Iraqi Journal of Market Research and Consumer Protection
(2023) 15(2): 156-161
DOI: http://dx.doi.org/10.28936/jmracpc15.2.2023.(15)
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USE OF PCR TECHONLOGY IN DIAGNOSIS OF *Botrytis cinerea* CAUSES SPOILAGE OF STRAWBERRY FRUITS AVAILABLE IN THE LOCAL MARKETS

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Received 21/ 5/ 2023, Accepted 22/ 6/ 2023, Published 31/ 12/ 2023

ABSTRACT

This study aimed to collect 20 samples of strawberry fruits imported from Turkey and Egypt, with ten samples for each of them, which are distributed in the local markets of the city of Baghdad, during the period from October 2022 to November of the same year. The results of conventional tests showed that the samples contained *Botrytis* spp. With a number of fifteen, and to confirm the return of these isolates to the fungus *Botrytis cinerea* specifically, the PCR technique was used as this technique showed obtaining 8 isolates out of 15, most of which belong to strawberry samples of Egyptian origin, in one package with a molecular weight of 700 bp when migrated on the agarose gel. This means that molecular diagnosis is superior to traditional methods, despite its importance as primary diagnostic methods.

Key words: Strawberry; Polymerase Chain Reaction; *Botrytis cinerea*; Local market.

استعمال تقنية PCR في تشخيص فطر *Botrytis cinerea* المسبب لتلف ثمار الفراولة المتوافرة في الاسواق المحلية

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الخلاصة

استهدفت هذه الدراسة جمع 20 عينة من فاكهة الفراولة المستوردة من تركيا ومصر وبعدد عشر عينات لكل منها المنتشرة في الأسواق المحلية لمدينة بغداد وخلال الفترة من تشرين الاول 2022 إلى تشرين الثاني لنفس العام. اظهرت نتائج الفحوصات التقليدية احتواء العينات على فطر Botrytis spp. وبعدد خمسة عشر، ولتاكيد عانديه هذه العزلات الى فطر *Botrytis cinerea* تحديدا تم الاستعانة بتقنية تفاعل البلمرة المتسلسل PCR اذ اظهرت هذه التقنية الحصول على 8 عزلات من اصل 15 تعود غالبيتها لعينات الفراولة ذات المنشأ المصري وبحزمة واحدة ذو وزن جزيئي 700 bp عند ترحيلها على هلام الآكاروز، مما يعني تفوق التشخيص الجزيئي على الطرائق التقليدية على الرغم من اهميتها كطرائق تشخيص اولية.

الكلمات المفتاحية: الفراولة، تفاعل البلمرة المتسلسل ، *Botrytis cinerea* ، الاسواق المحلية.

INTRODUCTION

The strawberry plant with the scientific name (Fragaria) belongs to the genus of strawberries, which falls under the pink family. Strawberries are characterized as juicy fruits with a fragrant and distinctive aroma, and they are of a bright red color, the northern hemisphere is the original home of the strawberry plant (**Manganaris *et al.*, 2014**). The growing seasons of the strawberry tree begin in June with a large crop to early July, the species that bear two crops produce one in early summer and the second in early fall. As for the breeding grounds of the strawberry tree, it is spread all over the world due to its distinctive characteristics, such as its delicious taste, aroma, and juice **(Afrin *et al.*, 2016**). "Botrytis" rot is a hidden fungal disease known as "gray rot" of a fungus class: "*Botrytis cinerea*" that infects many fruits, especially strawberry, and can cause great economic losses because it makes the fruits inedible **(Porquier *et al.*, 2019)**.Gray rot is one of the two main diseases affecting

strawberries along with powdery mildew, damage to the roots and tops of the fruits are signs that the infection is serious and can then cause the loss of entire crops as the infested fruits become unfit for consumption (**Cosseboom *et al.*, 2019; Garfinkel, 2021)**. The infection appears randomly on the plant and then spreads to all members of the strawberry tree if conditions are suitable for the development of the fungus, the infection often begins to appear on flowers at the end of the flowering stage and becomes necrotic and brown spots appear and turn to a gradual gray color, and for this reason it is called gray mold (**Almeida *et al.*, 2015**).As for the leaves, they are covered with brown spots, then the stems dry before causing the death of the branches, and sometimes the fungus reaches the roots, then brown necrosis can be seen when the roots are pruned longitudinally, and in general, damage to the root and its system leads to the death of the strawberry plant (**Konstantinou *et al.*, 2015**). In view of the great importance of the genus *Botrytis*, it has been the subject of multiple studies, including molecular technical studies, although the classification of the genus *Botrytis* depends largely on morphological characteristics, but many species of this genus are morphologically similar and are greatly affected by their culture conditions **(Liu *et al.*, 2019**).PCR molecular identification methods, which depend on DNA analysis, are more rapid and accurate in classification, so they have been widely used. **(Avenot *et al.*, 2020)**.The aim of the local study was to use the polymerase chain reaction test to detect the fungus *Botrytis cinerea* that causes spoilage of strawberry fruits in the local markets of the city of Baghdad.

