

Application of anthracnose resistance-associated molecular markers in the detection of resistant chili pepper cultivars in Vietnam

Vi An Ly, Thao Phuong Thi Truong, Thanh Hao Nguyen*



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ABSTRACT

Introduction: *Colletotrichum* species is responsible for anthracnose, a worldwide serious disease, causing an important loss in chili pepper production. Therefore, screening disease resistant and sensitive chili pepper cultivars in Vietnam is important not only for in-depth studies of disease resistance-associated molecular mechanisms but also for chili production improvement via molecular marker-assisted breeding in Vietnam. **Methods:** To this end, in this study, two *Colletotrichum* isolates were obtained from the infected fruits collected from chili pepper (*Capsicum annuum*) fields in Tra Vinh province. According to the morphology analysis and the sequencing results of the internal transcribed spacer (ITS) regions, these isolates were identified as *C. scovillei* and *C. acutatum*. In order to identify the anthracnose-resistant chili pepper cultivars, the pathogenicity test was conducted by infecting fully developed green fruits of eleven chili pepper cultivars with the two isolated *Colletotrichum* strains. **Results:** CN404 and HNCS were the two strongest anthracnose-resistant cultivars. Two chili pepper cultivars, TV3 and PN400, showed different resistance tendencies to each *Colletotrichum* isolates. Four different SSR molecular markers were used in this study to identify the potential molecular markers associated with anthracnose resistance traits in chili pepper cultivars. Among the four examined markers, HpmsE126 was detected in two anthracnose-resistant chili pepper cultivars, suggesting its close relation to the anthracnose resistance trait in chili pepper. **Conclusion:** Given that two of the three most anthracnose-resistant cultivars, CN404 and TV3, possess HpmsE126 marker, this marker can be used to detect anthracnose-resistant lines in chili pepper breeding in Vietnam.

Key words: Chili pepper, anthracnose, molecular marker

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INTRODUCTION

In Vietnam, chili pepper is an important crop with high economic value. However, the annual chili yield is greatly affected by diseases caused by bacteria and fungi. One of the most well-known diseases in chili pepper is the anthracnose disease caused by the *Colletotrichum* fungi species. These fungi were known to infect not only chili pepper but also other crops and orchard plants, such as apple, mango, coffee, etc.¹⁻⁴. Multiple *Colletotrichum* species have been found to be responsible for the anthracnose disease in chili pepper including *C. capsici*, *C. acutatum*, *C. gloeosporioides*, *C. dematium*, *C. nigrum*, *C. atramentarium*, *C. coccodes* and *C. scovillei*^{5,6}. Apart from *C. acutatum*, *C. capsici*, *C. gloeosporioides*, recent studies in Vietnam have revealed a number of *Colletotrichum* species infecting chili pepper including, *C. nigrum*, *C. siamense*, *C. fruticola* and *C. truncatum*^{7,8}. Among five domesticated chili pepper species, including *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens* and *C. pubescens*, *C. annuum* is the most

commercially important species but is highly susceptible to anthracnose disease. The anthracnose resistance was introduced to *C. annuum* by crossing with other *Capsicum* species^{9,10}. The conventional breeding and selection processes of disease-resistant cultivars are often time-consuming and require financial resources for pathogenicity testing on large-scale. Different QTL mappings of anthracnose resistance were performed in the cross between *C. annuum* and other *Capsicum* species discovered potential markers linked to anthracnose resistance trait^{11,12}. The identification of anthracnose resistance-associated molecular markers allowed the marker-assisted or based breeding, which is less time-consuming and more cost-effective compared to conventional breeding¹³⁻¹⁵. Given that anthracnose is one of the most destructive fungal diseases in chili-growing areas, including tropical Asian countries, e.g. Vietnam¹⁶, thus the isolation of new resistant cultivars via molecular marker-based breeding will help to improve chili production and further bring economic

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benefits from chili pepper cultivation. Therefore, the objectives of this study were (i) identifying the *Colletotrichum* species majorly causing the anthracnose disease in chili pepper in Tra Vinh province (Vietnam), (ii) finding the cultivars showing resistance to these pathogens among commercial chili pepper cultivars in Vietnam, and most importantly, (iii) determining the potential anthracnose resistance-associated molecular markers which can be potentially applied further in chili pepper breeding in Vietnam.

