The Efficacy of Semiselective Chemicals and Chloropicrin/1, 3-Dichloropropene–Containing Fumigants in Managing Apple Replant Disease in South Africa

M. Nyoni,¹ M. Mazzola,^{1,2} J. P. B. Wessels,³ and A. McLeod^{1†}

¹Department of Plant Pathology, Stellenbosch University, Matieland 7600, South Africa; ²Tree Fruit Research Laboratory, U.S. Department of Agriculture Agricultural Research Service, Wenatchee, WA 98801, U.S.A.; and ³ProCrop, Somerset West 7130, South Africa

Abstract

Apple replant disease (ARD) is a biological phenomenon that is encountered when old apple orchards are replanted, resulting in tree growth and yield reductions in young trees. Three ARD orchard trials were conducted, which showed that semiselective chemicals (fenamiphos, metalaxyl, imidacloprid, and phosphonates) used independently, two fumigant formulations (33.3% chloropicrin and 60.8% 1,3-dichloropropene [Pic33-1,3D] and 57% chloropicrin and 38% 1,3 dichloropropene [Pic57-1,3D]), and semiselective chemicals combined with Pic33-1,3D or Pic57-1,3D all contributed to significant increases in tree growth (trunk diameter and shoot length) relative to the untreated control 3 to 4 years postplanting. The treatments did not differ significantly from each other in improving tree growth. Yield was more indicative of treatment efficacy, but this varied between the three orchards. The Pic33-1,3D fumigant in combination with semiselective chemistries was the most consistent in significantly increasing cumulative yields. The Pic57-1,3D treatment was superior in increasing yields relative to the Pic33-1,3D treatment, because (i) it significantly increased cumulative yields in comparison with the Pic33-1,3D treatment in one orchard and (ii) in another orchard, a significant increase in yield was

obtained with Pic57-1,3D relative to the control treatment but not with the Pic33-1,3D treatment. The quantification of ARD causative agents 20 months postplant showed that *Phytophthora cactorum* contributed to disease development in all three orchards; significant negative correlations existed between the quantity of *P. cactorum* DNA detected in tree roots and tree growth and less often, yield. In two orchards, only some of the treatments that significantly reduced the quantity of *P. cactorum* DNA in tree roots relative to the control also resulted in a significant increase in tree growth. Some of the aforementioned trends were also evident for *Pratylenchus* spp. root densities in two of the orchards. There was a significant positive correlation between *P. cactorum* root DNA quantities and *Pratylenchus* spp. root densities. *Pythium* spp. and “*Cylindrocarpon*”-like DNA quantities detected in tree roots typically were not indicative of treatment efficacy. However, a significant positive correlation existed between these two pathogen groups, suggesting complex interactions not associated with pathogen quantities per se.

Keywords: causal agent, oomycetes, potassium phosphonate

Apple replant disease (ARD) is a root disease of apples (*Malus × domestica* Borkh.) that occurs when apple is replanted on soil previously planted to apple or related species. It is a soilborne disease that causes tree stunting and delayed apple fruit bearing (Mazzola and Manici 2012). Up to a 50% loss in orchard lifespan productivity and profitability can be incited by ARD (Rabie et al. 2001). The biological agents involved in ARD consist of a wide spectrum of organisms, including fungi, nematodes, and oomycetes. The occurrence or relative abundance of individuals that make up the causal pathogen complex can vary across ARD orchard sites (Manici et al. 2003; Mazzola 1998; Tewoldemedhin et al. 2011c).

The ARD causative agents are mostly similar worldwide, whereas some are absent or differ in species composition in South Africa. *Pratylenchus* spp. are known as causal agents of ARD worldwide (Dullahide et al. 1994; Jaffe et al. 1982b; Mazzola 1998; Stirling et al. 1995; Tewoldemedhin et al. 2011b). Although the multinucleates *Rhizoctonia solani* J. G. Kühn AG-5 and/or *Rhizoctonia solani* J. G. Kühn

AG-6 are highly virulent pathogens in the United States and Italy (Manici et al. 2003; Mazzola 1997), these agents have not been identified in South African ARD orchards. South African ARD orchard soils do contain binucleate *Rhizoctonia* anastomosis groups which have also been identified in other countries. The binucleate isolates can have a range of pathogenic to nonpathogenic interactions with apple (Manici et al. 2003; Mazzola 1997; Tewoldemedhin et al. 2011b). Manici et al. (2013) hypothesized that binucleate *Rhizoctonia* spp. and *Fusarium* spp. have a commensal or mutual symbiotic relationship with apple trees rather than a pathogenic relationship. “*Cylindrocarpon*”-like fungi can have a range of neutral or negative interactions with apple, although most isolates were found to be nonpathogenic to mildly virulent toward apple in different regions of the world (Braun 1991, 1995; Dullahide et al. 1994; Jaffe et al. 1982a; Manici et al. 2003, 2018; Mazzola 1998; Tewoldemedhin et al. 2011a). The taxonomy of *Cylindrocarpon*-like fungi has undergone major revisions, including changes in genus and species names (Cabral et al. 2012; Chaverri et al. 2011; Lombard et al. 2014). For the purpose of this study, reference will only be made to *Cylindrocarpon*-like fungi. In South Africa, selected oomycetes in the genera *Phytophthora* and *Pythium*, which occur worldwide, play an important role in ARD based on their widespread occurrence and high virulence toward apple (Tewoldemedhin et al. 2011b, 2011c).

Because of the complex composition of ARD causative agents, preplant broad-spectrum soil fumigants are the primary measure used to manage the disease. Chloropicrin combined with 1,3-dichloropropene is currently the most commonly used fumigant for the control of ARD (Cabrera et al. 2015; Mazzola and Manici 2012; Mazzola et al. 2015; Yao et al. 2006). The ratio of these two chemistries differs among fumigant products depending on whether the target organisms are fungi or nematodes. Chloropicrin mainly has a fungicidal activity, whereas 1,3-dichloropropene targets nematodes (Cabrera et al.

[†]Corresponding author: A. McLeod; adelem@sun.ac.za

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

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2015; Locascio et al. 1997). Preplant orchard soil fumigation is primarily limited to application in the planting row rather than the entire orchard block, which limits the overall treatment cost. Therefore, the fumigation effect is relatively short lived, likely as a result of rapid recolonization of fumigated soils from the untreated interrows (Mazzola et al. 2015). Nonetheless, fumigation is effective in managing ARD, because it allows for sufficient plant protection during the initial 1 to 2 years after orchard establishment when young apple trees exhibit significantly elevated growth suppression resulting from root infection by ARD causative agents in comparison with older trees (Mazzola and Manici 2012; Mazzola et al. 2015).

The use of systemic chemicals, including phosphonates, phenylamides, and fenamiphos, may have potential as tools for managing ARD in South Africa, because these pesticides target two of the major ARD causative agent groups: oomycetes and nematodes. Phosphonates and the phenylamides metalaxyl and mefenoxam (containing only the active R-enantiomer of metalaxyl) were reported to control *Phytophthora cactorum* (Lebert & Cohn) J. Schröon on apple under orchard conditions (Utkhede 1987; Utkhede and Smith 1995). Several pathogenic *Pythium* spp. associated with ARD were shown to be sensitive to metalaxyl in vitro (Mazzola et al. 2002). Autio et al. (1991) showed that the application of metalaxyl to an ARD site resulted in a significant increase in tree trunk and shoot growth but only in the first year of growth. The nematicidal activity of fenamiphos is well known, and the compound is registered for managing *Pratylenchus* spp. on nonbearing apple trees in South Africa. However, in several countries, excluding South Africa, it is no longer available because of environmental concerns (Wesseling et al. 2005).

The main aims of this study were to evaluate in three orchard trials (i) whether two fumigants varying in their ratio of chloropicrin/1,3-dichloropropene differed in ARD control efficacy and (ii) if semiselective chemicals (fenamiphos, metalaxyl, phosphonate, and imidacloprid) applied independently or in concert with soil fumigation could be used to manage ARD in South Africa. Because methyl bromide was only phased out in January 2015 in South Africa (Kapp et al. 2016), this treatment was also included in two of the trials to provide apple growers with information regarding the performance of new fumigants in comparison with the conventional fumigation treatment. To better understand the basis for treatment performance, the prevalence of a few ARD marker microbial pathogens (*Cylindrocarpus*-like fungi, *R. solani* AG-5, the genus *Phytophthora*, *Pythium ultimum* Trow, *Pythium irregulare* Buisman, *Pythium sylvaticum* W. A. Campb. & F. F. Hendrik, and *Phytophthora vexans* [de Bary] Abad, de Cock, Bala, Robideau, Lodhi & Levesque) and parasitic nematodes (*Pratylenchus* spp.) were monitored in orchard tree roots. A glasshouse apple seedling bioassay trial was conducted to gain additional insight into the identity of oomycete ARD causative agents involved and the relative potential for ARD development in the orchard soils.

