

## First Record of *Melanospora chionea* as a Possible Cause of Pink Root Rot Disease on Tomato Plants in Egypt

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### ABSTRACT

Tomato is one of the most important vegetable crops in the world. It is infected with several disease through the growth season, but new disease appeared as a new challenge to tomato productivity, causing pink root rot. Symptoms of pink root rot were observed on tomato (*Lycopersicon esculentum* Mill.) grown in Beni Sweif Governorate (Nasser, Sumosta, Beba and El-Wasta Counties) in summer 2013 as poor growth, chlorosis and then necrosis of the tip branches, by maturity. Typical symptoms on the infected root especially, epidermis were picked areas and both of cortex and vascular bundles were colored with pink along the infected tissues consistent with both those that were observed in the field. Based on morphological characteristics of the isolated fungus, disease symptoms and a pathogenicity test, *Melanospora chionea* was identified as the causal agent of pink root rot of tomato. Identification of this species was confirmed by sequencing of internal transcribed space (ITS region) of ribosomal RNA gene. *M. chionea* has not previously been reported on tomato. The host range of this disease was defined between numerous hosts belonging to *Fabaceae*, *Malvaceae*, *Cucurbitaceae* and *Solanaceae*. The aim of this work to determine and description of the disease and identification of the pathogen morphologically and genetically. More work is required to find appropriate methods for controlling this new disease.

**Keywords:** *Melanospora chionea*; Pink root rot; Morphological characteristics; Pathogenicity test; *Lycopersicon esculentum*; Egypt

### INTRODUCTION

The genus *Melanospora* is characterized by ostiolate ascomata having a thin translucent peridium and clavate prototunicate asci which deliquesce when mature [1]. Morphologically, species of *Melanospora* are similar to members of Chaetmiaceae and the Sordariaceae, but they have Diaporthe type centrum [2]. Most species of *Melanospora* are parasitic on or closely associated with other fungi. They have a wide host range including Basidiomycetes and both sexual and asexual Ascomycetes. The wilt disease fungus *Fusarium oxysporum* is one of the most common hosts, and some *Melanospora* species fuse with the host protoplasts to obtain nutrients, an interaction called fusion biotrophism [3].

Doguet [4] listed forty- five species of *Melanospora* and regarded others as synonyms. With the aid of the scanning electron

microscope (SEM), [5] further restricted the range of this genus based on ascospore morphology. Further species were added to the complex by Garcí a et al. [6,7], Hawksworth et al. [8], Krug [9], Nitzan et al. [10], Stchigel et al. [11,12] and Vujanovic and Goh [13]. Chaudhary et al. [14] published the new genus *Vittatispora* from India, which also belongs to the *Melanospora* complex, and the non-sporulating *Papulaspora* was demonstrated to represent sterile *Melanospora* ascomata by Davey et al. [15]. Li Fan et al. [16] published a new species of *Melanospora* parasitizing Chinese black and white truffles (*Melanospora subterranean*).

In this paper, the role of *M. chionea* as a potential pathogen causing pink root rot disease of tomato plants is demonstrated. Morphological identification of the fungus was confirmed by both microscopic images and performing gene sequencing of rDNA.

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## MATERIALS AND METHODS

For isolation from tomato root plants which collected from Beni Serif governorate (latitude and longitude at 29.0667 N and 31.0833 E), the roots were washed under tap water, chopped into 2 cm small pieces and surface sterilized in 0.5% NaOCl for two minutes then rinsed three times with sterilized distilled water, dried between sterilized filter paper and placed on PDA medium and finally kept in an incubator at  $25 \pm 2^\circ\text{C}$  under dark conditions.

For maintaining *M. chionea* with a highly and rapidly ascus fruits, the fungus was cultured by placing small agar blocks containing single-ascospore on V8 juice vegetable medium, incubated at  $30^\circ\text{C}$  for 14 days. The mycelia and spores were then scraped from the surface, suspended in water and sieved through 3 layers of cheesecloth under aseptic conditions. The number of colony forming units (CFU) in the spore suspension was counted using a haemocytometer (Thoma, Germany) and the concentration was adjusted using sterilized tap water for pathogenicity tests.