MATERIALS AND METHODS

Plant growth condition

One wild bird-eye chili pepper line (TV3) found in Tra Vinh province and ten commercial cultivars (CN404, HNCS, CP01, HNN1, SGX5, SGX6, HN7777, TN535, HNNS, and PN400) were used in this study. Seeds from eleven cultivars were sown on small soil pots. At the 4-leaf stage, individual seedlings were transferred to pots with 18 cm in diameter. Plants were watered every morning with 2 l of water. Nutrients were supplied to each plant every two weeks with 250 ml of 1/10 MS (Murashige & Skoog) mineral solution. Plants were kept in a net house to avoid insects. Plants that were infected by mealybug were isolated and treated with insecticide (Bihopper 207EC, Binh Dien – Mekong limited company) until recovered.

Isolation of pathogenic fungi species

Fruits showing anthracnose symptoms (e.g., rounded necrosis spots on the fruit) were collected from chili pepper fields in Tra Vinh province. Infected fruit samples were washed five times by shaking in sterile water and dried under a laminar airflow chamber prior to surface sterilization with 70% ethanol for 2 min. The 0.5 cm x 0.5 cm fruit tissues containing the infected region were transferred to potato dextrose agar (PDA, 4% potato starch, 2% dextrose, 1.5% agar) plates kept at 25 °C under 16 h light/8 h dark condition in an incubation room for seven days. Purified fungal strains were obtained after three consecutive sub-cultures on PDA plates. The pure fungal cultures were maintained on PDA plates for *Colletotrichum* identification and pathogenicity test.

Morphological analysis of isolated pathogenic fungi species

Pure cultures isolated from infected fruits were first morphologically analyzed to identify the putative *Colletotrichum* strains. The morphology of the fungi

strains was analyzed by observing fungal colonies grown on PDA plates, conidia form, and the appressoria formation. For appressoria preparation, a small piece of thin PDA medium was prepared on a sterile glass microscopy slide. A small spore fragment taken from the fungal culture was distributed to four corners of the PDA medium prepared on the slide that was then sealed by a glass coverslip and placed on a sterile wet filter paper in a petri dish to maintain the humidity. After 14 days, the appressoria, which developed under the coverslips, were collected and placed on a glass slide for observation under a light microscope¹. The identification of putative *Colletotrichum* strains was based on the description of Than *et al.* (2008)¹⁷.

Identification of pathogenic fungi species via sequencing

Based on the morphological characterization, the putative *Colletotrichum* samples grown on PDA medium were sent to the PHUSA Biochem LTD. company for sequencing, using the ITS1 primer (CTTGGT-CATTTAGAGGAAGTAA). The sequenced ITS regions were used for the sequence homology analysis. To identify *Colletotrichum* species, the sequencing results of the putative *Colletotrichum* samples were used to BLAST with sequences available from GenBank Nucleotide database of NCBI (National Center for Biotechnology Information).

Pathogenicity test

The two isolated *Colletotrichum* strains were used to study the anthracnose resistance of eleven chili pepper cultivars. *Colletotrichum* isolates were incubated on PDA medium at 25 °C for 14 days. To collect the fungal conidia, 5 ml of sterile water was added directly on the culture surface, followed by gently shaking of the petri dish. The conidia suspensions were collected to 1.5-ml tubes and centrifuged at 10000 rpm. The pellets that contained the concentrated conidia, was adjusted with sterile water to 10⁵ conidia per ml using a hemocytometer.

For the pathogenicity test, the experiment was performed twice to confirm the phenotype. In each replicate, two to six fully developed green fruits from each cultivar were injected with 10 µl of the conidia suspensions (10⁵ conidia per ml). Sterile water-infiltrated fruits were used as control. Three technical replicates were performed by injecting on three different places of a fruit of the cultivar that had medium or large fruits. To develop the disease, inoculated fruits were secured in plastic containers lined with wet paper towels and incubated for seven days at 25 °C. The

development of disease symptoms was observed, and the lesion diameter was measured every 24 h. The lesion diameter was determined by averaging the largest and the smallest diameters of each lesion. The mean of lesion diameters at seven days after injection were used to determine the resistance of eleven chili pepper cultivars to the particular fungal isolate. Data of lesion diameter from two replicates were statistically analyzed using analysis of variance ($P < 0.05$), followed by multiple range tests using Tukey's HSD test with SPSS 16.0 software (IBM).