Materials and Methods

Orchard trials. *Orchard sites.* ARD trials were conducted at three orchard sites (Grabouw [GRA], Witzenberg valley [WZB], and the Koue Bokkeveld [KBC]) situated in the Western Cape Province of

South Africa (Table 1). The region is characterized by a Mediterranean climate with cool, wet winters and warm, dry summers. The orchards were located in three different production regions. The orchard history of each of the orchards before planting is presented in Table 1. Orchards GRA and WZB were planted in 2013, and orchard KBC was planted in 2014. The orchards contained the M.793 or M.7 rootstocks grafted with Royal Beaut or the MM.109 rootstock and Early red one scion. All orchards had microsprinkler irrigation systems, whereas the row and tree spacing varied in the orchards (Table 1). Fertilization was conducted using standard grower practices. The soil type, pH, resistance and cation exchange capacity, and water holding capacity of each orchard soil are shown in Table 2. Orchards WZB and KBC had sandy loam soils, whereas Orchard GRA had a clay loam soil type (Table 2).

Trial treatments and design. The following treatments were applied in orchards GRA and WZB:

- (i) untreated control,
- (ii) preplant fumigation with 33.3% chloropicrin and 60.8% 1,3-dichloropropene [Pic33-1,3D],
- (iii) preplant fumigation with 57.0% chloropicrin and 38.0% 1,3-dichloropropene [Pic57-1,3D],
- (iv) semiselective chemicals (fenamiphos, imidacloprid, metalaxyl, and potassium phosphonate; independent semiselectives),
- (v) preplant fumigation with Pic33-1,3D plus semiselective chemicals (Pic33-1,3D/semiselectives), and
- (vi) methyl bromide.

In orchard KBC, the same treatments were applied as in orchards GRA and WZB except that the methyl bromide treatment was replaced with a treatment consisting of preplant fumigation with Pic57-1,3D combined with semiselective chemicals (Pic57-1,3D/semiselectives).

The fumigation treatments (Pic57-1,3D, Pic33-1,3D, and methyl bromide) were applied to the orchard rows before planting (12- to 20-m-long × 80- to 100-cm-wide plots) through shank injection (40-cm dept) followed by immediate tarping with impermeable black plastic for a period of at least 2 weeks. Fumigants were applied by a registered pesticide applicator (BioScience Research CC). Methyl bromide (980 g/kg methyl bromide and 20 g chloropicrin per 1 kg; Mebrom Chemicals) was applied at 50 g/m². The two different Pic-1,3-D formulations were applied at a dosage of 52 g/m². The two Pic33-1,3D formulations consisted of the products Agrocclone NE (Pic33-1,3D; Agroquimicos de levanter) and Agrocclone FE (Pic57-1,3D; Agroquimicos de levanter).

The semiselectives mixture consisted of the application of four pesticides, including metalaxyl, fenamiphos, imidacloprid, and phosphonates. Metalaxyl, fenamiphos, and imidacloprid were applied as a soil drench at planting followed by four to five sets of phosphonate applications over a 3-year period. The soil drench was applied shortly after planting when trees started budding and showed clear signs of growth initiation. The soil drench was applied as a 2-liter drench per tree, which contained 1.05 g imidacloprid (Confidor 70 WG; Bayer; 700 a.i. g/kg), 1 g fenamiphos (Nemacur 400 EC; Villa Crop Protection; 400 g a.i./liter), 2 g metalaxyl, and 12 g mancozeb (Metazeb 700 WP; Villa Crop Protection; 100 g/kg metalaxyl and

Table 1. Agronomic information of apple orchards that were used in apple replant disease management trials

Orchard name	Production region	History of old orchard that was removed (age, cultivar and rootstock, and year of orchard removal)	Fumigation date	Planting date	Rootstock	Variety	Row × tree spacing, m	Irrigation type
GRA	Grabouw	36 years, cultivars Granny Smith and Golden delicious on seedling rootstocks, 2010	16 September 2013	3 October 2013	M.7	Royal Beaut	4.5 × 2	Microsprinkler irrigation
WZB	Witzenberg valley	53 years, cultivars Golden delicious and Starking on seedling rootstock, 2013	10 September 2013	12 October 2013	MM.109	Early red one	3.75 × 1.25	Microsprinkler irrigation
KBC	Koue Bokkeveld	18 years, cultivar Braestars on M793, 2013	13 September 2014	6 October 2014	M.793	Royal Beaut	4 × 1.5	Microsprinkler irrigation

600 g/kg mancozeb). Metazeb is a cost-effective metalaxyl formulation currently used by growers. The alternative new formulations to Metazeb that do not contain mancozeb are more expensive, because they only contain mefenoxam, which is the active enantiomer of metalaxyl.

At the GRA and WZB orchards, the first set of potassium phosphonate (Phosguard 400 SL; 400 g a.i./L) applications was made in December 2013 (3 months after planting), which consisted of three foliar sprays (50 ml of 2 g a.i./liter per tree) applied at 1-week intervals. The second set of phosphonate applications was applied as a trunk paint (50 ml of 200 g a.i./liter solution per tree) in the second year of growth in October 2014 just after bud break (~13 months after planting). Trunk paint application was used, because leaves are required for foliar applications. The same dosage and frequency of applications were used for all subsequent phosphonate foliar and trunk paint treatments. The third set of phosphonate applications consisted of three foliar sprays conducted in December 2014. The fourth and fifth phosphonate application sets were made in the third year of growth and consisted of a trunk paint application in October 2015 followed by three foliar sprays conducted in December 2015. Foliar applications were made using a motorized mist blower backpack sprayer (SR 400; STIHL), and trunk paints were applied with a paintbrush from the soil surface upward onto stems (~30 cm). For orchard KBC, the phosphonate applications were made using the same methods and timing of application as for orchards GRA and WZB, except that all applications were made 1 year later, because the trial was planted 1 year after the GRA and WZB trials.

In all three orchards, treatments were replicated six times in a completely randomized design. Each replicate consisted of 10 trees.

Trial evaluations. *Tree growth.* All tree growth measurements were made on the center 8 trees of each 10-tree replicate. Tree growth was evaluated by determining shoot length and the increase in trunk diameter on an annual basis for 3 (orchard KBC) or 4 years (orchards GRA and WZB). In the first year of growth, the increase in total shoot length was determined. Subsequently, shoot growth was determined by measuring one of the 1-year-old shoots per tree from each of the center eight trees within a replicate. Growers pruned the leaders in the first overwintering year, and therefore, the increase in leader length could not be determined beyond the initial year.

Yield. The orchard sites differed in their first year of fruit bearing, with orchard GRA having an initial fruit yield in the fourth growing season, whereas orchards WZB and KBC had an initial fruit harvest in the third growing season. For each orchard, 2 years of yield data were obtained. The yield was determined by harvesting and weighing fruits from the center eight trees in each replicate, and values are expressed as kilograms of fruit per tree.

Relative percentage increases in trunk diameter and yield. To obtain an indication of the severity of ARD and the yield gain obtained in each orchard by managing ARD, the relative percentage increases in trunk diameter and yield were calculated, respectively. Only one management treatment, which performed the best (see below) across the three orchards, was selected for this purpose. The relative percentage increase in trunk diameter at each orchard was defined by the increase in trunk diameter of the Pic33-1,3D fumigant/semiselectives treatment (the best-performing treatment across the three orchards) compared with that attained by trees cultivated in nontreated soil after 3 years of growth. This was calculated as follows: (trunk diameter increment of fumigant-containing treatment – trunk diameter increment control) ÷ trunk diameter increment control × 100. The relative percentage increase in yield was calculated in a similar manner for each orchard.

