Mycologia Iranica 11(1): 101– 109, 2024 **Original Article** DOI:10.22092/MI.2024.367808.1294

Morphological and molecular characterization of *Neopestalotiopsis clavispora,* causing rose stem canker in Iran

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Abstract: Rose (*Rosa* spp.) is a widely cultivated perennial flowering plant grown in open fields and under controlled greenhouse conditions. This study focused on the isolation and characterization of a pathogen affecting *Rosa hybrida* plants in the Najafabad and Lenjan counties of Isfahan Province, Iran. Infected stems and crowns were collected from the greenhouses between July 2022 and September 2023. The cultivars affected were Samurai and Tanja. Fungal isolation was achieved, followed by morphological characterization and molecular identification by DNA sequencing of the internal transcribed spacer (ITS) regions, translation elongation factor 1-alpha (*TEF1-α*), and β-tubulin (*TUB*). Pathogenicity tests confirmed the ability of the isolate to induce stem canker and leaf spot symptoms in healthy Samurai and Tanja cultivars of rose (*R. hybrida*) plants, thereby fulfilling Koch's postulates. The isolates were identified as *Neopestalotiopsis clavispora* by the morphological and molecular characteristics. To our knowledge, this study reports the first identification of *N. clavispora* as a pathogen causing canker disease and dieback on roses in Iran, emphasizing the need for effective management strategies to protect rose health and mitigate economic losses in the ornamental horticultural sector.

Keywords: Plant disease, Phylogenetic analysis, Morphological identification, Rose stem canker, Pathogenicity.

INTRODUCTION

Roses (*Rosa* spp.) hold substantial economic value as ornamental plants, not just in cut flower production but also in garden cultivation and medicinal applications (Widrlechner, 1981). However, diseases such as stem canker and dieback pose significant threats to rose health. These conditions lead to lesions that can girdle the stem, resulting in wilting and browning of the upper foliage, ultimately affecting the terminal regions of the plant (Sweets, 1982). Currently, the fungal pathogens responsible for rose dieback and stem canker primarily include *Botryosphaeria dothidea* (Jia et al. 2019), *Diaporthe rosiphthora* (Caio et al. 2021), *Trichothecium roseum* (Wright et al. 2007), *Acremonium sclerotigenum* (Mirtalebi et al. 2016), and *Coniothyrium fuckelii* (Zaher et al. 2012). Additionally, emerging species from the genus *Neopestalotiopsis* (*Pestalotiopsidaceae*, *Sordariomycetes*), previously named as *Pestalotiopsis,* have been recognized for their role in causing canker and dieback in roses (Jiang et al. 2018). Steyaert (1949) proposed reclassification of the genus *Pestalotia*, breaking it into three genera: *Pestalotiopsis*, *Pestalotia*, and *Truncatella*. Furthermore, he subdivided *Pestalotiopsis* into four sections of *Monosetulatae*, *Bisetulatae*, *Trisetulatae*, and *Multisetulatae*, based on apical appendage characteristics. Guba (1961) further refined this classification by categorizing *Pestalotia* according to its conidial characteristics. Recent phylogenetic studies focusing on the 28S Large Subunit (LSU) nrRNA gene revealed three main monophyletic groups within *Pestalotiopsis*. This analysis led to the identification of two new genera: *Neopestalotiopsis* and *Pseudopestalotiopsis* (Maharachchikumbura et al. 2014). Morphologically, *Neopestalotiopsis* can be distinguished from the other genera by its unique median cell color and conidiophore structure (Maharachchikumbura et al. 2014).

One notable species, *N. clavispora*, has been associated with rose leaf blotch disease (Feng et al. 2014). It has also been reported to cause canker and twig dieback in southern highbush blueberries in Spain (Borrero et al. 2018).

