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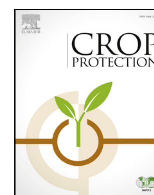

New disease caused by *Neoscytalidium dimidiatum* devastates tomatoes (*Solanum lycopersicum*) in Turkey

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ABSTRACT

A novel disease of tomato (*Solanum lycopersicum* L.) was observed in the Southeast Anatolia Region of Turkey. Symptoms were blight of all aerial parts of the plant, including stems, branches, leaves, petioles, flowers and fruits, defoliation, root rot, inner stem necrosis, and plant death. The disease was found in 13.9% of surveyed fields, with an incidence varying from 3% to nearly 75% (average 21.2%) of the plants in symptomatic fields. The average severity of blight on stem in fields with the symptomatic plant surveyed was 1.4%. A Botryosphaeriaceae species, identified as *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers using morphological and cultural features, was consistently isolated from symptomatic roots, inner stems, and blighted leaves, shoots, stems, fruits and flowers. The partial nucleotide sequence data for three gene loci, including nuclear rDNA internal transcribed spacer (ITS), large subunit (LSU) genes and the translation elongation factor 1-alpha (TEF-1α), confirmed the morphological identification. Furthermore, sequence data of actin genes from *N. dimidiatum* was, for the first time, deposited to the GenBank. Koch's postulates were fulfilled by testing the susceptibility of different tomato tissues (leaves, stems, inner stems and roots of tomato seedlings, and detached tomato fruits and flowers) to *N. dimidiatum* inoculation. To our knowledge, this is the first report of *N. dimidiatum* on tomato.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most economically important, widely grown and consumed vegetables in the world (FAOSTAT, 2016) and in Turkey. Turkey is the fourth biggest tomato-producing country after China, India and the USA, with 12.6 million tons of fruits produced on 188,270 ha area (FAOSTAT, 2016). In Turkey, tomato is widespread, but the mass production is mainly in the Mediterranean region. Productions in fields, glasshouses, polytunnels and household gardens supply tomato all year round. In Şanlıurfa, Diyarbakır and Mardin provinces, located in the Southeast Anatolia Region, tomato can generate significant income for small farmers, and areas under cultivation have constantly been expanding for the last 10 years. Tomato production in this region is estimated to cover approximately 12,481 ha in area, with 581,796 tons produced, which constitutes 4.6% of the whole production in Turkey (TÜİK, 2017).

A wide range of microbial pathogens and insect pests attack different parts of tomato plants, causing significant crop losses. In 2016, a tomato foliar blight and root rot disease of unknown etiology was observed in fields of Şanlıurfa, Diyarbakır and Mardin provinces. In 2017, the production of tomato was drastically reduced by this unknown, destructive and widespread disease in these provinces of the Southeast Anatolia Region. Morphological traits on the basis of symptom development and the presence of arthrospores and pycnidia within lesions were found compatible with characteristics of botryosphaeriaceous fungi. The accurate identification of the causal agent of this disease will constitute a fundamental step for the development of appropriate and effective disease management strategies. Therefore, the present work was conducted to characterize this fungal agent via morphological and molecular techniques, as well as to evaluate the disease incidence, severity and symptoms on tomato plants. Koch's postulates were used throughout the study to determine whether the fungal agent is the responsible pathogen for producing symptoms on different tomato tissues.

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Table 1
Origin of *Neoscytalidium dimidiatum* isolates obtained in three provinces of the Southeast Anatolia Region from tomato plants with symptoms of blight, number of plantings where they were sourced, and incidence and severity of Neoscytalidium blight in symptomatic fields.

Province	Sampling location (no. of symptomatic fields)	Incidence (%)[a]	Severity (%)[b]	No. of isolates retained											
				shoot	flower	branch	leaf petiole	leaf	stem	inner stem	pedicel	sepal	fruit	seed	root
Şanlıurfa	Göktepe (13)	41.4	5.7	1	2	2	2	6	5	1	2	2	2	1	2
	Karabahçe (5)	9.7	1.2	1					4					2	
	Sedatlı (9)	20.2	1.4		1		1		6		2			1	1
	Siverek Road (4)	11.0	0.8			1			5		1				
	Yaylak (4)	12.8	0.7	1				1	4						
	Çobandere (5)	18.3	1.7	1	1	1		3	3		1	2	1		2
	Kefirli (2)	11.3	0.6	1	1	1	1	1	1	1			1	1	1
Diyarbakır	Hani (4)	9.8	0.4	2				2	2						
Mardin	Kızıltepe (1)	3.0	0.1					2	1						
Total	47	21.2	1.4	7	5	5	4	15	31	2	6	4	4	5	6

[a] Average percentage of plants showing disease symptoms in symptomatic fields at each location. For each field in a location, 150–300 plants were counted.
[b] Average percentage of stem surface with blight in symptomatic fields at each location. For each field in a location, 150–300 plants were inspected for the severity of stem blight symptoms.

