The 'Cylindro' complex of avocado root pathogens

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Abstract
Species of *Cylindrocladium* sp., *Cylindrocarpon* sp. are known to be pathogens of many agronomic and forestry crops worldwide, and in avocado one or both genera have been isolated from roots of either mature or nursery trees in South Africa, Israel, Spain, Chile and Australia since the early 1970s. More recently *Cylindrocladium* sp., *Cylindrocarpon* sp. and *Cylindrocladiella* sp. fungi have been isolated from diseased roots of young avocado trees which had infrequently declined or died soon after transplanting into orchards in Australia. Glasshouse studies with young seedlings were undertaken to establish pathogenicity of these fungi.

Fourteen weeks after transplanting into potting mix containing infested media, plants from *Cylindrocladium* amended pots were significantly smaller, and roots were the least healthy based on area of black lesions on roots and mass, than from all other treatments. Interestingly, plants from *Cylindrocladiella* amended pots were significantly taller and roots were healthiest, although not significantly, than those from all other treatments. Plants from *Cylindrocarpon* amended pots had intermediate height and root health. The three fungi were re-isolated from roots of plants originally inoculated with the respective genera, completing Koch's postulates.

Morphological examination and DNA sequencing of ITS, β-tubulin and histone 3 gene regions were used to confirm isolate identity as *Cylindrocladium parasiticum* (teleomorph *Calonectria ilicicola*), *Cylindrocarpon liriodendri* (teleomorph *Neonectria liriodendri*), and an undescribed *Cylindrocladiella* sp.

EL COMPLEJO CYLINDRO EN PATÓGENOS DE LA RAÍZ DE AGUACATE

Especies del género *Cylindrocladium* y *Cylindrocarpon*, son patógenos que se han encontrado en muchos cultivos agronómicos y forestales. En cultivos de aguacate, ambos géneros se han aislado tanto en árboles maduros como en plantas en invernaderos en países como Sur África, Israel, España, Chile y Australia desde los años 70s. Recientemente, en Australia, el hongo de estos dos géneros se ha aislado de las raíces de árboles jóvenes de aguacate muertos después de haber sido transplantados al campo. Estudios con plántulas de aguacate fueron realizados para establecer la patogénesis de este hongo.

Basados en la masa y área negra de la raíz y después de 14 semanas de haber sido transplantadas las plantas a medio infectado con *Cylindrocladium*, éstas fueron significativamente más pequeñas y las raíces poco saludables con respecto a los demás tratamientos. Curiosamente, las plantas con *Cylindrocladiella* fueron significativamente más altas y las raíces más saludables, pero no considerablemente en comparación con los demás tratamientos. Las plantas con *Cylindrocarpon* presentaron tamaño y raíces saludables de

rango intermedio. Los tres hongos fueron re-aislados de las raíces de plantas inoculadas con los géneros respectivos, cumpliendo las premisas de Koch.

Estudios morfológicos y de secuenciación de ADN de ITS de tres regiones en los genes de β-tubulina e histona, fueron usados para identificar las especies *Cylindrocladium parasiticum* (teleomorf *Calonectria ilicicola*), *Cylindrocarpon liriodendri* (teleomof *Neonectria liriodendri*) y una especia no descrita de *Cylindrocladiella*.

Calonectria ilicicola, *Gliocladiopsis* sp. and *Neonectria liriodendri* were isolated from diseased roots of young avocado trees. Pathogenicity studies with seedlings of three avocado cultivars, Velvick, Hass and Reed, demonstrated that *Calonectria ilicicola* is a severe root rot pathogen, reducing the portion and biomass of healthy roots, and reducing plant heights over time. *C. ilicicola* was re-isolated from diseased roots. *Neonectria liriodendri* and *Gliocladiopsis* sp. were not pathogenic and plant heights were increased after *Gliocladiopsis* sp. amendment compared to all other treatments in trials with 'Velvick' and 'Hass'.

