Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Adesemoye at al., J Plant Pathol Microbiol 2016, 8:1
DOI: 10.4172/2157-7471.1000391

Research Article Open Access

Association of *Neonectria macrodidyma* with Dry Root Rot of Citrus in California

Adesemoye AO[1], Mayorquin JS[2], Peacock BB[2], Moreno K[2], Hajeri S[3], Yokomi R[4] and Eskalen A[2]*

[1]*Department of Plant Pathology, University of Nebraska-Lincoln, West Central Research and Extension Center, North Platte, NE, USA*
[2]*Department of Plant Pathology and Microbiology, University of California, Riverside, CA, USA*
[3]*Citrus Pest Detection Program, Central California Tristeza Eradication Agency, Tulare, CA, USA*
[4]*USDA, ARS, CDPG, Parlier, CA, USA*

Abstract

During yearly surveys that started in 2010 to identify pathogens associated with dry root rot disease of citrus in California, samples with root rot symptoms were collected in Tulare County. Small pieces of tissue from root samples were plated onto potato dextrose agar amended with 0.01% tetracycline and incubated at 25°C. Pure cultures of fungal isolates were identified by morphology and sequence analysis of the Internal Transcribed Spacer and Beta Tubulin regions. *Neonectria macrodidyma* (*Cylindrocarpon macrodidymum*) was first recovered in 2011 and has subsequently been recovered multiple times from citrus samples. The pathogen appeared to be widely distributed in association with citrus dry root rot and possibly interacts with *Fusarium solani, Phytophthora nicotianae* and *P. citrophthora*, the major soil borne pathogens that were frequently identified from plant samples with root and/or crown rot of citrus in California. The fungal genus *Cylindrocarpon* (Teleomorph: *Neonectria wolenw=Dactylonect ria=Ilyonectria*) contains ubiquitous soil borne pathogens that cause black foot disease on a wide range of hosts, including grapevine, strawberry, apple, and conifers. Hosts typically become infected through natural wounds on roots and other below ground parts. In this report, we present strain UCR3312, which is the most recently isolated pathogenic strain in 2015. Considering the potential damage that this organism may cause to the citrus industry, detailed studies are recommended to better understand its distribution, epidemiology, and the general pathogen biology to improve the disease management practices.

Keywords: *Neonectria macrodidyma*; Citrus; Dry root rot

Introduction

Dry root rot of citrus usually affect only a few trees in an orchard but once symptoms appear, the tree hardly recovers. Affected trees show chlorosis, poor vigor and degenerate for several years before they suddenly wilt and die (Figure 1). Examination of roots and lower trunk usually shows staining from which *F. solani* are frequently recovered. It was generally agreed that *F. solani* was the primary cause of dry rot. The fungus, *Fusarium solani*, is known to be a facultative pathogen that feeds on dead organic matter, but, under certain conditions, becomes pathogenic and causes disease on various hosts [1]. It is unclear how dry rot develops, but stress factors such as, but not limited to, root damage, gophers, Phytophthora root rot, wet soils and excess fertilizers and rootstock incompatibilities are critical in initiating the disease [1]. Whether horticultural practices such as frequent microjet irrigation or close plant spacing can exacerbate the problem is unknown.

Since the beginning of this decade, trees collapsing with dry root rot appear to be increasing and is becoming a significant economic problem in some orchards in central California. The condition seems to develop on younger trees from 3 to 7 years in age and, often, on trifoliate and hybrid rootstocks. Increasing chemical measures to control *Phytophthora* does not appear to be very helpful; affected trees are removed and replaced by young citrus trees. Given the difficulty of managing dry root rot, we hypothesize that the disease is more complex and involve more pathogen than *Fusarium solani* and *Phytophthora* sp. As *Neonectria macrodidyma* is known to cause black foot disease in containerized nursery trees for grapevines, apples and forest, it is important to determine if it also has any role or association with citrus root rot in California. This paper presents our findings through a survey on the role of *N. macrodidyma* in citrus dry root rot disease.