2. Materials and methods

2.1. Field observations

Field surveys were carried out in May–September 2017 in 338 commercial tomato fields in three provinces (Şanlıurfa, Diyarbakır and Mardin) of the Southeast Anatolia Region of Turkey. Development of the disease symptoms and the location where the disease occurred were noted. In each province, all disease-positive fields were re-visited within 3 weeks of harvest during July and August, when fruit were ripened. In each field, 10 locations on a W-shaped path were selected, and at each location, 15–30 plants were randomly chosen to evaluate for incidence and severity of the disease on the plant (at least 150 plants were inspected from each field visited). Disease incidence was assessed as the percentage of plants with symptoms of the total 150–300 plants inspected. The severity of the disease was calculated as the average of percent affected areas on the stem surface.

2.2. Isolation and morphological characterization of isolates

Diseased samples were collected from tomato plants at 42 sites in Şanlıurfa, four sites in Diyarbakır, and one site in Mardin. Isolations were made separately from blighted shoots, flowers, branches, leaf petioles, leaves, stems, pedicels, sepals and fruits, necrosed stem piths and vascular tissues, as well as from decayed fruits, seeds and roots. The fungus was also cultured from conidia, mycelium and black pycnidia formed on the diseased tissues of tomato, showing typical morphology of Botryosphaeriaceae fungi. Symptomatic tissues, 3×5 mm in size, were cut with a sterile scalpel, and their surfaces were disinfected with 2% NaOCl for 2 min and rinsed twice in sterile distilled water (sdw), dried on sterile filter paper for 10 min and placed in Petri dishes containing 2% potato dextrose agar (PDA; Merck, Darmstadt, Germany) supplemented with 12 $\mu g\ ml^{-1}$ tetracycline. Petri dishes were then incubated at 25 $\pm$ 1 °C in the dark for 3–7 days until fungal colonies were large enough to be examined. To obtain pure cultures, single hyphal tips and spores from the colonies were transferred to fresh PDA and pine needle agar (PNA) prepared with 20 mm length, autoclaved-sterile pine needles. The cultures were then incubated for 1–3 weeks depending on the development of fungal culture in each medium. Mycelia, conidia and pycnidia prepared from the cultures were mounted in sterile water and squeezed between the slide and cover slip. The length and width of 50 conidia per isolate were measured at 400 × magnification under a light microscope (Nikon Model Eclipse E200). Conidial shape, color, and the presence of septa were also recorded. Isolates were identified based on the description of their pycnidial and mycelial anamorphs (Crous et al., 2006; Phillips et al., 2013; Huang et al., 2016). All isolates were then deposited at the culture collection of the Plant Pathology Laboratory at the GAP Agricultural Research Institute, Şanlıurfa.

2.3. DNA extraction, PCR amplification and sequencing

The isolates designated ND94 and ND121, obtained from tomato shoot and stems, respectively, were grown on 2% PDA for one week at 25 °C for DNA extraction. Pure culture mycelia were collected from the medium with a sterile scalpel, and total genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Germany). Primers ITS6 and ITS4 were used to amplify the nuclear rRNA operon spanning the 3′ end of the 18S rRNA gene, internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, ITS2, and the 5′ end of the 28S rRNA gene with the amplification conditions of 3 min at 94 °C, 40 cycles of 94 °C 30 s, 55 C 45 s, 72 °C 1 min, and finally 72 °C 10 min (Cooke et al., 2000). Part of the translation elongation factor 1-alpha gene (TEF-1α) was amplified by PCR with primers EF1-728F and EF1-986R (Carbone et al., 1999). The primer pairs NL1 and NL4 were used to amplify approximately 650 bp of the large subunit (LSU) gene of rRNA (O'Donnell, 1993). The ActF/ActR primer pair was used to amplify 210- 220-bp partial sequences of the fungal actin gene (Tang et al., 2012).

Amplicons were sequenced after purification in both directions using the same primers in an automated sequencer using the Sanger method by Medsantek (Istanbul/Turkey). Trimming and assembly of the sequences were performed with MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 (Kumar et al., 2016). Gene sequences were deposited in GenBank, with the accession numbers MH114588-MH114595 (Table 3).

2.4. Phylogenetic analysis of the ITS and LSU rRNA, TEF-1α and actin gene sequences

The amplified sequences were subjected to BLAST queries using the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) nucleotide database to check for homologies among the assembled consensus sequences and for preliminary identification of the isolates used in the analysis. In the database, there was no available sequence for actin gene of *N. dimidiatum*. Available LSU gene sequences, on the other hand, were retrieved from plant-based sources. Due to these inconveniences, sequences for two gene regions (ITS and TEF-1α) were combined for each isolate (ND94 and ND121) for multi-locus sequence typing (MLST). They were aligned, and the phylogenetic tree was regenerated with the selected sequences of the available allied taxa obtained from GenBank (Table 2) with MEGA7 software.

Phylogenetic analyses were conducted with the ITS, LSU and TEF-

Table 2
The sequence coverage and identity results of fungal isolates from tomato after BLAST analysis.

