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First record of *Dactylonectria macrodidyma* causing black root rot on strawberry

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Abstract: Strawberry is a major fruit cultivated in Kerman greenhouses. During visiting strawberry cultivation greenhouses, black root rot symptoms were detected on strawberry plants. In order to identify the causal agents of the disease, symptomatic tissues were collected and transferred to the laboratory. *Cylindrocarpon*-like isolates were consistently recovered from infected tissues. Based on morphological characteristics as well as sequence data, the causal agent was identified as *Dactylonectria macrodidyma*. Colonies of *D. macrodidyma* on PDA were brown with yellow (honey) pigmentation at the margins. Macroconidia on SNA medium were 1–3 (–4) septate, straight, cylindrical (sometimes widening toward the tip), apical cell slightly bent to one side, 40 (±11) × 6.3 (±1.8) µm with free-standing, slender, unbranched conidiophores. Microconidia with 0–1 septum, ellipsoid and ovoid 10.5 (±3.2) × 4.1 (±1.6) µm. The results of pathogenicity tests showed that the tested isolates were pathogenic to strawberry. According to the knowledge, this is the first report of *D. macrodidyma* on strawberry.

Keywords: Greenhouse, ITS-rDNA, Kerman province, morphology, root disease.

INTRODUCTION

Strawberry (*Fragaria ananassa* Duch.) is a major fruit crop in Iran. Strawberry was imported to Iran about 100 years ago from France (Eshghi et al. 2007). It is cultivated in fields and greenhouses. According *to* agriculture statistic book of Iran (2019), the greenhouse strawberry production area has been approx. 522 ha in 2019, from which 266 ha were located in Kerman province (including southern parts of Kerman). Root and foot diseases caused by fungi as well as post-harvest diseases are major constraints to strawberry production worldwide, including Iran (Embaby 2007, Ayoubi et al. 2016, Fang et al. 2011, Petrasch et al. 2019). Botrytis gray mold (Petrasch et al. 2019), wilt disease caused by *Verticillium dahliae* Kleb. (Harris & Yang 1996), red stele, caused by *Phytophthora fragariae* Hickman (Newton et al. 2010), crown rot (vascular collapse) and leather fruit rot caused by *Phytophthora cactorum* (Leb. and Cohn) Schröeter (Stensvand et al. 1999) are major fungal diseases on this crop. *Botrytis cinerea* Pers., *Colletotrichum* spp. and *Rhizopus stolonifer*, are of great importance among fungal diseases of strawberry in Iran. Black root rot disease on strawberry has been reported to be associated with many fungal species, including *Ceratobasidium fragariae* (Wilhelm et al. 1972), *Pythium* spp., (Watanabe et al. 1977), *Gnomoniopsis fructicola* (Moročko-Bičevska et al. 2019), *Fusarium* spp. (Koike & Gordon 2015) and *Dactylonectria torresensis* (Weber & Entrop 2017). The disease symptoms appear as deterioration and black necrosis of the root system and decline in productivity. The aim of this study was to identify the causal agent of strawberry black root rot, which was observed during visits to greenhouse strawberry productions in Kerman.

MATERIALS AND METHODS

Sample collection and fungal isolation

During visiting strawberry cultivation greenhouses in different locations in Kerman County, several plants with typical symptoms of black root rot were observed. Symptomatic plants showing stunted growth, leaf margins necrosis and black root rot were collected and transferred to the laboratory. Plant materials were washed under running tap water for 30 minutes to remove excess soil particles and spores of fast-growing contaminant fungi on the tissue's surface. Small segments of symptomatic tissues were surface disinfected in 70% EtOH for 10 s, 1% NaOCl for 1 min, and rinsing in sterile deionized H_2O for 1 min. Plant materials were dried on sterile filter papers. Tissue pieces (3–5mm long) were placed on potato

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dextrose agar (PDA), amended with streptomycin sulfate at 100 mg/L and incubated at 25°C for seven days in darkness. Single conidial cultures were prepared and stores on PDA slants at 10°C. The isolates were deposited in (Kerman Graduate University of Advanced Technology, Kerman, Iran) fungal culture collection and stored in 15% glycerol at -80°C.