Plant DNA extraction and PCR amplification

The genomic DNA of chili pepper was extracted using the CTAB method¹⁸. The DNA quality and purity were determined by nanodrop (ND-2000, Thermo Scientific). To verify the presence of anthracnose resistance-associated markers in different chili pepper cultivars, polymerase chain reactions (PCRs) were performed using primers listed in Table 1. PCRs using Taq DNA polymerase and other components were prepared according to the manufacturer's instruction (BIO-21105, Bioline). The thermal cycle was started with a denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C 30 s, 60 °C 30 s, 72 °C 1 min, and ended by a 2-min extension step at 72 °C. Reactions were held at 4 °C. Depending on the product size, 2 to 4% agarose gel was used to migrate DNA under 150 V constant voltage for 50 min. DNA was then visualized under UV light, and DNA size was determined according to the 100-bp ladder (SM0241, Thermo Scientific).

RESULTS

Isolates obtained from infected fruits

From the infected fruits collected from chili pepper fields in Tra Vinh province, five different fungal isolates were obtained. Among these isolates, two putative *Colletotrichum* strains, named 18T and 21T, were identified. The morphology analysis was performed according to the morphological descriptions of *Colletotrichum* species outlined by Than *et al.* (2008)¹⁷.

Colonies of the 18T strain grown on PDA showed pale orange color with fluffy aerial mycelia on the colony surface (Figure 1 A). Conidia of the 18T isolate were smooth-walled, transparent, colorless straight, cylindrical form with uneven ends. The size of the conidia was 10 – 14- μm long and 3 – 4- μm wide. The 18T appressoria were ovoid to ellipsoidal, 6 – 9- μm long, 4 – 6- μm wide, with medium to dark brown color. On the other hand, the colonies of the 21T strain grown on PDA were grey to pale orange with thin cottony

mycelia on the surface (Figure 1 B). The 21T isolate produced fusiform conidia with both acute ends, 7 – 13- μm long and 3 – 4- μm wide. Most of the 21T appressoria were irregularly shaped, 5 – 8- μm long, 3 – 4- μm wide.

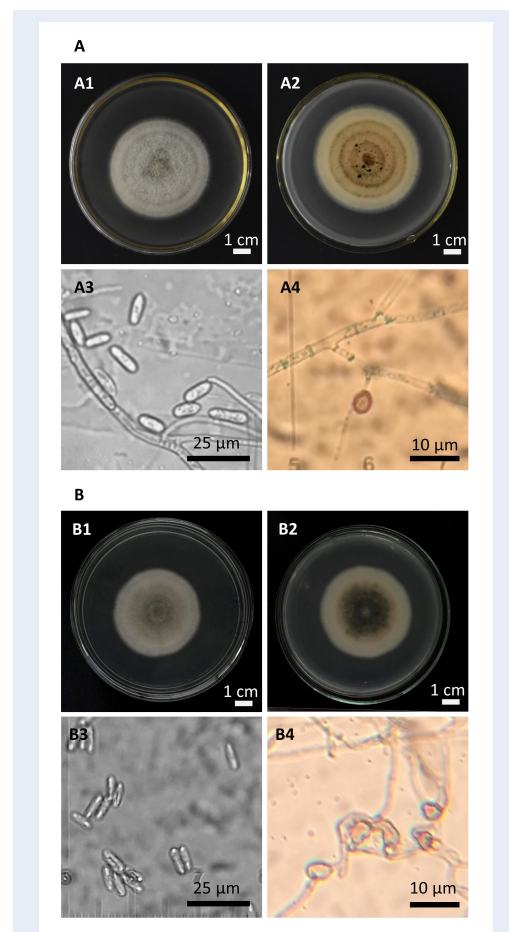


Figure 1: Morphology analysis of the *Colletotrichum* isolates on PDA medium. Morphology of the 18T isolate (A1 - A4) and the 21T isolate (B1 - B4). The morphological analysis based on the upper side (A1, B1) and lower side (A2, B2) of colonies grown on PDA medium, conidia (A3, B3) and appressoria (A4, B4).