Root sample collection. Root sampling was conducted 20 months after planting at a distance of 20 to 40 cm from the trunk on opposite sides of the tree row to a depth of 30 cm. Roots were sampled from 3 trees in the center of the 10-tree plots for each replicate. Roots from the three trees were pooled into one sample, and each root sample was divided into two groups for (i) quantitative polymerase chain reaction (qPCR) quantification of ARD microbial pathogens and (ii) nematode analysis.

qPCR quantification of ARD microbial pathogens from roots. qPCR assays were used to assess the DNA quantity in tree roots of the genus *Phytophthora*, *Cylindrocarpon*-like fungi, *P. sylvaticum*, *P. vexans*, *P. irregulare*, *P. ultimum*, and *R. solani* AG-5. For all qPCR assays, standard curves were established. A gBlock fragment was used for constructing the *R. solani* AG-5 standard curve. The gBlock fragment, synthesized by Integrated DNA Technologies Inc., consisted of a 131-bp fragment that matched the sequence base pairs (44 to 175 bp) of *R. solani* AG-5 Genbank accession number JQ946296. The synthesized gBlock fragment was dissolved in TE buffer (Integrated DNA Technologies Inc.) to a final concentration of 1.568×10^{12} copies per 1 μ l, which was fivefold serially diluted to a concentration of 2.56 copies per 1 μ l, yielding 14 points for the standard curve. For the remaining pathogens, standard curves were constructed using genomic DNA. Isolates of the different species were obtained from the Stellenbosch University culture collection. One isolate each of *P. cactorum* (STEU 7204), *P. vexans* (STEU 6741), *P. irregulare* (STEU 7193), *P. ultimum* (STEU 2187), *P. sylvaticum* (STEU 7199), and *Cylindrocarpon macrodoidum* was grown in pea broth (Goodwin et al. 1994). The mycelia harvested from the broth cultures were lyophilized, and ~20 mg mycelia was powdered in 2-ml tubes each containing 0.5-g glass beads (2 mm) by shaking for 10 min in a Retsch MM301 mixer mill. DNA was extracted from the powdered mycelia using the Nucleo-Spin PLANT II kit (Macherey-Nagel GmbH and Co) according to the manufacturer's instructions. Extraction buffer PL1 was used, and in the last step, DNA was eluted by applying two 50- μ l aliquots of elution buffer. The genomic DNA was quantified using a Nanodrop spectrophotometer (ND1000; Nanodrop Technologies). A standard curve for each pathogen was constructed from fivefold serial dilutions of genomic DNA, which yielded 8 to 12 DNA concentration points for the various standard curves depending on the pathogen: *P. cactorum*, 11.37 ng to 5.83 fg (10 points); *P. vexans*, 24.76 ng to 12.6 fg (10 points); *P. sylvaticum*, 35.8 ng to 18.8 fg (10 points); *P. irregulare*, 26.6 ng to 0.54 fg (12 points); *P. ultimum*, 3.7 ng to 47 fg (8 points); and *C. macrodoidum*, 13.56 ng to 6.90 fg (10 points). The standard curve qPCR reactions for the genus *Phytophthora*, *Cylindrocarpon*-like fungi, *P. sylvaticum*, *P. irregulare*, and *R. solani* AG-5 were Syber Green-based reactions. The Kapa Syber fast qPCR master mix (Sigma-Aldrich, SA) was used to a final concentration of 1 $\times$ in a final reaction volume of 20 μ l. For each of the various qPCR reactions, the primers, primer concentrations, and additional magnesium chloride added to reactions are shown in Table 3. Syber Green amplifications consisted of an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturing at 95°C for 10 s, the relevant annealing temperature and times (Table 3), and extension at 72°C for 20 s. The exception was the *P. cactorum* assay, which used a 27-s extension time. Amplifications were conducted in a Rotor-Gene 6000 machine (Qiagen GmbH). Standard curve reaction concentrations were all assayed in triplicate. All assays included a nontemplate water control.

Table 2. Soil properties of orchards where apple replant disease management options were investigated

Orchard ^x	pH	Classification	Resistance (Ohm) ^y	CEC (Cmol [+]/kg) ^y	Clay (%)	Silt (%)	Sand (%)	Water-holding capacity (mm/m) ^z
Orchard GRA	5.2	Clay loam	200	10.09	39	38	23	59.55
Orchard WZB	5.4	Sandy loam	1,400	17.46	13	20	67	109.20
Orchard KBC	4.8	Sandy loam	790	8.68	19	14	67	94.92

^x GRA, Grabouw; KBC, Koue Bokkeveld; WBZ, Witzenberg valley.

^y Cation-exchange capacity (CECs) (Cmol [+]/kg) are the total exchangeable cations, a measure of the soil's ability to retain and supply nutrients, specifically the positively charged nutrient ions called cations. These include the cations calcium, magnesium, potassium, and ammonium and many of the micronutrients.

^z Water-holding capacity (millimeters per meter) is the depth of water held between field capacity and permanent wilting point per meter depth of soil.

The *P. vexans* and *P. ultimum* qPCR assays were probe-based assays. The primers and probes, which were labeled with 6-carboxy-fluorescein (6-FAM) and an Iowa Black dark quencher, are shown in Table 3, and were synthesized by Integrated DNA Technologies Inc. The qPCR reactions consisted of the Kapa probe mastermix (Sigma-Aldrich) at a final concentration of 1× in a total reaction volume of 20 µl. The primer and probe concentrations used for each of the qPCR reactions are shown in Table 3. The qPCR amplification consisted of denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension temperature and times as indicated in Table 3. Amplifications were conducted in a Rotorgene, with each standard curve concentration assayed in triplicate.

All pathogen standard curves generated were linear ($R^2 = 0.98$ to 0.99). The reaction efficiencies, M-slope values, and limits of detection for all assays are shown in Table 3.

DNA was extracted from the fine feeder roots (~70 mg) of trees. The root samples were placed into 2-ml tubes for lyophilization. The lyophilized roots were fragmented using a sterile plastic pestle, and a subsample of 20 mg was used for DNA extraction. DNA was extracted by first powdering the roots in 2-ml tubes using 0.5-g glass beads (2 mm) and shaking for 10 min in a Retsch MM301 mixer mill. DNA was extracted using the NucleoSpin PLANT II kit (Macherey-Nagel GmbH and Co) according to the manufacturer's instructions as described above for the extraction of DNA from microbial mycelial cultures.

Quantification of ARD pathogens in root DNA extracts was conducted by qPCR using the same reaction and amplification conditions that were used for constructing the standard curves. The root DNA extract was diluted fivefold to eliminate qPCR inhibition that was detected when undiluted DNA was utilized. Each qPCR reaction contained 2 µl of the diluted DNA in 20-µl reactions, and each sample was assayed in duplicate reactions. Two standard curve control (calibrators) samples were included in all runs, which allowed for the importation of standard curves for pathogen quantification. Pathogen quantities were expressed as absolute pathogen DNA quantities (picograms per milligram_{DW}) using the formula

Absolute pathogen DNA quantity

$$= \left(\frac{\text{qPCR pathogen DNA concentration} \times \text{total volume of extracted genomic DNA} \times \text{dilution factor}}{\text{milligrams of roots used in DNA extraction}} \right)$$

as described by Moein et al. (2019b). Because the *Phytophthora* qPCR assay is a genus-based assay, the identity of the *Phytophthora* spp. amplified in qPCR reactions was determined through sequence

analysis of the qPCR products (470 bp). This was conducted on three randomly selected root DNA samples from the untreated control samples in each of the trials. The identities of the sequences were determined through BLAST analyses in the Phytophthora-ID database (version 2.0; <http://phytophthora-id.org/index.html>) (Grünwald et al. 2011).

Nematode extraction and quantification. Approximately 5 g of washed roots of each replicate treatment, sampled as described above, were sent for parasitic nematode analyses at Nemlab (Durbanville, South Africa). Nematodes were extracted using the centrifugal sugar flotation method (Jenkins 1964).

Apple seedling glasshouse bioassay. *Bioassay establishment and treatments.* In each orchard, soil samples were collected before treatment applications. Soil samples were collected to a depth of 30 cm at ~6 to 10 different sites across the orchard. The soils for each orchard were pooled into one sample. A subset sample of each orchard soil was steam pasteurized for 2 h at 80°C on 2 consecutive days using a Systec Pasteurizer (VE150). The pasteurized soil was ventilated for at least 2 days before use.

Four-week-old cultivar Golden delicious apple seedlings were produced from germinated seed as previously described (Tewoldemedhin et al. 2011a). The 4-week-old seedlings were planted into untreated orchard soil, pasteurized soil, and 15% untreated soil in pasteurized soil. Each treatment was replicated six times in a completely randomized block design. Each replicate consisted of a 1-liter planting bag containing three apple seedlings. The length and weight of seedlings were recorded just after and before planting.