Morphological characterization

For morphological examination of colony characteristics and growth, isolates were grown on PDA, water agar (WA), synthetic poor nutrient agar (SNA) at 24°C in darkness (Schroers et al. 2008) and examined after 7–20 days of incubation. Morphology of conidia and chlamydospores were determined. An average of 30 conidia was measured. Microphotographs of fungal features were taken using a Dinoeye microscope camera USB lens (The Microscope Store, LLC., USA). Colony diameters were measured on three replicate plates on PDA after 7 days.

DNA extraction, PCR and Sequencing

For molecular identification at the species level, three representative isolates were selected for sequencing of ITS rDNA regions. Fresh fungal mycelia were scraped off from 7-day-old PDA plates of single spore cultures, homogenized using liquid nitrogen and Genomic DNA was extracted using a CTAB extraction procedure (Zhang et al. 2010). A standard polymerase chain reaction (PCR) protocol was used to amplify ITS rDNA regions with primers ITS1 and ITS4 (White et al. 1990). Amplifications were performed in a Biometra TAdvanced Thermal Cycler (Biometra, Göttingen, Germany) with an initial denaturation of 5 min at 95°C followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 59°C, 60 s extension at 72°C and a final extension of 5 min at 72°C. The quantity and quality of PCR products were evaluated on 1% agarose gels. The PCR products sequencing was performed by Bioneer (Bioneer Co., Korea). The sequences generated in this study were deposited in GenBank and the accession numbers were obtained (Table 1).

Phylogenetic analysis

The obtained sequences were manually edited using Geneious v. 7 (Biomatters)s and compared with those in the GenBank database using a basic local alignment search tool (BLAST) (Altschul et al. 1990). Generated sequences were added to sequences retrieved from GenBank according to the reliable published papers and included in the phylogenetic analysis (Table 1). The sequences were aligned using Geneious version 7 (Biomatters, USA). Phylogenetic relationships and identification of the isolates in the species level were performed using PAUP* 4.0a133 (Swofford 2002) for parsimony. Gaps were treated as missing data. To assess the branch support, bootstrap analysis with 1000 replicates using a heuristic search was performed. *Nectria balansae* (GenBank accession no. HM484857) was used as an outgroup taxon.

Pathogenicity test

Representative isolates were tested for pathogenicity confirmation on strawberry. Potted symptomless strawberry plants (*Fragaria ananassa* cv. Paros) were surface-sterilized by dipping the roots in 0.5% (w/v) NaOCl for 30 s and washed with sterile distilled water for 2 min. Ten-day-old fungal cultures growing on PDA were used to obtain conidial suspensions. Spore concentration was adjusted to 5×10^{6} spores mL^{-1} using a hemocytometer. Plants were inoculated by immersing in a conidial suspension of

Table 1. Strains used in the phylogenetic analysis.