The species of the two putative *Colletotrichum* isolates were identified by sequencing the ITS region using the ITS1 primer. The sequencing result successfully confirmed the prediction that was based on the morphology observation. By comparing with the *Colletotrichum* sequence database from NCBI, the ITS sequences of the 18T and 21T isolates showed the highest similarity to *Colletotrichum scovillei* (Accession number: MH603141.1) and *C. acutatum* (Accession number: MF629920.1), respectively (data not

Table 1: List of molecular markers and the respective primers used in this study

Marker name	Primer sequence (5' to 3')	Product size	Reference
HpmsE032	ATGCGCAAAGGGAGAAAATTCA	282 bp	Suwor et al. (2017)
	CGAACTAACCGTTCATGGTGGG		
HpmsE116	TCTCTCTACATCTCTCCGTTGA	245 bp	Yi et al. (2006)
	ACTCCCATACGGTGTCTCGTTC		Sun et al. (2015)
HpmsE126	TGGGTATTTCTTCTGCTGGAG	108 bp	
	TCCTTCCAGGAACTGATGG		
HpmsE047	AACCCGTGTTCAATCCCCAAAT	252 bp	Jayaram et al. (2015)
	TGGCCATACCACCAGCAGTAGA		

shown). Therefore, together with the morphology observation, the 18T and 21T isolates were identified as *C. scovillei* and *C. acutatum*, respectively. These two fungal strains were later used in the experiment to determine the anthracnose resistance of different chili pepper cultivars.

Pathogenicity testing

Fruits from 11 chili pepper cultivars were infected with the two isolates of *Colletotrichum* species, *C. scovillei* and *C. acutatum*. Both isolates were pathogenic and could successfully infect most of the cultivars. The infected areas were all rounded-shape with concentric acervuli (Figure 2 A). The lesion diameters of the eleven cultivars injected with the two *Colletotrichum* isolates were described in table 2. The water-injected fruits significantly showed no lesion in eleven cultivars at seven days after injection (Figure 2). When injected with the *Colletotrichum* conidia, the lesion diameters were all significantly larger than their corresponding water-injected controls (Figure 2 B). There were also significant differences in lesion diameter among eleven cultivars. The two cultivars, including CN404 and HNCS showed the strongest resistance to both *Colletotrichum* species with the smallest infection diameter, smaller than 3 mm. In these cultivars, larger lesion diameters when infected by *C. acutatum* compared to that infected by *C. scovillei* indicated that they were more resistant to *C. scovillei*. Additionally, TN535 and HNNS were the two most susceptible cultivars to both *Colletotrichum* species due to their large lesion diameters when infected by these two pathogens. The resistance level to *C. scovillei* was similar to *C. acutatum* in each cultivar, with the exception of the TV3 and PN400 cultivars (Figure 2 B). TV3 was strongly resistant to *C. scovillei* (3 mm in lesion diameter), but moderately

resistance to *C. acutatum* (6 mm in lesion diameter). The PN400 cultivar, on the other hand, was susceptible to the *C. scovillei* (11.5 mm in lesion diameter) but showed the moderate resistance to *C. acutatum* (7.5 mm in lesion diameter).

Potential anthracnose resistance-associated molecular markers

Four different PCR-based molecular markers (SSR markers), were tested to determine the potential molecular marker related to the anthracnose resistance in Vietnamese chili pepper cultivars. PCRs were performed for eleven chili pepper cultivars, and the summary of results was presented in table 2. Among the four tested molecular markers, HpmsE032, HpmsE047, and HpmsE116 were detected in both resistant and susceptible cultivars indicating these markers are not associated with the anthracnose resistance caused by defined *Colletotrichum* species (Figure 3 B-D). Interestingly, the HpmsE126 marker was observed only in two of three anthracnose resistant cultivars, CN404 and TV3 (Figure 3 A). These two chili pepper cultivars carrying HpmsE126 showed very strong resistance to anthracnose since their lesion diameter was significantly lower than the lesion diameter in the cultivars without this marker. The data analysis also suggested that having HpmsE126 marker might contribute more to *C. scovillei* resistance than *C. acutatum* resistance (Figure 4). Among the markers showing differences between cultivars, HpmsE126 was observed in the two cultivars, CN404 and TV3, which had a very strong resistance to anthracnose, especially the disease caused by *C. scovillei*. As a result, the HpmsE126 might be related to the resistance to anthracnose and would be a potential molecular marker used in chili pepper breeding. Given that these chili pepper cultivars showed