Gene	Isolate	% Coverage/% Identity	Identical Organism/Accession no Deposited in GenBank
Actin	ND94	99/85	*Botryosphaeria dothidea*/AY972117
		100/84	*Macrophomina phaseolina*/KF951804
	ND121	100/84	*Botryosphaeria dothidea*/AY972117
		100/84	*Macrophomina phaseolina*/KF951804
LSU	ND94	100/99	*Neoscytalidium dimidiatum*/JF719036
	ND121	100/99	*Neoscytalidium dimidiatum*/JF719036
ITS	ND94	100/99	*Neoscytalidium dimidiatum*/KP054960
	ND121	100/99	*Neoscytalidium dimidiatum*/KY013660
TEF-1 alpha	ND94	100/99	*Neoscytalidium dimidiatum*/KF553898
	ND122	100/100	*Neoscytalidium dimidiatum*/KX464763

Table 3
Strains, hosts, geographic origins, gene regions and NCBI GenBank accession numbers of *Neoscytalidium dimidiatum* species used for phylogenetic analysis.

Voucher /culture	Country	Host	GenBank accession no			
			ITS	TEF-1 alpha	LSU	Actin
ND94	**Turkey**	**Tomato**	**MH114590**	**MH114594**	**MH114592**	**MH114588**
ND121	**Turkey**	**Tomato**	**MH114591**	**MH114595**	**MH114593**	**MH114589**
CBS 499.66	Mali	*Mangifera indica*	AY819727	EU144063	DQ377925	–
CBS 251.49	USA	*Juglans regia*	KF531819	KF531797	DQ377923	–
CBS 204.33	Egypt	*Prunus* sp.	AY819728	EU144064	–	–
UCRDC284	USA	Citrus limon	KF620370	KF620406	–	–
IRNHM-NAT	Iran	Elm tree	KP054960	KU095813	–	–
DE1606	China	*Dioscorea esculenta*	KY013660	KY349086	–	–
282	Brazil	*Jatropha curcas*	KF369269	KF553902	–	–
7E63	USA	English walnut	KC357303	KC357315	–	–
PD104	USA	Fig	GU251107	GU251239	–	–

Fig. 1. Field symptoms of blight disease on tomato (*Solanum lycopersicum* cv. Hazara) caused by *Neoscytalidium dimidiatum* in the Kefirli location.

1α sequences individually and with a combined dataset together with selected reference sequences from the NCBI database. Maximum likelihood analyses were performed with MEGA7 software. A bootstrap analysis (50% majority rule, 1000 replicates) was performed (Kumar et al., 2016) to determine the confidence levels of the tree-branching points (Felsenstein, 1985). The tree was rooted to *Macrophomina phaseolina* isolate CMM3543.

2.5. Susceptibility of different tomato tissues to N. dimidiatum inoculation

The susceptibility of leaves, stems, inner stems and roots of potted 6-week-old tomato cv Hazara plants, as well as detached fruits and flowers of cv Hazara, was tested against the isolate ND94, which was obtained from a blighted shoot. For the pot experiments, seeds were sown in multipots filled with commercial steam-sterilized peat:perlite (2:1, v:v) mixture. Plants were removed from the seedling trays at the first true leaf stage (3-day-old seedlings). Seedlings were then transplanted (one per pot) into 12-cm diameter plastic pots filled with commercial steam-sterilized peat:perlite (2:1, v:v) mixture and maintained in a growth chamber under a 16-h/8-h (light/dark) photoperiod at 26 °C with a relative humidity of 55%. All plants were watered to field capacity once a week to maintain adequate moisture for plant growth and were fertilized every month with 200 ppm of water-soluble fertilizer (20-10-20, N—P—K). A conidial suspension was prepared by rubbing the surface of the plate with a sterilized paintbrush (with bristles), and the suspension was then filtered through sterile cheesecloth and suspended to 1×10^5 conidia ml^{-1} in sdw.

Inoculated plants were maintained at the same growth chamber conditions and inspected daily for symptoms for two months. All experiments were conducted twice at different times in the year following the survey study, in 2018.

Fig. 2. Blight and root rot symptoms on young tomato plants.

Fig. 4. Shoot and fruit blight symptoms observed on a tomato plant in the Çobandere location.

2.5.1. Leaves

The leaves of tomato plants were sprayed with 70% ethanol and allowed to air dry. The suspension was then sprayed on the leaves and suckers (twenty seedlings). Ten seedlings sprayed with sdw served as control. All foliar parts of the plants were enclosed in plastic bags for 16 h to maintain high relative humidity. The bags were then removed, and the plants were kept in growth chambers.

2.5.2. Stems

In a second experiment, stems of tomato (10 seedlings) 5–6 cm above the collar were wounded via making shallow cuts (3–5 mm in length) with a sterile scalpel in the stem of each plant. Inoculation was made by inserting 5-mm mycelial plugs obtained from a 7-day-old PDA culture. Sterile agar plugs (5-mm PDA) were used for ten control seedlings. Inoculated areas of the stems were covered with sterile moist

Fig. 3. a. Brown discoloration of the pith and stem xylem and root rot on young tomato plants b. Necrotic vascular tissue in the upper portion of older plants caused by *Neoscytalidium dimidiatum.* (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5. a. b. Stem and leaf lesions or blight.

Fig. 6. a. Leaf and flower blight b. Flower blight.

Fig. 7. a. Blight of pedicels, sepals, and fruits b. Shoot and fruit blight symptoms observed on a tomato plant c. Fruit rot.

cotton balls and wrapped with Parafilm to maintain high humidity during the inoculation period (45 days).