In Iran, the authors noted the rising incidence of *Pestalotiopsis*-like fungi in different hosts, which they attributed to various species, including *P. trachycarpicola* on croton (Atashi Khalilabad & Fotouhifar 2022), *P. theae* on bananas (Ketabchi, 2014), *N. asiatica* on almonds (Ayoubi & Soleimani 2016a), *N. mesopotamica* and *N. iranensis* on strawberry (Ayoubi & Soleimani 2016b), *P. disseminata* on feijoa (Naeimi et al. 2015), and *P. biciliata* on Eucalyptus (Amirmijani et al. 2024). Mirabolfathi and Ershad (2004) conducted the first and only report of *Pestalotiopsis* sp. as a causal agent

Submitted 01 Mar. 2024, accepted for publication 03 Apr. 2024
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of stem canker in Iranian roses. The fungal pathogens responsible for stem canker and dieback present significant challenges in horticulture. Understanding their characteristics, alongside developing effective management strategies, is crucial for maintaining rose health. Thus, this study aims to deepen our understanding of *N. clavispora*, particularly its association with stem canker in *Rosa* spp. in Iran through morphological comparisons and phylogenetic analysis.

MATERIALS AND METHODS

Sampling and fungal isolation

From July 2022 to September 2023, 40 samples of infected rose stems exhibiting symptoms of canker and dieback were collected from various greenhouses in the Najafabad and Lenjan counties of Isfahan Province, Iran. Approximately 0.5 cm segments of the stems, encompassing both healthy and diseased tissue, were excised using sterilized scalpels. The samples underwent sterilization by dipping them in 70% ethanol for 1 minute, followed by treatment with sodium hypochlorite (NaOCl) solution at a concentration of 5% for 1 minute, and were then rinsed with sterile distilled water. After surface sterilization, the tissue pieces were placed on potato dextrose agar (PDA) plates and incubated at 25 °C under a 12-hour light/dark cycle for five to seven days to promote fungal growth. To obtain pure cultures of the isolates, the hyphal tip method (Brown, 1924) was employed. A small portion of the actively growing mycelium was carefully collected from the edge of the colony and transferred to a new water agar (WA) plate. Once growth was established, single hyphal tips were subcultured onto fresh PDA plates to eliminate any remaining contaminants and ensure a pure isolate.

Morphological characterization

Morphological characteristics of the fungal colonies were examined following incubation. The colony morphology including color, diameter, texture, and growth pattern was meticulously recorded (Maharachchikumbura et al. 2014). Slide mounts were prepared with the fungal samples stained with lactic acid, and images were captured using a high-resolution BH2 Olympus light microscope equipped with a TrueChrome 4K Pro camera. This facilitated the assessment of morphological characteristics such as conidial size and the presence of apical and basal appendages. The captured images were subsequently imported into Mosaic v. 2 software (https://sios.net.au/software/mosaic-2 which enables precise measurements of various fungal structures. To ensure accuracy, measurements of conidia were obtained from at least 20 conidia per isolate.

DNA extraction, and PCR amplification

For molecular identification, genomic DNA was extracted from the fungal mycelia using the CTAB method, as described by Murray and Thompson (1980).

.Table 1. Strains and GenBank accession numbers were used for phylogenetic analysis in this study