Species	Isolate	Source	Country	GenBank accession no.
Ilyonectria europaea	CBS 129078	*Vitis vinifera*	Portugal	JF735294
Neonectria robusta	CBS 308.35	*Panax quinquefolium*	Canada	JF735264
Neonectria radicicola	CBS 264.65	Grapevines		AY677273
Cylindrocarpon cylindroides	CBS 503.67	grapevines		AY677261
Neonectria ramulariae	CBS 182.36	*Malus sylvestris*		JF735314
Ilyonectria crassa	CBS 139.30	*Lilium* sp.	Netherlands	JF735275
Ilyonectria robusta	KARE1740	-	California	MK400320
Cylindrocarpon sp.	JAT1366	-	Canada	AY295306
Cylindrocarpon pauciseptatum	CBS 120172	Vineyard	Slovenia	EF607086
Cylindrocarpon pauciseptatum	CBS 120173	Vineyard	Slovenia	EF607088
Dactylonectria valentina	Cy-FO-133	Forest Nurseries	Spain	KY676881
Dactylonectria amazonica	MUCL55430	Rain Forests	Ecuador	MF683706
Dactylonectria onectriaamazonica	MUCL55433	Rain Forests	Ecuador	MF683707
Dactylonectria macrodidyma	CBS 112615	type material, grapevine		AY677290
Dactylonectria macrodidyma	dmE	*Vitis riparia*	Canada	MF567498
Dactylonectria macrodidyma	KARE423	perennial fruit and nut crops	California	MK400300
Dactylonectria macrodidyma	7PDA-Ate	*Quercus ilex*	Spain	KX343141
Dactylonectria macrodidyma	IJK90-13	*Vitis vinifera*		MN540296
Dactylonectria macrodidyma	**O3-1**	**Strawberry**	**Iran**	**MZ254760**
Dactylonectria macrodidyma	**O3-2**	**Strawberry**	**Iran**	**MZ254761**
Dactylonectria macrodidyma	**O3-3**	**Strawberry**	**Iran**	**MZ254762**
Dactylonectria polyphaga	MUCL55209	Rain Forests	Ecuador	MF683689
Ilyonectria capensis	KARE1920	perennial fruit and nut crops	California	MK400330
Ilyonectria capensis	KARE1921	perennial fruit and nut crops	California	MK400331
Nectria balansae	G.J.S. 86-117	living woody vine	French Guiana	HM484857

the isolate (10^6 conidia mL^{-1}) for 20 min. Control plants were inoculated with sterile distilled water. Plants were incubated at 20°C for 24 h, then transferred to a greenhouse and inspected daily for symptoms.

RESULTS AND DISCUSSION

Cylindrocarpon-like isolates were consistently isolated from infected tissues. The species identification was based on morphological and molecular criteria. A total of 12 isolates of *D. macrodidyma* were obtained from roots of strawberries showing black root rot symptoms from Kerman strawberries cultivation greenhouses. Colonies of *D. macrodidyma* on PDA were brown with yellow (honey) pigmentation at the margins. Conidiophores arising laterally from the aerial mycelium unbranched or sparsely branched and 1–4-septate. Phialides cylindrical tapering towards the tip. Macroconidia on SNA medium were 1–3 (–4) septate, straight, cylindrical (sometimes widening toward the tip), apical cell slightly bent to one side, 40 (±11) × 6.3 (±1.8) µm with free-standing, slender, unbranched conidiophores. Microconidia were 0–1 septate, ellipsoid and ovoid 10.5 (±3.2) × 4.1 (±1.6) µm. Chlamydospores in short, intercalary chains (Fig. 1). Morphological characteristics are corresponding with published descriptions of *D. macrodidyma* (Haleen et al. 2004). The results of BLAST search against sequences in GenBank and phylogenetic analysis confirmed the species identification. In the phylogenetic tree (Fig. 2), the isolates obtained from strawberries grouped with *D. macrodidyma* in a well-supported clade.

The results of pathogenicity tests showed that the tested isolates were pathogenic to strawberry and the first symptoms were observed 15 days after inoculation while control plants remained healthy and asymptomatic. *Dactylonectria macrodidyma* was consistently re-isolated from symptomatic tissues. The inoculated plants showed identical symptoms to those observed in strawberry cultivation greenhouses. The inoculated plants showed stunted growth, necrosis in leaf margins and black root rot symptoms with necrotic lesions on roots (Fig. 3).

Species of *Cylindrocarpon* / *Dactylonectria* are plant pathogens causing black foot and root diseases (Vitale et al. 2012, Dos Santos et al. 2014, Adesemoye et al. 2016). This study represents the first report of *D. macrodidyma* on strawberry in Iran. However, the distribution of this disease in other strawberry production areas remains to be investigated. The impact of this pathogen on strawberry production in Kerman greenhouses is not clear yet. Black foot rot resulting from *Dactylonectria* has been wildly studied in nurseries and vineyards of grapevines (Halleen et al. 2003, 2004, Cabral et al. 2012).