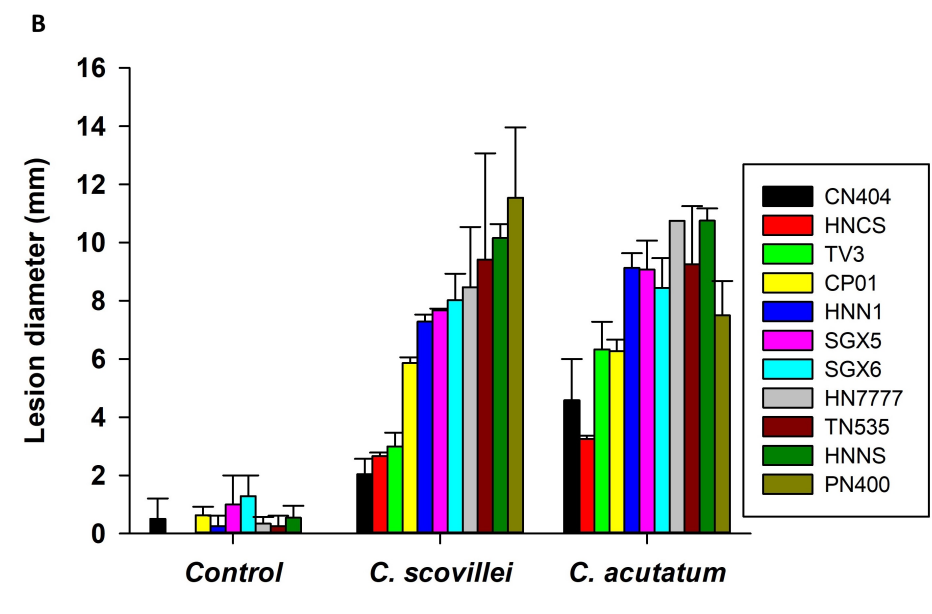
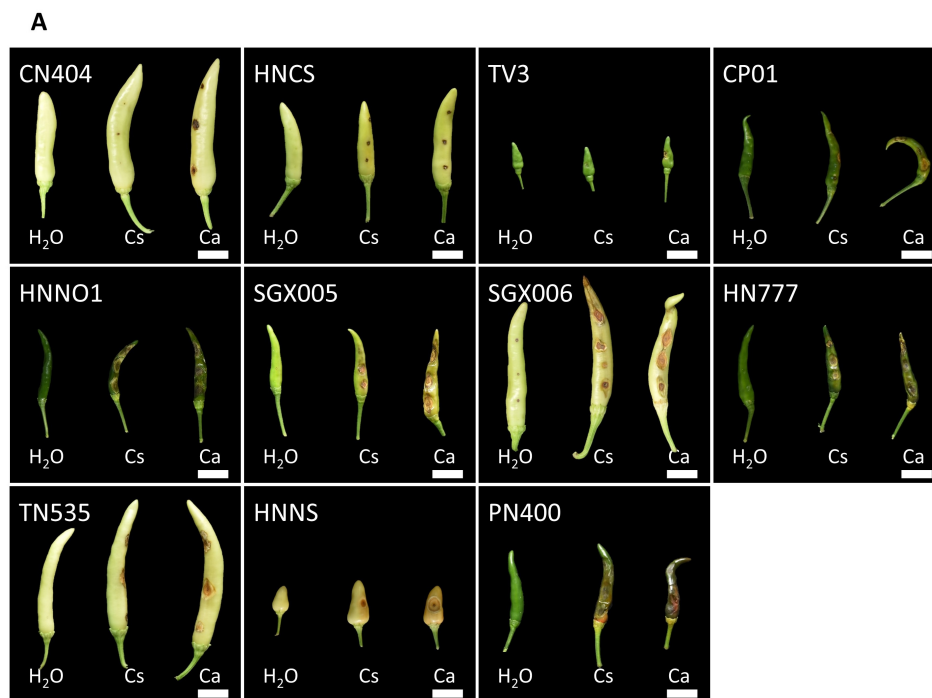


Figure 2: Anthracnose symptoms on chili fruits from different cultivars at 7 days after injection. (A) Disease symptoms developed on chili fruits from eleven cultivars after injection with either *C. scovillei* (Cs) or *C. acutatum* (Ca) isolates. The water-injected fruits were used as control (H2O). Scale bars represent 1 cm. (B) Comparison of lesion diameter caused by the two *Colletotrichum* isolates on eleven chili pepper cultivars. Mean represents data from two independent replicates. Error bars represent the standard deviation.

