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A multi-phasic approach reveals that apple replant disease is caused by multiple biological agents, with some agents acting synergistically

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ABSTRACT

Apple replant disease (ARD) has been reported from all major fruit-growing regions of the world, and is often caused by a consortium of biological agents. The aim of this study was to investigate the etiology of ARD in South Africa in six orchard soils, using a multiphasic approach under glasshouse conditions. This approach first involved determining the ARD status of the soils by monitoring apple seedling growth responses in non-treated soil versus growth in pasteurized soil, as well as in 15% non-treated soil that was diluted into pasteurized soil. Subsequently, the potential for specific organisms to function as causal agents of ARD was investigated using (i) biocide applications, (ii) quantitative real-time PCR (qPCR) analyses of ARD 'marker' microbes (*Pythium irregulare*, *Pythium sylvaticum*, *Pythium ultimum*, *Pythium vexans*, *Rhizoctonia solani* AG-5 and the genera *Cylindrocarpon* and *Phytophthora*), (iii) nematode analyses, (iv) isolation of actinomycetes and (v) pathogenicity testing of actinomycetes individually, and when co-inoculated with *P. irregulare* or *Cylindrocarpon macrodidymum*. The analyses showed that the soils could be grouped into low, moderate and severe ARD soils, with each group containing two soils. Several lines of evidence suggested that actinomycetes are not involved in ARD in South Africa. Multiple biological agents were determined to contribute to ARD including (i) oomycetes (*Phytophthora* and *Pythium*) that are important based upon their widespread occurrence, and the fact that metalaxyl application improved seedling growth in four soils (ii) the genus *Cylindrocarpon* that was also widespread, and for which a synergistic interaction with *P. irregulare* was demonstrated and (iii) occasionally parasitic nematodes, mainly *Pratylenchus penetrans*, *Pratylenchus scribneri* and *Pratylenchus delattrei*, since fenamiphos application improved seedling growth in two orchards. qPCR analyses of the ARD marker microbes showed that *R. solani* AG-5 is absent from South African orchards, and that *P. ultimum* is widespread, even though the latter species could not be detected in previous isolation studies. The other marker microbes were also widespread, with the exception of *P. sylvaticum*. qPCR quantification of the marker microbes could not be correlated with the severity of ARD in any manner. qPCR analyses did, however, show that possible root pruning pathogens such as *P. irregulare*, *P. sylvaticum* and *P. ultimum* had much lower DNA concentrations in seedling roots than *P. vexans* and the genera *Cylindrocarpon* and *Phytophthora*.

1. Introduction

Young apple trees that are planted on sites that were previously cultivated with apple or closely related species often exhibit poor growth. It is generally assumed that this poor growth is most severe on sites that were planted to apple for extended periods of time (Mai and Abawi, 1981). However, symptomatic trees have also been noticed after apples had been grown in soil for only one year (Savory, 1966). Microbial communities consistent with replant disease have also been documented to develop within three years of orchard establishment (Mazzola, 1999). The phenomenon of poor growth on replanted apple soils is characterized by its persistence in soil, and its lack of spread through replant sites. Therefore, the effect is most evident when trees are replanted into the old tree rows (Hoestra, 1968; Jensen and Buszard, 1988; Mazzola, 1998b; Rumberger et al., 2004; Leinfelder, 2005).

Symptoms associated with poor tree growth are neither distinctive nor always dramatic (Jackson, 1979; Sewell, 1981). The

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most obvious aboveground symptom is the uneven growth of young trees within an orchard. However, when severe disease pressure is encountered, poor growth may be exhibited by the majority of trees in the orchard and death of young trees may occur (Traquiar, 1984). Below-ground symptoms include small root systems that have a significant reduction in lateral root development and functional root hairs (Savory, 1966; Hoestra, 1968; Caruso et al., 1989). Trees that are affected often also begin cropping fruit 2 to 3 years later than unaffected trees and fail to attain comparable yields (Mazzola, 1998a).

