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Identification of shallot pathogens in Vĩnh Châu town of Sóc Trăng province

Bui Thanh Thu, Tran Quoc Tuan and Nguyen Dac Khoa*

Biotechnology Research and Development Institute, Can Tho University, Vietnam

**Correspondence: Nguyen Dac Khoa (email: ndkhoa@ctu.edu.vn)*

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ABSTRACT

Shallot (Allium ascalonicum) is an important crop of Vĩnh Châu town, Sóc Trăng province of Vietnam. This study aims at identifying the contemporary pathogens in shallot fields in this region. The identification was done using the Koch's postulates, morphological observation and molecular techniques. A collection of 124 infected shallot samples was obtained from three cropping seasons during 2015-2016 at three major shallot producing areas of Vĩnh Châu town. From these samples, a total of 49 bacterial and 118 fungal isolates were obtained. Using the Koch's postulates, 160 isolates were confirmed to be shallot pathogens. Based on morphological observation and molecular techniques, i.e., PCR using specific primers and sequencing of the 16S rRNA genes, the pathogens were identified as Erwinia carotovora (soft rot), Pseudomonas aeruginosa (bulb rot), Aspergillus niger (black mold), Colletotrichum gloeosporioides (anthracnose) and Fusarium oxysporum (basal rot). Among these, E. carotovora and F. oxysporum appeared to be the predominant pathogens causing bulb rot in the shallot fields of Vĩnh Châu town.

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1 INTRODUCTION

Shallot (*Allium ascalonicum*) is cultivated in many countries around the world (Sintayehu *et al.*, 2014). It is an important crop of Vĩnh Châu town, Sóc Trăng province of Vietnam, which covers approx. 6,000 hectares of the region. It has been the main source of farmers' income here (Dang Thi Cuc, 2008). However, shallot cultivation in recent years has been facing different diseases such as soft rot, bulb rot, basal rot, black mold and anthracnose. They cause significant damages to shallot quality and yield, especially during storage, and reducing its commercial values. In 2005, about 50% of shallot growing areas in Vĩnh Châu town was damaged and lost due to bulb rot diseases (Dang Thi Cuc, 2008). Identification of pathogens causing diseases of shallot is necessary to develop effective methods to control the diseases. This study aims at identifying the contemporary pathogens in shallot fields in Vĩnh Châu town, Sóc Trăng using the Koch's postulates, morphological observation and molecular techniques, i.e., PCR using specific primers and sequencing of the 16S rRNA genes.

2 MATERIALS AND METHODS

2.1 Collection of shallot samples and pathogen isolation

Diseased shallots were collected from shallot fields in Ward 1, Ward 2 and Vĩnh Hải commune of Vĩnh Châu town, Sóc Trăng province. The shallot samples were then classified into two groups including bacteria- and fungi-infected samples based on the disease symptoms. Rot diseases caused by bacteria

were identified using water-soaking symptoms (Schwartz and Bartolo, 1995). After removing leaves, roots and the outer scales of shallot samples, the samples were surface sterilized using 70% ethanol in three minutes then rinsed with sterile distilled water. Bacterial and fungal pathogens were isolated from the samples according to Burgess *et al.* (2008).

Bacterial isolation: A small piece of shallot was cut at margin of necrotic tissue and macerated with sterile distilled water for 15 minutes for the bacteria oozing out. The bacteria then were streaked on nutrient agar (NA) plates [5 g peptone, 3 g beef extract, 5 g NaCl, 15 g agar and distilled water per 1 L of the medium, pH 6.8 (Shivaji *et al.*, 2006)] and incubated at 25 ± 2°C for 24-48 hrs. Based on the morphological characteristics, different isolates were transferred to new NA plates until pure culture.

Fungal isolation: Small segments of shallot were cut at margin of necrotic tissue and placed on potato dextrose agar (PDA) plates [200 g of potato infusion, 20 g of dextrose, 20 g of agar and distilled water per 1 L of the medium (Shurleff and Averre, 1997)]. The plates were incubated at 25 ± 2°C for 48 hrs. for fungal growth. The fungi were then isolated from single-spore method.

Bacterial isolates and spores of fungal isolates were stored in glycerol 50% at -20ºC.

2.2 Pathogenicity test

The disease-free shallot sets (immature bulbs) of the susceptible cultivar (local variety) were provided by the Plant Protection Department of Sóc Trăng Province.

