# Assessing the effect of rootstock and number of trunk injections on *Phytophthora cinnamomi* root DNA quantities in avocado

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**ABSTRACT**

Tolerant rootstocks and phosphonate fungicides form part of an integrated management strategy of Phytophthora root rot (PRR) in avocado; a disease caused by *Phytophthora cinnamomi* (Pc). The aim of our study was to evaluate whether the effect of rootstock tolerance and phosphonate trunk injections (once or twice during the season) on PRR could be assessed using DNA-based pathogen quantification. Quantitative real-time PCR (qPCR) analysis was used to investigate Pc quantities in roots and soil from three asymptomatic phosphonate orchard trials and one rootstock trial (only Pc root quantities were evaluated). In the phosphonate trials, no significant differences were evident in Pc root and soil DNA quantities for trees receiving one or two phosphonate trunk injections (preventative dosage of 0.3 g a.i./m$^2$) for both of the investigated time points (May and October 2018). However, qPCR analysis in roots was able to reveal the importance of root phosphite concentrations since a significant negative correlation was found between root phosphite- and Pc root DNA concentrations for the May sampling point. The 2x trunk injection treatment consistently yielded significantly higher root phosphite concentrations than the untreated control, whereas the 1x trunk injection treatment did not. In the orchard where rootstocks were evaluated, the more PRR-tolerant R0.06 rootstock had a significantly lower ($P$ = 0.0793) Pc root DNA concentration than Dusa® at the 90% confidence interval in the November (2017) sampling month. However, there were no significant differences between the rootstocks in Pc root DNA quantities for the March and May 2018 samplings. DNA-based pathogen quantification in roots and soil should, in future, also be evaluated in symptomatic orchards with higher Pc inoculum, since the mainly asymptomatic orchards used in the current study might have contained Pc inoculum levels that were too low for Pc DNA quantities to reveal the effect of management strategies.

**INTRODUCTION**

Management of Phytophthora root rot (PRR) in avocado requires an integrated management strategy, which is accurately described by the 'Pegg wheel'. The 'Pegg wheel' covers six management principles, including soil selection, irrigation management, chemical control, inorganic nutrition, organic amendments and tolerant rootstocks (Wolstenholme and Sheard, 2010). Chemical control of PRR mainly consists of the use of phosphonates, especially since the discovery of phosphonate trunk injections by Darvas *et al.* (1984) (Dann *et al.*, 2013).

In South Africa, in a preventative management strategy, avocado producers typically apply phosphonate trunk injections twice a season in accordance with the two root flush windows. One application is made in summer (after the spring foliar flush has hardened off) and the other in fall (usually after harvest when the summer foliar flush has hardened off) (McLeod *et al.*, 2018). This application strategy has become problematic due to strict maximum residue level (MRL) regulations for fosetyl-Al (fosetyl + phosphonic acid) in fruit, which was imposed by the European Union (EU) in 2014 for avocado (McLeod *et al.*, 2018). Many producers have been unable to maintain EU standards despite following phosphonate label recommendations, thus leading to market access problems. Exceedances of the EU MRL is likely due to the phosphonate applications made in summer, since small fruit are present on trees that serve as a strong sink for phosphite (breakdown product of phosphonates in plants). Several growers in South Africa have thus enquired whether applying only one trunk injection during the fall application window using the preventative dosage (0.3 g a.i./m$^2$ canopy) is sufficient for managing PRR.

This approach is likely to reduce fruit residues, but it is unknown whether it will reduce the efficacy of PRR management in the long-term.

Rootstock resistance is considered an essential preventative management strategy for PRR control. However, despite several decades of intensive investigations into the identification of *Phytophthora*-resistant avocado rootstocks (Kremer-Köhne and Köhne, 2007; Smith *et al.*, 2011), only tolerant rootstocks have been discovered thus far. Westfalia Technological Services (WTS) at Westfalia Fruit has been very successful in identifying PRR-tolerant rootstock selections in their rootstock programme. Two prominent rootstocks that have been discovered by this programme include the Dusa® rootstock (also known as R0.09) and more recently rootstock R0.06 (Engelbrecht and Van den Berg, 2013), which is potentially superior to Dusa® in terms of PRR tolerance (Van Rooyen, 2017).