2.5.3. *Inner stems*

Susceptibility of inner stems was assessed by injecting 50 µl of conidial suspensions (1×10^5 conidia ml^{-1}) into 0.4 cm depth of tomato plant stems (ten stems) 12 cm above the collar with a sterile 2-mm sterile syringe. The controls (ten stems) were inoculated with sdw. Plants were examined 2 months after inoculation by cutting the stem longitudinally.

2.5.4. *Roots*

The susceptibility of roots was tested by transplanting tomato plants (ten seedlings) into 12-cm diameter plastic pots (one per pot) filled with commercially available steam-sterilized peat:perlite (2:1, v:v) mixture. The soil was then inoculated with the mycelia and conidia grown on autoclaved wheat kernels. A 3-g wheat kernel containing fungal mycelia and conidia was placed at the base of the tomato root area. Wheat kernels without the fungal mycelia served as controls. For the preparation of inoculum, wheat kernels (100 g) and distilled water (200 ml) were mixed well in 500-ml conical flasks and autoclaved at 121 °C for 40 min. The flasks were inoculated with ten 5-mm mycelial plugs. Then, the flasks were incubated at 28 °C in the dark for 3 weeks. The flasks were shaken by hand daily to disperse the fungus. After incubation, the wheat kernels with hyphae were naturally dried and gently ground by a sterile pestle.

2.5.5. *Fruits*

Fruit susceptibility was tested on ten fully mature tomato fruits, which were surface-sterilized with 70% ethanol, rinsed with sterile

Fig. 8. a. Rot growing from the tomato fruit pedicel observed on field conditions, internal collapse and rotten seeds.

distilled water and dried with paper tissues. A 5-mm mycelial plug cut from the margin of a 7-day-old culture was placed onto fruit wounds, which were made with a sterile blade. The wounds were then sealed with Parafilm. Wounded but un-inoculated fruits served as controls. In another set, fruits were inoculated by injecting 1 ml of the conidial suspension (1×10^5 conidia ml^{-1}) through a 1-ml syringe into the equatorial center of the fruits. Control fruits were injected with 1 ml sdw. All fruits were incubated in an incubator in the dark at 27 °C for a week. The fruits were visually examined daily for symptom appearance.

2.5.6. Flowers

Ten freshly cut tomato shoots (ca. 15 cm in length) containing flower clusters (three healthy and opened flowers) were placed individually into sterile 250-ml bottles filled with 125 ml sdw, and the top of each bottle was wrapped with Parafilm to prevent dehydration and to support the shoot. Ten μl of the conidial suspension (1×10^5 conidia ml^{-1}) was injected through a sterile 1-mm syringe into the center of the flower (five shoots, 15 flowers) by wounding and inoculating the tube in one action. Non-wounded flowers were inoculated with a conidial suspension (1×10^5 conidia ml^{-1}) sprayed on the petals (five shoots, 15 flowers). Control flowers (ten shoots, 30 flowers) were inoculated in a similar manner with sdw.

Re-isolation of the fungus from diseased parts of inoculated leaves, stems, inner stems (vascular tissues and pith), roots, fruits, and flowers was performed to complete the steps of Koch's postulates. Edges of the lesions were cleansed with 2% NaOCl for surface disinfection, and tissues were washed in sdw and incubated on 2% PDA at 25 °C in the dark for 5 days. Fungal growth emerging from plant tissues was identified based on morphology.

3. Results

3.1. Field observations

Diseased plants showed symptoms of blight (irregular browning to blackening) with necrotic lesions on all foliar parts, including leaf, petiole, shoot, stem, flower, and peduncle, yellowing, withering, defoliation, pith necrosis, and fruit (cluster) and root rot (Fig. 1). The disease, in general, was observed in 47 out of 338 fields surveyed (13.9%). In particular, the disease was observed in 42 out of 250 fields (16.8%) in Şanlıurfa, 4 out of 67 fields (6.0%) in Diyarbakır, and 1 out of 21 fields (4.8%) in Mardin provinces, compromising an area of approximately 3000 da. The incidence of plants with blight symptoms varied between 3 and 75% in these symptomatic fields. On average, 21.2% of the plants were affected by the disease (Table 1). The severity of the disease in symptomatic fields at each location ranged from 0.1 to 5.7%. The average severity of the disease in symptomatic fields surveyed was 1.4% (Table 1).

On young tomato plants, symptoms were blight of large areas of the crown, stem and leaves, and root rot (Fig. 2). Longitudinal cuts into stems of these plants revealed vascular and pith discoloration extending all over the stem (Fig. 3a). Symptoms on older tomato plants appeared as water-soaking followed by browning of large areas in the upper stem (Fig. 4) that extended to plant leaves. Water-soaked lesions on stems, darkening and death of buds and flowers, and stem lesions or blighting continuously expanded downwards. At later stages, the symptoms progressed towards the branches, petiole, and lower stem, which resulted in collapse, dieback, dry out and defoliation (Fig. 5). Irregular brown-to-black stem lesions at different sizes evolved into brown discoloration and resulted in necrosis in the xylem and cortex (Fig. 3b). Leaves of affected plants in some fields initially had irregular, small, gray-to-dark brown spots with and without a yellow halo, appearing similar to bacterial specks, and then withered in the upper parts of the plant. Lesions coalesced and became irregularly circular or angular, usually turning into a brown color (Figs. 5 and 6a). The blight of flowers, including petals, stamens and styles (Fig. 6a and b) and resembling *Monilinia* spp. infections on stone fruits, was also a common symptom. Fruits were heavily affected at all stages of growth. Small fruits were blighted on the outer skin, and their pedicels and sepals were also blighted (Fig. 7a); abscission and rot of immature and mature fruits were common (Figs. 4 and 7a, b, c). The internal collapse of fruit developing from pedicel and seed discoloration within fruit was also observed (Fig. 8). Sporulation was noticed on completely rotten fruits