Apple replant disease (ARD) or soil sickness, refers to situations where poor growth of trees is caused by a biological component (Savory, 1966; Mai and Abawi, 1981; Traquiar, 1984; Gilles and Bal, 1988). In general, demonstration of an ARD-inducing soil requires that (i) soil pasteurization or fumigation improves tree growth relative to non-fumigated soil (Hoestra, 1968; Covey et al., 1979; Mai and Abawi, 1981; Jaffee et al., 1982a; Slykhuis and Li, 1985) and/or (ii) dilution of the soil into pasteurized soil with as low as 10% of the original field soil can still cause stunting of trees (Hoestra, 1968; Jaffee et al., 1982a).

Numerous biological agents that vary across orchards have been implicated in ARD, but among these only a few pathogenic nematodes, fungi and oomycetes species have been reported world-wide. *Pratylenchus penetrans* Cobb is considered to be the primary nematode species involved in ARD (Mai et al., 1957; Jaffee et al., 1982b; Merwin and Stiles, 1989; Utkhede et al., 1992a; Dullahide et al., 1994; Van Schoor et al., 2009). Within the fungal genus *Cylindrocarpon*, which in general is considered to have low virulence, *Cylindrocarpon destructans* (Zinnsm.) Scholten and *Cylindrocarpon lucidum* Booth have been reported as the dominant and pathogenic species associated with ARD in a few regions of the world (Jaffee et al., 1982a; Braun, 1991, 1995; Dullahide et al., 1994; Mazzola, 1998a; Manici et al., 2003). In South Africa, four pathogenic *Cylindrocarpon* species have been reported including *Cylindrocarpon macrodidymum* Schroers, Halleen & Crous, *C. destructans*, *C. liriodendri* Halleen, Schroers, Groenewald, Rego, Oliveira & Crous and *C. pauciseptatum* Schroers, Zerjav, Munda, Halleen & Crous, with *C. macrodidymum* being the most wide-spread (Tewoldemedhin et al., 2011a). Several *Rhizoctonia* species have been associated with ARD, but only the multinucleate *R. solani* Kühn AG-5 and AG-6 were shown to be highly virulent, whereas a few of the binucleate anastomosis groups have exhibited low virulence toward apple (Mazzola, 1997; Manici et al., 2003). Although *Fusarium* (mainly *Fusarium oxysporum* Schlectend) is frequently associated with ARD, its role as a pathogen of apple is controversial. Most studies were unable to demonstrate that *Fusarium* isolates are pathogenic (Merwin and Stiles, 1989; Dullahide et al., 1994; Mazzola, 1998a; Manici et al., 2003; Tewoldemedhin et al., 2011b). However, *F. tricinctum* (Corda) Sacc. and some isolates of *F. solani* (Mart.) Sacc. and *F. avenaceum* (Fr.) Sacc. have been shown to be pathogenic, with the latter two species having low virulence (Dullahide et al., 1994; Manici et al., 2003; Tewoldemedhin et al., 2011b). In most countries several pathogenic *Phytophthora* species have been identified, with *P. cactorum* (Leb. and Cohn) Schröeter being the dominant species (Sewell, 1981; Matheron et al., 1988; Utkhede et al., 1992a; Mazzola, 1998a; Tewoldemedhin et al., 2011b). In contrast, not all *Pythium* species are pathogenic with some even promoting the growth of apple seedlings (Mazzola et al., 2002). The most virulent species that have been identified and have also been frequently associated with ARD include *P. intermedium* de Bary, *P. irregulare* Buisman, *P. sylvaticum* Campbell & Hendrix, *P. ultimum* Trow and *P. vexans* de Bary (Sewell, 1981; Jaffee et al., 1982a; Dullahide et al., 1994; Mazzola, 1998a; Mazzola et al., 2002; Tewoldemedhin et al., 2011b).