Cylindrocarpon (Teleomorph: *Neonectria wolenw=Dactylonectria=I lyonectria*) are ubiquitous soil borne fungal pathogens that cause black foot disease on a wide range of hosts, including grapevine, strawberry, apple, and conifers. *Cylindrocarpon* species usually infect their hosts through wounds on the roots and other below ground portions of the rootstock and stress may induce the disease. *Neonectria macrodidyma* and three other members of the genus (*C. destructans*, *C. liriodendra, or C. macrodidyma*) have been reported in association with black foot disease of grapevine in nurseries and vineyards in many countries, including Australia, Canada, Chile, New Zealand, South Africa, Spain, Tasmania, United States, and Uruguay [2-4].

Neonectria macrodidyma is a pathogen of economic importance to containerized seedlings and the nursery industry in California [5,6]. It is also important in apple [7], pine and other conifer seedlings, and the forest nurseries [8,9]. *Neonectria macrodidyma* is a necrotic plant pathogen and a new name was recently proposed as *Dactylonectria macrodidyma* (syn. *Ilyonectria macrodidyma*) [10]. In addition to *Phytophthora nicotiana* (syn. *P. n.* var. *parasitica*), *P. citrophthora*, and *Fusarium solani,* which are major soilborne pathogens causing root and/or crown rot on citrus in California, *N. macrodidyma* and other *Cylindrocarpon* spp. are suspected to be unidentified threats.

Materials and Methods

Sample collection and isolation

In yearly surveys that started since 2010 for dry root rot disease of citrus in California, samples with crown and root rot symptoms were collected in Tulare County**.** Samples were collected from six orchards

***Corresponding author:** Akif Eskalen, Department of Plant Pathology and Microbiology, University of California, Riverside, CA 92521, USA, Tel: 9518273499; E-mail: akif.eskalen@ucr.edu

Received December 27, 2016; **Accepted** January 20, 2017; **Published** January 22, 2017

Citation: Adesemoye AO, Mayorquin JS, Peacock BB, Moreno K, Hajeri S, et al. (2016) Association of *Neonectria macrodidyma* with Dry Root Rot of Citrus in California. J Plant Pathol Microbiol 8: 391. doi: 10.4172/2157-7471.1000391

and 10 trees per orchard in a Z-pattern. The roots were transported on ice to the laboratory at the University of California, Riverside (UCR). In order to isolate fungi, 2 mm^2 pieces of symptomatic tissues were plated onto potato dextrose agar amended with 0.01% tetracycline (PDA-tet) with incubation in the dark for four days at 25°C. Pure cultures of fungal isolates were obtained by serial transfer of excised hyphal tips of fungal colonies growing out of the root pieces. Mycelial plugs of isolates were stored in 1 ml of sterile deionized water in a 1.5 ml microtube and maintained in 4°C refrigerator.

Morphology and molecular identification of isolates

Isolates obtained were identified through anamorph morphology characteristics [3,4,11,12]. Digital images of 20 conidia of isolates were captured with a Leica DFC420 camera mounted on a compound microscope (Olympus BX40). The length and width of the conidia were determined using the SPOT Imaging Software (v4.7.0.35, Diagnostic Instruments, Inc., MI). The conidia characters were compared with those in previous reports [3,4,12] and isolates were subjected to molecular identification.