Introduction

In recent years, several isolates of *Calonectria, Gliocladiopsis* sp. and *Neonectria* were obtained from the diseased roots of young avocado trees that had declined or died soon after transplanting into orchards in Australia. Since 1972, there have been three specimens of nectriaceous fungi including *Cylindrocladium* sp. and *Cylindrocladiella parva* (as *Cylindrocladium parvum*) associated with avocado in Queensland (unpublished). There are no records of pathogenicity testing of these isolates in avocado or any other host. The literature on the pathogenicity of these fungi in avocado is limited, with most reports concentrating on *Cylindrocarpon* sp.

Studies from several countries have reported isolation of *Cylindrocarpon* sp. from roots of avocado trees exhibiting symptoms of tree decline eg. chlorotic or brown leaves, leaf drop, tree death and from roots of nursery trees which either had symptoms of severe root rot or which often appeared healthy (Besoain and Piontelli 1999; Darvas 1978; Zilberstein *et al.* 2007). The causal fungus in these studies was *Neonectria radicicola* (anamorphic synonym *Cylindrocarpon destructans*) based on morphological and molecular characterisation.

The current study was conducted to confirm identity of the fungi that had been recently isolated from diseased roots of avocado nursery trees, and to establish pathogenicity of three of these fungi.

Materials and Methods

Initial isolation of fungi

A selection of nine young vegetatively cloned avocado trees and seedlings collected from a commercial nursery were examined. The plants were chosen due to their apparent ill-thrift compared to others, but none had severe foliar dieback. The root systems of most samples were severely necrotic with very few healthy white feeder roots (eg. Plate 1). Root tissue was selected and plated onto selective agar and resulting fungal cultures examined.

Calonectria sp. (*Cylindrocladium* sp.) alone was most frequently isolated. There were various other combinations of *Neonectria* (*Cylindrocarpon* sp.), *Calonectria* sp. and *Gliocladiopsis* sp either alone or as mixed infections. One plant was also infected with *Phytophthora cinnamomi*. Single germinated conidial isolates were established, which were subsequently used in pathogenicity tests.

Plate 1. Roots of young avocado plant removed from the pot without disturbing potting media. Note the dark brown/black necrotic root in the centre, and other roots with brown lesions.

Preparation of inoculum and amendment of planting mix at seedling transplantation

Inoculum was prepared by growing on a sand:bran:water mixture for about a week, then mixing with equal volumes of vermiculite. For Trials 1 and 2, with 'Velvick' and 'Hass' seedlings, this inoculum mix was placed in the lower 5cm of plastic pots (8cm diameter, 16cm high), and a single seedling planted using Searle's premium potting mix for fill. The unamended control pots received uncolonized media/vermiculite mix.

In Trial 3, with 'Reed' seedlings, pots for transplantation were prepared by one third filling with potting mix, then amending with 4 mycelial plugs per pot of the test fungus (or sterile agar media as the unamended control), seedlings transferred and pots filled with potting mix.

Pots were kept in controlled environment cabinets or glasshouse/polyhouse run at approx. 22-24°C day, 18°C night watered daily with de-chlorinated water and fertilized as required.

For each trial, plant heights after transplanting were measured, and at the conclusion of the trial root systems were examined visually for % of healthy roots. Fresh root pieces were plated onto selective agar medium, and examined for fungal growth. Root mass was determined after severing the roots below the seed, washing to remove potting mix, and weighing after drying at 60°C.

Molecular and morphological characterisation

Cultural characteristics were observed after 14 days growth on PDA in the dark at 24°C. Morphological characteristics were observed after 7-10 days growth on carnation leaf agar (CLA) in the dark at 24°C. The internal transcribed spacer (ITS), part of the β-tubulin-2 (BT), ITS4 and histone 3 (H3) genes or regions were amplified with suitable primers. Purified PCR products were sequenced and the sequences obtained were compared to the nucleotide sequence database (GenBank) using the nucleotide query BLASTn accessed via the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

Results

In each trial, amendment with *Calonectria* sp. significantly reduced plant heights and percentage of healthy roots, compared to plants growing in unamended mix, or in *Neonectria* sp. or *Gliocladiopsis* sp. amended media (Figure 1). Root mass was also reduced but not always significantly. Roots from plants receiving *Calonectria* sp. showed large areas of severe dark brown or black necrosis, and also smaller brown necrotic lesions (Plate 2).