Species	isolates	GenBank Accession Numbers		
		ITS	*TUB2*	*TEF1α*
Neopestalotiopsis acrostichi	*MFLUCC 17-1755	MK764273	MK764339	MK764317
Neopestalotiopsis brachiata	*MFLUCC 17-1555	MK764274	MK764318	MK764340
Neopestalotiopsis chrysea	LSCKS81	OQ392362	OQ410711	OQ410712
Neopestalotiopsis clavispora	*CBS_447.73	KM199374	KM199443	KM199539
Neopestalotiopsis clavispora	RS-PC-67	MZ097377	MZ097380	MZ090098
Neopestalotiopsis clavispora	MZ097377	MZ090100	MZ097382	MZ097379
Neopestalotiopsis clavispora	**Na3**	**PQ586978**	**PQ606055**	**PQ606056**
Neopestalotiopsis cubana	*CBS 600.96	KM199347	KM199438	KM199521
Neopestalotiopsis ellipsospora	*GZCC15-0085	KU500017	KU500010	KU500013
Neopestalotiopsis formicarum	*CBS 362.72	KM199358	KM199455	KM199517
Neopestalotiopsis macadamiae	*BRIP 63737c	KX186604	KX186654	KX186627
Neopestalotiopsis musae	*MFLUCC 15-0776	KX789683	KX789686	KX789685
Neopestalotiopsis saprophytica	*CBS_115452	KM199345	KM199433	KM199538
Neopestalotiopsis sonneratae	*MFLUCC 17-1744	MK764279	MK764345	MK764323
Neopestalotiopsis zimbabwana	*CBS 111495	MW422813	KM199456	KM199545
Pestalotiopsis trachicarpicola	*MFLUCC12-0266	JX399002	JX399033	JX399066
Pseudopestalotiopsis cocos	*CBS 27229	MH855069	KM199467	KM199553

CBS: culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Center, Utrecht, The Netherlands; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; GZCC: Guizhou Provincial Culture Collection Center, Guizhou, China; BRIP: Queensland Plant Pathology Herbarium, Australia.

Ex-type strains are labeled with the superscript *. Isolate in boldface was sequenced in this study

The internal transcribed spacer (ITS) region of rDNA was amplified using ITS1 and ITS4 primers (White et al. 1990). EF1-728F and EF-2 primers were employed to amplify the partial sequence of the translation elongation factor 1-alpha (*TEF*) gene (O'Donnell et al. 1998; Carbone & Kohn, 1999). The partial β-tubulin (*TUB*) gene was also amplified using the specific T1 and Bt2b primers (Glass & Donaldson, 1995; O'Donnell & Cigelnik, 1997). PCR reactions were performed in a total volume of 10 µl, containing 5 µl of 2× *Taq* master mix Red, 0.5 µl each of forward and reverse primers (10 pmol/µL), 3 µl of nuclease-free water, and 1 µL of genomic DNA (15 ng/µl). The thermal cycling conditions included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52°C (ITS), 57°C (*TEF*), or 55°C (*TUB*) for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min (Weir et al. 2012; Maharachchikumbura et al. 2014). The PCR products were electrophoresed on 1% agarose gel and visualized under UV light using a Vilber Lourmat SSM-930 gel documentation system.

Sequencing, and phylogenetic analysis

The amplicons were directly sequenced by Pishgam Biotech Company using Sanger sequencing. The obtained sequences were edited using chromas 2.6.6 software (https://www.technelysium.com.au/chromas.html) and then compared with those available in the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) to confirm the identity of the sequences.

The phylogenetic tree was constructed by incorporating the newly obtained sequences alongside those from ex-type specimens cited in earlier studies (Maharachchikumbura et al. 2014; Daengsuwan et al. 2021). For phylogenetic analysis, the sequences were aligned using Clustal W (Thompson et al. 1994). *Pseudopestalotiopsis cocos* (CBS 272.29) and *Pestalotiopsis trachicarpicola* (MFLUCC 12-0266) were used as outgroups (Table 1). A maximum likelihood approach was then employed to construct the phylogenetic tree, applying the General Time Reversible (GTR) model to account for nucleotide substitution rates in MEGA v. 6 (Tamura et al. 2013). Statistical support for the inferred relationships was assessed through bootstrap analysis with 1,000 replicates.

