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Identification of rose black spot pathogen in Sa Đéc city, Đồng Tháp province of Vietnam

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ABSTRACT

Black spot is one of the most destructive diseases of roses, causing premature defoliation, thus progressively weakening the plant and even leading to death. This study aimed at identifying the pathogen causing black spot on Rosa chinensis Jacq. cv. nhung (Hồng Nhung) and R. chinensis Jacq. var. minima (Hồng Tỉ Muội) in Sa Đéc city, Đồng Tháp province, Vietnam, using a combination of conidia morphology and pathogenicity tests including detached leaf and intact plant techniques. A total of 32 infected leaf samples with the black spot typical symptoms were collected from six rose cultivation areas. The morphological characterization of the conidia obtained from these samples was elliptical, hyaline, two-celled and had vacuole-like structures, similar to those produced by Diplocarpon. In pathogenicity test, the symptoms were observed on cv. nhung but not var. minima in the detached leaf technique while symptoms were observed on both cultivars in intact plants technique. These included black spots with perforated edges, aggregating into bigger patches. The infected leaves could yellow and defoliate at 21 days after inoculation. Compared to the previous pathogenic studies and description of diseases on roses, the fungal pathogen was identified as fungus Diplocarpon rosae.

1. INTRODUCTION

Roses (family Rosaceae, genus *Rosa*) have been cultivated worldwide for millennia and are currently one of the most important ornamental flowers in floriculture (Goody, 1993; Gachomo, 2005). Thousands of rose species, hybrids and cultivars have been bred and grown for their beauty. Their economic importance also relies on the use of rose petals as a source of natural fragrances and flavorings for perfume, food and medicinal industries (Guterman *et al.*, 2002; Roberts *et al.*, 2003; Baldermann *et al.*, 2009). In Vietnam, roses are the most popular year-round perennials which are planted and harvested in several parts of the country (Nguyen, 1998; Van & Nga, 2007). Sa Đéc, a provincial city of Đồng Tháp province is a major cultivation area and also the largest supplier of roses in Southwest Vietnam. Here, *Rosa chinensis* Jacq. cv. *nhung* (Hồng Nhung) and *R. chinensis* Jacq. var. *minima* (Hồng Tỉ Muội) are among the most widely cultivated cultivars, which have been grown for centuries, representing a significant portion of local farmers' income and labor (Tung & Phuong, 2014).

Roses are susceptible to several pests and diseases, among which, black spot is one of the most common and destructive diseases (Thuy, 2012). Symptoms are variable depending on rose varieties, fungal strains and environmental conditions, but initial

expressions include purplish or black circular spots with perforated edges appearing on the upper leaf surface with a diameter of up to 18 mm. In severely infected plants, these spots usually merge into large and black patches and leaf tissues may turn yellow around the leaf patches. Upon infection, the pathogen can cause premature defoliation, thus progressively weakens the plant and even leads to death in susceptible varieties (Dong *et al.*, 2002; Gachomo, 2005; Horst & Cloyd, 2007; Whitaker *et al.*, 2010). The disease is caused by *Diplocarpon rosae* Wolf [asexual stage: *Marssonina rosae* (Lib.) (Wolf, 1912; Whitaker *et al.*, 2007). The fungus can be cultured on artificial media but grows very slowly (Palmer *et al.*, 1966). Conidia were hyaline, straight to slightly curved, obovoid, unequally two-celled and ranged from 12~20 × 4~6 µm in size (Lee *et al.*, 2011).

This paper presents the identification of the pathogen causing black spot on two cultivars, *i.e.*, *R. chinensis* Jacq. cv. *nhung* (Hồng Nhung) and *R. chinensis* Jacq. var. *minima* (Hồng Tỉ Muội), in Sa Đéc city, Đồng Tháp province based on pathogenicity and morphology of the fungal conidia. This would provide a basis for further studies to control of the pathogen.

2. MATERIALS AND METHODS

2.1. Sample collection

Infected rose leaves with typical symptoms of black spot (purple to black spots, leaves turn yellow around the spots) on cv. *nhung* and var. *minima* cultivars were collected from six rose cultivation areas in Sa Đéc city, Đồng Tháp province. At each collecting site, infected leaves were obtained randomly from 5–10 plants (2 leaves/plant) by clipping at the petiole with sterilized scissors in infected field. The samples were placed in paper bags and information of the samples was recorded. The bags were kept at 25°C and transferred to the Molecular Biology Laboratory, Biotechnology R&D Institute, Can Tho University for pathogen isolation and identification.

