DEVELOPMENT OF SPECIES-SPECIFIC PRIMERS FOR THE IDENTIFICATION OF *Colletotrichum musae* IN BANANAS

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ABSTRACT

Bananas (*Musa* spp.) is an important crop which has high economic and nutritional values, but it is also very susceptible to diseases, especially anthracnose caused by *Colletotrichum* spp. In particular, *Colletotrichum musae* is the most common pathogen causing banana anthracnose and leading to yield loss. Therefore, developing a method for identification of *C. musae* has received a lot of attention from scientists, recently. In this study, *Colletotrichum* spp. were isolated from anthracnose lesions of different banana cultivars collected in Viet Nam including Can Tho, Da Lat, Hau Giang, Soc Trang and Vinh Long. Pathogenicity of fungal strains was tested using 3 common banana cultivars in Vietnam (Gia, Xiem and Cau). Specific primers for *C. musae* were designed on the glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene sequence to accurately identify *C. musae* causing anthracnose on bananas. In this study, 11 strains of *Colletotrichum* spp. were isolated from anthracnose lesions. Of which, the G2 strain showed the highest pathogenicity (lesion diameter: 19.33 mm) while the CA strain had the lowest pathogenicity (lesion diameter: 7.44 mm). The two primers CM1 (5' CCGCTGTAATCTACATCTC 3') and CM2 (5' CTGATATGAGTGATAGCATGTA 3') were designed to generate the 115-bp DNA amplicon, a specific product for *Colletotrichum musae*. PCR results showed that 6/11 isolated fungal strains were identified as *C. musae.* These strains have the highest pathogenicity in the pathogenicity test, especially the G2 strain.

PHÁT TRIỂN MỒI ĐẶC HIỆU MỚI NHẬN DIỆN NẤM *Colletotrichum musae* TRÊN CHUỐI

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TỪ KHÓA

Bệnh thán thư
Colletotrichum musae
Colletotrichum spp.
GADPH
Mồi đặc hiệu

TÓM TẮT

Chuối (*Musa* spp.) là cây trồng quan trọng, có giá trị kinh tế, dinh dưỡng cao và rất dễ mắc bệnh, đặc biệt là bệnh thán thư gây ra bởi nấm *Colletotrichum* spp. Trong đó, nấm *Colletotrichum musae* là tác nhân gây bệnh thán thư phổ biến nhất trên cây chuối dẫn đến những thiệt hại nặng nề. Vì vậy, việc nhận diện *C. musae* hiện nay nhận được rất nhiều sự quan tâm. Trong nghiên cứu này, các chủng *Colletotrichum* spp. được phân lập từ vết bệnh thán thư trên các giống chuối khác nhau được thu thập ở Việt Nam như thành phố Cần Thơ, thành phố Đà Lạt, tỉnh Hậu Giang, Sóc Trăng và Vĩnh Long. Khả năng gây bệnh của các chủng nấm đã được thử nghiệm trên 3 giống chuối phổ biến ở Việt Nam (chuối Già, chuối Xiêm và chuối Cau). Để xác định chính xác *C. musae* gây bệnh thán thư trên chuối, cặp mồi đặc hiệu để nhận diện nấm *Colletotrichum musae* đã được thiết kế dựa trên vùng gen glyceraldehyd-3-phosphate dehydrogenase (GADPH). Trong nghiên cứu này, 11 chủng *Colletotrichum* spp. đã được phân lập. Chủng G2 có khả năng gây bệnh cao nhất (đường kính vết bệnh: 19,33 mm) trong khi chủng CA có khả năng gây bệnh thấp nhất (đường kính vết bệnh: 7,44 mm). Cặp mồi được thiết kế CM1 (5' CCGCTGTAATCTACATCTC 3') và CM2 (5' CTGATATGAGTGATAGCATGTA 3') khuếch đại đoạn DNA có kích thước 115 bp. Dựa trên kết quả PCR khi sử dụng cặp mồi được thiết kế có 6/11 chủng nấm được xác định là *C. musae*, các chủng này có khả năng gây bệnh cao nhất trong thử nghiệm lây bệnh nhân tạo, đặc biệt là chủng G2.