Pathogenicity test

To fulfill Koch's postulates, pathogenicity tests were conducted by inoculating healthy Samurai and Tanja cultivars of rose (*R. hybrida*) plants with isolated fungi. Conidial suspensions were prepared by collecting conidia from cultures grown on potato dextrose agar (PDA) for seven days and diluting them in sterile distilled water to achieve a concentration of 10^6 conidia/mL. Small wounds were created on the tissues of six, eight-week-old healthy rose plants, and 20 µL of conidial suspension was applied to the wounds on both stems and leaves. Two control plants were treated with sterile distilled water to account for external factors that affect plant health (Bhunjun et al. 2021). The inoculated plants were covered with plastic bags and maintained in a controlled environment with 16-hour light cycles and temperatures ranging from 20 to 25 °C. Over four weeks, symptoms were monitored, noting the emergence of stem canker, wilting, and leaf necrosis. Small pieces of necrotic tissue from the edge of each lesion were cut and placed on PDA. The pathogen was isolated from inoculated plants and compared with the primary recovered isolate to confirm Koch's postulates.

Fig. 1. The observed symptoms caused by the fungus *Neopestalotiopsis clavispora* in the rose greenhouses of Najafabad and Lenjan counties of Isfahan Province a, b. on the Samurai cultivar; c. on Tanja cultivar.