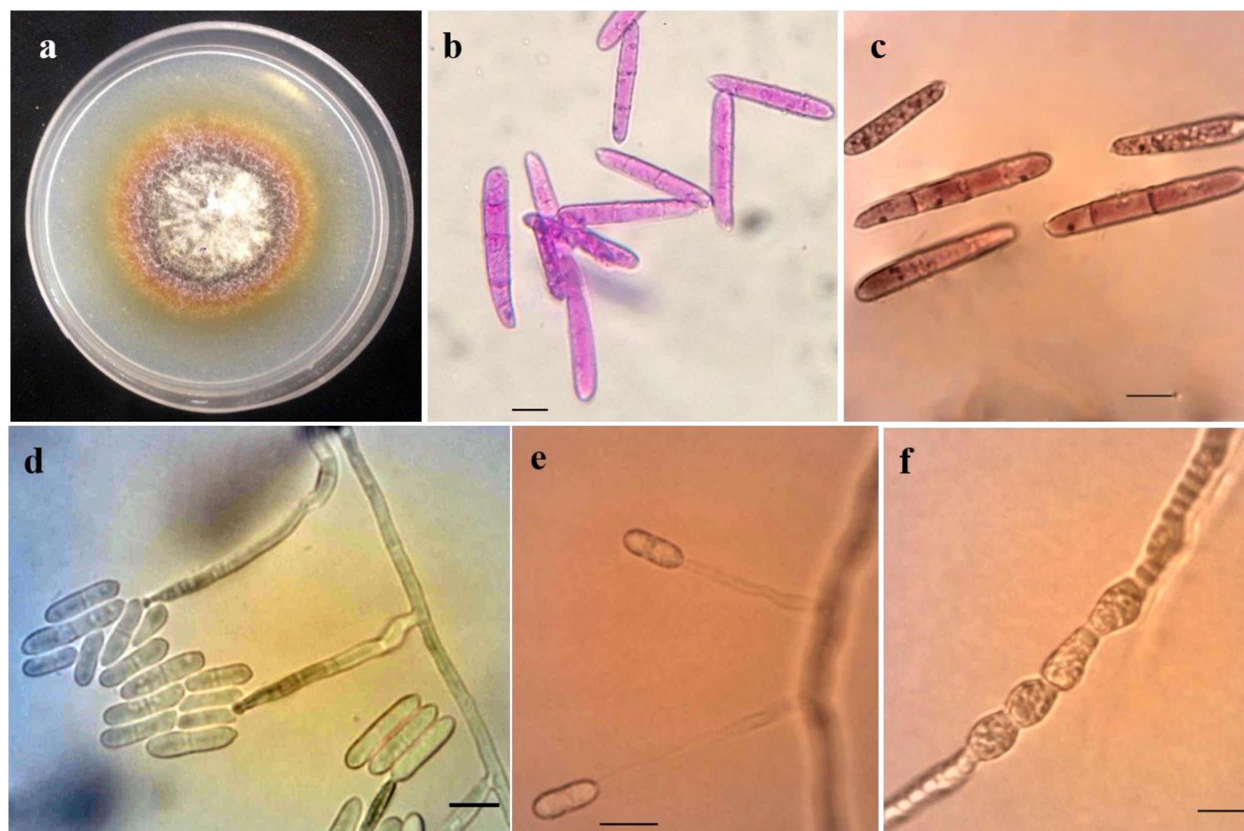

Fig. 1. *Dactylonectria macrodidyma*. a. Colony after 14 days at 24°C; b-c. macroconidia; d-e. macroconidia and microconidia; f. chlamydospores. — Scale bars = 10 µm.