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1. Introduction

Bananas (*Musa* spp.) are a popular fruit, which originated from Southeast Asia, belonging to the genus *Musa* [1]. Bananas bring not only economic value but also significant benefits to human health. However, anthracnose caused by *C. musae* is the most aggressive disease during the postharvest of bananas [2]. *Colletotrichum musae* is believed to be the main cause of most anthracnose on bananas [3]. This plant pathogen has been shown to belong to the species complex *C. Gloeosporioides* [4], which has a wide range of genetic and biological diversity [5]. Other species within the *C. gloeosporioides* complex were also reported to cause anthracnose on bananas such as *C. chrysophilum*, *C. fructicola*, *C. gloeosporioides*, *C. scovillei*, *C. siamense*, *C. theobromicola* and *C. tropicale* [6]. *C. musae* causes a latent, subcuticular infection in the field during early stages of fruit development; still, disease symptoms on the banana peel (sunken brown-black lesions) only develop after harvest, usually during fruit ripening [7]. Anthracnose symptoms initially appear as small spots, after a while, ripe bananas will develop sunken brown spots with orange fungal colonies [8]. *C. musae* may form lesions on fruits without skin bruising but produces larger lesions when fruits are damaged [9].

Because *C. musae* is a major pathogen on bananas leading to post-harvest losses, *C. musae* has received much attention from researchers. Several molecular methods have been developed to detect *Colletotrichum* spp. in plant tissue or to determine phylogenetic relationships [10]. According to Cannon *et al*. (2000) [11], DNA analysis is the most reliable basis for classifying *Colletotrichum* fungi because DNA is not affected by environmental factors. Ribosomal DNA (rDNA) genes are widely used in identifications and classifications of fungal pathogens. Moreover, the amplification of target DNA via PCR with specific primers is a more precise method than conventional microscopy techniques [12]. According to Han *et al.* (2015) [13], the GAPDH has been used to distinguish *Colletotrichum* spp. Several single genes or gene combinations, such as glutamine synthetase and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), can be used to differentiate *Colletotrichum* species [14]. Vieira *et al*. (2020) [15] suggested that GAPDH gene region-based identification is one of the most effective methods for *Colletotrichum* fungi, or more specifically *C. musae* in this study.

The study was carried out to isolate strains of *Colletotrichum* spp. from anthracnose lesions appearing on banana samples collected in some provinces in Vietnam, and to design primers to identify *Colletotrichum musae*, the major causative agent of banana anthracnose, based on the GAPDH gene sequence.

2. Materials and methods

2.1. Isolation of fungi

Different banana cultivars with symptoms of anthracnose were collected from Can Tho city, Da Lat city, Hau Giang, Soc Trang, and Vinh Long province. Anthracnose lesions were cut into small pieces (2 × 2 mm) and placed on PDA (Potato Dextrose Agar) media, the fungi colonies that appeared after the incubation period were isolated in fresh sterile PDA medium.

2.2. Morphology characteristics

2.2.1. Colonies

Discs of each fungus (6 mm) obtained from cultures were transferred to new Petri dishes containing PDA media and incubated at 28ºC. After 7 days, macroscopic features of colonies such as the colour of the upper surface and reverse side, texture of the colony and rate of colony growth (mm/day) were recorded.

2.2.2. Mycelia, appressoria, conidia

A glass slide culture was used based on the method of Johnston and Jones (1997) [16]. After 7 days of incubation, the morphological characteristics of the mycelia, appressoria and conidia were recorded using a microscope with a magnification of 1000 times.

2.3. Pathogenicity test

The pathogenicity tests were carried out by inoculations of isolates on 3 common banana cultivars in Vietnam: Gia, Xiem and Cau. Bananas fruits were surface-sterilized with ethanol 96%, 3 wounds were made on each fruit (5 mm depth), 10 µL of fungal conidial suspension (10^5 conidia/mL) were inoculated into the wounds, and sterile distilled water was used as a control.

Inoculated banana samples were incubated at 28°C. After 3-5 days, the diameters of the lesion were recorded. The fungi were re-isolated from the inoculated banana fruits showing typical anthracnose symptoms and their morphology and colony characteristics were confirmed.