2.2. Microscopic examination

Leaf samples were washed under running tap water for 5 min to remove soil debris and dirt and blot-dried on sterile paper towels. The samples were subsequently surface-sterilized with 70% (v/v) ethanol solution for 1 min and then 3% (v/v) H_2O_2 solution for 1 min, followed by rinsing in sterile distilled water three times for 10 sec for each. A 3 × 3 mm^2 piece at the edge of the typical lesion was cut and observed under a microscope at ×400 magnification (Zeiss Primo Star) with 1-2 drops of sterile distilled water. Leaf samples infected with *D. rosae* conidia were stored aseptically in plastic bags at −20°C as a source of inoculum for pathogenicity tests. In addition, the conidia were obtained and cultured on the potato dextrose agar (PDA) medium [200 g potato, 20 g dextrose and 20 g agar for 1 liter] supplemented with ampicillin for the morphological observation.

2.3. Rose cultivation

Rose cultivars: Two rose cultivars, *i.e.*, *Rosa chinensis* Jacq. cv. *nhung* and *R. chinensis* Jacq. var. *minima*, were obtained from Sa Đéc city, Đồng Tháp province. They were propagated by the layering method as described by Dong *et al.* (2002).

Soil preparation and plant cultivation: Alluvial soil was first treated with calcium carbonate and then amended with rice husk ash and coir compost in a mass ratio of 1:1:1. Each pot (10 × 17 cm) was filled with the mixture (2/3 volume of the pot) and 30 g NPK 13-13-13 + TE as basal fertilization (Binh Dien Fertilizer Joint Stock Company, 2017). One healthy rose plant was grown in each pot under greenhouse conditions with a mean temperature of 27±2°C under a 12-hour light/12-hour dark regime.

2.4. Pathogenicity test using the Koch's postulates and pathogen identification

Inoculum preparation: Conidia of *Diplocarpon rosae* were harvested directly from infected leaves as described by Yokoya *et al.* (2000). The infected-leaf samples were thawed at 25°C and then washed with sterile distilled water to harvest conidia. The number of conidia per milliliter was counted with haemocytometer (BLAUBRAND® Neubauer improved, Sigma-Aldrich, Germany) and adjusted to 10^5 conidia/mL.

Pathogenicity test using the detached leaf technique: The youngest fully opened leaves were collected and rinsed under running tap water for 10 min. The leaves were then surface-sterilized (as described in Section 2.2 Microscopic examination) and then placed on a 1.5% (w/v) water agar (WA) plates (1 leaf per plate) separated by a layer of sterilized Whatman filter paper with the adaxial side up as described by Palmer *et al.* (1966). The leaves were inoculated by pipetting 30 µL of the conidial suspension on the adaxial surface and incubated at 25°C. The same amount of sterile distilled water was used as the control and each treatment had three replicates.

Pathogenicity test using the intact plant technique: the technique was carried out as described by Gachomo (2005) with some minor modifications when rose plant had at least six fully opened leaves on each stem. Each stem was sprayed with five milliliters of the conidial suspension (5 x 10^5 conidia/mL) and wrapped with sterile nylon bags for adequate moisture content to promote disease development. In untreated control plants, the same amount of sterile distilled water was sprayed instead. Each treatment had three replicates on three independent rose plants.

Disease examination: After inoculation, symptom development was observed and recorded daily. The symptoms were compared to the typical symptoms of black spot and the pathogen was determined.

Pathogen identification: The pathogen was identified based on factors including *(1)* the typical symptoms observed in the detached leaf and intact plant techniques and *(2)* the microscopic morphology of fungal mycelia and conidia.

3. RESULTS AND DISCUSSION

3.1. Microscopic examination and provisional identification of the fungal pathogen

Based on typical symptoms of black spot, a total of 32 leaf samples were collected, of which 18 samples were from cv. *nhung* and 14 from var. *minima* (**Fig. 1**). The conidia harvested from those samples were similar; they were elongated-ovoid to elliptical, hyaline and two-celled, 19.4-24 x 5.5-6.0 µm. The cytoplasm of each cell had two vacuole-like structures (**Fig. 2A**). The fungus appeared to grow slowly on PDA medium. After 40 days of inoculation, mycelium was circular, fluffy and whitish on the front side and yellowish on the reverse side. The mycelium then turned regularly to light mouse grey after more than three months of inoculation (**Fig. 3**).