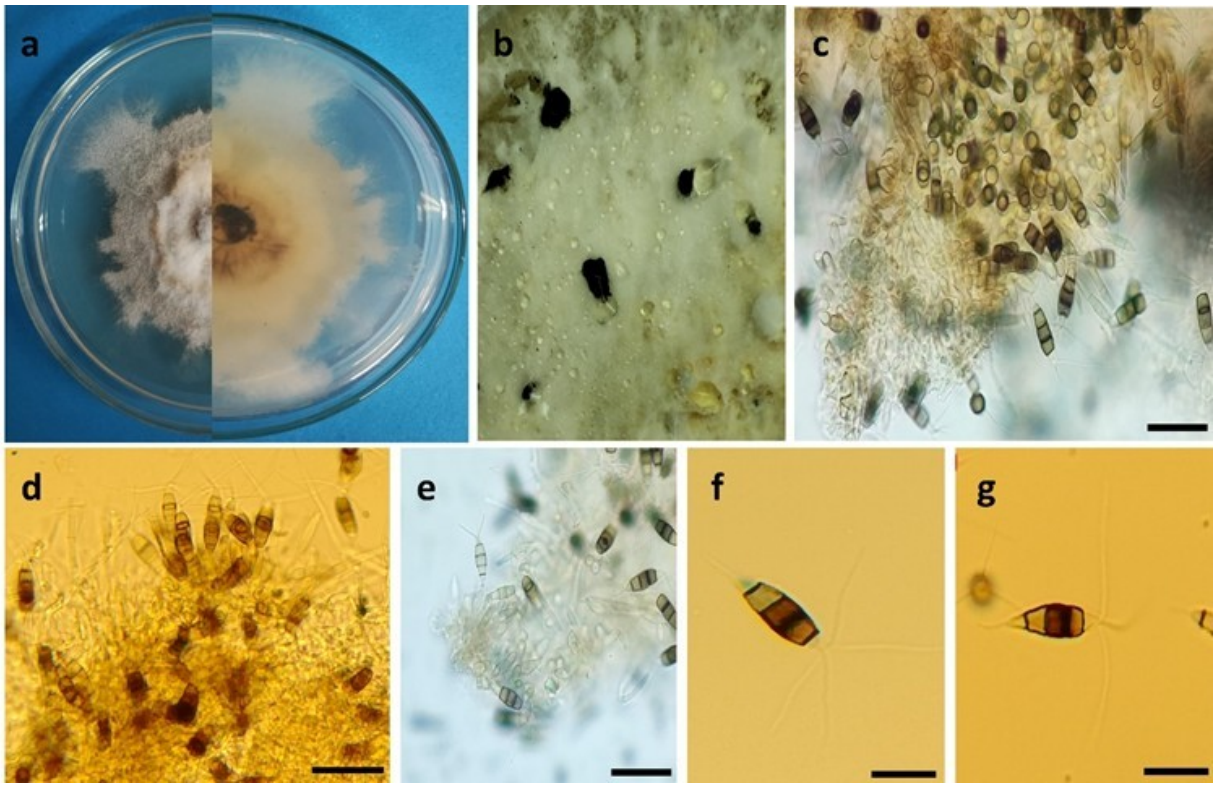

Fig. 2. Morphological characteristics of *Neopestalotiopsis clavispora*. a. Colony characteristics of fungi on PDA media; b. Conidiomata sporulating on PDA; c-e. Conidiogenous cells; f, g. Conidia. Scale bars: c-e = 100 µm; f, g = 10 µm.

RESULTS

Morphological characteristics and DNA sequence analysis

In this study, two isolates of *Neopestalotiopsis* (Na3 and Ln1) were identified as the causal agents of canker affecting the stems of rose varieties: Samurai (Ln1) and Tanja (Na3) (Fig. 1). The morphological characteristics (Fig. 2) led to the initial identification of the pathogen as belonging to the genus *Neopestalotiopsis* (Maharachchikumbura et al. 2014). Blast search and sequence analysis revealed a striking similarity, with over 99% sequence homology between isolate Na3 and multiple sequences of *Neopestalotiopsis clavispora*. The resulting phylogenetic tree displayed robust clustering of isolate Na3 with the type strain of *N. clavispora* (CBS_447.73), supported by a high bootstrap value of 100% (Fig. 3). The new sequences have been deposited in GenBank under the following accession numbers: PQ586978 (ITS), PQ606055 (*TUB*), and PQ606056 (*TEF1-α*).

Based on morphological and molecular characteristics the causal agent of rose canker obtained from single conidia was identified as *Neopestalotiopsis clavispora* and characterized as follows:

Neopestalotiopsis clavispora (G.F. Atk.) Maharachch., K.D. Hyde & Crous, stud. Mycol. 79: 138 (2014).

Cultures were incubated on potato dextrose agar (PDA) at 25 °C under a 12-hour day/night regime. Following incubation, the fungal colonies on PDA appeared white with irregular edges, exhibiting a wavy surface and masses of black conidiomata (Fig. 2a). Small black spots, identified as acervuli, were observed on the surface of the aerial mycelial layer (Fig. 2b). The whitish mycelia produced black, smooth, globular acervuli containing slimy spore masses. The colony diameter reached 70 ± 5 mm after a seven-day incubation period. Conidia were fusiform or clavate, either straight or slightly curved, consisting of five cells and four septa, measuring 18.4–27 × 6.3–8.2 µm. The three median cells were multicolored, featuring progressively darker walls; the second cell from the base was pale brown, the third cell was darker brown, and the fourth cell was the darkest. The basal cells were conical and hyaline, each featuring a single hyaline appendage at the base, measuring 5.8–6.8 µm. The apical cells were hyaline and subcylindrical, bearing two or three hyaline appendages, and measuring 19.2–29.5 µm (Fig. 2f, g).

Pathogenicity test

Seven days post-inoculation, isolate Na3 exhibited symptoms including stem cankers and leaf spots. Over the following 14 days, these cankers and leaf spots progressed to a necrotic state, characterized by the formation of black globular acervuli filled with spore masses. In contrast, no visible symptoms were observed in the control stems and leaves (Fig. 4). Koch's postulates were fulfilled through the successful re-isolation and microscopic identification of *N. clavispora*.