2.4. Species-specific primers designing

2.4.1. DNA extraction

DNA extractions from pure fungal cultures were performed following the method of Al-Samarrai and Schmid (2000) [17]. The DNA concentration was estimated by electrophoresis on 1% agarose gel, supplemented with Safeview and visualized with a UV transilluminator.

2.4.2. Primer design for Colletotrichum musae

Species-specific primers were designed and gene reference sequences were received from the NCBI database (Table 1). The ClustalW tool in BioEdit software was used to align the sequences, regions that were conserved within *C. musae* but differed from the others were found. These regions were used for designing primers with the Primer3Plus tool and the specificity of primers was checked by the Primer-BLAST tool.

Table 1. *Accession number of GAPDH gene sequences of fungal species*

Fungal species	Accession number
C. chrysophilum	MK948849; ON192534; ON457932
C. fructicola	MN535099; MK675257; MK675259
C. gloeosporioides	KX379458; KC913204; MK532362
C. musae	MW343722; MT396830; MT396824; MH681323; MK369689; MF188165; MF188167; KX417772; KF242180
C. scovillei	KP145175; KJ018661
C. siamense	MK393451; OP115980
C. theobromicola	MZ391870; MN027904; MH155179
C. tropicale	OL799316; MN607955; MH151149

2.4.3. Test of primer specificity

The isolates' GAPDH gene regions were amplified by PCR using designed primers. The amplification was performed in 25 µL volumes of 15.75 µL sterile double-distilled water; 5 µL Buffer 5X; 1 µL each primer with a primer concentration of 10 µM; 0.25 µL *Taq* DNA polymerase 5 U/µL; 2 µL DNA 50-100 ng/µL.

The PCR program was optimized and consisted of the initial denaturation phase at 94°C for 3 min; followed by 35 cycles of denaturation step at 94°C for 30s, primer annealing at 55°C for 30s and elongation at 72°C for 60s; final extension was maintained at 72°C for 10 min. PCR products were electrophoresed on a 2.5% agarose gel, stained with Safeview and observed under UV light.

2.5. Statistical analyses

All statistical analyses were performed by using Minitab 16 software to calculate the mean, coefficient of variation (%CV), standard deviation (SD) and Tukey's test of the experimental means.

3. Results and discussion

3.1. Isolation and morphology characterization of fungi

Eleven strains of *Colletotrichum* spp. were isolated from anthracnose lesions in banana samples (Figure 1). Morphology characteristics of all fungal strains were described in Table 2 after 7 days of culture.

Most of the isolates formed orange cottony colonies with flattened mycelia, some of them appeared black or orange drops on the surface of the medium (G1, SA, CO and TQ), and the rest fungal colonies were purple (LB), grey-black (BO) or milky white (CA, X1, X2) (Figure 1). The cultures had sparse, cottony mycelia. The mycelium was hyaline, slender, branched and had septa and many oil droplets inside. All fungal strains grew quickly, covering the whole surface of the petri dish (9 cm) in 4-11 days. Conidia were aseptate, hyaline, straight cylindrical, rounded at both ends and without septa except strains X1 and TQ (Figure 2, Table 2). Appressoria of 11 isolates were brown or dark brown, rounded or irregularly lobed.

The findings in this study are in agreement with the results of Sutton and Waterson (1970) [18], Lim *et al*. (2002) [19], Abd-Elsalam *et al*. (2010) [20] and Sakinah *et al*. (2014) [21]. Traditionally, identification of *C. musae* was based on morphological characteristics such as the abundant sporulation, straight, cylindrical conidia and irregularly shaped appressoria [22]. However, the identification of *C. musae* based only on that can lead to misidentification because morphological characteristics are easily influenced by environmental factors such as humidity, temperature, and precipitation [23]. The morphological characters of *C. musae* are often overlapping and ambiguous among the gloeosporioides species complex [24]. In order to identify *C. musae*, applying molecular biology techniques is necessary.