Molecular identification of isolates was done by sequencing the internal transcribed spacer (ITS) and β-tubulin regions. Polymerase chain reaction (PCR) was performed in a MyCycler (Bio-Rad Laboratories, Inc., Hercules, CA) using primers Bt2a and BT2b for the β-tubulin and ITS4 and ITS5 for the internal transcribed spacer ITS1-5.8S-ITS2 regions [4,13,14]. The 25 µl PCR reaction contained 12.5 µl of GoTaq Green Master Mix (Promega, Madison, WI), 9.3 µl of PCR-grade water, 0.6 µl of 10 µM of each primer set, and 2 µl DNA templates. The reaction protocol included an initial preheat at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 45 s; and final extension at 72°C for 5 min. The PCR products were verified using 1% agarose gel and the gels were documented with Dark Reader **DR88X** transilluminator (Clare Chemical Research, Dolores, CO). The PCR products were purified using the Isopure PCR purification kit (catalog # CM-0100-100, Denville Scientific Inc, Metuchen, NJ). The quality and quantity of the PCR products were estimated using NanoDrop Spectrophotometer ND-1000 (Thermal Scientific, Wilmington, DE). Sequencing was done at the Institute for Integrative Genome Biology of UCR. The forward and reverse sequences were edited using Sequencher software 4.6 (Gene Codes, Corp.) and aligned with Cap3 [15]. The sequences were compared in the *Basic Local Alignment Search* (BLAST) search.

Pathogenicity test

An assay was set up to test the pathogenicity of the recently characterized *Neonectria* (*Ilyonectria*) *macrodidyma* strain UCR3312. Trifoliate seedlings (Carrizo) maintained in a lathhouse were stem wound inoculated with a number one cork-borer at approximately 1.5 cm above the soil line. Mycelial plugs were taken from a one week old culture of strain UCR3312, inoculated onto the wounded seedlings, and wounds were wrapped with parafilm covered in petroleum jelly. The control plants were similarly wounded but the inoculation was done with a freshly prepared plain agar plug without any pathogen. Each trial had five replicates and the test was repeated. Plants were destructively sampled after three months of inoculation and lesion length was measured and re-isolations were made to fulfill Koch's postulates.

Results and Discussion

Symptoms observed in the field

Various symptoms were observed in the field during the period of the survey, including wilting or drying of shoots or whole plants (Figure 1), necrosis, abnormal development, reduction of root biomass, cankers near the crown and black discoloration of wood (Figure 2), which were similar to the description by Menkis and Burokien [9]. Also observed were bark peeling and discoloration of bright, salmon pink pads from the dead bark that darkened with age (Figure 3). It should be noted that these symptoms are however not unique to this pathogen as *Fusarium solani* was recovered from other citrus trees that had similar symptoms.

Characteristics of isolates and the Conidia size

The cultures of this isolate (strain UCR3312) obtained from Tulare County was similar to those obtained previously, *it* grew slowly on *PDA* producing orange red pigment that diffused into the agar and was noticeable at the underside of the agar medium. Observation of the conidia revealed morphological characteristics that matched previous

Figure 1: Trees showing several symptoms in the field.

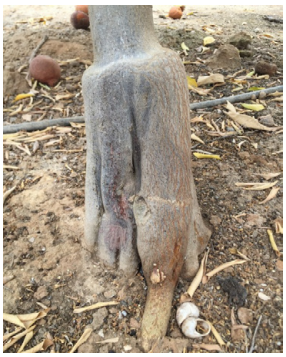

Figure 2: Sample canker symptoms on many trees: (i) showing wide lesions and canker at the crown and the grafted region (on the left) and (ii) advancing dead areas through the cross section of a tree (on the right).

Figure 3: Canker and damages to the phloem (on the left) and Bark peeling (on the right). Trees showed bright, salmon pink pads from the dead bark that usually turn black with age.

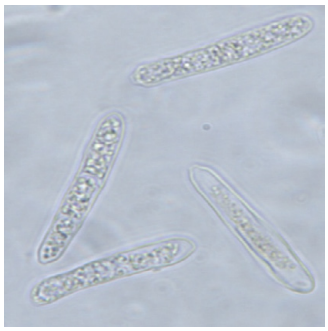

Figure 4: Macroconidia of *Neonectria macrodidyma* under a compound microscope (100X).