### Molecular identification of fungal isolates

Fungal isolates were grown in sterile Petri plates containing autoclaved Czapek's Dox agar provided with 0.5% yeast extract (CYA) medium and incubated for 7 days at  $28^\circ\text{C}$  [17]. Cultures were sent to the Molecular Biology Research Unit, Assiut University for DNA extraction using Patho-gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. The fungal DNA was then sent to SolGent Company, Daejeon South Korea for polymerase chain reaction (PCR) and rRNA gene sequencing. PCR was performed using ITS1 (forward) and ITS4 (reverse) primers which were incorporated in the reaction mixture. Primers have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5' - TCC TCC GCT TAT TGA TAT GC - 3'). The purified PCR products (amplicons) were sequenced with the same primers [18].

The ITS dataset included 15 sequences, three of which are *Melanospora damnosa* AUMC 14376 and *M. chionea* AUMC 14438 and AUMC 14440 and 11 are sequences for the nearest strains of *Melanospora* downloaded from GenBank and including the available type species, in addition to sequence for *Sarocladium implicatum* as an out group. DNA sequences of *Melanospora damnosa* AUMC 14376 and *M. chionea* AUMC 14438 and AUMC 14440 were edited with the DNA STAR computer package (DNA star version 5.05). Assembled sequences of the three *Melanospora* strains obtained in this study were aligned with those downloaded from GenBank using MAFFT [19]. [20] Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using PhyML 3.0 [21] The robustness of the most parsimonious trees was evaluated by 100 bootstrap replications [22]. The best optimal model of nucleotide substitution for the ML analyses was determined using alkaline information criterion (AIC) as implemented in Model test 3.7 [23]. The phylogenetic tree was visualized using BioNJ [24], and edited using Fig. tree version 1.4.3 [25].

### Pathogenicity test

The pathogenic capabilities of the isolated fungi were determined using seeds of tomato commercial cultivar (super jahal). In summer 2018, seeds of tomato were sown in plastic pots (30 cm) having sterile moist sand and were placed in greenhouse and watered as needed. When the seedlings reached the 15-25 cm tall with 3-5 true leaves they were gently removed from pots and the roots were dipped individually in a spore suspension ( $10^6$  spores/ml) of the five isolates obtained previously from different counties of Beni Sweif governorate for 30 min. the inoculated seedlings were then transplanted in a potting mix consisting of clay, sand and peat (1:1:1). Seedlings were dipped in tap water used as control. The pots were placed in greenhouse and arranged as a randomized complete block design with three replicates. Three months after inoculation, the plants were assessed for disease symptoms and gently uprooted and the roots examined for signs of infection

### Host range

Host range studies were conducted on fourteen crop plants from six botanical families (*Molwaceae*, *Asteraceae*, *Brassicaceae*, *Cucurbitaceae*, *Fabaceae* and *Solanaceae*) were selected for testing. Sterile soil in pots (20 cm) was inoculated with ascospores suspension of a single isolate of *M. chionea* immediately after incubated at  $30^\circ\text{C}$  for 10-15 days on V8 medium to allow colonization. Ascospores concentrations as determine by dilution at  $10^6$  /ml. seeds of the host range plants were soaked 1hr in an ascospores suspension and then planted in the pots (3 replicates) previously colonized with the same isolate. Plants were grown in a greenhouse for 8-10 weeks. Plants were then uprooted to observe the pink root rot symptoms.

## RESULTS AND DISCUSSION

Symptoms on inoculated plants appeared as poor growth, chlorosis and then necrosis of the tip branches, by maturity. Typical symptoms on the infected root especially, epidermis were picked areas and both of cortex and vascular bundles were colored with pink along the infected tissues consistent with both those that were observed in the field, and those reported for this disease (Figures 1 and 2). Koch's Postulates were fulfilled by re-isolation of the tested fungus.