Seedlings were grown for 3 months under glasshouse conditions of $26 \pm 2^\circ\text{C}$ and a humidity range of 60 to 70%. Irrigation was applied according to seedling size; during the first month, 2-min cycles were applied twice a day using a drip irrigation system, resulting in 200 ml per bag a day. From the second month onward, two 5-min irrigation cycles were used daily, which were equivalent to 500 ml of water applied per bag; 100 ml of water-soluble classic Multifeed (Plaaskem, Pty Ltd) fertilizer was applied every 10 days to each planting bag. The Multifeed fertilizer contained nitrogen (90 g/kg), phosphorous (82 g/kg), potassium (158 g/kg), manganese (900 mg/kg), zinc (350 mg/kg), boron (1,000 mg/kg), molybdenum (70 mg/kg), iron (750 mg/kg), manganese (300 mg/kg), and copper (75 mg/kg). Foliar pathogens and pests were managed on a 2-week basis by applying Agromectin EC (abamectin: 18 g a.i./liter; Arysta LifeScience Pty Ltd), Arcastin Flo (cyhexatin: 600 g a.i./liter; Sipcam Southern Africa Pty Ltd), Topaz 200 EW (penconazole: 200 g a.i./liter; Syngenta Pty Ltd), Nimrod EC (bupirimate: 250 g a.i./liter; Makhteshim-Agan SA Pty, Limited), and Mospilan 20 SL (acetamiprid: 222 g a.i./liter; Plaaskem Pty Ltd) using a laboratory spray bottle.

Table 3. Real-time quantitative polymerase chain reaction (PCR) primers, probes, and amplification conditions used for quantifying apple replant disease microbial pathogens (*Pythium irregulare*, *Phytophthora vexans*, *Pythium ultimum*, the genus *Phytophthora*, *Pythium sylvaticum*, and *Cylindrocarpon*-like fungi) from apple roots

Target species	Primers (nM)		Probe (nM)	Annealing ^x		MgCl ₂ ^y	Efficiency	M slope	Limit of detection (fg per reaction)	Reference
				Temperature	Time					
<i>P. sylvaticum</i>	Syl1F (200)	Syl1R (200)	—	65	15 ^z	500 nM ^z	0.93	-3.3	18.8 fg	Schroeder et al. 2006
<i>P. irregulare</i>	PirF1 (300)	PirR1 (900)	—	60	5	100 nM ^z	0.96	-3.4	0.54 fg	Spies et al. 2011
Genus <i>Phytophthora</i>	Yph1F (250) ^z	Yph2R (250) ^z	—	62	20 ^z	—	1	-3.10	5.80 fg	Schena et al. 2008
<i>P. ultimum</i>	PulF2 (300)	PulR2 (300)	PulP2 (150)	—	—	—	0.98	-3.5	47.0 fg	Spies et al. 2011
<i>P. vexans</i>	PV390P (300)	PV455P (300)	PV412P (200)	—	—	—	0.95	-3.4	12.6 fg	Moein et al. 2019b
<i>Cylindrocarpon</i> -like fungi	YT1F (300)	CylR (300)	—	—	—	300 nM	0.92	-3.2	6.90 fg	Tewoldemedhin et al. 2011a
<i>Rhizoctonia</i> AG5	RSAG5F (900)	RSAG5R (300)	—	60	15	—	0.98	-3.4	2.56 copies	Mazzola and Zhao 2010

^x Annealing temperatures and extension times used in assays. All assays were Syber Green based, with the exception of the *P. ultimum* and *P. vexans* assays, which were probe based.

^y The qPCR master mix used in all assays contained a final concentration of 2.5 mM MgCl₂, and therefore, most assays did not require additional MgCl₂, except for the *P. sylvaticum* and *P. irregulare* assays.

^z Values were modified from the published assay.

Seedling growth assessments. After 3 months, the lengths and weights of seedlings were determined, and the increases in length and plant fresh weight (shoots and roots) were determined. Plant growth data from the untreated control and the pasteurized treatments were used to calculate the percentage increase in length or weight for each orchard. Relative increase in plant growth assessments was determined as described above.

Isolation and identification of oomycetes. Oomycetes were isolated from the washed roots of seedlings that had been cultivated in the untreated orchard soils. Twenty feeder roots were plated from each replicate bag onto PARP medium (Jeffers and Martin 1986), to which 0.8 ml/liter Benomyl (500 g benlate per 1 kg; Villa Crop Protection) was added to suppress Zygomycete fungal growth. Plates were incubated at room temperature for 2 to 3 days. Hyphal tips emerging from roots were transferred to potato dextrose agar (Biolab Diagnostics) amended with 0.04 g/liter streptomycin, and plates were incubated for 3 to 7 days at 25°C. After a pure culture was confirmed, fungal mycelium was scraped from the plates, and DNA was extracted using a slightly modified cetrimonium bromide (CTAB) method (Lee and Taylor 1990).

Oomycete isolates were first grouped into polymerase chain reaction (PCR)-restriction fragment-length polymorphism (RFLP) groups. The internal transcribed spacer (ITS) region was amplified as previously described (Tewoldemedhin et al. 2011b), and the PCR products were digested using a double-enzyme restriction digest containing *Hinf*I and *Hha*I (Mazzola et al. 2009). DNA fragments were separated on 3% agarose gels containing ethidium bromide, and fragments were visualized under ultraviolet illumination. Isolates that yielded similar PCR-RFLP patterns were placed in the same PCR-RFLP group. The PCR products of isolates representing the different PCR-RFLP groups were sequenced by the Central Analytical Facility at Stellenbosch University. The identity of the sequences was determined by BLAST analyses in GenBank (<https://www.ncbi.nlm.nih.gov/Genbank/>). For *Pythium* species identification,

only reference sequences submitted by Levesque and de Cock (2004) or published sequences of described new species were utilized. *Phytophthora* sequences were submitted to the *Phytophthora*-ID database Version 2.0 (<http://phytophthora-id.org/index.html>) for species identification (Grünwald et al. 2011).

Nematode quantification and qPCR quantification of ARD marker microbial pathogens. Nematodes and microbial pathogens were quantified from the fine feeder roots of seedlings from each replicate bag (one planting bag containing three seedlings) as described above.

Statistical analyses. Tree growth data (shoot length and increase in trunk diameter), yield, pathogen DNA quantities, and ARD severity estimates (percentage increase in seedling growth in the bioassay trial; in the orchard trials, the trunk diameter and yield) were subjected to an analysis of variance using the General Linear Models Procedure of SAS statistical software (Version 9.4; SAS Institute Inc.). The Shapiro–Wilk test was used to test for deviation from normality (Shapiro and Francia 1972). The pathogen DNA concentration data deviated significantly from normality, and therefore, the data were $\ln(x + 1)$ transformed, resulting in the data being normally distributed. Fisher's least significant difference test was calculated at the 95% confidence level to separate means for significant effects. Levene's variance ratio test was used to calculate variation within replications (Levene 1960). Pearson correlation analyses were conducted on (i) increase in trunk diameter and shoot length, (ii) ARD causative agents and tree growth (shoot length and increase in trunk diameter) and yield, and (iii) ARD causative agents with each other using XLStat (Version 2014; Addinsoft).

Results

Orchard trials. Tree growth. In the final year of tree growth measurement (year 3 or 4), in all three orchards, the applied treatments yielded significantly higher increases in trunk diameter and shoot length than the untreated control (Table 4). There were, in general, no significant differences between the applied treatments for shoot

Table 4. The effect of various soil fumigation and semiselective chemical treatments on apple tree growth (shoot length and increase in trunk diameter) and yield in three apple replant disease orchard trials^w

Orchards	Treatments ^x	Increase in shoot length (cm) in the third or fourth year of growth ^y	Increase in trunk diameter (mm) 3 or 4 years postplanting ^z	2-Year cumulative yield (kg per tree)
Orchard GRA	Untreated control	39.05 c	30.35 c	11 c
Orchard GRA	Pic33-1,3D	65.42 ab	39.86 ab	17.69 abc
Orchard GRA	Pic57-1,3D	62.86 ab	41.86 ab	21.38 ab
Orchard GRA	Methyl bromide	68.18 a	42.75 a	24.08 a
Orchard GRA	Independent semiselectives	61.25 ab	37.83 b	17.06 bc
Orchard GRA	Pic33-1,3D plus semiselectives	60.36 b	39.37 ab	18.64 ab
Orchard GRA	<i>P</i> value	<0.0001	<0.0001	0.0360
Orchard WZB	Untreated control	38.32 b	14.30 c	1.62 b
Orchard WZB	Pic33-1,3D	72.78 a	20.54 ab	1.53 b
Orchard WZB	Pic57-1,3D	62.91 a	20.83 ab	1.79 b
Orchard WZB	Methyl bromide	68.14 a	21.62 ab	3.47 ab
Orchard WZB	Independent semiselectives	63.43 a	19.64 b	1.87 b
Orchard WZB	Pic33-1,3D plus semiselectives	65.13 a	25.10 a	4.21 a
Orchard WZB	<i>P</i> value	<0.0001	0.0018	0.0228
Orchard KBC	Untreated control	20.48 b	22.16 b	14.35 c
Orchard KBC	Pic33-1,3D	38.15 a	27.60 a	29.77 b
Orchard KBC	Pic57-1,3D	38.22 a	29.48 a	37.36 a
Orchard KBC	Independent semiselectives	35.46 a	27.37 a	28.81 b
Orchard KBC	Pic33-1,3D plus semiselectives	35.78 a	28.94 a	37.36 a
Orchard KBC	Pic57-1,3D plus semiselectives	39.29 a	29.07 a	35.78 a
Orchard KBC	<i>P</i> value	<0.0001	0.0045	<0.0001