Cultures of *Calonectria* sp. (Plate 3), *Neonectria* sp. and *Gliocladiopsis* sp. were consistently re-isolated from diseased roots from respective amendment treatments.

The molecular and morphological characterization confirmed the identity of re-isolated fungi as *Calonectria ilicicola, Neonectria liriodendri* and an undescribed *Gliocladiopsis* sp.

Figure 1. Proportion of healthy roots of 'Velvick', 'Hass' and 'Reed' seedlings grown in amended media for 14, 19 and 6.5 weeks, respectively, compared with roots from seedlings grown without amendment

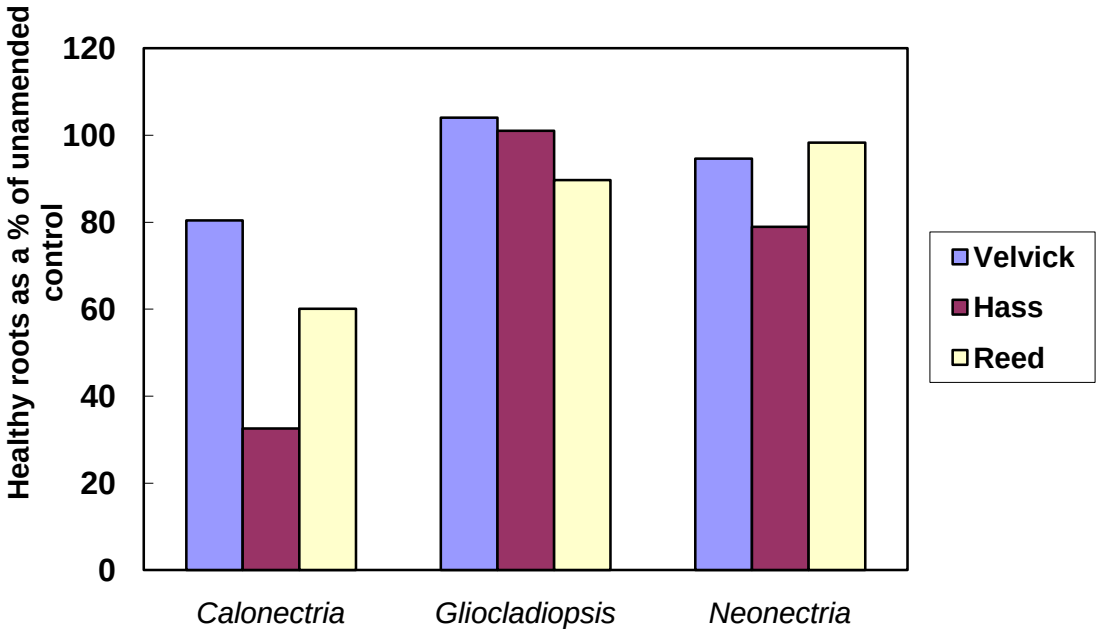

Plate 2. Roots from 'Velvick' plants receiving *Calonectria* sp. amendment to potting media 14 weeks earlier