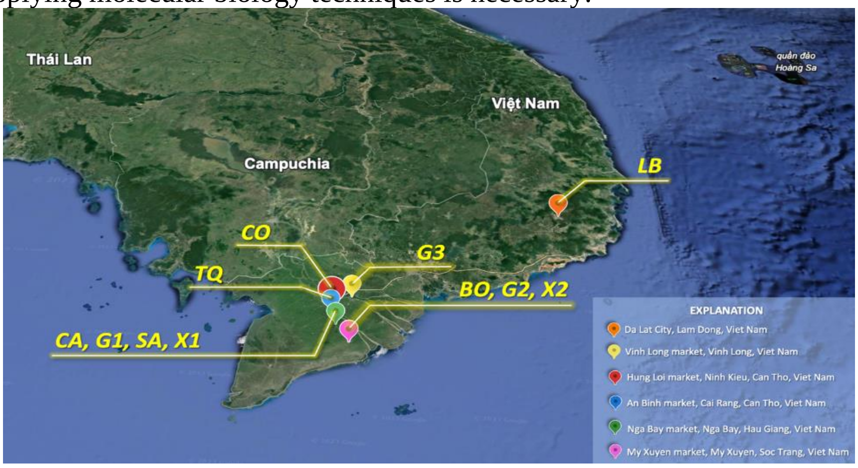

Figure 1. *Fungal strains isolated from banana from different locations*

Table 2. *Morphology characterization of isolated fungal strains*

Fungal strains	Colony colour	Appressoria	Conidial colour	Conidial shape
BO	Grey-black	Irregularly lobed	Hyaline	Straight cylindrical
CA	Milky white	Rounded	Hyaline	Straight cylindrical
CO	Orange	Irregularly lobed	Hyaline	Straight cylindrical
G1	Orange	Rounded	Hyaline	Straight cylindrical
G2	Orange	Rounded	Hyaline	Straight cylindrical
G3	Orange	Rounded	Hyaline	Straight cylindrical
LB	Purple	Rounded	Hyaline	Straight cylindrical
SA	Orange	Irregularly lobed	Hyaline	Straight cylindrical
TQ	Orange	Irregularly lobed	Hyaline	Straight cylindrical
X1	Milky white	Rounded	Hyaline	Straight cylindrical
X2	Milky white	Rounded	Hyaline	Straight cylindrical

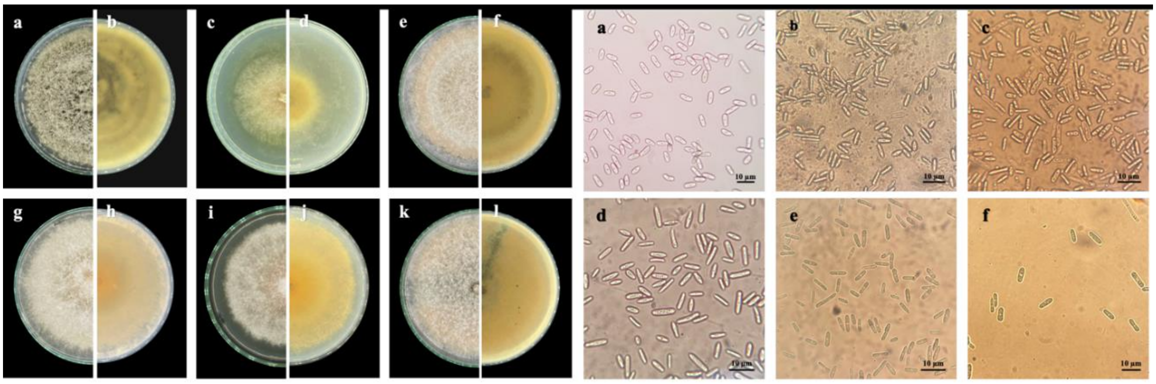

Figure 2. *The colony and conidia morphology of some fungal strains on potato dextrose agar medium. Left side: Colonies of some fungal isolates (BO, CO, G1, G2, G3, TQ) (a, c, e, g, i, k: uper view of colony. b, d, f, h, j, l: reverse view of colony). Right side: Conidia of some fungal isolates were observed using an Olympus microscope (Olympus Coporation) with 1000 magnification. a: BO, b: CO, c: G1, d: G2, e: G3, f: TQ*

3.2. Pathogenicity test

All 11 isolates were highly pathogenic (Table 3). Most of the lesions were sunken, circular, necrotic and dark brown, with white mycelia appearing on the lesions in all 3 bananas (Figure 3). These results were similar to the previous studies on banana anthracnose symptoms caused by *C. musae* [19], [20]. Some inoculated bananas appeared whitish mycelia grew from the lesions. The G2 strain had the highest pathogenicity (19.33 mm), whereas the CA strain had the lowest pathogenicity (7.44 mm) (Table 3).