Table 2: Lesion diameter caused by two *Colletotrichum* isolates in each cultivar and the detected molecular markers

Cultivar	Lesion diameter*		HpmsE126	HpmsE032	HpmsE047	HpmsE116
	<i>C. scovillei</i>	<i>C. acutatum</i>				
CN404	2.04 a	4.58 ab	X	X	X	X
HNCS	2.67 ab	3.25 a		X	X	X
TV3	3.00 ab	6.33 abc	X	X	X	X
CP01	5.86 abc	6.28 abc		X	X	X
HNN01	7.28 abc	9.13 cd		X	X	X
SGX005	7.66 abc	9.08 cd		X	X	X
SGX006	8.02 bc	8.44 bcd		X	X	X
HN7777	8.47 c	10.75 d		X	X	X
TN535	9.42 c	9.25 cd		X	X	X
HNNS	10.16 c	10.75 d		X	X	X
PN400	11.54 c	7.50 bcd		X	X	X

* Different letters indicate statistically significant difference (ANOVA, Tukey's HSD test, P < 0.05)

stronger resistance to *C. scovillei* compared to *C. acutatum*, the HpmsE126 marker might be more related to the *C. scovillei* resistance in chili pepper. Interestingly, it must be noticed that the HNCS cultivar was among the best anthracnose resistant cultivars in this study, although the HpmsE126 marker was not found in this cultivar (Figure 3 A).

DISCUSSION

In addition to *C. acutatum* isolation, which was previously identified in studies in Vietnam^{7,8}, different *Colletotrichum* species causing anthracnose disease in chili pepper in Vietnam, *C. scovillei*, was firstly identified in this study. This *Colletotrichum* species was previously reported to cause chili anthracnose disease in Japan, Brazil, China, and Korea^{6,19-21}. Recently, it was shown that in South Asia and South-East Asia, except Sri Lanka, *C. scovillei* was the prevalent anthracnose-causing pathogen and showed very high aggressiveness when infecting non-wounded fruits²². In the study of Oo *et al.* (2017), all 36 tested cultivars belonging to *C. annuum*, *C. chacoense*, and *C. baccatum*, were susceptible to the *C. scovillei* isolates⁶. Crossing between *C. annuum* and *C. baccatum* conferred to the resistance to *C. scovillei* in hybrid sweet pepper was reported in 2012⁶. Interestingly, among the tested cultivars in this study, three cultivars, including CN404, HNCS, and TV3, showed strong resistance to the *C. scovillei* isolate obtained from Tra Vinh province. These cultivars could be a valuable *C. scovillei* resistance source for chili pepper breeding.

According to the findings in this study, it is likely that the *C. scovillei* resistance trait has already been introduced into commercial chili pepper cultivars in Vietnam.

Most of the present commercial chili pepper cultivars belong to the *C. annuum* species. While *C. annuum* is commercially important, this species is highly anthracnose susceptible²³. From the 1990s, the potential anthracnose resistance sources were identified in other chili pepper species, including *C. baccatum* (PBC80, PBC81) and *C. chinense* (PBC932)⁹. In 2014, by studying the backcross BC₁ population derived from the hybrid between *C. annuum* line 77013 and the *C. chinense* PBC932 using QTL analysis method, different markers, including HpmsE116 and HpmsE126, were identified to be closely related to the resistance of chili pepper fruit to *C. acutatum*^{24,25}. In this current study, only the HpmsE126 was found to be related to moderate anthracnose resistance caused by *C. acutatum*. Interestingly, plants carrying this marker also showed additional strong resistance to *C. scovillei*, suggesting that a common mechanism might be shared between *C. acutatum* resistance and *C. scovillei* resistance. Although HpmsE126 was considered as a minor QTL related to anthracnose resistance, in the current study, this marker was more efficient in identifying the anthracnose-resistant cultivars compared to the other markers. Other markers, HpmsE032 and HpmsE047, were proven to be closely related to the anthracnose resistance^{26,27}. However,