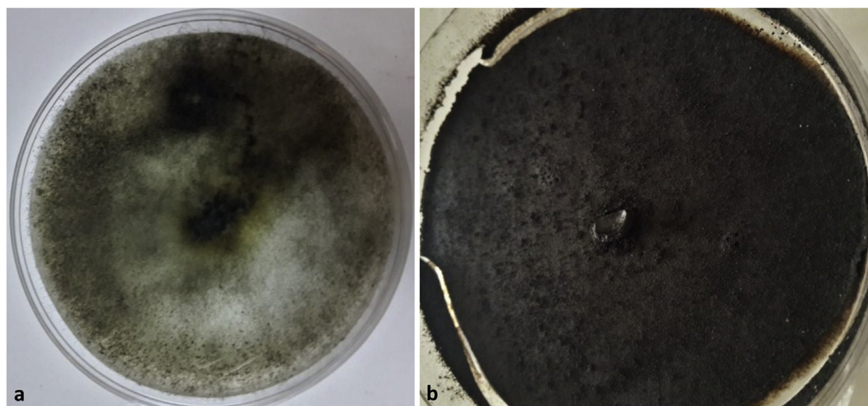

Fig. 9. Colony of *Neoscytalidium dimidiatum* on PDA, a. Incubation for 5 days and b. for 20 days at 25 °C in the dark.

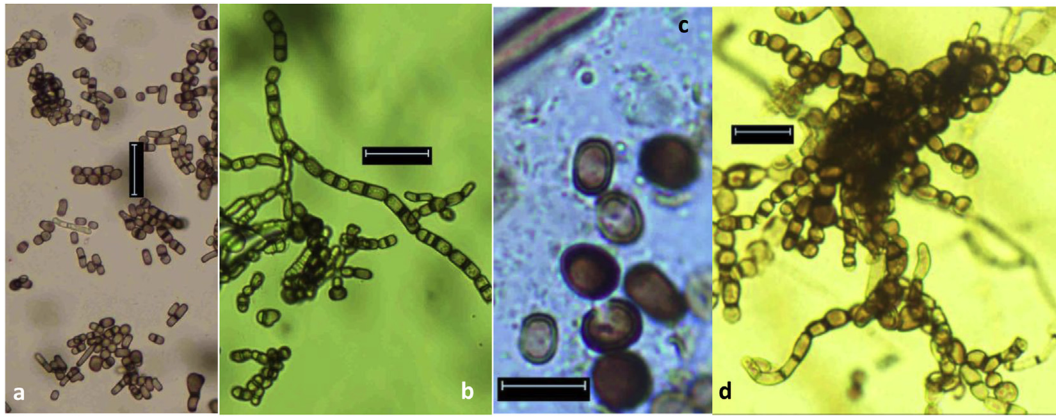

Fig. 10. a. Size-and-shape-variable arthroconidia b. Contiguous arthroconidia c. Thick-walled arthroconidia d. Arthric chains of conidia growing on PDA Scale bars = 30 µm.

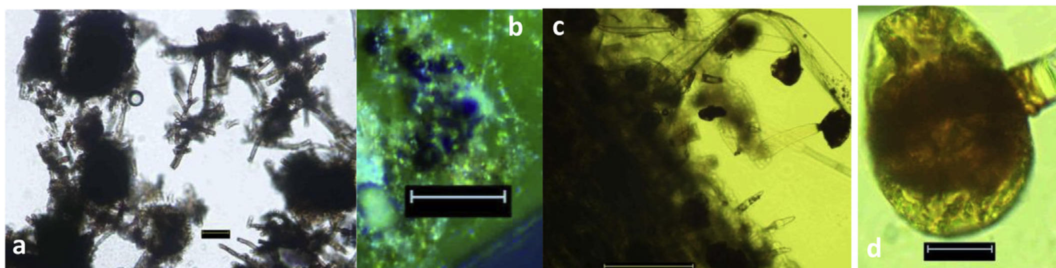

Fig. 11. a. Conidiomata formed on 30-day-old PDA culture b. Conidiomata (pycnidia) formed on pine needles in culture c. Black conidiomata formed on and in the surface of stem lesions d. Close-up of conidiomata formed on stem lesions Scale bars: (a, d) = 100 µm, (b, c) = 1000 µm.