There are interactions between banana varieties and isolated fungal strains, specifically that the pairs of banana varieties - fungal strains such as Xiem - BO, Cau - G2 and Xiem - X1 had the highest lesion diameter (19.00-22.67 mm) (Figure 4). From these artificially inoculated bananas, the causal agent was re-isolated and identified morphologically as *Colletotrichum* spp.

The identification of the fungus, especially *C. musae,* that causes anthracnose on bananas is a crucial issue. Banana ripeness can affect the growth of *C. musae* on bananas, conidia germination, appressoria formation as well as the first appearance of mycelia on the anthracnose lesions. Expression and development of anthracnose symptoms may be related to plant metabolic changes from hypoxic to more oxidised states [25]. In ripe bananas, nutrients are no longer processed and synthesised immediately, which leads to a higher rate of pathogen colonisation in ripe bananas. In addition, Zhang *et al*. (2011) [26] suggested that the faster the growth rate of an isolated fungal strain, the higher the rate of conidial germination and appressoria formation leading to higher pathogenicity.

Table 3. *Average lesions diameter after 5 days of inoculation (mm)*

Fungal strains	Average lesions diameter after 5 days of inoculation (mm)
BO	16.78^{b}
CA	7.44^{e}
CO	16.00^{b}
G1	16.33^{b}
G2	19.33^{a}
G3	16.89^{b}
LB	8.44^{de}
SA	17.44^{b}
TQ	16.56^{b}
X1	10.33^{c}
X2	9.56^{cd}

[a]Mean values followed by the same lowercase letter are not significantly different according to Tukey's test at 5% significance level.

Figure 3. *Pictures of some bananas after 5 days of inoculation. a, b, c: Control samples on Gia bananas, Xiem bananas, Cau bananas. d, e, f: The disease appears on Gia bananas, Xiem bananas, Cau bananas*

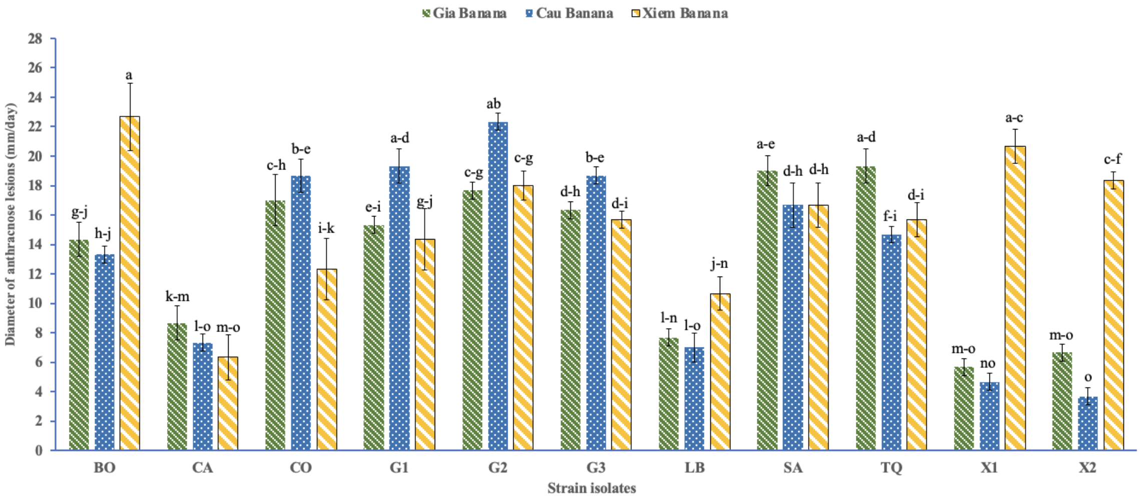

Figure 4. *Diameter of lesions after 5 days of inoculation (Mean values in the same column followed by the same lowercase letter are not significantly different according to Tukey's test at 5% significance level)*

3.3. Primer design for Colletotrichum musae

In order to accurately identify *C. musae* causing anthracnose disease on bananas, in addition to identification by morphological features above, molecular biology techniques were applied to identify *C. musae* based on specialized primer pair.