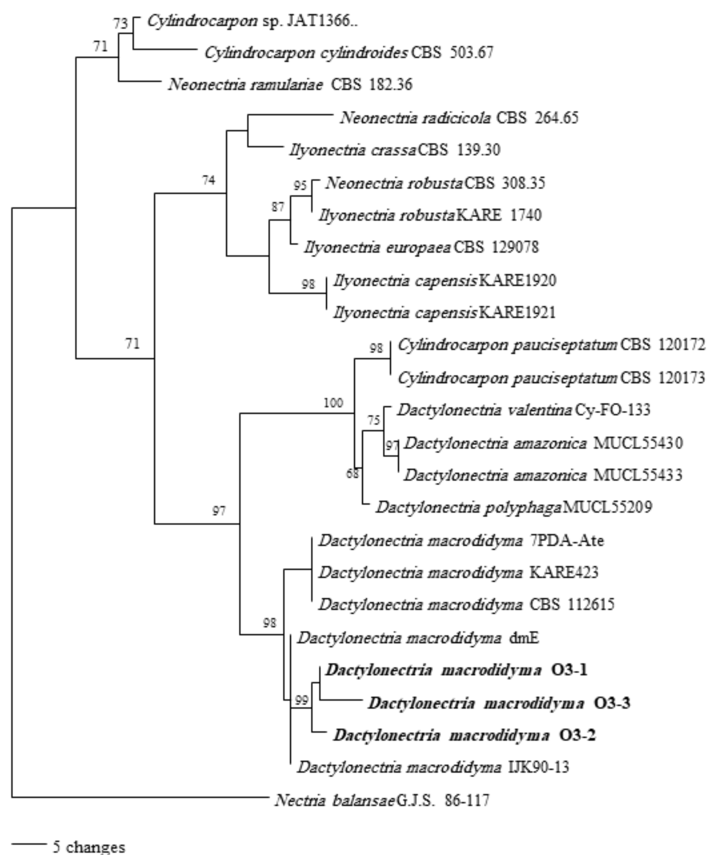

Fig. 2. Parsimony tree based on aligned sequences of ITS region. Bootstrap values (1000 replicates) indicated at the nodes. The scale bar indicates the number of nucleotide changes. The tree was rooted in *Nectria balansae*.

Fig. 3. Disease symptoms on strawberry including necrosis in leaf margins (upper) and black root rot symptoms with necrotic lesions on roots (beneath).

According to Halleen et al. (2003), the main practice for disease management is using a clean potting medium collected from unaffected areas where the disease has not been observed. Greenhouse staff should pay attention to root decay symptoms and discard seedlings that exhibit symptoms.

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نخستین گزارش از قارچ *Dactylonectria macrodidyma* به عنوان عامل پوسیدگی سیاه ریشه توت‌فرنگی

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چکیده: توت‌فرنگی یکی از مهم‌ترین محصولاتی است که در استان کرمان به صورت گلخانه‌ای کشت می‌شود. در طی بازدیدهایی که از این گلخانه‌ها صورت گرفت، نشانه‌های پوسیدگی سیاه ریشه در گیاهان توت‌فرنگی مشاهده شد. برای شناسایی عامل این بیماری، بافت‌های گیاهی دارای نشانه جمع‌آوری و به آزمایشگاه منتقل شد. جدایه‌های دارای آنامورف از نوع *Cylindrocarpon* از تمامی بافت‌ها جداسازی شدند. عامل بیماری بر مبنای صفات ریخت‌شناختی و داده‌های مولکولی، گونه *Dactylonectria macrodidyma* تعیین شد. پرگنه‌های روی محیط PDA به رنگ قهوه‌ای با حاشیه‌ی زرد عسلی بودند. ماکروکنیدیوم‌ها روی محیط SNA دارای ۱-۳ (گاهی ۴) دیواره، مستقیم، سیلندری (گاهی در نزدیکی نوک کمی عریض‌تر)، سلول انتهایی کمی به یک سمت خمیده به ابعاد ۴۰ (± ۱۱) در ۶/۳ (± ۱/۸) میکرومتر و دارای کنیدیوفورهای آزاد، باریک و تک شاخه بودند. میکروکنیدیوم‌ها ۰-۱ سلولی، بیضی شکل تا سوسیسی شکل به ابعاد ۱۰/۵ (± ۳/۲) در ۴/۱ (± ۱/۶) میکرومتر بودند. نتیجه آزمون بیماری‌زایی نشان داد که جدایه‌های مورد آزمون روی توت‌فرنگی بیمارگر بودند. نتیجه این مطالعه اولین گزارش از *Dactylonectria macrodidyma* روی توت‌فرنگی در ایران است.

کلمات کلیدی: گلخانه، ITS-rDNA، استان کرمان، ریخت شناسی، بیماری ریشه

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