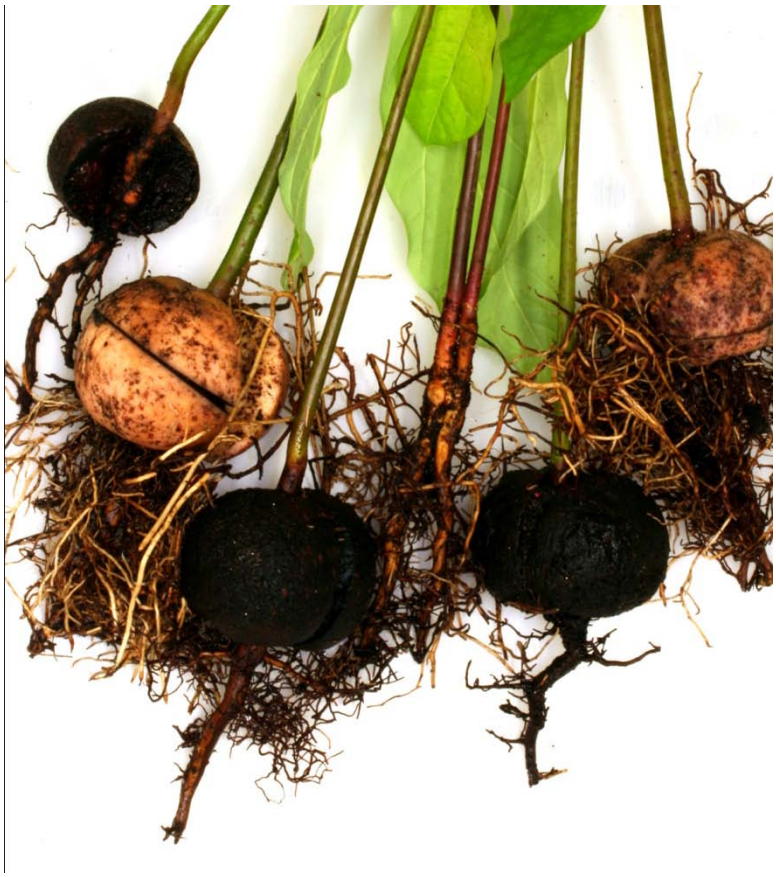

Plate 3. Colonies of *Calonectria ilicicola* growing from root pieces of avocado 5 days after transferring to selective media

Discussion

This study has demonstrated that *Calonectria ilicicola* is a severe root rot pathogen of young avocado trees. The fungus consistently reduced the portion of healthy roots compared to controls and the other test fungi, in separate trials with three cultivars. In two trials, amendment of potting media with this fungus impacted negatively on plant heights over time, and root biomass. *C. ilicicola* could be reliably re-isolated from diseased roots, fulfilling the requirements of a pathogen according to Koch's Postulates. This is the first report demonstrating pathogenicity of *C. ilicicola* on avocado.

Calonectria ilicicola has a wide international distribution and host range. It causes peg, pod and root necrosis of peanuts (Bell and Sobers 1966), and red crown rot in soybean (Berggren and Snow 1989). The pathogen also causes collar rot of papaya seedlings and young replants (Nishijima and Aragaki 1973), and the papaya isolate was also extremely pathogenic on three species of *Acacia*, peanut and several *Eucalyptus* spp.. Clean planting material is the most critical step in successful prevention of black root rot disease, caused by *Calonectria ilicicola*, in avocado. Although nursery trees may look healthy, root examination followed by rapid diagnosis, will quickly determine whether this pathogen, or another insidious root pathogen like *Phytophthora cinnamomi* is present.

Neonectria liriodendri and *Gliocladiopsis* sp. used in these trials were not pathogenic to avocado roots in the current study. *Neonectria liriodendri* has been established as the causal agent of black foot disease, a root rot, of grapevines in many parts of the world, including Australia (Whitelaw-Weckert *et al.* 2007). It has not previously been reported from avocado, and in this case it was most likely isolated as a ubiquitous rhizosphere inhabitant. *Cylindrocarpon destructans* represents a complex of fungi from which *N. liriodendri* was segregated (Halleen *et al.* 2006).. There are reports of isolates of *Cylindrocarpon destructans* as pathogenic on avocado (Besoain and Piontelli 1999; Darvas 1979; Zilberstein *et al.* 2007). Further surveys for nectriaceous pathogens associated with dieback of avocado are needed to resolve their pathogenicity and taxonomic status.

Acknowledgements

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