After aligning the GAPDH gene regions of the fungal strains (Table 1), only two species-specific sites distinguished *C. musae* from other *Colletotrichum* species (Figure 5). Based on that, two primers CM1 and CM2 were designed (Table 4). By using the Primer-BLAST tool, the result showed that the primers did not have similarities with DNA sequences of other organisms and it completely paired with the GAPDH gene region of *C. musae*.

Table 4. *Sequence, length and product size of primer pair CM1, CM2*

Primer's name	Sequence (5'-3')	Length (Nucleotide)	Product size (bp)
CM1	CCGCTGTAATCTACATCTC	19	115
CM2	CTGATATGAGTGATAGCATGTA	22	

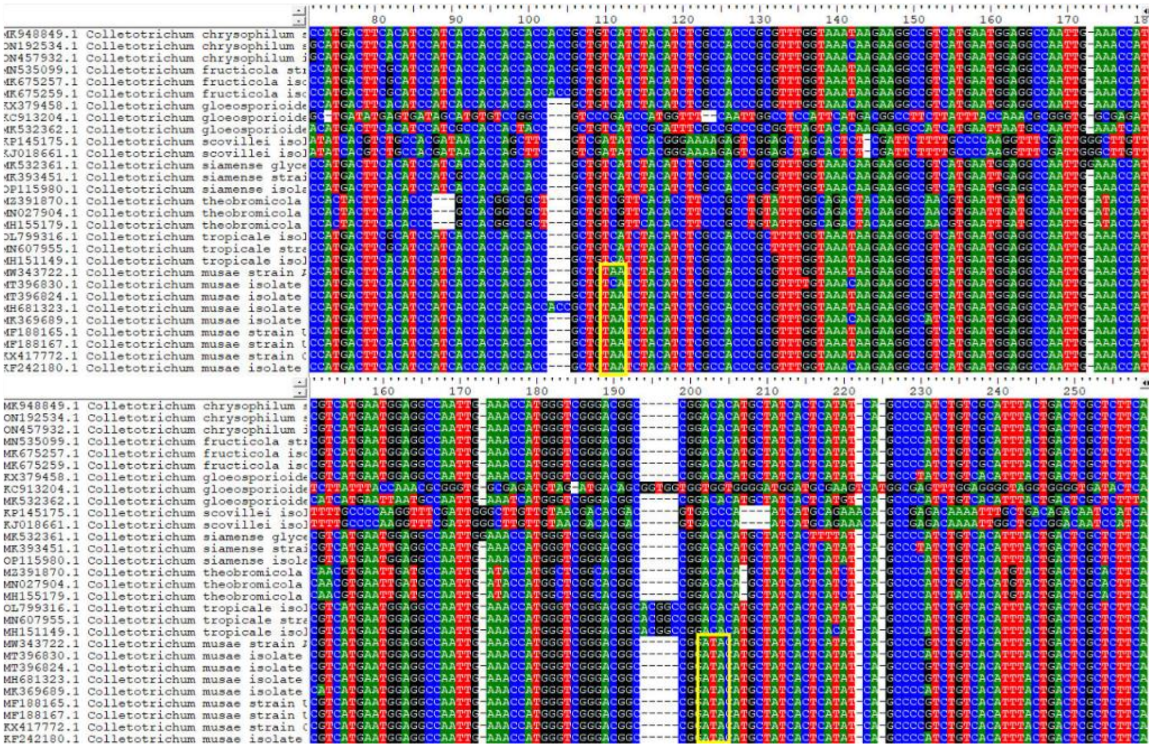

Figure 5. *Two species-specific sites distinguished C. musae from other Colletotrichum species*