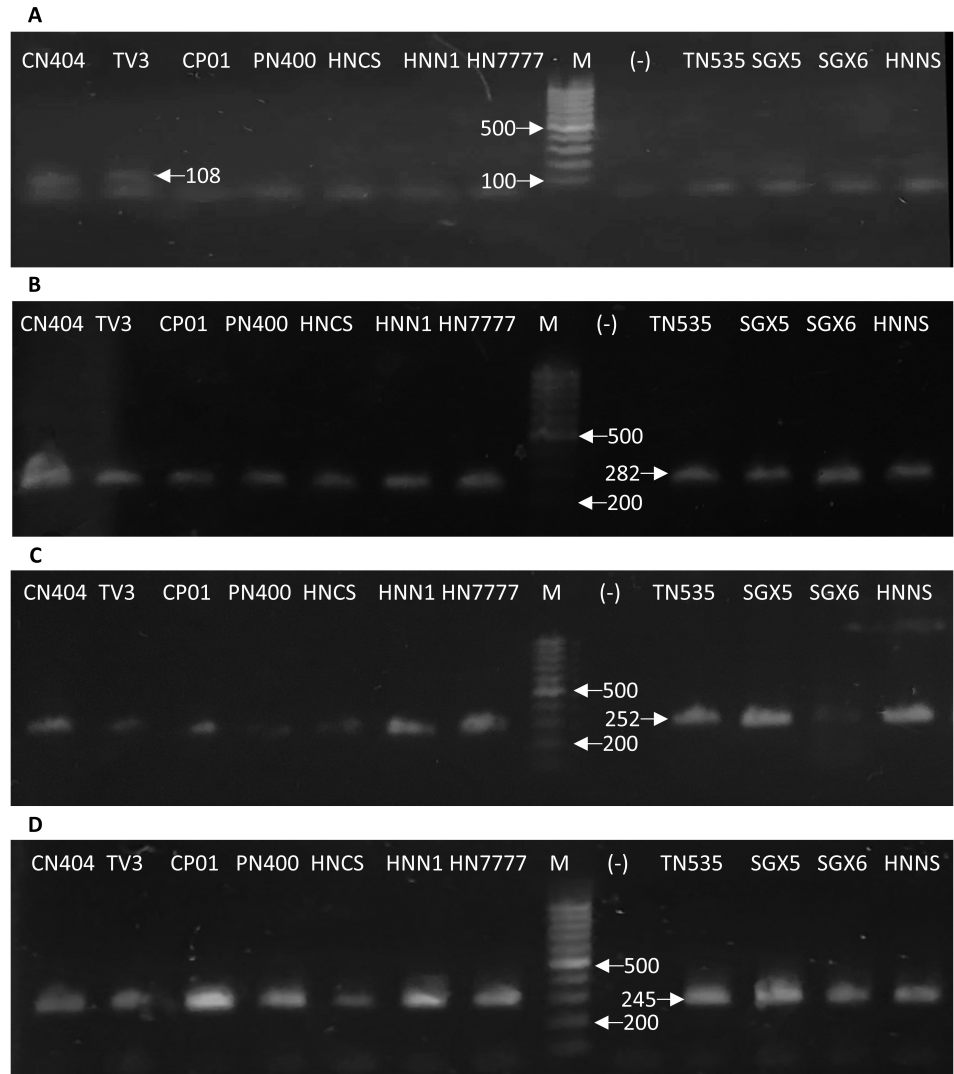


Figure 3: DNA electrophoresis of molecular markers amplified by PCRs in different chili pepper cultivars. Marker detection of HpmsE126 (A), HpmsE032 (B), HpmsE047 (C) and HpmsE116 (D) in eleven chili pepper cultivars. Lane M: 100-bp DNA Ladder, lane (-): DEPC-treated H₂O. All PCR products were migrated in 2% agarose gel.

in this study, these markers were not able to determine the anthracnose resistance variance in the tested chili pepper cultivars. It must be noted that the anthracnose disease in chili pepper is caused by different *Colletotrichum* species. HpmsE047 was determined as a disease resistance marker when tested with *C. gloeosporioides* and *C. capsici*^{26,28}. It is possible that chili pepper employs different molecular mechanisms to fight against different *Colletotrichum* species. In the case of HpmsE032, although this marker was proven to be a *C. acutatum*-resistant marker, its efficiency was only 65% suggesting the involvement of

other genes yet unidentified in the defence mechanism against *C. acutatum* and the combination of different marker would be more efficient in the detection of resistant cultivar²⁷. In 2011, Lee *et al.* identified two markers related to the resistance to *C. scovillei*/*C. acutatum* (CaR12.2) and *C. truncatum*/*C. capsici* (CcR9). The later marker was developed to SCAR marker (CcR9M1-SCAR) and was protected under the worldwide patent KR101010446B1^{13,28}. These markers could be the potential additional tools for the detection of anthracnose-resistant cultivars, although its application in Vietnam could be difficult