^w Values in columns are the average of six replicates (eight trees per replicate). For each orchard, values within a column followed by the same letter do not differ significantly ($P > 0.05$) according to Fisher's least significant difference test. Pic33-1,3D, 33.3% chloropicrin and 60.8% 1,3-dichloropropene; Pic57-1,3D, 57% chloropicrin and 38% 1,3 dichloropropene.

^x The semiselective treatments consisted of a soil drench applied at planting (metalaxyl, fenamiphos, and imidacloprid) followed by 3 years of potassium phosphonate applications. The fumigant treatments were all applied preplant. Two of the fumigants differed in their chloropicrin content; the Pic33-1,3D fumigant had 33.3% chloropicrin and 60.8% 1,3-dichloropropene, whereas the Pic57-1,3D fumigant had 57% chloropicrin and 38% 1,3 dichloropropene.

^y Shoot lengths for orchards Witzenberg valley (WZB) and Grabouw (GRA), which were planted in 2013, were taken in the fourth year of growth, whereas in the orchard Koue Bokkeveld (KBC), which was planted in 2014, shoot length was taken in the third year of growth.

^z Increases in trunk diameter values are for a 4-year period (2013 to 2017) for orchards WZB and GRA and a 3-year period for orchard KBC (2014 to 2017).

length and increase in trunk diameter. The exceptions for increase in trunk diameter were for the independent semiselectives treatment: (i) a significantly lower increase in trunk diameter was recorded relative to the methyl bromide treatment at orchard GRA, and (ii) in orchard WZB, a significantly lower increase in trunk diameter was evident relative to the Pic33-1,3D/semiselective treatment. For shoot length, the only applied treatment that had a significantly lower shoot length than the other treatments was the Pic33-1,3D treatment/semiselectives treatment, which had a significantly lower shoot length than the methyl bromide treatment at the GRA orchard (Table 4).

Yield. Not all of the applied treatments were consistent across the three orchards in significantly increasing yields relative to the untreated control (Table 4). The Pic33-1,3D/semiselectives treatment was the only treatment that resulted in a significant increase in yield relative to the untreated control in all three orchard trials. Pic57-1,3D significantly increased cumulative yields relative to the untreated control in two orchards (orchards GRA and KBC), whereas the methyl bromide treatment only did so in one of the two orchards where it was applied. Pic33-1,3D and the independent semiselectives treatments only resulted in significantly higher cumulative yields than the untreated control in the KBC orchard.

In some orchards, there were significant differences in cumulative yield between the Pic57-1,3D and Pic33-1,3D treatments and when semiselectives were added to these treatments (Table 4). Pic57-1,3D resulted in a significantly higher cumulative yield relative to Pic33-1,3D only in orchard KBC. The addition of semiselective chemicals to Pic33-1,3D resulted in significantly higher cumulative yields relative to the fumigant-only treatment in two of the orchards (WZB and KBC). The combined effect of Pic57-1,3D and the semiselectives was only evaluated in one orchard, where yield was not significantly increased.

In orchards GRA and WZB, preplant methyl bromide soil fumigation did not result in significantly higher cumulative yields relative to either of the Pic-1,3D fumigation treatments (Table 4).

Relative percentage increases in trunk diameter and yield. The relative percentage increase in trunk diameter between the untreated control and the Pic33-1,3D/semiselectives treatment after 3 years of growth varied in the three orchards (Table 5). The WZB orchard had a significantly higher relative percentage increase in trunk diameter than the KBC orchard (Table 5).

There were no significant differences ($P = 0.0982$) in the relative percentage increase in yield between the three orchards. The highest increase in cumulative yield was observed in orchard KBC (160.42%) followed by orchard WZB (158.72%) and lastly, orchard GRA (63.54%) (Table 5).

qPCR quantification of ARD microbial pathogens from roots. qPCR assays successfully detected several of the ARD microbial pathogens in roots of apple cultivated at the three orchard replant sites (Table 6). *Cylindrocarpon*-like spp., *P. cactorum*, and *P. irregulare* were detected in all three orchards. Although a genus-specific *Phytophthora* spp. assay was used, the *Phytophthora* species involved was identified as *P. cactorum* for all three trial sites through sequence analyses of a subset of the qPCR amplicons obtained from root DNA samples. *P. ultimum* was only detected in orchards GRA and WZB. *P. sylvaticum*, *P. vexans*, and *R. solani* AG-5 were not detected in root DNA samples obtained from any of the orchard sites (Table 6).

Significant differences ($P \leq 0.0338$) in the quantity in *Cylindrocarpon*-like spp. DNA in root samples were detected between the control and applied treatments at all three orchards (Table 6). The KBC orchard was the only orchard where the significant difference was because of treatments causing a reduction in *Cylindrocarpon*-like root DNA relative to the untreated control; tree roots from the Pic57-1,3D (14.35 pg/mg_{DW}) and the Pic33-1,3D/semiselective (11.30 pg/mg_{DW}) treatments had significantly lower DNA quantities of *Cylindrocarpon*-like spp. than the untreated control (62.25 pg/mg_{DW}). In contrast, roots of trees cultivated in methyl bromide-treated soil in orchard GRA had a

Table 5. Apple replant disease (ARD) severity and ARD microbial pathogens in three ARD soils evaluated under glasshouse conditions and under orchard conditions^u

Orchard	Apple seedling glasshouse bioassays				Orchard trials		
	% Increase seedling weight ^v	% Increase seedling length ^v	ARD oomycete pathogens detected isolation (% isolates) ^w	ARD pathogens detected qPCR (pg/mg _{DW}) ^x	% Increase trunk diameter ^y	% Increase yield ^y	ARD pathogens detected qPCR ^z
GRA	106.71 b	54.31 b	<i>P. cactorum</i> (100%)	<i>P. cactorum</i> (0.009), <i>P. irregulare</i> (0.006), <i>P. ultimum</i> (0.0006), <i>Cylindrocarpon</i> like (0.184), <i>P. vexans</i> (4.02)	42.78 ab	63.54	<i>P. cactorum</i> (40.330), <i>P. irregulare</i> (3.960), <i>P. ultimum</i> (0.002), <i>Cylindrocarpon</i> like (12.430)
WZB	63.28 c	65.45 b	<i>P. irregulare</i> (43%), <i>P. ultimum</i> (57%)	<i>P. cactorum</i> (0.004), <i>P. irregulare</i> (0.12), <i>P. ultimum</i> (0.016), <i>Cylindrocarpon</i> like (0.700), <i>P. vexans</i> (0.484)	63.98 a	158.72	<i>P. cactorum</i> (34.00), <i>P. irregulare</i> (0.028), <i>P. ultimum</i> (0.068), <i>Cylindrocarpon</i> like (1.300)
KBC	185.79 a	180.33 a	<i>P. irregulare</i> (21.4%), <i>Pythium</i> sp. B2A (64.2%), <i>P. heterothallicum</i> (14.2%)	<i>P. cactorum</i> (0.002), <i>P. irregulare</i> (0.084), <i>P. ultimum</i> (0.007), <i>Cylindrocarpon</i> like (0.3435)	30.62 b	160.42	<i>P. cactorum</i> (0.370), <i>P. irregulare</i> (0.040), <i>Cylindrocarpon</i> like (12.450)
P value	<0.0001	0.0004			0.0417	0.0982	

^u Pathogens listed are *Pythium irregulare*, *Phytophthora vexans*, *Pythium ultimum*, *Phytophthora cactorum*, *Pythium heterothallicum*. GRA, Grabouw; KBC, Koue Bokkeveld; WZB, Witzenberg valley.