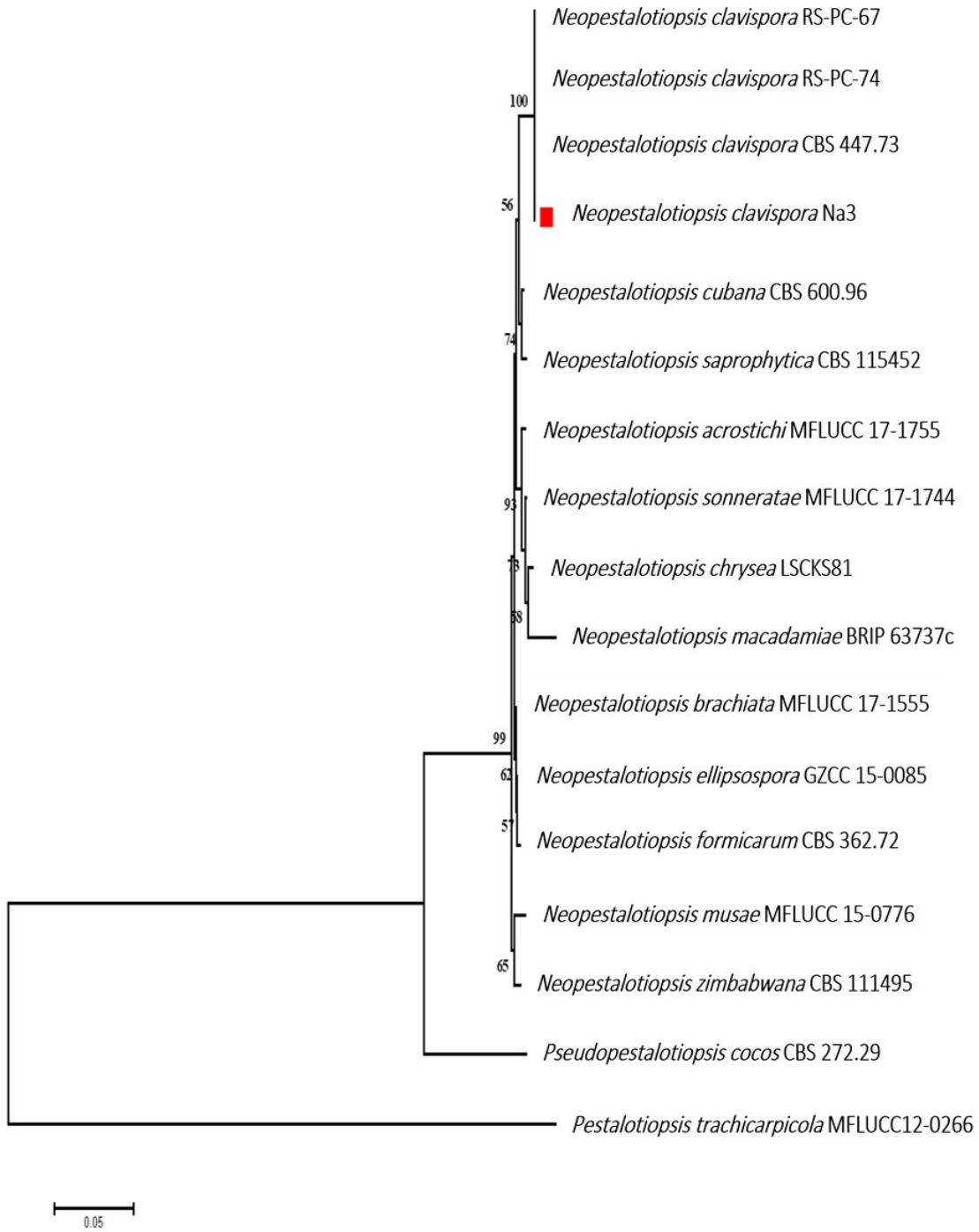

Fig. 3. Phylogram generated from maximum likelihood analysis based on combined ITS, *TEF1-α*, and *TUB* sequence alignments of 17 isolates of *Neopestalotiopsis*, *Pestalotiopsis*, and *Pseudopestalotiopsis*. Bootstrap values > 50% (1000 replicates) of the ML analysis were exhibited above/below the branches. The Iranian rose isolate is marked with a red square.