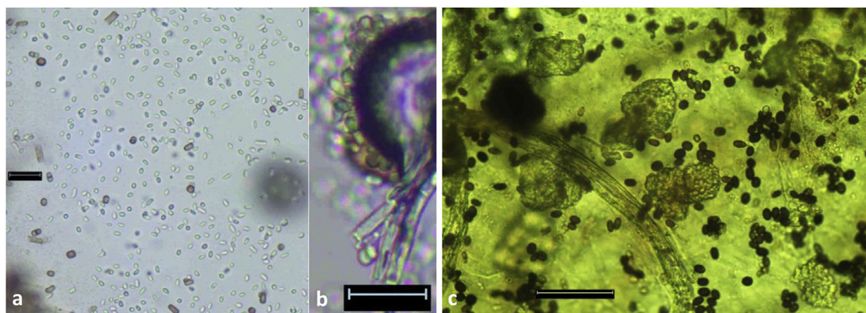

Fig. 12. a. Hyaline aseptate conidia produced by conidiogenous cells b. Close-up of lageniform to ampulliform conidiogenous cells of coelomycetous state formed on pine needles in culture c. Brown, ovoid to ellipsoidal conidia produced by conidiomata formed on the surface of older stem lesions. Scale bars: (a, b) = 30 µm, (c) = 250 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 7c). When the relative humidity had been high, tiny black specks were often visible within lesions of all plant organs. Plants that went through these stages eventually died at fields.

3.2. Isolation and morphological characterization of isolates

A total of 94 fungal isolates with identical cultural characteristics were isolated from symptomatic samples from 47 tomato fields in Şanlıurfa (42), Diyarbakır (4) and Mardin (1).

Fungal isolates grew quickly on PDA, and the colony diameter reached 6.5–8 cm at 25 °C in the dark after 3 days. Colony color was initially gray and gradually became olive green (Fig. 9a) or black (Fig. 9b) on the reverse and obverse sides of Petri dishes. Abundant gray-to-black, wooly aerial mycelium was produced. The aerial mycelia were composed of branched and septate (4–8 µm wide) brown hyphae constricted into 0- to 1-septate spore chains and disarticulated into arthroconidia (toruloid state). Dark brown, cylindrical–truncate or rod-shaped, thick-walled, 3.9 to 17.1 × 2.7–8.8 µm arthroconidia formed in arthric chains was the most common type of conidia observed (Fig. 10a, b, c, d). Ellipsoidal-oval or orbicular-dolioform conidia with a truncate base and an acutely rounded apex and muriform conidia were also observed (Fig. 10a). Abundant black pycnidia with globose base up to 300 µm formed on older PDA cultures (Fig. 11a), PNA (Fig. 11b), in and on stem lesions (Fig. 11c and d) and with at first aseptate, hyaline, and ellipsoid-cylindrical to nearly fusiform conidia (8–12 × 4–5 µm) (Fig. 12a) produced by holoblastic, lageniform to ampulliform conidiogenous cells (6–12 × 2.5–4 µm) (Fig. 12b). Phialoconidia later became brown and ovoid-to-ellipsoidal in shape (Fig. 12c). Based on these characteristics, the fungus was conditionally identified as *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers (Crous et al., 2006). Isolations from all blighted tissues showed almost 100% *N. dimidiatum* recovery, yielding only one colony type. The origins of the isolates and the numbers of plantations where they were sourced are shown in Table 1.

3.3. PCR and sequence-based identification of isolates

The PCR amplification and direct sequencing of the ITS region of isolates ND94 and ND121 resulted in the amplification of 499 bp and 559 bp sequences, respectively. The BLAST analysis of ITS sequences of both isolates confirmed the pathogen *N. dimidiatum* on tomato plants,

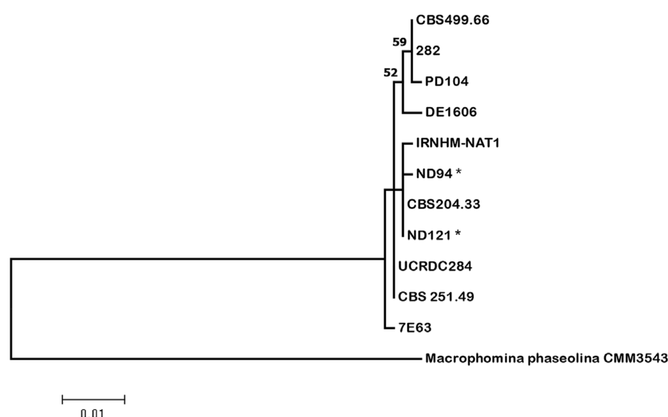

Fig. 13. The maximum likelihood tree showing the phylogenetic relationship (InL = −1351,03) between *Neoscytalidium dimidiatum* Turkey isolates and other available strains and isolates from GenBank based on combined sequences of internal transcribed spacer and translation elongation factor-1 alpha region sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches (Bootstrap values expressed as percentages of 1000 replications). Bootstrap support values (expressed as percentages of 1000 replications) for maximum likelihood greater than 50% are indicated above the nodes. *Macrophomina phaseolina* isolate was used as the outgroup. Isolates from this study are indicated with star.

providing 99% identity and 100% coverage with a known isolate of *N. dimidiatum* (accession number: KP054960). The TEF-1 alpha, LSU and actin gene regions of isolate ND94 were 251, 606 and 242 bp, and for isolate ND121, they were 170, 539 and 229 bp, respectively. The blast analysis of the ITS, TEF-1 and LSU gene sequences resulted in 100% coverage and 99–100% identity with the *N. dimidiatum* isolates deposited in GenBank (Table 2). Because there is no available sequence that belongs to the actin gene sequences of *N. dimidiatum* in GenBank, the sequences of actin gene BLASTed with the isolates of *Botryosphaeria dothidea* or *Macrophomina phaseolina* sequences showed 84–85% identity.