Only a few studies have focused on the role of prokaryotes in ARD, and in general their role is still controversial (Savory, 1966; Hoestra, 1968; Mazzola, 1998a; Dullahide et al., 1994). Although several bacterial genera and species have been associated and suggested as being involved in ARD, only isolates of *Bacillus subtilis* have been shown to limit plant growth (Catska et al., 1982; Utkhede et al., 1992b). However, the inoculum concentrations used in the latter study were inordinately high (8.8×10^9 to 1.2×10^{11} colony forming units/500 cm^3 soil), and most bacteria are likely to limit plant growth and development at these densities (Klement et al., 1990; Schaad et al., 2001).

Evidence for the involvement of actinomycetes in ARD is circumstantial (Savory, 1967; Hoestra, 1968; Westcott et al., 1986). Westcott et al. (1986, 1987) found through microscopic analyses that the extent of colonization of apple root epidermal tissue by actinomycete-like organisms was positively correlated with ARD severity, whereas roots in steamed soil were not infected by actinomycetes. More recently, Zhao et al. (2009) showed that certain actinomycetes, specifically *Streptomyces* species, which were isolated from brassicaceous seed meal amended apple soils, could alleviate *R. solani* AG-5 root infections but that some of the isolates by themselves caused leaf necrosis and a reduction in root biomass in young apple seedlings. The latter response was eliminated when older seedlings were employed in these assays. On the other hand, some of the *Streptomyces* isolates were able to suppress *R. solani* AG-5 infections and thus stimulate seedling growth (Cohen and Mazzola, 2006; Zhao et al., 2009).

A diverse range of approaches have been taken to elucidate the complex etiology of ARD. Most studies have used isolation studies along with pathogenicity testing (Jaffee et al., 1982a; Braun, 1991, 1995; Dullahide et al., 1994; Mazzola, 1998a; Manici et al., 2003). Additionally, the application of biocides has been used to suppress certain components of the pathogen complex in order to deduce the importance of specific groups (Mai and Abawi, 1978; Slykhuis and Li, 1985; Dullahide et al., 1994; Mazzola, 1998a). More recently, polymerase chain reaction (PCR) based techniques including DNA fingerprinting with denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphisms (T-RFLP), as well as sequencing of root infected clone libraries have also been used to characterize microbial populations in apple orchard soils (Rumberger et al., 2004, 2007; Yao et al., 2006; St. Laurent et al., 2008). Although these molecular techniques yield a vast amount of data on microbial community structure, the studies were unable to identify specific ARD causal agents nor specific genera or species to serve as predictors of potential growth reductions.

Real-time PCR or quantitative PCR (qPCR) is a powerful tool for detection and quantification of microbial genera or species of interest from soil and plant material (Schena et al., 2004; Lievens et al., 2005; Paulitz and Schroeder, 2005; Kernaghan et al., 2007; Ophel-Keller et al., 2008). Although several studies have conducted qPCR analyses on some of the known agents associated with ARD, they were on crops other than apple (Paulitz and Schroeder, 2005; Schroeder et al., 2006; Kernaghan et al., 2007, 2008; Hoagland et al., 2008; Schena et al., 2008). With regard to replant diseases in general, Bent et al. (2009) have recently shown that the amount of *P. ultimum* and *Sellaphora* DNA in roots from peach seedlings grown in replant soil was negatively associated with plant biomass, whereas *P. vexans* and *Aplanochytrium* did not show a significant association with plant biomass.

In South Africa, some information has been obtained on the biological agents involved in ARD. Van Schoor et al. (2009) showed that ARD in South Africa is primarily caused by a biological phenomenon. They reported that the genera *Pythium, Cylindrocarpon* and *Fusarium* were consistently isolated from replant soils, whereas *Rhizoctonia* and *Pratylencus* were inconsistently associated with ARD soils. However, no species level identifications or pathogenicty studies were conducted (Van Schoor et al., 2009).