Soil preparation: Alluvial soil was initially mixed with sand (in mass ratio 7:3) and autoclaved at 121°C, 1 atm for 30 minutes. One kg of the soil mixture was put in each round pot (15×25 cm) and covered with a layer of rice husk and rice husk ash.

Shallot planting: Shallot bulbs were removed old roots and planted in each pot (3 bulbs/pot) by pushing the bulbs into the ground so that their lower three-quarters were buried. Shallots were watered daily and fertilized with recommended dose following the guidelines of the Plant Protection Department of Sóc Trăng (Dang Thi Cuc, 2014).

2.2.1 Pathogenicity test under nethouse conditions

Bacterial diffusion was prepared by adding a loop full of 24-hour-old bacterial culture to 1 mL of sterile distilled water and homogenized by vortexing. At 30 days after planting (DAP), each bulb was inoculated by injecting 30 µL of bacterial suspension in (artificial wound located on) the shallot bulb (Lan *et al.*, 2013). Sterile distilled water was used in the control treatment.

The conidial suspension (10^5 conidia/mL) of each fungal isolate was prepared as described by Stankovic *et al.* (2007). The shallot bulbs were inoculated by soil drenching method at 30 DAP. Ten mililiter of the conidial suspension were thoroughly sprayed in each pot and sterile distilled water was used in the control treatment (Stankovic *et al.*, 2007; Nova *et al.*, 2011).

2.2.2 Pathogenicity test under storage conditions

Outer scales of the bulbs were removed to leave a single brown layer of skin, and its surface was sterilized with 70% ethanol.

Bacterial isolates were inoculated to shallot bulbs in the same method that were used in nethouse conditions. Thirty microliters of each bacterial suspension were injected in the middle of shallot bulb by using sterile syringe (Wright *et al.*, 1993).

A sterile cork borer was used to make a hole (5 mm diameter and 3 mm deep) on shallot bulbs, preparing for inoculation with fungal isolates. Thirty microliters of spore suspension (10^5 conidia/mL) of each isolate were inoculated in the hole (Prithiviraj *et al.*, 2004; Shehu and Muhammad, 2011).

A total of three bulbs were placed on Petri dish which was put inside a sealed plastic bag, with a moist cotton ball to maintain a high humidity atmosphere to facilitate infection (Taylor *et al.*, 2016). Sterile distilled water was used in the control treatment.

Symptoms observation and re-isolation

Inoculated shallot bulbs in Koch's postulates (Burgess *et al.*, 2008) under nethouse and storage conditions were kept under observation for four weeks and diseases symptoms were recorded. In addition, re-isolation of the pathogen from the newly diseased material was performed to complete the Koch's postulates.

2.2.3 Identification of pathogens

Morphological identification

Colony morphology of each isolate was observed and recorded. Furthermore, Gram staining were performed according to method described by Benson (2002), and the shape of bacteria were also observed under a microscope.

Fungal isolates were cultured on PDA medium at 25°C for 7-10 days for colony morphology observation. In addition, the shape of conidia, conidio-

phores, hyphae and other morphological characteristics of fungal isolates were also observed under an optical microscope. Morphological characteristics of the isolates were recorded and compared with morphological characteristics of the fungi described by Campbell *et al*. (2013) for genus identification.

PCR reaction using specific primers

Genomic DNA of bacterial and fungal isolates were extracted as described by Zakham *et al.* (2011) and Bayraktar and Dolar (2011). The specific primer sets were used for identification of *Erwinia carotovora, Pseudomonas* sp., *Aspergillus* sp., *Fusarium* sp., *Colletotrichum gloeosporioides* (Table 1). PCR reactions were set up following the procedures recommended.

Table 1: List of specific primers used for identification of shallot pathogens

Primer code	Specificity	Primer sequence	Amplified product size	References
Y1 Y2	*Erwinia carotovora*	TTACCGGACGCCGAGCTGTGGCGT CAGGAAGATGTCGTTATCGCGAGT	434 bp	Darrasse *et al.* (1994)
CFL.F CFL.R	*Pseudomonas* sp.	GGCGCTCCCTCGCACTT GGTATTGGCGGGGGTGC	650 bp	Dutta *et al.* (2014)
Asp1 Asp2	*Aspergillus* sp.	CGGCCCTTAAATAGCCCGGTC ACCCCCCTGAGCCAGTCCG	363 bp	Melchers *et al.* (1994)
FOF1 FOR1	*Fusarium* sp.	ACATACCACTTGTTGCCTCG CGCCAATCAATTTGAGGAACG	340 bp	Mishra *et al.* (2003)
MKCgF MKCgR	*Colletotrichum gloeosporioides*	TTGCTTCGGCGGGTAGGGTC ACGCAAAGGAGGCTCCGGGA	380 bp	Kamle *et al.* (2013)

16S rRNA sequencing of *Pseudomonas* sp.