The fungus involved in the pink root rot of tomato was identified morphologically on the basis of perithecial structure, dimensions, shape and measurements of ascospores. The mycokeys provided by Cannon and Hawksworth [5] and Marin-Flex [19] were consulted for identification of the isolated fungi with the help of mycologists at Assiut University Mycological Center (AUMC). Observations made with bright field light microscopy showed that, perithecia are superficial, solitary to gregarious, sub-globose to globose yellow to pale brown. Beak's length 120-130  $\mu\text{m}$ , slightly tapering, with a terminal ring of hyaline setae.

Ascospores are ellipsoidal to discoid, walls dark brown, with two terminal sessile pores measuring  $14-15 \times 10-11 \mu\text{m}$  as shown in Figures 3, 4 and 5. Images taken by scanning electron microscope (SEM) are illustrated in Figure 4. Microscopic

observations of the fungus were consistent with *Melanospora chionea* described by Cannon and Hawksworth [5] and Marin-Flex et al. [19] To the best of our knowledge, this is the first published report of pink root rot of tomato caused by *M. chionea*. Sequencing data of ITS region of rDNA extracted from the two fungal strains (Table 1 and Figure 1) revealed that they are closely related to *Melanospora damnosa* showing 98.20% similarity with *M. damnosa* strain KY575977 isolated from triennial branches stems of stumped hazelnuts. It also showed 98.89% identity with *M. damnosa* AUMC14376 (MT071833) recovered from *Phaseolus vulgaris* leaves and 98.19% similarity with *M. damnosa* CBS 113681 (KP981478).

The entire ITS data set comprised 15 sequences. The maximum parsimony dataset consisted of 583 characters with 405 constant characters (no gaps, no N), 324 variable characters which were parsimony-uninformative (80.0% of constant characters), and 295 characters were counted as parsimony informative (72.8% of constant). The GTR was the best fit model for nucleotides substitution. Maximum Parsimony analyses resulted in 2 most parsimonious trees with a tree length of 593 steps. Maximum likelihood analysis yielded one tree (Log-likelihood=-3852.02044) with a tree size of 4.77719.

However, the morphological characteristics of *M. chionea* showed some variations from *M. damnosa* particularly in the shape and dimensions of ascospores. In case of *M. chionea* the ascospores are smooth walled, discoid to ellipsoidal measuring 7.5-16 × 6-12 × 4-7 μm but those of *M. damnosa* are citriform to rhomboidal with larger dimensions measuring 18-28 × 10-14 μm [5]. *Melanospora verrucispora* showed also closer relationship (98.06% identity) with *M. chionea* but the distinct verrucose ascospores of *M. verrucispora* separates it from *M. chionea* [26].

**Sequences of *Melanospora chionea* AUMC14438 (MT336021) (length 566bp)**

GTAGGTGAAACTGCGGAGGGATCATTACAGAATCTGA  
CCATTTGTGTCATGGCTCTGCCAACCCTGTGAACTTTA  
TATACTTGACGTTGCCTCGGCGGAACCTGCCTTTTTG  
GCAGGCCGCCGG

CCGGCATATACGCAAACGCTCTGAAAAAGCTCCGCGC  
TCTATCTGAATAAGAAAACCTTTAACGAGTAAAACTTTT  
GGCAACGGATCTCTTGGCTCTGGCATCGATGAAGAAC  
GCAGCGAAATG

CGATACGTAGTGTGAATTGCAGAATTCAGTGAACCATC  
GAATCTTTGAACGCACCTTGC GGCCCGCGTAATCCG  
GCGGCCATGCCCGTCCGAGCGTCGTTTCGACCCTCG  
GGAGTTCTCCTCG

CAAGAGAAGTTCTCCCGCCTTGGGCCAGCGTGTTCG  
GCGGCCCGCAAACCAGCGGCGGCAGTACCGGCGATG  
TCCTCTGTGCAGTAGATTTATAGAAAACCTCGCATTGGT  
CTCCGGTAAGGC

