apple fruits. They have made use of *B. cinerea* transposable elements (boty and flipper) and IGS region as internal control for identification of this pathogen. They have also used this technique to distinguish between two subpopulations (vacuma and transposa) of *B. cinerea* and compared the sensitivity and cross reactivity of the assay with ELISA.
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ABSTRACT

Objective: To detect *Botrytis cinerea* (*B. cinerea*) latent infections on apples before storage, which is essential for effective control strategies in the fruit postharvest industry.
Methods: In the present study, a polymerase chain reaction detection method, based on primers designed on *B. cinerea* transposable elements (boty and flipper) and intergenic spacer region as internal control, were utilized to reveal the presence of symptomless infections on apple fruits. This molecular method proved to be highly specific and sensitive in detecting latent infections. It revealed the presence of the pathogen in 83% of the samples from infected apples with 10^4 conidia/mL, whereas those infected with 10^6 conidia/mL detected 94% as compared to the traditional method that revealed the pathogen in 40% and 66% of the samples inoculated with 10^4 and 10^6 conidia/mL respectively. Furthermore, the method characterized *B. cinerea* as subpopulation transposa–type by the presence of the transposable elements boty and flipper
Results: The results obtained from DNA quantification method were compared with enzyme–linked immunosorbent assay and these studies showed good correlation. Therefore our method has important advantages compared with others detection methods for *B. cinerea*, because the proposed methodology allowed distinguishes between its two subpopulations (vacuma and transposa) and this would allow establish possible appropriate control strategies.
Conclusions: Finally, the method can be an interesting alternative for its possible application in the phytosanitary programs of the fruit industry worldwide.

KEYWORDS
Botrytis cinerea, Latent infection, Transposable elements, Boty and flipper, Symptomless apples

1. Introduction

Botrytis cinerea Pers. (*B. cinerea*), the causal agent of gray mould, is regarded as one of the most efficient postharvest pathogens of apple fruits since it can spread at very low temperatures, shortening the duration of storage and marketing. This ubiquitous fungal pathogen is present often as latent infection. Latency is generally defined as the period between infection and the appearance of visible symptoms and can in the case of *B. cinerea* be

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long and variable[1,2]. Following the establishment of fungal hyphae, *B. cinerea* may become inactive for a long period of time, maintaining apples symptomless until the fungus is reactivated because of ripening and/or a conducive environment. Therefore, serious economic damage may come from harvesting apparently healthy apple fruits, in which infections did not advance far enough to be detected during packing operations or subsequent transport to distant markets, where the rots may become evident. Indeed, even single apple infections can lead to production of abundant sporulation, sufficient to contaminate and infect an entire batch of apple fruits. Consequently, an apparently healthy fruit can deteriorate suddenly due to the development of this latent infection[3,4].

The use of synthetic chemicals as fungicide is a primary method of control of gray mould on apple fruits in postharvest[5]. However, several fungicides are not used for postharvest treatment or have been removed from the market due to possible toxicological risks. Alternative methods of control are needed because of the negative public perceptions about the use of pesticides, development of resistance to fungicides among fungal pathogens, and high development costs of new chemicals[6].

However, early, rapid, and accurate detection of gray mold infection at harvest and during storage is, therefore, essential for devising disease prediction models, improving timing and efficacy of postharvest fungicides or alternative control applications[7]. To prevent the indiscriminate use of fungicides, a sensitive and reliable method to early determination of the fungus in fruit tissues becomes crucial. The ability to detect latent infections in fruit tissues should prove useful not only for early disease management but also for identifying infected fruit in postharvest. In addition, the quantification of the pathogen is necessary for the application of alternative methods of control, such as biological control using antagonist microorganisms or metabolites, because the success of those methods depend of the ratio antagonist-metabolites/pathogen[8,9].

The detection of this pathogen in apparently healthy fruits is generally made by cultures of fruit tissues on nutrient agar. However, this procedure is time consuming and can be misleading because of the presence of many competitive fungi with higher growth rate[10]. Therefore, with the development of molecular biology, the technology of polymerase chain reaction (PCR) has a very far-reaching effect on plant disease detection[11]. The PCR has been applied as an alternative technique that replaces microbiological methods in the detection and identification of some moulds from fruits samples[12,13].