Identification of *Pseudomonas* sp. isolate was performed by amplification of its 16S rRNA gene using universal primer set 27F/1492R (Weisburg *et al*., 1991). PCR products were sequenced by a commercial sequencing service provider (Phu Sa Biochem, Vinh Long province). Alignment of the obtained sequence with other 16S rRNA genes on the GenBank database (NCBI) was done using the standard Nucleotide Basic Local Alignment Search Tool (Nucleotide BLAST) where the percent similarity was used as a basis for bacterial identification.

3 RESULTS AND DISCUSSION

3.1 Isolation of fungi and bacteria from diseased shallot

A total of 124 diseased shallot samples were collected from shallot-cultivating fields in Vĩnh Châu town of Sóc Trăng province during three cropping seasons (from October 2015 to March 2016). Sixty percent of the total samples were collected in October 2015 when there was heavy rainfall along with high temperature. Therefore, the weather in this season might facilitate favorable conditions to pathogens causing the diseases since environmental factors were proven to have an important influence on the development of pathogens on shallot (Suhardi, 1993; Nguyen Duc Thang, 1999; Conn *et al.*, 2012; Dinakaran *et al.*, 2013).

Fifty-four out of 124 infected-shallot samples had the symptoms of bacterial wet rot with yellow leaves, rotten bulb, foul odor and lesion spreading deep inside the bulb. After the diseased symptoms were recorded, the samples were taken to isolate bacteria on NA medium, and a total of 49 (K1 to K49) bacterial isolates were recovered.

The remaining 70 fungi-infected samples had symptoms similar to the descriptions of fungal diseases on shallot by Vo Hoang Nghiem (2012). Based on symptoms, the samples were divided into three groups including anthracnose, basal rot, and black mold rot. A total of 118 (N1 to N118) isolates were obtained, in which 54 isolates isolated from basal rot samples, 34 isolates from anthracnose samples and 30 fungal isolates from black mold rot samples.

3.2 Pathogenicity test

3.2.1 Pathogenicity test under nethouse conditions

There were 42 out of 49 bacterial isolates being able to cause disease symptoms on shallot under nethouse conditions. Firstly, leaves turned yellow and wilt, scales at inoculated site were discolored. At 10 days after inoculation (DAI), lesions spread into inner scales and caused rot.

Bacterial isolates

The observed bacterial rot can be divided into two types based on disease symptoms. The first type consists of 20 isolates causing discoloration and softness of the infected scales (Fig. 1A) along with cream-colored liquid oozing out of dissected bulbs with foul smell. The second type had 22 isolates causing watery rot in inner scales of shallot with the

inner scales slightly shriveled and darker brown (Fig. 1B).

Fig. 1: Bulb longitudinal section showing extensive infection of the scales

A: soft rot and discoloration of infected bulb. B: brown rot of infected bulb

Fungal isolates: The result of pathogenicity test showed that all of 118 isolates were be able to cause disease on shallot under nethouse conditions. The infected shallots showed symptoms similar to those observed in the field. Specifically, after 10 DAI, shallots expressed three different types of symptoms.

Fifty-four out of 118 fungal isolates caused symptoms of yellowing, rotting of basal plate and discoloration of outer scale (Fig. 2A). These symptoms were consistent with description of Cramer (2000) about onion basal rot.

A total of 30 fungal isolates were shown to be the pathogens causing diseases of shallot of which symptoms under nethouse conditions included discoloration at infected site and development of black mold at the neck of shallot bulbs (Fig. 2B).

Fig. 2: Symptoms of diseases on shallot causing by fungal pathogens

A: shallot basal rot; B: black mold developed at shallot bulb neck. C: anthracnose lesion on shallot leaves

Beside yellowing and curling of leaves, shallots that were inoculated with 34 remaining isolates also were found white oval sunken spots on the leaves. In addition, many orange acervuli which consist a lot of conidia were formed on the spots (Fig. 2C). These recorded symptoms were similar to those in the study of Alberto (2014) on anthracnose of onion.