MATERIALS AND METHODS

Sample collection

A total of 20 Strawberry import samples (10 samples from Turkey and 10 samples from Egypt) collected different brands were randomly purchased from October to December 2022 were obtained from Baghdad's local markets.

Isolation and identification

Isolation and identification of fungi classified by two methods

Conventional diagnostic

The fungus was isolated from strawberry fruits, which showed symptoms of infection with gray mold, where a part of the fungus mycelium growing on the infected fruits was taken and placed on Potato Dextrose Agar (PDA), then the plates were incubated at a temperature of 25 °C. After the growth of the mycelium and the formation of conidia, purification was carried out by the single spore method (**Harper *et al.*, 2019**) whereby the conidia were collected using peptone water and placed in a sterile tube. Dilutions of the spore suspension were prepared, cultured on Petri dishes containing aqueous agar (distilled water with 2% agar) and incubated at 25°C for 5d to obtain a pure single-spore isolate of the fungus. The fungus isolate was preserved after purification on PDA tilted at 4ºC.

Molecular diagnostic

Mycelium preparation

The fungus was cultivated on (Potato Dextrose Broth) in conical flasks of 250 ml containing 50 mL of the medium, where the medium was inoculated with a volume of 200 µL of the spore suspension of the fungus, and incubated without stirring at 22 °C under alternating lighting conditions (24 h presence of darkness and light) for 12 d, then the mycelium growing on the surface of the medium was transferred to a 50 mL tube and centrifugation was performed (10000 rpm for 10 min), then the supernatant was discarded, and the mycelium was

washed with distilled water to get rid of the remnants of the culture medium (**Leroch *et al.*, 2013).**

DNA extraction

The fungus mycelium kept in liquid broth was crushed, then genomic DNA was extracted from 200 mg of mycelium powder, using a DNA isolation kit from the company DNeasy Plant Mini Kit (Qiagen) dedicated to isolating DNA from fungal cells according to the instructions attached to the kit(**Qin *et al.*, 2022)**.

Polymerase Chain Reaction Analysis

Amplification was done by using 25 µL reaction mix consisting of: 10 µL of Hotstar Taq Master Mix from (Qiagen), four nucleotides deoxyphosphate triphosphate dNTPs, PCR amplification reaction buffer with 3mL $MgCl_2$ and µl 2 of genomic DNA (100 ng/ µL),11 µL of sterile distilled water and 1µL of each 10 µM primer. The sequence of primers used in this study is C729 + (5'AGCTCGAGAGAGATCTCTGA-3') and (5'-CTGCAATGTTCTGCGTGGAA-3') C729- from Vivantis Company. The PCR amplification reaction done according steps: 1 cycle for 2 min at 94°C, 35 cycles each: 45 sec at 94°C for DNA strand separation, 50 sec at 50°C for primer encapsulation, then 50 sec at 94°C and finally one cycle for 5 min at a temperature of 72°C to elongate the chains. The PCR reaction products were transferred to a 1.5% agarose gel.

Sequence

The nucleotide sequence of the DNA fragment resulting from the PCR amplification reaction was determined using + C729 and - C729, which is 700 nucleotides long, as the PCR product was purified, then the chain was reacted using the chain device for genetic analysis, and then the result of the chain was read with the database in EMBL\GenBank.

STATISTICAL ANALYSIS

The Complete Randomized Design (CRD) was adopted. The averages of the treatments were compared using the Least Significant Difference (LSD) at a probability level of 0.05 (**Kirk**, **1995**).

RESULT AND DISCISION

The results of our study showed obtaining 15 pure isolates belonging to the type of fungus *Botrytis* spp. When diagnosed formally and microscopically, 10 of them belong to strawberries of Egyptian origin, and the other five isolates belong to strawberries of Turkish origin.

The morphological characteristics, number of fungal isolates, and color obtained from strawberry fruit infected with gray mold were consistent with the characteristic characteristics of *Botrytis* spp. this study was agreement with **(Van Kan *et al.*, 2014; MICLEA *et al.*, 2015)**, While **Sarven *et al.*** (**2020**) found the Different patterns of growth were observed on the PDA after incubation at 25ºC. The appearance of the fungal cultures was Cottony, Smooth and Radial. The coloration of the back side of the fungal culture was brown with a brown halo. When confirming the belonging of this fungus to Botrytis cinerea by **Ma *et al.*, (2018)** depending on the height of the fungus Conidia ranged from 8 to 13 µm, while other species ranged from 14.2 µm to 12.5 µm. Botrytis cinerea is a ubiquitous heterotrophic fungal

pathogen that attacks more than 200 species of ornamentals and forest trees in humid and temperate regions. Therefore, it is considered an important fungus that causes fruit damage.