3.4. Phylogenetic analysis

The phylogenetic division of most genera of Botryosphaeriaceae supports the monophyly of *Neoscytalidium* species (Huang et al., 2016). Therefore, a total of nine sequences of *N. dimidiatum* species obtained from GenBank and our two isolates were used to construct the phylogenetic tree (Table 3). The availability of the ITS, TEF-1 alpha and LSU sequences from the same isolate, as well as from different plant sources and countries, were the criteria for the determination of the sequences used in phylogenetic studies (Table 3). Individual and combined loci phylogenies were performed using all these sequences. Phylogenetic analysis of individual loci produced similar tree topology, and thus datasets for the ITS and TEF-1 alpha were combined and analyzed (Fig. 13).

Our isolates from tomato clustered with CBS204.33 and IRNHM-NAT1, which were isolated from *Prunus* from Egypt and elm from Iran, with lower 50 bootstrap value (Fig. 13). Among these isolates, no substantial diversity was detected by sequence and phylogenetic analyses.

3.5. Susceptibility of different tomato tissues to N. dimidiatum inoculation

3.5.1. Leaves

Almost two weeks after inoculation, water-soaking symptoms and dark brown irregular lesions were observed on all inoculated leaves (Fig. 14a and b), On suckers, the dying back gradually progressed downward, with leaves turning yellow, browning and abscising quickly, leaving a necrotic edge, and eventually the upper part of the plant completely blighted. All control leaves and shoots remained healthy. Koch's postulates were satisfied after re-isolating *N. dimidiatum*, which was identified morphologically, from the lesions of inoculated leaves and suckers. No disease occurred in 10 controls, and the pathogen was not recovered from these plants.

3.5.2. Stems

All the inoculated stems showed dark necrosis, often leading to bark cracking, at the inoculation site, whereas the wounded sites inoculated with sterile agar plugs and sdw did not change color. After 2 weeks, the average length of lesions caused by *N. dimidiatum* on stems was 33–45 mm. The symptoms described in field conditions were observed extending along the entire stem on all inoculated stems at 45 days post-inoculation, and black sporulation occurred at and near the point of inoculation sites, whereas control stems did not develop any symptoms. *N. dimidiatum* was re-isolated from the inoculated stems, and no fungus was isolated from the control.

3.5.3. Inner stems

Stems exhibited dark brown lesions at the inoculation site, and brown discoloration of the inner stem developed around the inoculation site, extending around seven and 5 cm above and below the site of infection, respectively, whereas no lesions were observed in control plants. Plants inoculated with sdw had green inner tissue. Isolates recovered from inoculated inner stems approximately 2 cm from the inoculation point had the same characteristics as the original isolate.

3.5.4. Roots

Early symptoms were yellowing of the lower leaves. As leaves wilted and died, there was progressive yellowing towards the top of the plants. The pathogen caused severe wilting after 2 weeks, and inoculated plants showed similar symptoms to those observed in the field. When the plants were uprooted after 2 months, the inoculated and exposed roots showed dark discoloration on the bark of infected primary roots and crown, and most fine roots were completely rotted. Dark discoloration of lower stem xylem was also observed. The fungus was re-isolated from symptomatic roots of inoculated plants, and had the same morphological conidial characteristics of the pathogen originally recovered from *S. lycopersicum*.

3.5.5. Fruits

Two days after inoculation, all wound-inoculated fruit showed rot symptoms. After 4 days, fruit rot symptoms started to appear on conidia-injected fruit. Profuse black conidia developed on inoculation points, and the fungus was re-isolated from these lesions. The infected fruit exhibited a watery internal collapse, developed a thin outer skin and became filled with a slimy mass. Seven days after inoculation, 100% of wound-inoculated and conidia-injected fruit became slimy and collapsed, and rot led to disintegrating of the fruit. In these fruits, seeds were also rotten, and the fungus was re-isolated from these seeds. The affected fruit had a rotten, foul-smelling odor. No symptoms developed on control fruits.

3.5.6. Flowers

Inoculated flower tubes developed blights 2–3 weeks after inoculation. Flowers generally collapsed as the fungus invaded through the pedicel, and no fruit developed on these flowers. Control flower clusters failed to develop any detectable disease symptoms, and they did not produce any fruits, since the flower tube was deformed during the sdw inoculation. *N. dimidiatum* was re-isolated from the inoculated flower tubes, and no fungus was isolated from the controls. On conidial spray-inoculated petals of the flowers, first, water-soaking symptoms appeared. Infected petals withered and turned brown. Blighting of the rest of the inflorescence and the pedicel occurred at later stages, leading to complete drying and no fruit development. No symptoms were observed on the control sdw-sprayed flowers, and small fruit development

Fig. 14. a. Leaf blight of tomato inoculated with *Neoscytalidium dimidiatum* b. A closer view of leaf blight.