3.2.2 Pathogenicity test under storage conditions

Bacterial isolates: At 10 DAI, 42 out of 49 bacterial isolates were capable of causing diseases of shallots under storage conditions. Similarly, bulb rot symptoms caused by these 42 isolates in storage conditions were also divided into two main disease types like those under nethouse conditions. Specifically, 20 isolates caused soft rot while the other 22 isolates caused brownish rot in infected bulbs. However, the lesions at the site of inoculation on the shallot bulbs have been shown to dry faster than those in nethouse conditions.

Fungal isolates: All of 118 fungal isolates caused rot symptoms on shallot bulbs under storage conditions after 10 DAI. The infected shallots inoculated with 30 fungal isolates from black mold shallot samples showed distinct symptoms. Specifically, clusters of black spores formed at inoculation sites and infected tissues were water-soaking and then became dry after 7 DAI.

Bulb rot symptoms caused by fungal isolates obtained from anthracnose and basal rot samples were similar. After 3 days of observation, shallots inoculated with these fungal isolates began to exhibit symptoms of discoloration of outer scales. At 7 DAI, the lesions were more widespread, and rotten-smell was emitted from the bulbs.

The same fungal and bacterial isolates were re-isolated from the diseased shallots to fulfill the Koch's postulates. After the pathogenicity test under nethouse and storage conditions, the results shown that all of 118 fungal isolates and 42 out of 49 bacterial isolates were pathogens causing diseases of shallot.

3.3 Identification of pathogens

3.3.1 Morphological identification

Bacterial pathogens

The results of Gram staining and microscopic morphology of 42 pathogenic bacterial isolates showed that they were rod shaped and belonged to the group of Gram-negative bacteria (Fig. 3A). After 4 days

on NA medium at 25°C, the colonies of these isolates was round and rose. The main difference between two groups of isolates causing bacterial rot in shallot was in the color of their colonies. Specifically, twenty isolates causing brownish of the shallot scales had an opaque colony (Fig. 3B). However, the 22 isolates that causing shriveled of the central part of infected shallot had yellow colonies (Fig. 3C).

Studies have proved that *Pseudomonas* sp., *Erwinia carotovora* and *Enterobacter cloacae* were capable of causing bacterial rot symptoms of onion (Schwartz and Bartolo, 1995; Vu Trieu Man, 2007; Black *et al.*, 2012). In addition, preliminary morphological observations and the disease symptoms caused by 42 bacterial isolates on shallot suggested that these isolates might be *Pseudomonas* sp. or *Erwinia carotovora*. Therefore, specific primers for *Erwinia carotovora* and *Pseudomonas* sp. were used in combination with morphology characteristics to identify 42 pathogenic bacterial isolates.

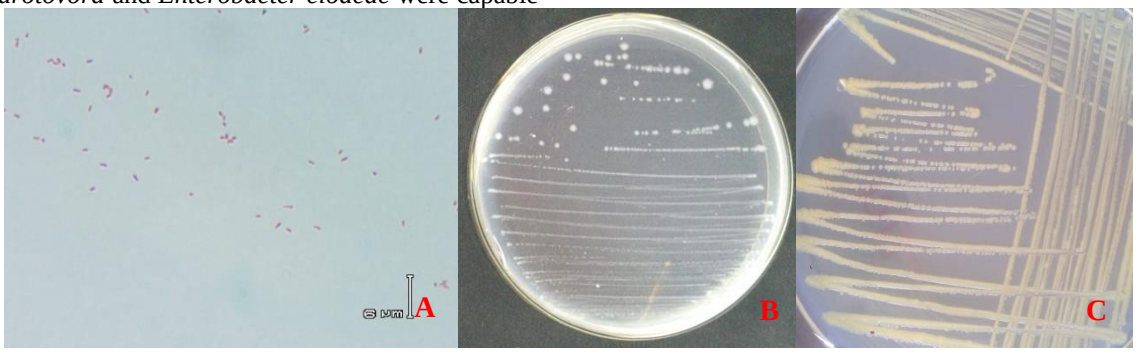

Fig. 3: Morphology of Gram-negative bacterial cells under microscope 100x (A) and morphology of colonies on Nutrient agar (B, C)