Figure 1. The black spot symptoms on rose leaves collected from infected fields in Sa Đéc city, Đồng Tháp province

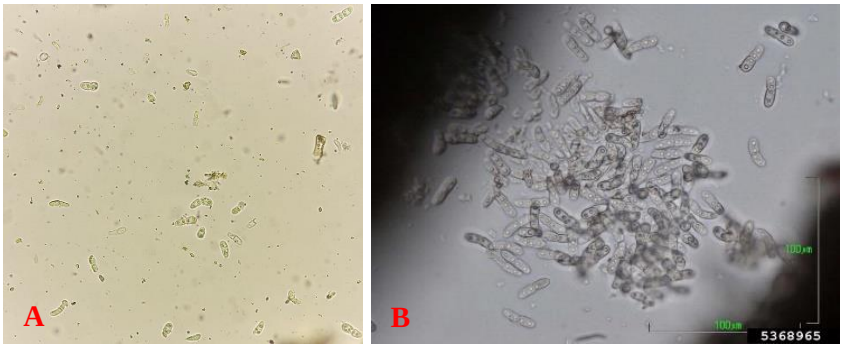

Figure 2. Morphology of the two-celled spores from black spot infected leaves under a light microscope at 400X magnification (A) compared to two-celled spores of the anamorph fungus *Marssonina rosae* (asexual stage of *Diplocarpon rosae*) (B) from the black spot on rose (Paul Bachi, University of Kentucky Research and Education Center)

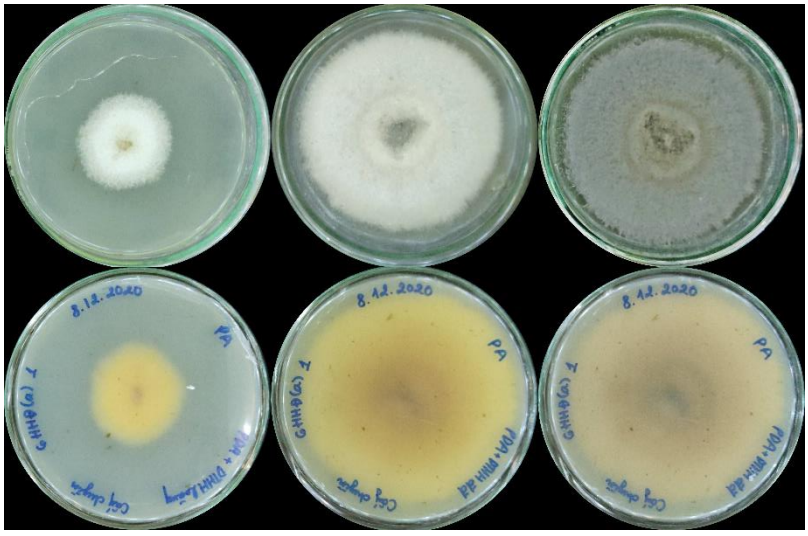

Figure 3. The colony morphology of *Diplocarpon rosae* on potato dextrose agar medium after 40, 76 and 107 days after inoculation (DAI)

These asexual two-celled spores and colony morphological characteristics were similar to the *D. rosae* as described by Wolf (1912), Rehman *et al*., (2012), and Chandel *et al*., (2017). Therefore, the fungal pathogen isolated from the samples could belong to the genus *Diplocarpon*.

Classification and identification of fungal pathogens have relied on morphological criteria including their mycelial colony and types of conidia (Guarro *et al.*, 1999). Despite its simplicity, the morphological approach has been criticized as many morphological characteristics are unstable and dependent on culture conditions; therefore, there has been inconsistency in the fungal taxonomy (Hennebert & Sutton, 1994). Other criteria, *e.g.*, physiological and biochemical tests and genotypes, have been used in fungal systematics and facilitated the accurate identification of fungi (Bridge and Hawksworth, 1990). In plant pathology, detection and identification of pathogens is an important step to control plant diseases. This can be achieved by a combination of morphological characterization and pathogenicity (physiological) tests using Koch's postulates (Burgess *et al.*, 2008). In a previous study, Hoc (2016) adopted this approach to identify the fungal pathogens causing diseases on shallots, *e.g.*, *Aspergillus niger* (black mold), *Colletotrichum gloeosporioides* (anthracnose) and *Fusarium oxysporum* (basal rot). In this study, the fungal pathogen causing rose black spot could be identified based on its morphology and pathogenicity.