(about the size of a lentil) was observed on these shoots. Isolations from symptomatic flower tissues yielded cultures of *N. dimidiatum;* however, no fungus was isolated from the controls.

In all repeated inoculation experiments, the symptoms observed were similar to those of the first experiments and those of the natural field infections.

4. Discussion

Emerging diseases caused by fungi and fungus-like oomycetes are steadily being reported on many crops (Farr and Rossman, 2018). Tomato is one of the most consumed summer vegetables in the world, either fresh or processed. The results of this study demonstrated that *N. dimidiatum* was the causal agent of the shoot, flower, stem, fruit and leaf blight, pith necrosis, and root rot of tomato plants in commercial fields of the Southeast Anatolia Region of Turkey. This species was isolated from all aboveground plant parts and roots of tomato. The pathogen was accurately identified on the basis of morphological features and using the ITS and LSU rDNA and TEF-1α sequencing. ITS, LSU and TEF-1α sequences of isolates from tomato sourced in southeastern Turkey were identical to reference sequences of *N. dimidiatum* and were clearly differentiated from all other currently recognized Botryosphaeriaceae species of the order Botryosphaeriales (Crous et al., 2006). All symptoms of the complex syndrome associated with the decline of tomato plants were reproduced in artificial inoculations using a *N. dimidiatum* isolate obtained from a blighted tomato shoot, and the pathogenicity tests were positive, confirming Koch's postulates. Observations in natural field conditions, together with controlled pathogenicity tests, demonstrated that this fungus had aggressive behavior and was responsible for killing tomato plants. Additionally, the isolates tested had a high ability to colonize the non-wounded healthy leaf, shoot, flower and root tissues, as well as the wounded stem, inner stem, fruit and flower tissues of tomato. The disease is currently causing serious losses to tomato plants in this region.

Neoscytalidium dimidiatum (Syn: *Fusicoccum dimidiatum*, *Torula dimidiata*, *Scytalidium dimidiatum*, *Hendersonula toruloidea, N. hyalinum*) (Crous et al., 2006; Phillips et al., 2013; Huang et al., 2016) was isolated from various substrates, including soil, woody plants and human (skin, nail and sinus) tissues, and it has been known as a plant pathogen (Punithalingam and Waterston, 1970; da Silva et al., 2016; Bakhshizadeh et al., 2014; Crous et al., 2006). The fungus was previously reported on a broad range of plant hosts in wide geographical locations throughout the world (Von Arx, 1987; Farr and Rossman, 2018). Recently, this pathogen was reported to cause brown spot (on the surface of fruit, in China, Lan et al., 2012), stem canker (in Taiwan, Chuang et al., 2012; in Malaysia, Mohd et al., 2013; in Costa Rica,

Retana et al., 2017), stem and fruit canker (in Florida, Sanahuja et al., 2016), and internal black rot (in fruit, in Israel: Ezra et al., 2013; in China, Yi et al., 2015) diseases of pitahaya (*Hylocereus* spp., a cactus genus with edible fruits). It has been identified as the causal agent of canker and gummosis of orange (*Citrus sinensis*) in Italy (Polizzi et al., 2009), dieback or canker of mango (*Mangifera indica*) in Australia (Ray et al., 2010; Sakalidis et al., 2011), dieback of common fig (*Ficus carica*) in Australia (Ray et al., 2010), collar and root rot of physic nut (*Jatropha curcas*) in Brazil (Machado et al., 2012), wood canker and dieback of grapevine (*Vitis vinifera*) in California and Brazil (Rolshausen et al., 2013; Correia et al., 2016), bot gummosis (Adesemoye et al., 2014) and branch canker of citrus (*Citrus* spp.) in California (Mayorquin et al., 2016), black root rot of cassava (*Manihot esculenta*) in Brazil (Machado et al., 2014), wood lesions and decline of willow (*Salix* spp.) and poplar (*Populus* spp.) in Iran (Hashemi and Mohammadi, 2016), dieback of lesser yam (*Dioscorea esculenta*) in China (Lin et al., 2017), leaf blight of white spider lily (*Hymenocallis littoralis*) (Nurul Nadiah et al., 2017), mother-in-law's tongue (*Sansevieria trifasciata*) (Kee et al., 2017) in Malaysia, and canker of ornamental figs (*F. nitida* and *F. benjamina*) in Egypt (Al-Bedak et al., 2018).

Neoscytalidium dimidiatum causes yield losses in tomato and other plants. Therefore, a better understanding of this destructive disease would lead to the establishment of better disease control strategies. The epidemiology of this disease is not known. It is possible that the fungus is seed, soil and/or air-borne, and it may also survive in the soil in association with infected tomato debris. Hygienic measures, such as removal of symptomatic plants or plant parts from fields, might reduce the proliferation and spread of the pathogen. Moreover, in some areas, the accumulation of dead plant biomass due to this disease could dramatically increase the severity and incidence of blights. To our knowledge, this is the first report of *N. dimidiatum* infecting and causing the death of tomato plants worldwide. The recent occurrence of the disease indicates that the disease may constitute a serious threat to tomato production and industry in Turkey, with a significant impact both on yields and on the long-term viability of plantations. The spread of *N. dimidiatum* pathogenic isolates in cultivations of tomato could exacerbate the damages.

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None.

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