In the present study, a sensitive and accurate detection system based on a PCR method has been specifically developed to detect *B. cinerea* in post-harvest apple fruits and utilized to evaluate the role of symptomless infections in the development of gray mould, in order to reduce the time required for diagnosis of latent infections.

2. Materials and methods

2.1. Fungal isolate and their maintenance

Isolate of *B. cinerea* were obtained from apples. *B. cinerea* (BNM 0527) was used in this study. The strain is deposited in the National Bank of Microorganisms (WDCM938) of the Facultad de Agronomía, Universidad de Buenos Aires (FAUBA). After monosporal purification, isolates were maintained on potato dextrose agar medium (PDA) at 4 °C.

2.2. Preparation of fruit extracts samples

Red delicious apples used in the assays were picked from a commercial orchard. To remove epiphytic microbial load, the fruits were surface-disinfected by immersion for 2 min in a 2% sodium hypochlorite solution, rinsed in sterile distilled water for 1 min, and left to dry at room temperature for 1 h. The surface-disinfected fruits were wounded by (3 mm×3 mm×3 mm) with a punch, inoculated with 20 μL of conidial suspension into each wound. The conidia were collected from a 7-day-old culture dish by flooding it with sterile distilled water. The conidial suspension was filtered through two layers of autoclaved gauze and the concentration was determined with a Neubauer chamber and adjusted with sterile-distilled water to approximately 10^4 and 10^6 conidia/mL, respectively. These conidial suspensions were used to infect the fruits. After inoculated, the fruits were air-dried for 2 h and incubated at (20±1) °C, 2 d after incubation and before appearance of visible symptoms, fruit tissues infected were removed and were ground to a fine powder in liquid N_2. Finally, fruit extracts samples were prepared by adding 0.1 g of powdered fruit tissue into 0.9 mL of 0.01 mol/L phosphate buffer solution (pH 7.2) and vortexed for 1 min to obtain a homogeneous suspension. The apple fruits untreated (uninfected) were used as control. Ten fruits were used for each assay with three wounds each. Each experiment was repeated three times. The samples were divided into two uniform groups which were: (I) uninfected (control); (II) artificially infected.

2.3. Traditional detection of B. cinerea latent infections

In order to detection *B. cinerea* (latent infection stage), traditional method was performed. The samples of Group I and Group II were incubated on PDA at (20±1) °C for 7 d. The pathogen was isolated after of incubation and was identified morphologically. Colony appearance, conidial shape, color and shape were examined.

2.4. PCR-detection of B. cinerea latent infections

DNA extractions; Genomic DNA was extracted from Group I and Group II according to the following procedure: The powder obtained of each sample, as reported above, was put into a tube of 1.5 and it was resuspended in 750 mL of cetyl trimethyl ammonium bromide buffer (2% cetyl trimethyl ammonium bromide, 100 mmol/L Tris pH 8, 10 mmol/L EDTA, and 0.7 mol/L NaCl). Then, 60 μg of proteinase K were added and the tubes were incubed for 30 min at 60 °C with occasional gentle mixing. Then, sodium acetate 300 μL was added (3 mol/L, pH 5.2) and gently mixed. Samples were incubated in ice for 15 min, centrifuged for 10 min at 14 000 r/min, and the supernatant was transferred to another tube. A total of 500 μL chloroform/isoamyl alcohol (24:1) was added, gently mixed by inverting tubes which were then centrifuged for 10 min at 14 000 r/min. Nucleic acid was precipitated from the aqueous layer by the addition of 500 μL ice-cold isopropanol, followed by incubation in ice for 60 min and centrifugation for 5 min at 14 000 r/min. And the supernatant was discarded. The pellet was washed with 500 μL of ice-cold ethanol 70% (v/v). DNA pellet was air dried for 30 min to discard ethanol. Finally, the DNA pellet was resuspended in 50 μL buffer TE (10 mmol/L Tris-HCL, 1 mmol/L EDTA pH 8.4), and then 1 μL of RNase was added and sample incubated at room temperature for 30 min (Möller *et al.*, 1992). Later each sample was stored at -20 °C.