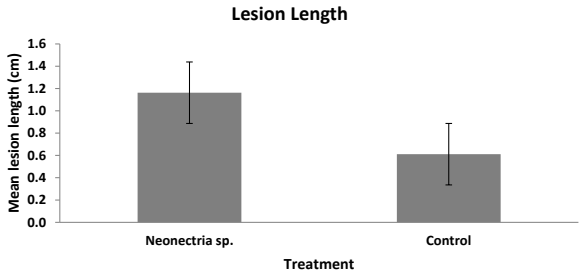

Figure 5: Mean lesion length on seedling from pathogenicity test after three months of inoculation with *Neonectria macrodidyma* strain UCR3312.

descriptions of *Neonectria* species [3,4,12]. The macroconidia had a mean measurement of 28.9 × 6.2 µm length and width respectively and were cylindrical, straight with one to three-septate (Figure 4) but the isolate did not produce microconidia. Morphological characters and the analysis of sequences for strain UCR3312 revealed the identity of the isolate as *N. macrodidyma* and the internal transcribed spacer (ITS) sequences were deposited in GenBank with Accession number KX712244. Previously in 2010, an isolate recovered (strain UCR519) was sequenced and deposited in the GenBank with Accession numbers JF934748 for ITS and JF934749 for β-tubulin. Other isolates that grew somewhat similar to *Neonectria* on the media were identified as *Fusarium solani* or *Fusarium* species through sequencing.

Following the initial isolation of *N. macrodidyma* (UCR519) during 2010/2011 survey and based on the preliminary pathogenicity assay on the strain, it was suspected that *N. macrodidyma* may be associated with dry root rot in citrus in California. However, the test was not repeated and no further study was conducted because that was the only isolate retained among those recovered from the same sample in only one orchard in Tulare County. It was believed that the pathogen may not be widely spread; thus, it generated very little interest. Systemic surveys continued and isolates with similar morphology was recovered at least once per year between 2012 and 2014, thus prompting a renewed interest. In 2015, a new *N. macrodidyma* isolate, (UCR3312) was recovered from samples obtained in another orchard in Tulare County. Pathogenicity assay was conducted and strain UCR3312 was pathogenic (Figure 5). This suggested that the pathogen may be more important and more spread than earlier believed. This organism may have been overlooked previously due to its slow growth on PDA-tet. There is a concern that *Neonectria macrodidyma* may be an important pathogen, which is receiving little attention in citrus production areas. In this regard, its relationship with the dry root rot complex including *P. nicotiana*, *P. citrophthora,* and *F. solani* needs to be studied in California. This is the first report of *N. macrodidyma* from a citrus orchard in California, though *Neonectria* spp. had been reported as a pathogen in nursery beds.

Symptoms from pathogenicity test of *N. macrodidyma* UCR3312

Seedlings inoculated with *N. macrodidyma* UCR3312 showed visible lesions with an average length of 1.2 cm (Figure 5). No lesions were observed in the control plants, only the superficial wound response was visible. *N. macrodidyma* was recovered from the fungal inoculated plants but not from the control seedlings. The pathogenicity of this strain shows that *N. macrodidyma* could play a role in dry root rot disease of citrus. It is important to mention that strain UCR519, which was pathogenic in a preliminary pathogenicity assay in 2011 was recently re-cultured from the freezer and re-tested and was found to have lost its pathogenicity.

Conclusion and Recommendation

The impact of this pathogen on the nursery industry and citrus production in California is not clear yet. Considering the potential damage that *Neonectria* spp. especially, *N. macrodidyma* may cause to the citrus industry, there is need for nurseries to take proactive measures. It is important to keep new containers sterile or as clean as possible. If any container is reused, it must be properly sterilized. Nursery operators should pay attention to root decay when selecting transplants and may discard seedlings that exhibit symptoms. Operators may choose to apply fungicides to seedlings that did not show noticeable or significant symptoms. While fungicide application is ineffective after symptoms are seen, pathogen impact could be reduced through fungicide root dips before transplanting.