3.2. Pathogenicity test of *Diplocarpon* sp. using the Koch's postulates and pathogen identification

D. rosae grows very slowly, it was furthermore reported that the fungus also lose its pathogenicity over time on artificial culture media (Palmer *et al.*, 1966; Gachomo, 2005), therefore conidia collected from infected leaves with fully developed the black spot symptom stored at -20°C was used for inoculation in the pathogenicity tests as described by Gachomo (2005).

3.2.1. Detached leaf

On *R. chinensis* Jacq. cv. *nhung*, symptoms appeared as small brown spots with an irregular margin on the upper leaf surfaces at 7 days after inoculation (DAI). These spots gradually turned brown to black and merged to form bigger patches with a diameter up to 8 mm, which could be observed by naked eyes (**Fig. 4A**). No symptom expression was recorded in the control treatment (**Fig. 4B**). However, these patches were smaller compared to those of black spot observed in the fields and were not surrounded with a yellow halo.

On *R. chinensis* var. *minima*, at 2 DAI, more than 50% of the leaf surfaces in both inoculated and uninoculated treatments turned dark brown and started wilting from the petiole, which likely resulted from oxidative stress due to excessive relative moisture content. Since necrosis occurred in all treatments at 3 DAI, the disease symptoms could

not be determined. Method modifications of the detached leaves of var. *minima* were made, where the leaves were placed only on a layer of sterilized moist Whatman filter paper only (without agar). However, symptom expression could not be observed neither as the leaves might suffer from drought exposure and developed symptoms including yellowing and wilting.

Figure 4. Disease symptoms on the detached leaf of *Rosa chinensis* Jacq. cv. *nhung* at 7 days after spraying inoculation of *Diplocarpon* sp. (A) and sterile distilled water (B)

Since conclusions on the fungal pathogenicity could not be made, the pathogenicity test was repeated using the intact plant technique under greenhouse conditions.

3.2.2. Intact plants

The expression patterns were highly similar on cv. *nhung* and on var. *minima* as symptoms started to develop at 7 DAI and became evident at 21 DAI. On inoculated leaves of cv. *nhung*, nearby dark-brown spots with distinct edges aggregated into larger patches of up to 10 mm in diameter with a yellow halo (leaf tissues) surrounding them (**Fig. 5**) whereas on var. *minima*, the spots were darker but smaller with "feathery edges" and the leaf tissues turned yellow more intensively (**Fig. 6**). In addition, abscission occurred in some plants of both cultivars and at higher degree in var. *minima*. Generally, pathogen inoculation using intact plant techniques under greenhouse conditions gave typical symptoms of black spot disease as observed in the fields. Furthermore, microscopic examination showed that the conidia of the fungus observed in the new host were the same as those of the originally inoculated pathogen (**Fig. 7**). Based on the descriptions of fungal pathogens of roses and their associated disease symptoms [Wolf, 1912; Rehman *et al*., (2012); Yasin *et al*., 2016; and Chandel *et al*., (2017)], the fungal pathogen was eventually identified as *Diplocarpon rosae*.

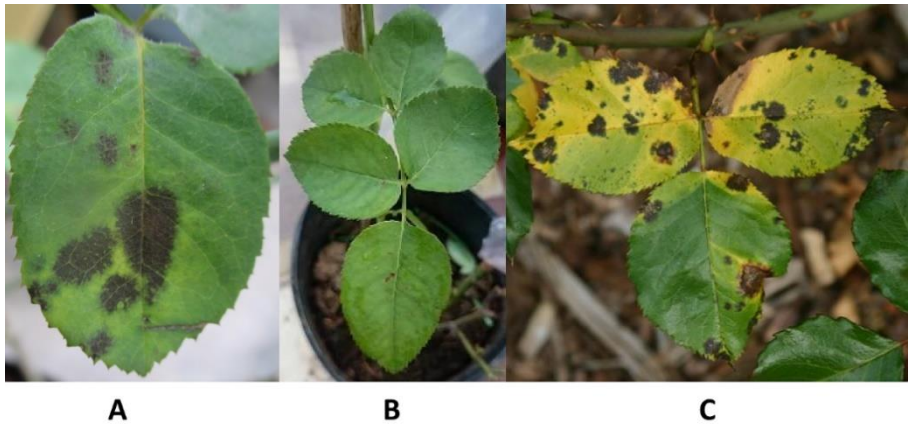

Figure 5. Disease symptoms on *Rosa chinensis* Jacq. cv. *nhung* (A) inoculated with *Diplocarpon* sp. at 21 DAI under greenhouse conditions in comparison with those of control treatment using sterile distilled water (B) and those on the same cultivar in the fields (C)