TTGCCTTACAACCAACTTCTTTAGGTTCGACCTCGGATC  
GGGTAGGGATACCCGCTAAACTTAAGCATATC

***Melanospora chionea* AUMC14440 (gb: MT336022) (length 568 bp)**

CTGCGGAGGGATCATTACAGAATCTGACCATTTGTGTG  
ATGGCTCTGCCAACCCTGTGAACTTATATACTTGTACG  
TTGCCTCGGCGGAACCTGCCTTTTTGGCAGGCCGCC  
GGCCGGCATATAC

GCAAACGCTCTGAAAAAGCTCCGCGCTCTATCTGAAT  
AAGAAAACCTTTAACGAGTAAAAACCTTTTGGCAACGGAT  
CTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAAT  
GCGATACGTAG

TGTGAATTGCAGAATTCAGTGAACCATCGAATCTTTGA  
ACGCACCTTGC GGCCCGCGTAATCCGGCGGCCATG  
CCCGTCCGAGCGTCGTTTCGACCCTCGGGAGTTCTCC  
TCGCAAGAGAAG

TTCTCCCGCCTTGGGCCAGCGTGTTCGCGGCCCG  
CAAACCAGCGGCGGCAGTACCGGCGATGTCTCTGT  
GCAGTAGATTTATAGAAAACCTCGCATTGGTCTCCGGTA  
AGGCTTGCCTTACA

ACCAACTTCTTTAGGTTCGACCTCGGATCGGGTAGGGA  
TACCCGCTAAACTTAAGCATATCAATAAGCGGAGGA

**Table 1:** Percentage identity and coverage of ITS sequences of *Melanospora* species accessed from GenBank showing close relationship with *M. chionea* obtained in the current study.

Fungal species isolated in the present study	Closest strains accessed from the GenBank					
	Name	Strain No.	Accession No.	Identity (%)	Coverage (%)	Source
<i>Melanospora chionea</i> AUMC14438	<i>Melanospora damnosa</i>	-----	KY575977	98.41	99	Stem of stumped hazelnut, China
(gb: MT336021)	<i>Melanospora damnosa</i>	CBS113681	KP981478	98.05	99	Spain
	<i>Melanospora damnosa</i>	AUMC14376	MT071833	99.04	91	Blighted seedlings of <i>Phaseolus vulgaris</i> , Egypt
<i>Melanospora chionea</i> AUMC14440 (gb: MT336022)	<i>Melanospora verrucispora</i>	NRBC31375	KP981501	98.41	99	Spain



**Figure 1:** Maximum Likelihood (ML/MP combination) phylogenetic tree of *Melanospora damnosa* AUMC 14376 and *M. chionea* AUMC 14438 and AUMC 14440 associated to other *Melanospora* related genes in the ITS gene sequence. Sequence of the *Melanospora* species in this study are in blue color. The tree is rooted to *Sarocladium implicatum* Zbf-S9 as an outgroup T = Type strain.

### Host range

Observations of the host range trials in Table 2 showed that tomato isolate was pathogenic on eggplant, common bean, pea, cowpea, watermelon, muskmelon, roselle, cabbage, chamomile and Snake cucumber beside tomato. Symptoms on all species included poor growth, chlorosis and then necrosis of the tip branches, by maturity. Typical symptoms on the infected root especially, epidermis were picked areas and both of cortex and vascular bundles were coloured with pink along the infected tissues (Figure 4).