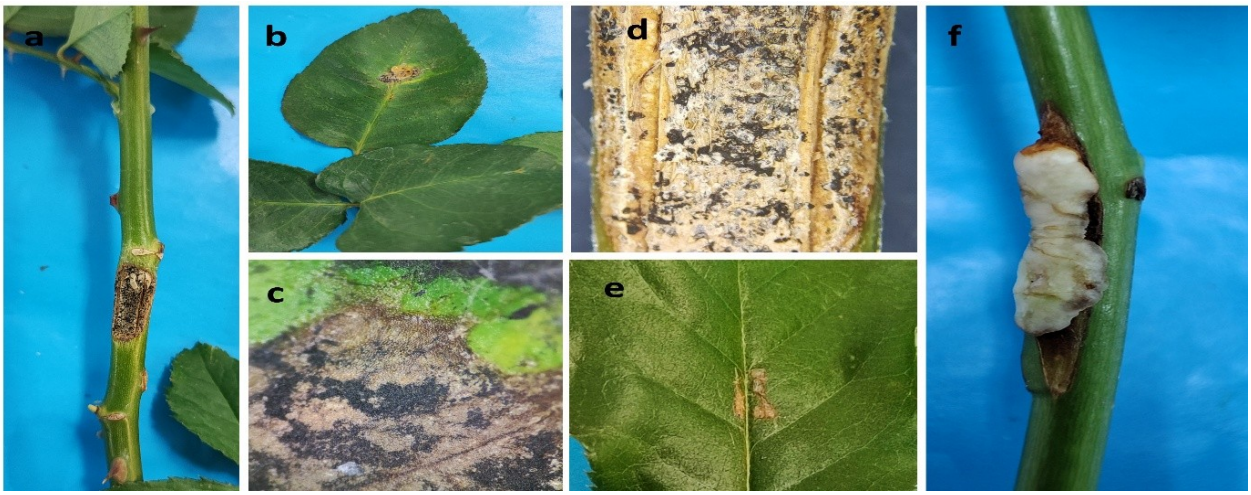

Fig. 4. Lesions of infection by *Neopestalotiopsis clavispora* (Na3) on the stems and leaves of Tanja varieties with small black acervuli at 10 days post-inoculation under the greenhouse condition (20 to 25 °C). (a, d) The stem surface symptoms were inoculated with a conidial suspension. (b, c) Leaf surface symptoms inoculated with a conidial suspension. (e, f) Control stems and leaves.