The primers used in the PCR assays were: a) Intergenic spacer (IGS), 5′-ATG AGC CAT TCG CAG TTC-3′ (Accession No.J01353), unit of nuclear ribosomal DNA (rDNA) present in *B. cinerea*, to determine the transposable elements status of each isolate (whether they were of vacuma or transposa type b) flipper, with the primers F-300, 5′-GCA CAA AAC CTA CAG AAG A-3′ (Accession No.U74294) and c) boty, with the two primers botty-R, 5′-CCC AAT TTA TTC AAT GTC AG-3′ (Accession No.X81791) and botty-L, 5′-TAA CCT TGT CTT TGC TCA TC-3′ (Accession No.X81790). Each PCR mixture contained 6 μL of primers, 2.5 μL of dNTP, 2.5 μL of DNA, 2.5 μL of Mg^{+2}, and 0.5 μL of Taq polymerase in a total volume of 50 μL. The PCR program consisted of an initial denaturation step of 94 °C by 4 min; 35 cycles of 95 °C by 60 seconds, 60 °C by 60 seconds, and 72 °C by 210 seconds; and a final elongation of 4 min at 72 °C. Finally, the products were analyzed on 2% agarose gel, stained with ethidium bromide and then observed under UV light.

2.5. Enzyme-linked immunosorbent assay (ELISA) for the B. cinerea quantification

Twenty-five microliters of extracts from groups I and II and 25 μL of the monoclonal antibody IgG mouse anti-*B. cinerea* (15 μg/mL in 0.01 mol/L phosphate buffer solution, pH 7.2) were added to wells and incubated for 10 min at 37 °C. In this step, *B. cinerea* present in the fruit sample was allowed to compete by the specific monoclonal antibody with the immobilized purified *B. cinerea* antigens on surface of microtiter plates. After that, the plates were washed three times with PBST. Then, 50 μL of the anti-mouse IgG-HRP conjugate (diluted 0.75:1500 in 0.01 mol/L phosphate buffer, pH 7.2) were added and incubated for 5 min at 37 °C. The plate was washed again three times with PBST and finally, 50 μL of substrate solution (orthophenylenediamine 4 mg/5 mL; PCB 0.1 mol/L phosphate citrate, 10 μL H_2O_2) per well, were incorporated, and incubated for 3 min at room temperature. After 3 min, the reaction was stopped with 50 μL of 8 mol/L H_2SO_4. Absorbance values were determined using a microplate reader at 490 nm. The stock solution of substrate was prepared freshly before the experiment and stored in the darkness for the duration of the experiment.

2.6. Cross-reactivity studies

For the cross reaction study, the phytopathogenic fungi most common in postharvest diseases were assayed, *Penicillium expansum* (CEREMIC 151-2002), *Aspergillus niger* (NRRL 1419), were isolated from apple fruits. Single spore cultures were incubated on PDA for 7 to 10 d at (20 ±1) °C. The conidial suspension was filtered through two layers of autoclaved gauze and the concentration was determined with a Neubauer chamber and adjusted with sterile-distilled water to approximately 10^6 conidia/mL (for each fungus). These conidial suspensions were used to infect the fruits. Fruit extracts samples were prepared as described above.

PCR and ELISA assay were used as described our method above, except that the groups I uninfected; II artificially infected were replaced for extracts apple fruits artificially infected with *Penicillium expansum* and *Aspergillus niger*.

3. Results

3.1. Molecular detection method of B. cinerea

The infected apples after 2 d of incubation for concentrations of 10^4 and 10^6 conidia/mL, (Group II), there were not visible signs of disease, those fruits were considered as latent infection stage, whereas showed initial symptoms after 5 d in 100% of the fruits. At Day 7, sporulation of the pathogen was clearly evident.

The specificity of the primers was assessed against samples of Groups I and II. The primers used in this study (IGS, transposable elements boty and flipper) successfully amplified DNA from samples Group II (Figure 1).