^v The percentage increase was calculated as follows: (pasteurized treatment – untreated control)/pasteurized treatment × 100.

^w ARD microbial pathogens were obtained in a glasshouse study from the roots of apple seedlings grown in untreated soil. The percentages of the isolates that each species represented of all of the oomycetes isolates obtained are shown in parentheses.

^x The quantity of microbial ARD pathogens detected in apple seedling roots of the untreated control using quantitative polymerase chain reaction (qPCR) analysis. The amount of pathogen DNA is indicated in parentheses as pg/mg dry weight (DW) of roots.

^y Percentage increase in trunk diameter was calculated for the third year of growth for all orchards as follows: (increase in trunk diameter of the low chloropicrin fumigant combined with semiselective treatment – increase in trunk diameter of the untreated control)/increase in trunk diameter of the 33.3% chloropicrin and 60.8% 1,3-dichloropropene fumigant combined with semiselective treatment × 100. The percentage increase in yield was calculated in a similar manner except that the cumulative yield of the relevant treatments was used.

^z The ARD microbial pathogens detected in orchard tree roots of the control treatment using qPCR. The amount of pathogen DNA detected using qPCR is shown in parentheses.

significantly higher *Cylindrocarpon*-like spp. concentration (195.70 pg/mg_{DW}) than the untreated control (62.150 pg/mg_{DW}). In orchard WZB, three treatments had significantly higher *Cylindrocarpon*-like DNA quantities than the untreated control. These included Pic33-1,3D, Pic57-1,3D, and independent use of semiselective treatments (Table 6).

P. irregulare DNA quantities in tree roots were only significantly different between treatments in orchard WZB ($P = 0.0197$) (Table 6). However, the differences were not because of a reduction in pathogen quantity in treatments relative to the untreated control. The Pic57-1,3D treatment had a significantly higher *P. irregulare* concentration (5.89 pg/mg_{DW}) than the untreated control (0.140 pg/mg_{DW}).

P. ultimum DNA concentrations detected in apple roots in orchards GRA and WZB were low (<0.340 pg/mg_{DW}), and the pathogen was not detected in root samples from the KBC orchard (Table 6). There were no significant treatment effects ($P > 0.1795$) in the quantity of *P. ultimum* DNA detected in tree root samples from the GRA and WZB orchards.

The quantity of *P. cactorum* DNA detected in tree roots differed significantly among treatments at the GRA and KBC ($P < 0.0090$ or 0.0008) orchards. In orchard GRA, all of the applied treatments with the exception of the Pic33-1,3D and methyl bromide fumigation treatments possessed significantly lower *P. cactorum* root DNA concentrations than the no treatment control. In orchard KBC, the quantity of *P. cactorum* DNA detected in the root samples from the untreated control was significantly higher than that detected for all other treatments (Table 6).

Nematode extraction and quantifications. *Pratylenchus* spp. was the only plant parasitic nematode genus identified in root samples from the study orchards. *Pratylenchus* spp. infestations were observed in orchards WZB and KBC but not in orchard GRA (Table 6). *Pratylenchus* spp. root densities were significantly different among treatments only at the WZB orchard ($P = 0.0260$). At the

WZB orchard, all treatments significantly reduced *Pratylenchus* spp. root densities (<47/5-g roots) relative to the control (548/5-g roots).

Correlation between tree growth and yield with ARD causative agent quantities. Tree growth (shoot and trunk diameters) and/or yield in some of the orchards had a significant negative correlation with root DNA quantities of *P. cactorum* and *P. ultimum*, and *Pratylenchus* spp. root densities (Table 7). There were no significant correlations between *P. irregulare* and *Cylindrocarpon*-like fungal DNA quantities detected in tree roots and relative tree growth (data not shown).

The importance of *P. cactorum* in limiting tree performance at all three orchards was evident from several significant negative correlations between *P. cactorum* DNA quantities in tree roots and certain tree growth parameters and yield (Table 7). In all three orchards, there were significant negative correlations ($r = -0.340$ to -0.632 ; $P < 0.042$) between shoot length and *P. cactorum* root DNA quantities in the third and sometimes, fourth year of growth. Increase in trunk diameter was significantly negatively correlated with *P. cactorum* root DNA quantities in all orchards in the third year of growth ($r = -0.333$ to -0.410 ; $P < 0.047$). Yield was only significantly negatively correlated with *P. cactorum* root DNA quantities in orchard KBC ($r = -0.455$; $P = 0.005$) (Table 7).

P. ultimum root DNA quantities only showed negative correlations with tree growth in orchard WZB. The contribution of *P. ultimum* to disease development at the WZB orchard was evidenced by the significant negative correlation between the quantity of pathogen DNA detected in tree roots and increase in trunk diameter in the third year of growth ($r = -0.458$; $P = 0.030$) (Table 7).

In orchards WZB and KBC, significant negative correlations were observed between *Pratylenchus* spp. root densities and tree growth or yield (Table 7). In orchard WZB, shoot length and increase in trunk diameter were significantly correlated with *Pratylenchus* spp. root densities in the third and fourth year of growth ($r = -0.393$ to

Table 6. The effect of various soil fumigation and semiselective chemical treatments on the DNA quantities of apple replant disease pathogens (*Cylindrocarpon*-like fungi, *Pythium irregulare*, *Pythium ultimum*, and *Phytophthora cactorum*) and *Pratylenchus* spp. densities in the roots of apple trees in three apple replant disease orchard trials²

Orchards and treatments	<i>Cylindrocarpon</i> -like fungi (pg/mg _{DW})	<i>P. irregulare</i> (pg/mg _{DW})	<i>P. ultimum</i> (pg/mg _{DW})	<i>P. cactorum</i> (pg/mg _{DW})	<i>Pratylenchus</i> spp. (5-g roots)
Orchard GRA					
Untreated control	62.150 b	19.8	0.010	201.650 a	ND
Pic33,1,3D	50.700 b	5.9	0.015	107.495 abc	ND
Pic57-1,3D	57.250 b	0.025	0.140	0.000 c	ND
Methylbromide	195.700 a	0.900	0.200	101.400 ab	ND
Independent semiselectives	54.250 b	2.500	0.015	0.000 c	ND
Pic33-1,3D plus semiselectives	36.950 b	2.200	0.025	54.550 bc	ND
<i>P</i> value	0.0172	0.1518	0.744	0.0090	ND
Orchard WZB					
Untreated control	6.500 bc	0.140 b	0.340	170.00	548 b
Pic33,1,3D	18.950 ab	0.150 b	0.095	30.450	47 a
Pic57-1,3D	54.850 ab	5.890 a	0.075	16.450	42 a
Methylbromide	6.050 bc	0.060 b	0.015	3.650	75 a
Independent semiselectives	24.000 ab	0.150 b	0.035	18.600	30 a
Pic33-1,3D plus semiselectives	0.950 c	0.205 b	0.010	12.750	10 a
<i>P</i> value	0.0338	0.0197	0.1795	0.3273	0.0260
Orchard KBC					
Untreated control	62.250 a	0.200	ND	1.850 a	231
Pic33,1,3D	18.250 a	0.050	ND	0.000 b	95
Pic57-1,3D	14.350 b	0.150	ND	0.350 b	31
Independent semiselectives	109.600 a	0.150	ND	0.000 b	70
Pic33-1,3D plus semiselectives	11.300 b	0.100	ND	0.300 b	21
Pic57-1,3D plus semiselectives	62.250 a	0.150	ND	0.050 b	50
<i>P</i> value	0.0019	0.4832	ND	0.0008	0.5339

² Root samples used for pathogen and *Pratylenchus* spp. quantification were obtained 20 months after planting. Sampling was conducted at a distance of 20 to 40 cm from the tree trunk on opposite sides of the tree at a depth of 30 cm. Pathogen quantities are expressed as picograms of pathogen DNA per milligram of dry weight (DW) of root, which were determined through quantitative polymerase chain reaction analyses. Values in columns are the average of six replicates. For each replicate, roots sampled from three trees were pooled into one sample. For each orchard, values in columns followed by the same letter do not differ significantly according to Fisher's least significance difference test at the 95% significance level. ND indicates that the organisms were not detected. *Pythium sylvaticum*, *Pythium vexans*, and *Rhizoctonia solani* AG-5 were not detected in any of the orchards. GRA, Grabouw; KBC, Koue Bokkeveld; Pic33-1,3D, 33.3% chloropicrin and 60.8% 1,3-dichloropropene; Pic57-1,3D, 57% chloropicrin and 38% 1,3 dichloropropene; WBZ, Witzenberg valley.