However, the response of squash, potato and pepper showed no infection. Although, most species of *Melanospora* are parasitic on or closely associated with other fungi, but *Melanospora subterranean* is the first record of *Melanospora* species parasitizing Chinese black and white truffles (*T. indicum* and *T. huidongense*), and its host range indicates that the new species might be a disease threat to commercially exploited European truffles including *Tuber melanosporum* and *Tuber magnatum*. [16]. Also, *M. zamiae*, the causal agent of crown rust of maize was detected in only one accession (IC-587520) of *C. speciosus* from Giridih, Jharkhand. The report of the Technical Working Group 1 of Australia listed this as a pathogen of quarantine significance and

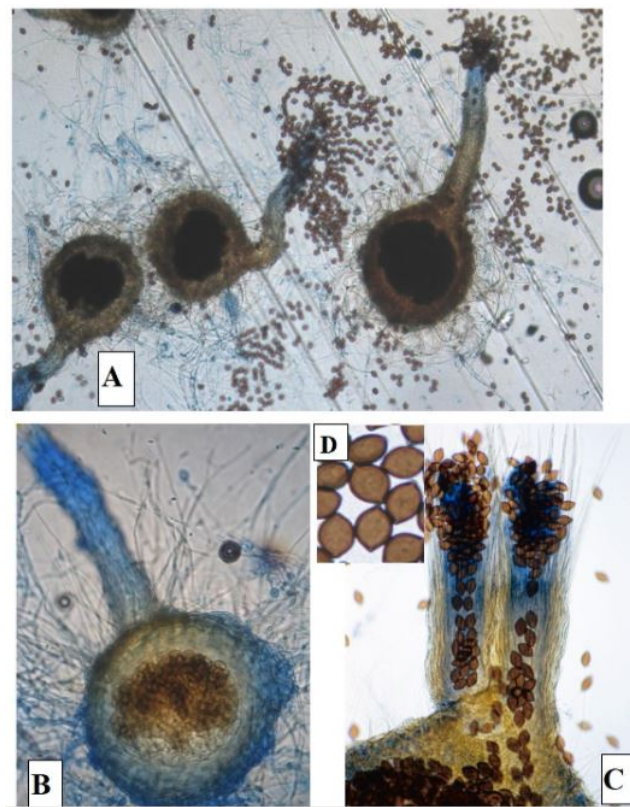
highlighted the risk associated with the bulk maize import from USA [27].

**Table 2:** Susceptibility of various species infection by *Melanospora chionea*.

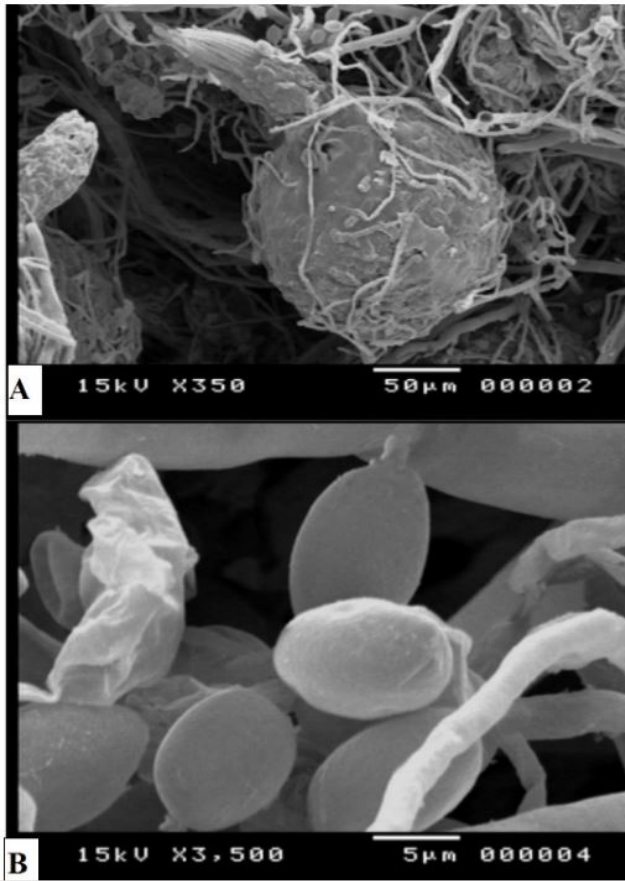
N	Family	Plant species, Common name	Disease reaction
1	<i>Solanaceae</i>	<i>Solanum melongena</i> Linn Egg plant	+
		<i>Lycopersicon esculentum</i> Mill. Tomato	+
		<i>Capsicum annum</i> Linn. Pepper	-
		<i>Solanum tuberosum</i> Linn. Potato	-
2	<i>Fabaceae</i>	<i>Phaseolus vulgaris</i> Common bean	+
		<i>Pisum sativum</i> Pea	+
		<i>Vigna unguiculata</i> subsp. <i>unguiculata</i> Cowpea	+
3	<i>Cucurbitaceae</i>	<i>Cucumis melo</i> Naud Muskmelon	+
		<i>Cucumis melo</i> var. <i>flexuosus</i> Snake cucumber	+
		<i>Citrullus lanatus</i> Watermelon	+
		<i>Cucumis sativus</i> L. Cucumber	-
4	<i>Brassicaceae</i>	<i>Brassica oleracea</i> var. <i>Capitata</i> Cabbage	+
5	<i>Asteraceae</i>	<i>Matricaria chamomilla</i> chamomile	+
6	<i>Molvaceae</i>	<i>Hibiscus sabdariffa</i> Roselle	+