Fungal pathogens

Morphological characteristics of fungal isolates causing basal rot revealed that fungal hyphae growing on the PDA medium were white and formed abundantly on the surface of agar plate. Besides, these isolates all had sickle-shaped macroconidia with 3-5 septa (Fig. 4A) and single cell microconidia in accordance with description of Campbell *et al.* (2013) about the main characteristics for *Fusarium* genus identification. Since basal rot of onion was reported to be caused by *Fusarium oxysporum* in the major growing areas of the world (Cramer, 2000), specific primers for *Fusarium oxysporum* were used to identified these basal rot fungal pathogens.

Thirty-four fungal isolates which caused symptoms of anthracnose in the pathogenicity test were identified as *Colletotrichum* sp. To be more specific, conidia of these isolates were hyaline, single-celled and cylindrical (Fig. 4B), which was similar to the description of Le Hoang Le Thuy and Pham Van Kim (2008). Because pathogen causing anthracnose of onion were identified as *Colletotrichum gloeosporioides* (Alberto, 2014), specific primers for *Colletotrichum gloeosporioides* were used in identification of the pathogens causing anthracnose of shallot in Sóc Trăng province.

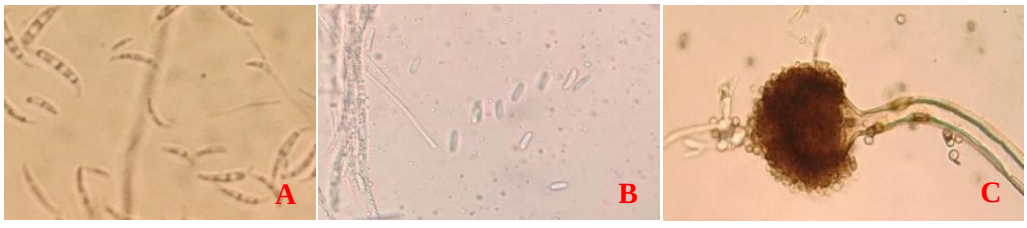

Fig. 4: Conidial morphology of *Fusarium* sp. (A), *Colletotrichum* sp. (B) and *Aspergillus* sp. (C)

Thirty fungal isolates causing black mold of shallot had a distinct colony appearance with black conidial heads covering a flat white mycelium. A closer look of these isolates under microscope revealed that brown globose conidia were produced abundantly on heads of conidiophore (Fig. 4C). The described morphological characteristics fit well to the description of *Aspergillus* sp. by Black *et al.* (2012). According to Schwartz and Bartolo (1995), *Aspergillus niger* was identified as the pathogen causing black mold of onion under storage conditions. Therefore,

Asp1/Asp2 primers which were specific for *Aspergillus niger* were used to identified these fungal pathogens.

3.3.2 PCR reaction using specific primers

Bacterial pathogens

The genomic DNA samples from the 42 pathogenic bacterial isolates were subjected to PCR analysis using the Y1/Y2 primers and CFL.F/CFL.R primers, which were specific for *E. carotovora* and *Pseudomonas* sp., respectively. The results showed that 20 out of 42 bacterial isolates had products with the size of 434 bp which were similar to the results of Darrasse *et al.* (1994), when using Y1/Y2 primer set to identify *E. carotovora* (Fig. 5B). Meanwhile, using *Pseudomonas* sp. specific primers (CFL.F/CFL.R), PCR products of 650 bp were generated from 22 remaining bacterial isolates (Fig. 5A).

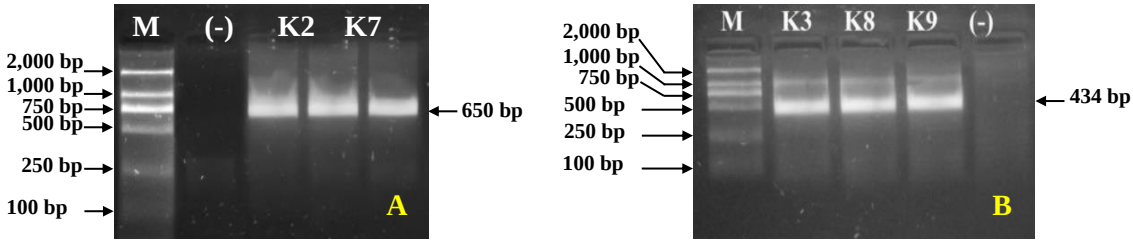

Fig. 5: Bands of 650-bp PCR products amplified by primer set CFL.F/R (A) and 434-bp PCR products amplified by the primer set Y1/Y2 (B) on 1.5% agarose gel