−0.516; $P < 0.042$). Additionally, in orchard KBC, *Pratylenchus* spp. root densities were significantly correlated with yield ($r = -0.433$; $P = 0.008$ (Table 7).

Correlation between different ARD causative agent quantities. Correlation analyses between the different microbial pathogen DNA root quantities and *Pratylenchus* spp. root densities yielded interesting associations in two orchards. Significant positive correlations were evident between *P. cactorum* root DNA quantities and *Pratylenchus* spp. root densities at orchard WZB ($r = 0.942$; $P < 0.0001$) and orchard KBC ($r = 0.43$; $P = 0.009$). The quantity of *Cylindrocarpon*-like spp. DNA detected in tree roots was positively correlated with *P. irregulare* root DNA quantity ($r = 0.714$; $P < 0.0001$) at the WZB orchard and *P. ultimum* ($r = 0.432$; $P = 0.009$) at orchard GRA.

Apple seedling glasshouse bioassay. Seedling growth assessments. For all three orchard soil bioassay trials, there were significant differences between the untreated control and pasteurized and 15% diluted soil treatments for length ($P < 0.01389$) and weight ($P < 0.01389$) (Supplementary Table S1). The pasteurized soil significantly enhanced seedling length and weight relative to the untreated control soil in all three orchard trial soils.

The relative percentage increase in seedling weight and height in response to pasteurization differed among the three orchard soils (Table 5). Pasteurization of the KBC orchard soil resulted in significantly higher percentage increases in seedling growth (180.33 and 185.79% in weight and length, respectively) than the WZB and GRA orchard soils. The relative percentage increase in seedling weight was significantly lower in the WZB orchard relative to the other two orchards.

Isolation and qPCR identification of oomycetes. Isolations from roots of seedlings cultivated in certain orchard soils revealed the presence of additional oomycete species beyond those identified through qPCR analyses (Table 5). This was owing to the fact that only a limited number of oomycete species were included in qPCR analyses. In orchard KBC, isolation studies revealed the presence of *Pythium heterothallicum* (14.2% of isolates) and *Pythium* sp. complex B2A (64.2% of isolates). In all three orchards, qPCR analyses from seedling roots in the bioassay revealed a few additional oomycete pathogens than those that were detected in the orchard tree roots (Table 5). In orchards GRA and WZB, this consisted of *P. vexans* DNA. In orchard KBC, *P. ultimum* DNA was detected in seedling roots but not orchard tree roots.

qPCR identification of *Cylindrocarpon*-like spp. In all three orchard soils, *Cylindrocarpon*-like spp. were identified in the roots of the bioassay seedlings, similar to what was observed in the orchard trial tree roots (Table 5).

Nematode extraction and quantifications. The genus *Pratylenchus* was the only plant parasitic nematode genus identified in the seedling roots. The seedling roots grown in orchards WZB and KBC had relatively high *Pratylenchus* spp. root densities of 250 and 590 per 5 g of roots, respectively. Orchard GRA had a very low *Pratylenchus*

spp. seedling root density (10/5-g roots), which is consistent with *Pratylenchus* spp. not recovered from tree roots collected at this orchard site.

Discussion

In this study, several treatments were evaluated for the management of ARD in three apple replant orchards (GRA, WZB, and KBC) situated in different production regions in South Africa. All treatments, including the fumigants Pic33-1,3D, Pic57-1,3D, and methyl bromide, a semiselective chemical mixture (metalaxyl, imidacloprid, fenamiphos, and potassium phosphonate) applied independently or combined with Pic-1,3D fumigants, improved tree growth (shoot length and increased in trunk diameter) to a similar level that was significantly higher than that of the untreated control. Although the semiselective chemical treatment used independently did not consistently improve tree growth performance to the level of methyl bromide, it was as effective as the two Pic-1,3D fumigants (Pic33-1,3D and Pic57-1,3D). The latter fumigants are currently the standard ARD management practice available to growers in South Africa (Kapp et al. 2016).

Cumulative yield data were more useful for distinguishing differences in efficacy among the evaluated treatments. However, relative cumulative yield responses of any given treatment differed among the three orchards, making it difficult to identify the best-performing treatment. The Pic33-1,3D fumigant combined with semiselectives was the most consistent treatment in improving cumulative yield relative to the untreated control in all orchard trials. In the WZB orchard, all soil fumigation treatments failed to significantly increase cumulative yields. This is most likely because of the inability of the fumigants to affect ARD inoculum that was present in the numerous undecomposed tree roots that were present in the orchard soil at the time of fumigation. In the WZB orchard, the fumigants were applied in the same year in which the old orchard trees were removed, which did not provide enough time for the decomposition of tree roots. The two Pic-1,3D fumigants resulted in cumulative yields that were similar to those attained in response to methyl bromide fumigation, but the treatments differed in efficacy from each other. Based on this study, the Pic57-1,3D formulation is considered a superior option to Pic33-1,3D for ARD management, because it resulted in (i) a significantly greater cumulative yield than Pic33-1,3D in the KBC orchard and (ii) a significant increase in cumulative yield relative to the untreated control in the GRA orchard, whereas Pic33-1,3D did not. The higher chloropicrin concentration in the Pic55-1,3D fumigant most likely contributed to improved control of ARD causative fungi and oomycetes. The formulation, however, still contains enough 1,3-dichloropropene for lesion nematode suppression as encountered at the KBC orchard. There are no other studies that have specifically compared 33.3% chloropicrin-containing fumigant formulations with 57% chloropicrin-containing fumigant formulations for the management of ARD or replant disease on other tree crops. The preponderance of ARD studies have used Pic-1,3D formulations

Table 7. Pearson correlation analyses between apple tree growth parameters (shoot length and increase in trunk diameter) and yield with DNA quantities of apple replant disease microbial pathogens (*Phytophthora cactorum* and *Pythium ultimum*) in tree roots and *Pratylenchus* spp. root densities in three apple ARD orchard trials.^z

Orchard	Pearson correlation coefficient (P value)				
	Third-year tree growth		Fourth-year tree growth		Cumulative yield
	Increase trunk diameter	Shoot length	Increase trunk diameter	Shoot length	
<i>P. cactorum</i>					
Orchard GRA	−0.358 (0.032)	−0.340 (0.042)	NS	−0.355 (0.034)	NS
Orchard WZB	−0.410 (0.030)	−0.413 (0.029)	NS	−0.452 (0.016)	−0.455 (0.005)
Orchard KBC	−0.333 (0.047)	−0.632 (<0.001)	ND	ND	NS
<i>P. ultimum</i>					
Orchard WZB	−0.458 (0.03)	NS	NS	NS	NS
<i>Pratylenchus</i> spp.					
Orchard WZB	−0.49 (0.009)	−0.49 (0.009)	−0.393 (0.042)	−0.516 (0.006)	NS
Orchard KBC	NS	NS	NS	NS	−0.433 (0.008)

^z GRA, Grabouw; KBC, Koue Bokkeveld; ND, not done; NS, nonsignificant; WZB, Wittenburg valley.

containing either 35 or 17% chloropicrin (Braun et al. 2010; Mazzola and Brown 2010; Parker et al. 2014; Rumberger et al. 2004).

Investigations into the causative agents associated with orchard tree roots indicated that several agents were often present. However, the DNA root quantities or densities of only a few were consistently negatively correlated with tree growth or yield. *P. cactorum*, *P. irregulare*, and *Cylindrocarpon*-like fungi were associated with tree roots in all three trials. *Pratylenchus* spp. and *P. ultimum* were associated with tree roots in two orchards. A previous study in South Africa reported that *P. cactorum*, *P. ultimum*, *P. irregulare*, and *Cylindrocarpon*-like fungal DNA were present in all of the investigated ARD orchards, whereas *Pratylenchus* spp. occurred in 67% of the orchards (Tewoldemedhin et al. 2011c). Worldwide *Pratylenchus* spp. are known to be associated inconsistently with ARD orchard soils (Mazzola and Manici 2012). Based on correlation analyses between causative agent quantities and tree growth and yield, *P. cactorum* contributed to disease development at all three orchards. This was also true in the two orchards where *Pratylenchus* spp. (WZB and KBC) were present but only in one orchard where *P. ultimum* occurred in tree roots.