3.4. Test of primer specificity

The amplification using primers CM1 and CM2 was positive for 6 of 11 isolates (G1, G2, G3, CO, BO and TQ), generating fragments of 115 bp and negative for the rest isolates. *C. fructicola*, *C. aenigma* and *Fusarium solani* were used as control (Figure 6). The designed primers did not amplified in other *Colletotrichum* species, which clearly discriminated *C. musae* the pathogen of banana anthracnose. Silva *et al*. (2017) [27] successfully used GAPDH sequence to identify and classify *Colletotrichum* spp. causing postbloom fruit crop disease. In the study of Tao *et al*. (2012) [28], the GAPDH gene was selected to develop a species-specific DNA marker, *C. kahawae* was quickly and accurately identified.

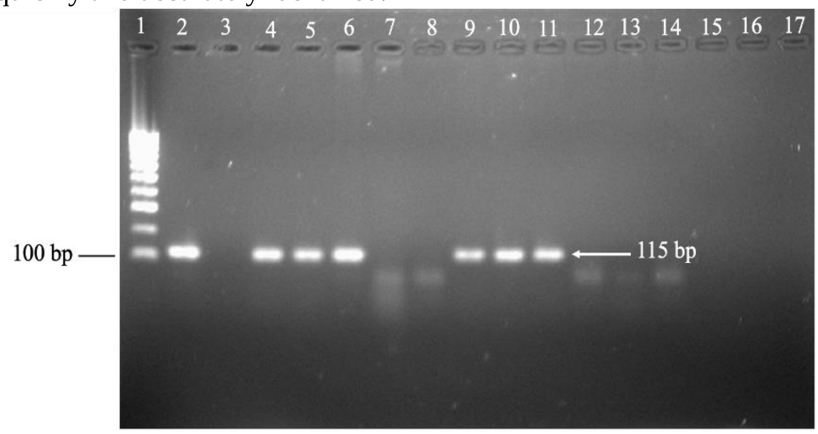

Figure 6. *Electrophoresis results of PCR products on 2.5% Agarose gel. Lane 1: Bioline's HyperLadder 100 bp standard scale. Lane 2: positive control (strain of fungi C. musae is provided by Laboratory of Microbiology, Institute of Food and Biotechnology, Can Tho University, Vietnam). Lane 3: negative control (sterile distilled water). Lanes 4-14: strains G1, G2, G3, X1, X2, CO, BO, TQ, CA, LB and SA. Lanes 15-17: strains of fungi C. fructicola, C. aenigma and Fusarium solani.*

Six fungal strains identified as *C. musae* by molecular markers also had high pathogenicity in the pathogenicity analysis (Table 3). Anthracnose symptoms caused by these strains appeared very early and spread to the whole banana faster than the other strains. According to Balendres *et al*. (2019) [29], in the pathogenicity test of *C. musae* and other fungal strains of *C. gloeosporioides* species complex on the 3 tested banana cultivars, only *C. musae* showed the expression of anthracnose disease. Huang *et al*. (2021) [6] also reported that *C. musae* caused the most severe anthracnose symptoms in bananas compared with *C. siamenes*, *C. fructicola*, *C. cliviicola* and *C. karstii* strains isolated from bananas. It was shown that *C. musae* is the causative agent of anthracnose disease on bananas, their ability to cause disease on bananas is higher than that of some other fungal species. These factors are useful in the identification of *C. musae* based on morphological characteristics and pathogenicity. This study combined the results from the morphology and molecular analyses to have enough evidence for the accurate identification of *C. musae*.

4. Conclusion

A total of 11 fungal strains showing morphological characteristics of *Colletotrichum* spp. were isolated from anthracnose lesions on banana samples collected from different provinces. The GAPDH gene region was amplified and validated to find the conserved sequences among the tested strains. A specific primer pair was designed for detecting *C. Musae*. Six out of 11 fungal strains (G1, G2, G3, CO, BO and TQ) were possitive for PCR analysis with the designed primers which indicate that they are *Colletotrichum musae*. This result provides a rapid and sufficient method for early detection and management of banana anthracnose causing by Colletotrichum musae.

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