(A) - M: Ladder DL2000; K2, K7 and K10: Erwinia carotovora; (-): negative control. (B) - M: Ladder DL2000; K3, K8 and K9: Pseudomonas sp.; (-): negative control

It was determined that 22 bacterial isolates causing internal brown rot of shallot belong to genus *Pseudomonas*. However, there are many *Pseudomonas* spp. causing different diseases of onions. In particular, bacterial blight was caused by *P. syringae*, leaf streak and bulb rot by *P. viridiflava*, soft rot with shriveled of the internal scales by *P. gladioli* (Black *et al.*, 2012) and brown rot by *P. aeruginosa* (Mishra *et al.*, 2014). Therefore, *Pseudomonas* sp. K27 was chosen for further identification using 16S rRNA sequencing. The 16S rRNA gene segment of the isolate was sequenced (750 nucleotide) and aligned to other bacterial 16S rRNA genes in the GenBank database (NCBI). *Pseudomonas aeruginosa* (accession number: AY486361.1) was the best hit to K27 with 98% similarity.

Fungal pathogens

Thirty isolates causing shallot black mold were identified as *Aspergelus niger* since it created a specific amplification product of 363 bp size with primer set Asp1/Asp2 (Fig. 6A). Similarly, thirty-four isolates causing anthracnose of shallot were identified as *Colletotrichum gloeosporioides* after these isolates formed PCR products of the same size as description of Kamle *et al.* (2013) when amplified with primer set MKCgF/MKCgR (Fig. 6B). Furthermore, all of 54 isolates of *Fusarium* sp. causing shallot basal rot had specific product with primers FOF1/FOR1 that were designed to differentiate *Fusarium oxysporum* from other species of the *Fusarium* genus (Fig. 6C).

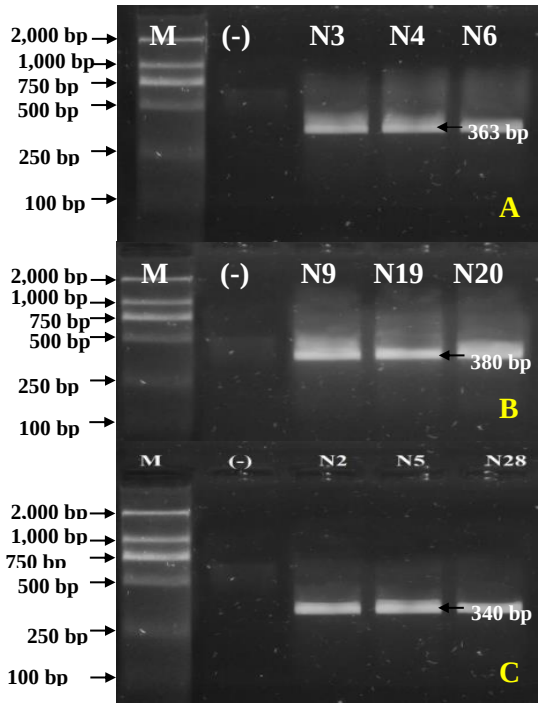

Fig. 6: Bands of 363-bp PCR products amplified by primer set Asp1/Asp2 (A), 380-bp PCR products amplified by the primer set MKCgF/R (B) and 340-bp PCR products amplified by the primer set FOF1/FOR1 (C) on 1.5% agarose gel

(A) – M: Ladder DL2000; N3, N4 and N6: Aspergillus niger; (-): negative control. (B) – M: Ladder DL2000; N9, N19 and N20: Colletotrichum gloeosporioides; (-): negative control. (C) – M: Ladder DL2000; N2, N5 and N28: Fusarium oxysporum; (-): negative control.

4 CONCLUSIONS

Five species that were identified as pathogens causing diseases on shallot in Vĩnh Châu town of Sóc Trăng province included *Erwinia carotovora*, *Pseudomonas aeruginosa*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides* and *Aspergelus niger*; of which *E. carotovora* and *F. oxysporum* appeared to be predominant pathogens.

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