When confirming belonging of these isolates to the fungus *Botrytis cinerea* in the imported strawberry samples specifically at the molecular level using the Polymerase Chain Reaction technique when migrating them on the agarose gel, appearance of 8 out of 15 pure isolates of the Egyptian strawberry samples in one package for these isolates with a molecular weight of 700bp. (Figure 1)

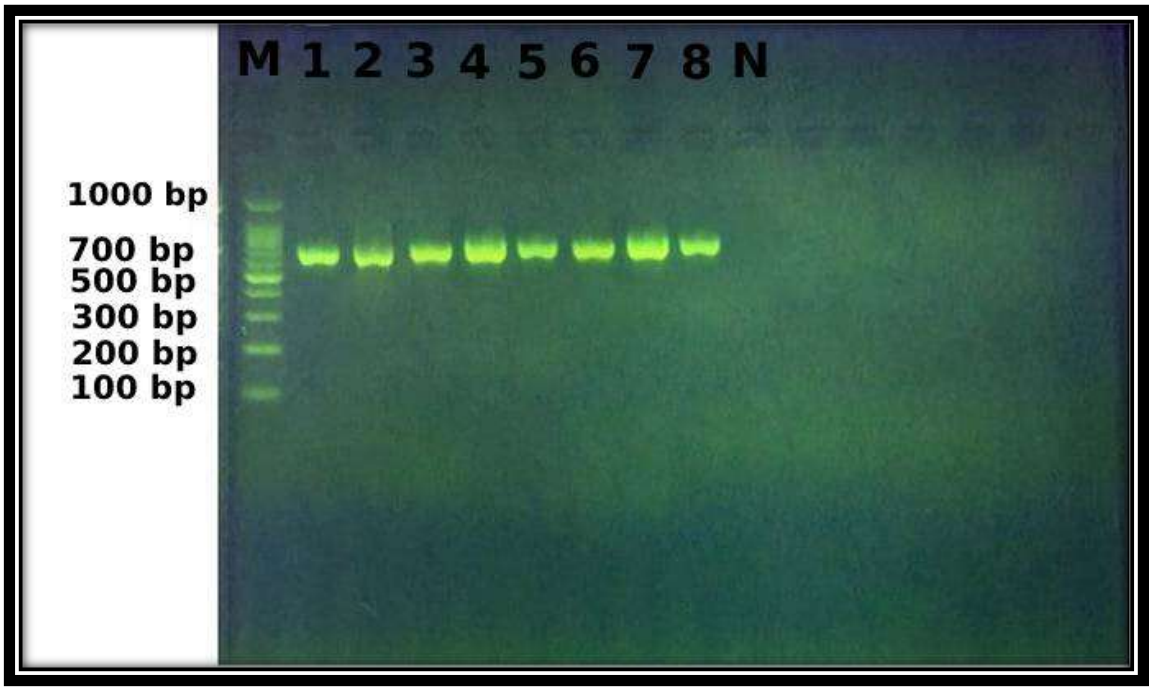

Figure (1): Electrophoresis of PCR reaction product (700bp) on Agarose gel of local isolates from strawberry fruit. Lanes: (M) DNA, Lader (100-1000 bp), L 1,2,3,4,5,6,7,8 (*Botrytis cinerea*), N (control).

The fast detection of minute amounts of target DNA is now available because to the specificity and sensitivity of PCR. *Botrytis cinerea* specificity 700-bp fragment has been extracted and sequenced in the presented study employing PCR methods. A 20-mer primer pair was created on the sequence and utilized in PCR on DNA obtained from strains of fungi and the strawberry plant to determine its specificity this study was agreement with (**Behr *et al.*, 2013** ;**Muñoz *et al.*, 2016).** These results indicate the importance of PCR-based technology for the detection of *B. cinerea* in strawberry plants compared to the regular method of diagnosis, which involves growing contamination samples on agar plates. PCR can be used in following studies to identify the pathogen's latent infections in strawberry and other plants. The PCR assay used to measure infection need exhibited fungal penetration to plant and detected lower amount of *B. cinerea* inoculum in infected tissue in early time (**Zhou *et al.*, 2014**; **Banani *et al.*, 2016).**

CONCLUSION

The results of this study showed that the molecular diagnosis of *Botrytis cinerea* fungus isolates in imported strawberry fruits using the PCR technique contributed to identifying the fungus that causes gray rot, in addition to the accuracy of this examination and the shortening of time compared to the traditional method.

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