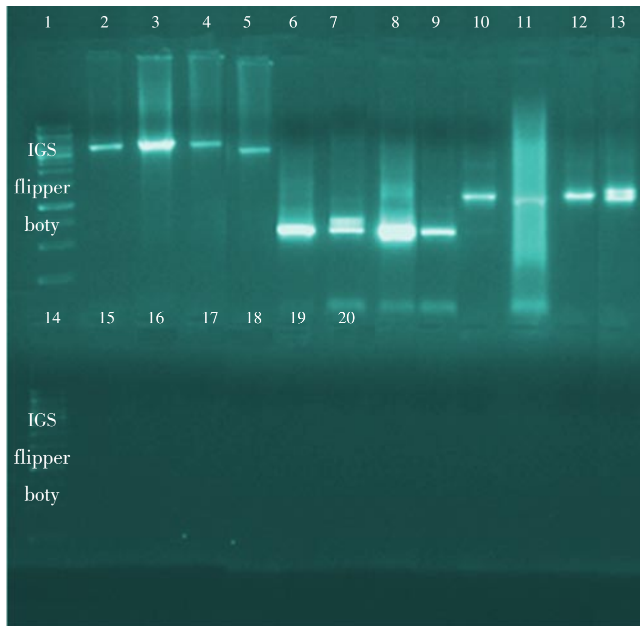

Figure 1. Lane 1: standard molecular weight marker.
Lanes 2 and 3: correspond to a molecular marker IGS for Group II, after 2 and 7 d incubation from apples artificially inoculated with 10^4 conidia/mL.
Lanes 4 and 5: correspond to a molecular marker IGS for Group II, after 2 and 7 d incubation from apples artificially inoculated with 10^6 conidia/mL.
Lanes 6 and 7: correspond to the boty transposable element for Group II, after 2 and 7 d incubation from apples artificially inoculated with 10^4 conidia/mL.
Lanes 8 and 9: correspond to the boty transposable element for Group II, after 2 and 7 d incubation from apples artificially inoculated with 10^6 conidia/mL.
Lanes10 and 11: correspond to the flipper transposable element for Group II, after 2 and 7 d incubation from apples artificially inoculated with 10^4 conidia/mL.
Lanes 12 and 13: correspond to the flipper transposable element for Group II, after 2 and 7 d incubation from apples artificially inoculated with 10^6 conidia/mL.
Lane 14: standard molecular weight marker.
Lanes 15 and 16 correspond to a molecular marker IGS for Group I, after 2 and 7 d incubation from apples uninoculated.
Lanes 17 and 18 correspond to a correspond to the boty transposable element for Group I, after 2 and 7 d incubation from apples uninoculated.
Lanes 19 and 20 corresponds to the flipper transposable element for Group I, after 2 and 7 d incubation from apples uninoculated.

Concentrations of DNA were detected spectrophotometrically by measuring absorbance changes at 260 nm (Table 1), showed good integrity by the high molecular weight bands on electrophoresis (data not shown).

The infected apples without visible signs of disease (2 d of incubation), showed a DNA concentration ranging from 13.22 to 18.29 µg/mL DNA of *B. cinerea* per sample, for infected apples with concentrations of 10^4 and 10^6 conidia/mL respectively.

The proposed method PCR-based was able to detect the presence of gray mold in 83% of the samples from infected apples with 10^4 conidia/mL, whereas those infected with 10^6 conidia/mL detected 94% (Table 1). Furthermore, the presence of both transposable elements (boty and flipper) indicates that *B. cinerea* can be molecularly identified as subpopulation transposa-type according to the proposed by Giraud *et al.* (1997, 1999). Table 1 shows the detection percentage of *B. cinerea* in Group II after 7 d of incubation, it can be seen there is no significant difference between the apples detection with disease symptoms and without them.

3.2. Traditional detection of B. cinerea

The method developed was compared with the traditional detection, this was performed on PDA medium. The control samples (Groups I), obtained from uninfected fruits, in which the absence of *B. cinerea* was confirmed after 7 d of incubation (Table 1).

The samples of Group II, obtained from artificially inoculated with 10^4 conidia/mL revealed 40% of the presence of *B. cinerea* by microscopic and morphological observations whereas those obtained from artificially inoculated with 10^6 conidia/mL, the presence of gray mould was confirmed by the same way that the previously mentioned in 66% (Table 1). Indeed, a high incidence of other fungal genera was detected by technique traditional, in Group I, and II, like *Penicillium* spp. (23%), *Aspergillus* spp. (19%), *Cladosporium* spp. (11%), among the most relevant. Table 1 shows the detection of *B. cinerea* after 7 d of incubation, where it is compared with the detection by PCR.