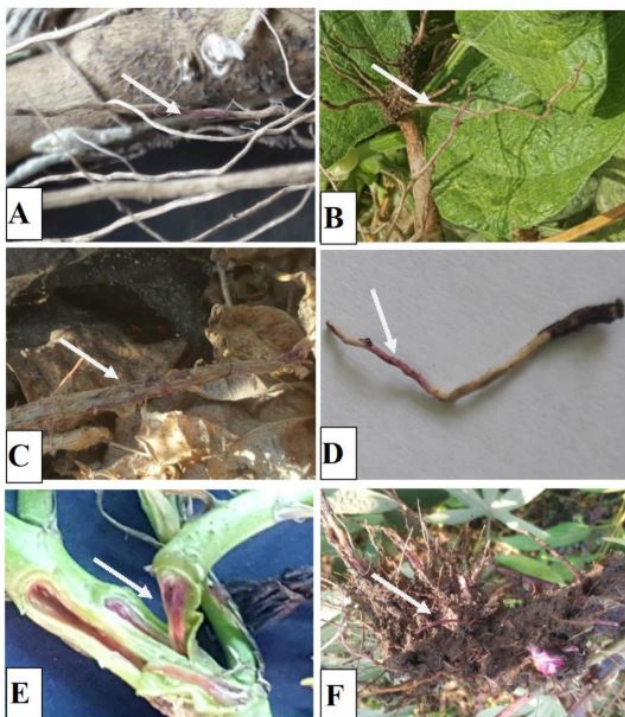
**Figure 2:** Symptoms of pink root rot of tomato plants in the field showing chlorosis and necrosis (A and B). Symptoms of pink coloration of secondary roots (C) and main root (D).



**Figure 3:** Mature perithecia 100x (A), Perithecia with ascospores 200x (B), perithecial beak with setae and discharging ascospores 400x (C). Higher magnification of ascospores 1000x (D).



**Figure 4:** Scanning Electron Microscope images of *Melanospora chionea* showing Perithecial ascoma (A, 350x) with a crown of smooth-walled setae; and discoid, smooth-walled ascospores with depressed germ pores (B, 3500x).



**Figure 5:** (A-F). Symptoms of pink root rot on eggplant, bean, cowpea, cucumber, chamomile and roselle respectively infected by *M. chionea*.

## CONCLUSION

Our results were similar with the previously on Chinese black and white truffles and maize as the pathogen but it differs in infection and symptoms. As the author is far aware, there is no available information on tomato pink root rot disease with *Melanospora chionea* in Egypt. Therefore it can be concluded that the present work is a first record of tomato pink root rot disease by *M. chionea*, so we will complete survey this disease in Egypt, epidemiology of this disease and we will carry up more required work to find appropriate methods for controlling this new disease.

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