DISCUSSION

Through comprehensive morphological and molecular analyses, coupled with pathogenicity assessments, this study demonstrates that *N. clavispora* is associated with rose canker and dieback disease. To our knowledge, this is the first report of *N. clavispora* as a causative agent of rose canker in Iran. Another species of the genus *Neopestalotiopsis,* including *N. asiatica,* has been identified as the causal agent of leaf spot on sweet almond (*Prunus dulcis*) in Isfahan province, Iran (Ayoubi & Soleimani 2016a). In another study, a new species of *Neopestalotiopsis*, named *N. iranensis*, was isolated from rotted strawberry (*Fragaria ananassa*) fruits and lesions in Kurdistan province, Iran, characterized by distinct morphological features and confirmed through phylogenetic analysis, alongside the first report of *N. mesopotamica* as a pathogen of strawberries (Ayoubi & Soleimani 2016b). Morphologically, *Neopestalotiopsis* can be distinguished from *Pseudopestalotiopsis* and *Pestalotiopsis* by its distinctive versicolorous median cells. Additionally, *Neopestalotiopsis* feature is the indistinct conidiophores that are often reduced to conidiogenous cells. While these three genera share overlapping morphological traits, distinguishing them based solely on morphology can be challenging (Maharachchikumbura et al. 2014; Liu et al. 2017). *Neopestalotiopsis* spp. exhibit widespread distribution and an extensive host range (Reddy et al. 2016), typically initiating pathogenesis via insect vectors, wounds, or natural openings (Keith et al. 2006; Daengsuwan et al. 2020; Pornsuriya et al. 2020). Notably, *N. clavispora* has demonstrated a remarkable capacity to infect over 50 plant species across 27 families, inducing severe foliar diseases (Qiu et al. 2020). Its pathogenic effects extend to various plant hosts, manifesting as canker and twig dieback in blueberry (Borrero et al. 2018), flower blight in *Anthurium andraeanum* (Daengsuwan et al. 2020), root and crown rot in strawberry (Obregón et al. 2018), leaf spot in macadamia (Santos et al. 2019), and apple leaf spot (Shi et al. 2024). Furthermore, seven *Neopestalotiopsis* species: *N. clavispora*, *N. palmarum*, *N. rosicola*, *N. concentrica*, *N. subepidermalis*, *N. rosae*, and *N. versicolor,* have been implicated in leaf blotch and stem canker of roses (Feng et al. 2014; Maharachchikumbura et al. 2014; Jiang et al. 2018; Peng et al. 2022). *Neopestalotiopsis clavispora* is distinguishable from other *Neopestalotiopsis* species causing rose diseases through molecular analyses and morphological characteristics. Specifically, *N. clavispora* possesses longer and fewer tubular apical appendages compared to *N. rosicola* (19-32 µm vs 12-23 µm), differs from *N. subepidermalis* in having a smaller basal appendage (3-5.5 µm vs 7-7.5 µm), and exhibits larger conidia than *N. concentrica* (20-24×6.5-8.5 vs 14-18.5 ×4.5-5 µm) (Maharachchikumbura et al. 2014, Peng et al. 2022).

ACKNOWLEDGMENTS

The authors acknowledge the financial support of Isfahan University of Technology.

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تعیین خصوصیات مورفولوژیکی و مولکولی *Neopestalotiopsis clavispora* عامل بیماری شانکر ساقه گل رز در ایران

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چکیده: رز (*Rosa* spp.) یک گیاه گل‌دار چندساله است که به‌طور گسترده در فضای باز و شرایط گلخانه‌ای کشت می‌شود. اکثر بیماری‌های رایج این گیاه ناشی از بیمارگرهای قارچی هستند که خسارات اقتصادی قابل‌توجهی را به همراه دارند. در این مطالعه، طی بازه زمانی تیر ۱۴۰۱ تا شهریور ۱۴۰۲، نمونه‌برداری از ساقه‌های دارای علائم شانکر و سرخشکیدگی ارقام رز هلندی (*Rosa hybrida*) تانجا و ساموراییی در شهرستان‌های نجف آباد و لنجان استان اصفهان انجام شد. جداسازی قارچها با استفاده از روش‌های خالص‌سازی و کشت نمونه صورت گرفت و سپس ویژگی‌های ریخت‌شناسی و تجزیه و تحلیل نواحی ITS، *TEF1-α* و *TUB* مورد بررسی قرار گرفت. آزمون‌های بیماری‌زایی توانایی جدایه‌ها را در ایجاد علائم شانکر و لکه‌برگی تأیید کردند. همچنین، با جداسازی مجدد گونه‌های قارچی از علائم نکروتیک، اصول کخ اثبات شد. بر اساس ویژگی‌های مورفولوژیکی و مولکولی، جدایه‌ها به‌عنوان *Neopestalotiopsis clavispora* شناسایی شدند. بر اساس یافته‌های این پژوهش، این نخستین گزارش از *N. clavispora* به عنوان عامل شانکر و سرخشکیدگی گل رز در ایران است.

کلمات کلیدی: بیماری گیاهی، آنالیز فیلوژنتیکی، شناسایی مورفولوژیکی، شانکر ساقه رز، بیماریزایی.

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تاریخ دریافت: ۱۴۰۲/۱۲/۱۱ تاریخ پذیرش: ۱۴۰۳/۰۱/۱۵