3.3. ELISA assay

The method developed was compared with ELISA assay with the purpose of confirming the presence of gray mold. The results of quantitative ELISA were compared with DNA quantification method and these studies showed good correlation (Table 1). The method was applied for the quantitative determination of *B. cinerea* on samples of Group I and Group II. Because the developed method was based in a competition between *B. cinerea* purified antigens immobilized onto the surface of the microtiter plates, and *B. cinerea* antigens present in fruit tissues, the absorbance at 490 nm was inversely proportional to the amount of the *B.*

Table 1

Detection of *B. cinerea* by traditional and PCR method.

Samples antigen		Days of incubation	Visible symptoms infection	[c]Lesion diameters (mm/rot)	Transposable elements		PCR method	Traditional method	[d]DNA-*B. cinerea* (μg/mL)	[d]*B. cinerea* (μg/mL)
					boty	flipper				
Group I	[a]Control	2	-	0	-	-	0%	0%	not detected	not detected
		7	-	0	-	-	0%	0%	not detected	not detected
Group II	10^4 conidia/mL	2[b]	-	0	+	+	83%	40%	13.22±0.33	15.23±0.98
		7	+	15.57±1.45	+	+	86%	89%	28.22±0.63	27.23±0.48
	10^6 conidia/mL	2[b]	-	0	+	+	94%	66%	18.29±0.73	17.23±0.98
		7	+	24.57±2.39	+	+	95%	91%	47.22±0.93	51.23±1.07

[a]-Negative control (uninfected fruits); [b]-Latent infection stage; [c]-Diameters of the lesion measured in the fruit samples at 2 d and 7 d of incubation (20 °C) respectively; [d]-μg/mL, mean±SD: standard deviation.

cinerea antigen present in the fruit.

The ELISA assay proved to be sensitive enough to detect *B. cinerea* with an concentration ranging from 15.23 to 17.23 μg/mL *B. cinerea* antigen per sample (Table 1), with a linear regression coefficient r=0.983 and a detection limit (DL) of 1.09 μg/mL. The DL was considered to be the concentration that gives a signal three times the standard deviation (SD) of the blank. The precision of the ELISA assay was checked with control of *B. cinerea* purified antigens. Non-inoculated controls, Group I, did not produce any quantitative determination.

3.4. Cross-reactivity studies with fungi isolated from fruits

The cross reactivity tests for both PCR and ELISA resulted in no crossreactions. PCR-method proved not to cross-react with other fungal genera phylogenetically related because unamplified of IGS and transposable elements.

ELISA test, the monoclonal antibody for *B. cinerea* resulted in no crossreactions, the absorbance read at 490 nm corresponded to maximum value indicating that the sample did not contain competitive antigens and that the antibody was specific.

4. Discussion

Serious damages are inflicted by *B. cinerea* on apple fruits in postharvest. The control is particularly complicated by legislation on the use of pesticides, the growing concern for the possible risks for human and environmental health, and the appearance of resistant strains to the most widely used products[7]. Furthermore, the efficacy of *B. cinerea* to remain latent in the apple tissues makes it difficult to establish control strategies.

Thus, an early and accurate diagnosis of *B. cinerea* in symptomless apples might be extremely useful for a proper management of the disease or, alternatively, to choose the lots of apples that can be stored safely for long periods.

In the present study, molecular techniques (PCR) were utilized to evaluate incidence and role of latent infections in the development of postharvest apple rots. Furthermore, were compared to conventional culturing techniques and by indirect competitive ELISA.

When fungal DNA was isolated from asymptomatic apples the method was able to detect the presence of *B. cinerea* in 83% and 94% of the samples from fruits infected with 10^4 and 10^6 conidia/mL, respectively, whereas that traditional method was efficient able detect gray mold in 40% and 66% of the samples from fruits infected with 10^4 and 10^6 conidia/mL, respectively, however required one week to detection. The low sensitivity of the traditional method is probably the result of the competition against *B. cinerea* of several other microorganisms commonly associated with apples rot. The competition of bacteria was excluded by using a semi-selective medium amended with antibiotics, however many other microorganisms include filamentous fungi like *Penicillium* spp., *Aspergillus* spp., *Cladosporium* spp. and yeasts could have prevented the growth of *B. cinerea* or, in the case of fast growing microorganisms, could have just concealed its presence. These difficulties could be partially solved by using stronger selective media; however, traditional method will always remain a time-consuming process, in which skilled expertise is required for fungal isolation and identification by microscopic observation.