Figure 6. Disease symptoms on *Rosa chinensis* Jacq. var. *minima* (A) inoculated with *Diplocarpon* sp. at 21 DAI under greenhouse conditions in comparison with those of control treatment using sterile distilled water (B) and those on the same variety in the fields (C)

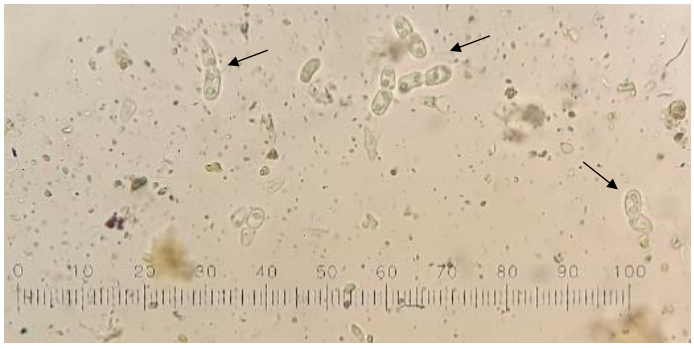

Figure 7. The conidia of *Diplocarpon rosae* (under a light microscope at 400 × magnification) were detected on inoculated rose leaves using the intact plant technique

The detached leaf technique has been extensively used in studies on rose black spot because it can form similar symptoms compared to the intact plant technique (Gachomo, 2005). However, the similarity in symptom expressions between the two assays was not reproduced in this study, partly due to the use of different rose cultivars, *i.e.*, cv. *nhung* and var. *minima*, which could be more vulnerable to stress (*e.g.* excessive moisture). In addition, it is thought that the degeneration after a short period of time may have facilitated the earlier infection of this fungus in detached leaves than in intact plants, resulting in the difference in symptoms between the two techniques in cv. *nhung*.

Different studies on other plant-microbe systems also revealed differences in defense-response and symptom expressions between the two techniques (Sharma, 1984; Liu *et al.*, 2007). Initially, the detached leaf technique was used to apply for the Koch's postulates because it offers several important advantages, *e.g.*, saving of plant materials, cost, time and space, the consistency due to similar size and age of leaves, the requirement of smaller volume and the identical conditions of the incubation, furthermore easy handling and preventing pathogen spread (Parke *et al.*, 2006). Nevertheless, the prominent disadvantage of the detached leaf assay is the disruption of the relationship between the root system (*e.g.*, uptake of water, nutrient and minerals and association with soil microorganisms) and the foliar system (*e.g.*, photosynthesis and transpiration). Therefore, some host defense responses may be compromised, these responses are not exclusively related to the reaction against pathogen infection but to general environmental stresses (Foster *et al.*, 1980; Parke *et al*, 2006; Liu *et al.*, 2007). This may result in inaccurate evaluation of symptom expression and identification of the pathogen.

The intact plant technique can overcome the limitations of the detached leaf technique. This was evident in our study as the inoculated rose plants showed typical symptoms of black spot disease as those observed in the fields, providing reliable conclusions on the fungal pathogenicity. However, the intact plant technique is more labor- and time-consuming and requires more plant materials and space to conduct. Hence, the choice of evaluation method depends on the objectives of the study considering the advantages and disadvantages of each technique. Our findings suggested that both detached leaf and intact plant techniques could be suitably deployed in cv. *nhung* for disease assessment while intact plant assay is recommended for studies on var. *minima*.

4. CONCLUSION

A total of 32 rose leaf samples with typical symptoms of rose black spot disease were collected from Sa Đéc city, Đồng Tháp province, Vietnam. Microscopic examination of the samples showed the presence of elongated-ovoid, hyaline and two-celled conidia containing vacuole-like structures in all 18 samples of cv. *nhung* and 14 samples of var. *minima*. The detached leaf technique worked better on cv. *nhung* as compared to var. *minima.* This however needs to be further tested. In the intact plant technique, both cultivars showed typical symptoms of rose black spot. They were small brown-black spots with perforated edge aggregating into bigger patches at 21 DAI. The disease symptoms also included yellowing of leaf tissues and early defoliation. Therefore, the fungal pathogen was eventually identified as *Diplocarpon rosae* based on its conidial morphology and pathogenicity.

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