The effect of the various treatments on ARD causative agents was also investigated by determining if the treatments incited a significant reduction in quantities relative to the untreated control. A 20-month postplant timepoint was selected, because several of the treatments consisted of the application of semiselective chemicals. The latter included the use of phosphonate applications until 3 years postplant. At the 20-month sampling point, three of the five sets of phosphonate applications were completed. There were only a few cases where treatments significantly reduced ARD causative agents in roots relative to roots from the non-treated soil. These involved *P. cactorum* (orchards KBC and GRA), *Pratylenchus* spp. (orchard KBC), and *Cylindrocarpon*-like fungi (orchard KBC; Pic57-13D and Pic33-1,3D/semiselectives treatment). The suppression of *P. cactorum* and *Pratylenchus* spp. by the independent use of semiselectives was evident in all of the aforementioned orchards. It is likely that an earlier timepoint could have revealed a clearer indication of the specific effect of the soil fumigant treatments on the ARD causative agents. ARD is known to affect trees within the first year of growth (Mazzola and Manici 2012). Furthermore, the effect of fumigants has previously been reported to not extend beyond 1 year postplant (Auvil et al. 2011), and *Pythium* spp. and *Pratylenchus* spp. can recolonize the fumigated tree row within 1 year postplant (Mazzola et al. 2015). The results from this study suggest that this is not always the case for all ARD soils because the effect of fumigants on some ARD causative agents were still evident 20 months postplant.

Synergistic interactions between ARD causative agents and their effect on treatment efficacy and tree growth are also important to consider. For example, it has been reported that *P. irregulare* and *Cylindrocarpon*-like fungi act synergistically to cause ARD (Braun 1991; Tewoldemedhin et al. 2011c). In this study, a significant positive correlation was documented between *Cylindrocarpon*-like spp. and *P. irregulare* or *P. ultimum* in two orchards, supporting the synergistic interaction between these two groups of organisms. Thus, although no significant correlation was observed between tree growth and root infection by *P. irregulare*, which has previously been reported to be highly virulent (Tewoldemedhin et al. 2011b), and *Cylindrocarpon*-like fungi, these pathogens likely did contribute to ARD severity but in a manner unrelated to their root DNA quantities per se. This is also supported by a report indicating that no correlation existed between apple seedling stunting and *P. irregulare* root DNA quantities and root colonization under glasshouse conditions (Moein et al. 2019b). In these orchard trials, the significant positive correlation between *P. cactorum* root DNA quantities and *Pratylenchus* spp. root densities suggests the potential for a synergistic interaction in orchards where they cooccur. This has previously been reported for replant disease occurring in red raspberry (Gigot et al. 2013) and the tobacco/*Phytophthora parasitica* Dastur/*Pratylenchus brachyurus* (Godfrey 1929) Filpjev & Schuurmans Stekhoven 1941 system (Inagaki and Powell 1969). It is likely that wounds caused by *Pratylenchus* spp. facilitate infection by *Phytophthora* spp. (Inagaki and Powell 1969). In other systems, however, nematode infections

were reported to negatively affect *Phytophthora* spp. infections. This was observed in the tobacco/*P. parasitica*/*Pratylenchus penetrans* system (Mc Intyre and Miller 1978) and the citrus/*Tylenchulus semi-penetrans* Cobb 1913/*Phytophthora nicotianae* Breda de Haan system (El-Borai et al. 2002).

A few important causative agents that were not investigated in this study in orchard tree roots might have also influenced treatment efficacy. In the KBC orchard, *P. heterothallicum* and *Pythium* complex B2A might have been involved, because they were recovered from roots of seedlings cultivated in this soil in the glasshouse bioassay study. *Pythium* sp. complex B2A is a species complex that includes several species (*Pythium dissotocum*, *Pythium coloratum*, *Pythium lutarium*, *Pythium marinum*, *Pythium diclinum*, *Pythium* aff. *dictyosporum*, and *Pythium* sp. group F and *P. sp. tumidum*) that cannot be differentiated based on ITS sequence data (Robideau et al. 2011). Among these listed species, *P. dissotocum* and *P. coloratum* have been identified as pathogens of apple (Braun 1995; Sewell 1981; Tewoldemedhin et al. 2011b). In orchards GRA and WZB, *P. vexans* might also have been functioning as a causative agent, because it was detected in glasshouse seedling roots but not in orchard tree roots. *P. vexans* has previously been reported to occur in established apple tree roots in South Africa and incite disease on apple seedlings (Tewoldemedhin et al. 2011b, 2011c). Future studies should further investigate the pathogenicity of *P. vexans* toward 1-year apple trees and its occurrence at an earlier timepoint postplant under orchard conditions. Although several binucleate *Rhizoctonia* groups, such as AG-I, AG-F, AG-G, and AG-Q, in general have low relative virulence (Mazzola 1997; Tewoldemedhin et al. 2011a), some isolates of AG-G, which do occur in South Africa (Tewoldemedhin et al. 2011a, 2011c), can be highly virulent (Mazzola et al. 2015). The quantities of binucleate *Rhizoctonia* groups in tree roots thus also warrant investigation in the future.

This is the first study to show that the independent use of semiselective chemicals can consistently improve tree growth in ARD orchards and cumulative yield to a lesser extent. Cumulative yield was only significantly increased in the KBC orchard, and it was not increased to a level that was equivalent to that attained with fumigants. The semiselectives in some instances when combined with the Pic33-1,3D fumigant can furthermore significantly increase cumulative yields in comparison with the independent use of the fumigant. The efficacy of semiselective chemicals might be twofold: owing to the suppression of ARD causative agents associated with planting material or the prevention of pathogen reinfestation of the fumigated tree row. ARD causative agents are known to be associated with planting material in South Africa (Moein et al. 2019).

The only other study that has investigated the potential of metalaxyl and fosetyl-Al (alkyl phosphonate) for managing ARD was a study by Autio et al. (1991) in Massachusetts, U.S.A. The products were reported to not be highly effective when applied independently during the first year of growth, because a significant increase in trunk and shoot growth was only observed in the first year and not in the subsequent two growing seasons. The short-lived performance in the efficacy of the treatments was most likely because of ARD causative agents continuing to affect tree growth in the second year. Therefore, chemical applications must not only be made in the first year. In this study, phosphonates were applied until 3 years postplant. Another study that supports the efficacy of metalaxyl in reducing ARD severity comes from observations that the application of a mefenoxam soil drench to a *Brassica napus* seed meal treatment can significantly improve apple tree growth and yield in ARD orchards provided that *P. penetrans* was not present (Mazzola and Brown 2010; Mazzola and Mullinix 2005).

The mode of action of the semiselective chemicals and the relative importance of the different compounds in the mixture will be difficult to determine. It will furthermore not be productive to evaluate whether each chemical can single handily control ARD, because ARD is known to be a disease complex that cannot be controlled using single products. This was clearly shown by Mazzola and Brown (2010), where single *Brassicaceae* seed meal product applications were ineffective in suppressing ARD pathogens; only certain

mixtures of *Brassicaceae* species seed meals were effective. It is reasonable to expect that the metalaxyl, phosphonates, and fenamiphos are most likely involved in suppression of oomycetes and *Pratylenchus* spp., because they are known to function in this manner. Whether imidacloprid, applied for woolly apple aphid control, played a role in suppressing the ARD agents is unknown, but it cannot be excluded. Imidacloprid is also known as a resistance inducer on citrus against bacterial diseases when applied at the correct dosage (Francis et al. 2009). Phosphonates are also increasingly being seen as plant resistance inducers (Jackson et al. 2000; Massoud et al. 2012). Future studies can, therefore, investigate the effect of the semiselective mixture on the induction of host plant defense responses of apple roots challenged with the ARD causative agents. Host plant defense induction is known to be important in ARD disease suppression (Shin et al. 2016; Weiss et al. 2017).

In summary, the study showed that, considering all of the unknown variabilities associated with ARD orchard sites, the best management option consisted of fumigation with Pic33-1,3D combined with semiselective chemicals. Because the Pic57-1,3D fumigant was superior in a few instances to the Pic33-1,3D fumigant, growers should in the future use Pic57-1,3D. The effect of combining the latter fumigant with semiselective chemicals is currently uncertain, because this treatment was only evaluated in one trial, where it did not provide a benefit in cumulative yield. Considering the high levels of ARD causative agents present in South African plant material (Moein et al. 2019a), the addition of semiselective chemicals to trees on fumigated soil might furthermore be warranted. The inclusion of fenamiphos in the semiselective mixture is problematic, because this nematocide is likely to be lost from the South African market in the near future, such as has occurred internationally. Therefore, future studies should investigate the inclusion of alternative nematicides, such as fluapyram (Bayer), in the mixture.

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