In the present study, transposable elements boty and flipper were detected out by PCR from samples symptomless apples infected with 10^4 and 10^6 conidia/mL. Molecular analysis of *B. cinerea* have identified two sub populations, based on the presence or absence of two transposable elements, vacuma (isolates without either transposable elements) and transposa (isolates with the transposable elements boty and flipper), these results confirm the presence of the two transposable elements and its molecular characterization in the transposa type subpopulation[14]. Also, a primer designed to target a species-specific portion

IGS region (ribosomal region 18S) was utilized. Further, it proved not to cross-react with other fungal genera phylogenetically related.

Moreover, with the purpose of quantifying antigens of *B. cinerea* from samples of symptomless apples infected with 10^4 and 10^6 conidia/mL, the ELISA method was realized, allowing determining very low levels of *B. cinerea* antigens in apparently healthy fruits.

Therefore, the validation procedures showed that the method developed was reliable and accurate and that was possible to correlate the quantities DNA and antigens of *B. cinerea* present in fruit samples with the number of inoculated conidia and the incidence of the disease on apples.

The routine detection of latent *B. cinerea* is difficult, whatever the employed method[15], such as *in vitro* culture, ELISAs and electrochemical immunosensors[16-19]. However, the specificity and sensitivity of PCR method allow the rapid detection of small amounts of target DNA[10]. Although, there are reports PCR-based for the latent stage detection of *B. cinerea* in plants and grape fruit[7,15], detection of gray mould in latent infection stage from apples through presence of transposable elements has not been reported to date. Our method proved to be more appropriate for the detection of latent infection since the results showed a high degree of presence of *B. cinerea* in the symtomless apple samples as compared with the traditional detection. Furthermore, in the present paper one important advantage compared with other methods of detection (in vitro culture, ELISAs, immunosensors) is that the proposed methodology allows identify *B. cinerea* subpopulations and provide valuable information to establish new control strategies as for example highest growth rate, higher virulence and resistance at fungicides that present the different subpopulations[20].

In conclusion, results obtained in the present study suggest that the proposed methodology employing PCR can be used as an alternative method of *B. cinerea* detection in storage apples before visual decay symptoms are apparent (latent infection). Furthermore, these findings confirmed early experiments of detection of transposable elements boty and flipper in latent infection stage of *B. cinerea*. This assay represents a small breakthrough in understanding the infection pathway of pathogen in symptomless apples. This procedure might be a valuable tool for the early and accurate diagnosis of the pathogen in apple fruits and provide information, not only for choosing the less contaminated packs to be long stored or shipped, but also to establish control strategies. Moreover the amount and quality of DNA extracted was enough for the detection by PCR of transposable elements boty and flipper. Finally, the proposed methodology can be an interesting alternative for its possible application in the phytosanitary programs of the fruit industry worldwide.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

This method is useful for rapid identification of *B. cinerea* its two subpopulations (vacuma and transposa) in postharvest apple fruits. This might help early detection and adopt bio friendly control measure that are crucial from public health point of view.

Research frontiers

Although PCR method of *B. cinerea* detection has already been described, this method has added to the simultaneous detection of two subpopulations (vacuma and transposa) that is particularly helpful for identifying the lineages involved in infection of post harvest apple.

Related reports

There are reports related to PCR method of *B. cinerea* detection in other fruits. However, authors have made nice comparative analysis of the PCR and ELISA methods that has proved the efficacy and effectiveness of this PCR based method.

Innovations and breakthroughs

As mentioned above PCR technique to detect *B. cinerea* is not novel but the effort authors made in using this technique in simultaneous identification of two sibling with an internal

control is interesting.

Applications

This method is useful for rapid identification of *B. cinerea* its two subpopulations (vacuma and transposa) in postharvest apple fruits. This might help early detection and adopt bio friendly control measure that are crucial from public health point of view.

Peer review

Authors of this manuscript have presented an interesting work on PCR based detection of *B. cinerea* in postharvest apple fruits. They have made use of *B. cinerea* transposable elements (boty and flipper) and IGS region as internal control for identification of this pathogen. They have also used this technique to distinguish between two subpopulations (vacuma and transposa) of *B. cinerea* and compared the sensitivity and cross reactivity of the assay with ELISA.

It's an interesting work and might be of help in early detection and implementation of bio friendly control measure in protection of postharvest apples that are crucial from economic as well as public health point of view.

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