This is the first report of *Neonectria* (*Cylindrocarpon*) species in California citrus though the pathogen has been reported in grapevine in California [6]. This organism has been recently renamed *Ilyonectria (Dactylonectria) macrodidyma* as some other members of the Nectriaceae are being renamed. This pathogen as well as strain UCR519 produced macroconidia (Figure 4) but not microconidia, contrary to the description of type species by Lombard et al. [10]. The pathogen's spread in California citrus is not clear yet. Is there a correlation between infections in grapevine and citrus and, if so, what is the relationship of the species on these two hosts and other hosts?

Comprehensive studies should be conducted to answer these questions and provide understanding on the distribution and epidemiology of this pathogen in California and other U.S citrus growing regions. Investigation should include variations among species of *Neonectria,* that are present in the state; host specificity, potential phenotypic and/or genotypic differentiation in comparison to previous reports from other locations [3]. It is also essential that further studies be conducted to provide understanding on the type of interactions existing between this pathogen and other pathogens involved in dry root rot of citrus. All these information will help improve disease management practices directed at citrus dry root rot.

References

1. Adesemoye A, Eskalen A, Faber B, O'Connell N (2011) Current knowledge on Fusarium dry root rot of citrus. Citrograph 2: 29-33.

2. Abreo E, Martinez S, Bettucci L, Lupo S (2010) Morphological and molecular characterization of *Campylocarpon* and *Cylindrocarpon* spp. associated with black foot disease of grapevines in Uruguay. Australas Plant Pathol 39: 446-452.

3. Alaniz S, León M, Vicent A, García-Jiménez J, Abad-Campos P, et al. (2007) Characterization of *Cylindrocarpon* species associated with black foot disease of grapevine in Spain. Plant Dis 91: 1187-1193.

4. Halleen F, Schroers HJ, Groenewald JZ, Rego C, Oliveira H, et al. (2006) *Neonectria liriodendra* sp. nov., the main causal agent of black foot disease of grapevine. Stud Mycol 55: 227-234.

5. Gimenez-Jaime A, Aroca A, Raposo R, Garcıa-Jimenez J, Armengo J (2006) Occurrence of fungal pathogens associated with grapevine nurseries and the decline of young vines in Spain. J Phytopathol 154: 598-602.

6. Petit E, Gubler WD (2005) Characterization of *Cylindrocarpon* species, the cause of black foot disease of grapevine in California. Plant Dis 89: 1051-1059.

7. Tewoldemedhin YT, Mazzola M, Mostert L, McLeod A (2011) *Cylindrocarpon* species associated with apple tree roots in South Africa and their quantification using real-time PCR. Eur J Plant Pathol 129: 637-651.

8. Beyer-Ericson L, Damm LE, Unestam T (1991) An overview of root dieback and its causes in Swedish forest nurseries. Eur J Forest Pathol 21: 439-443.

9. Menkis A, Burokien D (2011) Distribution and genetic diversity of the root-rot pathogen *Neonectria macrodidyma* in a forest nursery. Forest Pathol 42: 79-83.

10. Lombard L, Van Der Merwe NA, Groenewald JZ, Crous PW (2014) Lineages in *Nectriaceae*: re-evaluating the generic status of *Ilyonectria* and allied genera. Phytopathol Mediterr 53: 515-532.

11. Barnett HL, Hunter BB (2006) Illustrated genera of imperfect fungi (4thedn). The American Phytopathological Society (APS) Press, Minesota, USA pp: 130-131.

12. Brayford D, Honda BM, Mantiri FR, Samuels GJ (2004) *Neonectria* and *Cylindrocarpon*: The Nectria mammoidea group and species lacking microconidia. Mycologia 96: 572-597.

13. Adesemoye AO, Mayorquin JS, Wang DH, Twizeyimana M, Lynch SC, et al. (2014) Identification of species of Botryosphaeriaceae causing Bot gummosis in citrus in California. Plant Dis 98: 55-61.

14. Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91: 553-556.

15. Huang X, Madan A (1999) CAP3: A DNA sequence assembly program. Genome Res 9: 868-877.