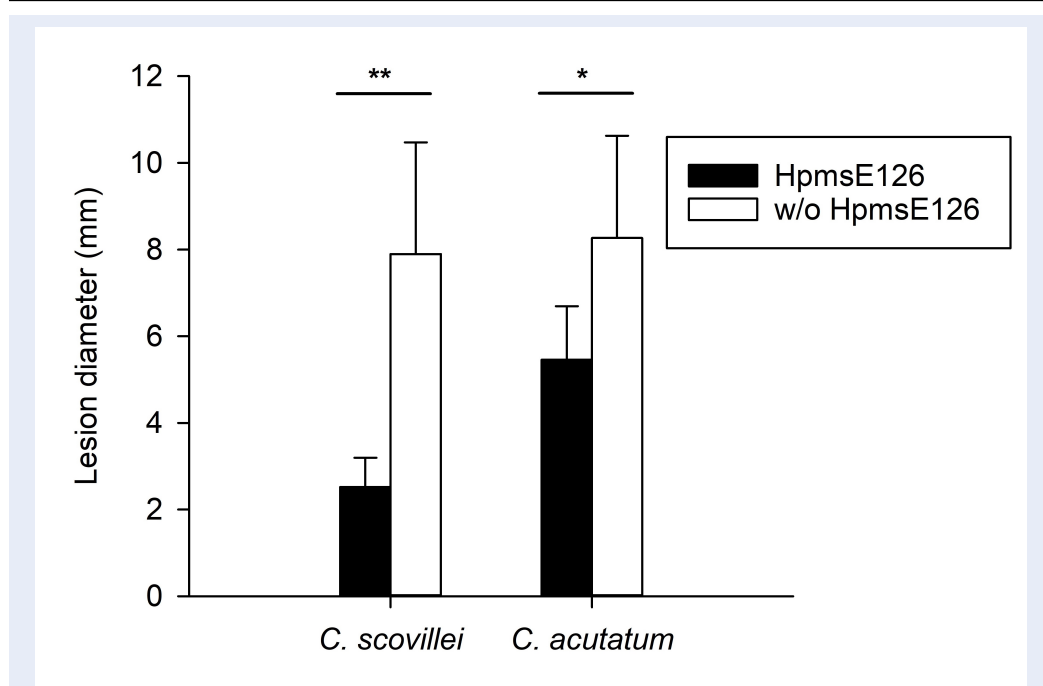


Figure 4: Comparison of lesion diameter between cultivars having HpmsE126 marker and cultivars without HpmsE126 marker. Data is presented as mean \pm SD. Means were compared using Student's t-test. Statistical difference is presented by either (*) ($P < 0.05$) or (**) ($P < 0.01$).

due to patent protection. In this current study, the strong resistance to both *C. scovillei* and *C. acutatum* of the HNCS cultivar without HpmsE126 marker supported the involvement of other gene clusters and other mechanisms in the resistance to *C. scovillei* and *C. acutatum* and the possibility to combine with other molecular markers in the determination of anthracnose resistance caused by *C. scovillei* and *C. acutatum* in chili pepper. The findings also support that the CN404 cultivar could be a very good commercial chili pepper cultivar for chili production and cultivation, especially in the regions affected by *C. scovillei* and *C. acutatum*. Additionally, the wild bird-eye chili pepper cultivar found in Tra Vinh province could be a potential anthracnose resistance source, which can be applied for chili pepper breeding.

CONCLUSION

In this study, from the infected fruits collected from chili pepper fields in Tra Vinh province, two *Colletotrichum* isolates, *C. scovillei* and *C. acutatum*, were successfully isolated and identified using morphological and sequencing approaches. Among eleven tested chili pepper cultivars infected by these *Colletotrichum* isolates, two cultivars were identified to have the resistance against both of these pathogens. Moreover, the

marker analysis found the HpmsE126 SSR marker in the two *C. scovillei* resistant cultivars. The HpmsE126 might be an efficient molecular tool to identify the anthracnose-resistant cultivars for chili pepper breeding.

ABBREVIATIONS

ITS Internal transcribed spacer
 SSR Simple Sequence Repeats
 SCAR Sequence Characterized Amplified Region
 NCBI National Center for Biotechnology Information

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

All the experimental works were performed by VA Ly. TPT Truong was involved in the pathogen isolation and molecular biology works. The project design and manuscript writing were performed by TH Nguyen.

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