Limited information is available regarding the extent of Pc root colonisation in rootstocks R0.06 and Dusa®, since studies have mainly focused on their host defence responses during pathogen attack (Engelbrecht and Van den Berg, 2013; Van den Berg *et al.*, 2018). *Phytophthora cinnamomi* root infection and colonisation have only been investigated in Dusa®, where it was shown that less Pc root colonisation occurred in Dusa® in comparison to the susceptible R0.12 rootstock (Engelbrecht *et al.*, 2013). This observation was made using artificial inoculation of avocado seedlings under glasshouse conditions, followed by an assessment of Pc root colonisation through quantitative real-time PCR (qPCR) analysis. The extent of Pc root colonisation in rootstock R0.06 has not yet been reported. However, reduced and delayed zoospore germination has been reported for infected R0.06 roots as opposed to the moderately tolerant R0.10 and susceptible R0.12 rootstocks (Van den Berg *et al.*, 2018), thus suggesting that root colonisation might be less in R0.06.

The efficacy of management strategies against PRR may be better understood through investigations into pathogen DNA concentrations within host roots and rhizosphere soil. DNA extraction and qPCR quantification from plant roots are readily conducted (Engelbrecht *et al.*, 2013). However, qPCR quantification from soil DNA is challenging, due to the presence of variable quantities of PCR inhibitors in soils as well as variable DNA extraction efficacies (Daniell *et al.*, 2012). It is, therefore, important that an artificial internal DNA standard is used when conducting qPCR analysis of soil pathogens. In the latter approach, co-amplification of the targeted microbe and the foreign gene is conducted, which can be used to determine and adjust for PCR inhibition and DNA loss (Fall *et al.*, 2015).

The first aim of our study was to determine whether qPCR quantification of Pc from avocado orchard tree roots and rhizosphere soil can differentiate between the efficacy of two phosphonate treatments (1x versus 2x trunk injections). The efficacy of the phosphonate trunk injection treatments was also assessed by measuring root phosphite concentrations. The second aim was to compare Pc DNA concentrations from the roots of two rootstocks (Dusa® and R0.06) differing in PRR tolerance under orchard conditions.

## MATERIALS AND METHODS

### Phosphonate trials

*Orchard selection and trial layout*

The trials were conducted in three orchards, without obvious symptoms of PRR decline, which were located in two production regions (Mooketsi and Letaba) in Limpopo, South Africa. Two of the orchards (BL and EL) were situated in Letaba, and one (FM) was situated in Mooketsi. The scion/rootstock combinations included Carmen/Dusa® (BL) and Maluma-Hass/Duke 7 (EL and FM). None of the orchards were mulched.

Three treatments were evaluated: (i) untreated control, (ii) 1x trunk injection and (iii) 2x trunk injections. A total of eight trees (single replicates) were selected for each treatment at each orchard (i.e. 24 trees per orchard). The trial design was a completely randomised design. For the 1x trunk injection treatment, trees were injected in fall (April 2018). For the 2x trunk injection treatment, trees were injected in summer after the spring foliar flush had hardened off (November 2017) and in fall (April 2018). Each trunk injection was applied at a preventative dosage of 0.3 g a.i./$m^2$ (Avoguard® 500 SL; Nulandis, Kempton Park, South Africa) according to the registered label recommendation.

*Root and soil sampling*

Roots and soil were sampled from each treatment replicate in two different months (May 2018 and October 2018); 4 and 23 weeks, respectively, after the April 2018 injections.

*Root DNA extraction and qPCR analysis*

Root DNA extraction and qPCR amplification targeting the *Ypt1* gene were conducted as previously described (Masikane, 2017).

*Soil DNA extraction and multiplex qPCR analysis*

Soil DNA was extracted using the NucleoSpin® Soil kit (Macherey-Nagel GmbH and Co., KG, Düren, Germany). Prior to starting the soil DNA extraction kit protocol, the required amount of SL1 DNA extraction buffer was spiked with a plasmid containing the exogenous internal foreign positive control (EIPC) DNA fragment (Fall *et al.*, 2015) to a final concentration of 1.2 x $10^3$ copies/µl.

A probe-based multiplex qPCR assay reaction was optimised that co-amplifies the EIPC plasmid DNA fragment and the pathogen's *Ypt1* protein gene within one qPCR reaction. The relative pathogen DNA concentration (in ng/$mg_{DW}$) was calculated by using the formula:

$$\left(\frac{\left(\frac{\text{pathogen DNA concentration of sample}}{\text{EIPC gene copy number of sample}}\right) \times \text{EIPC gene copy number used for spiking}}{\text{mg soil used in DNA extraction}}\right)$$

(Moein *et al*., 2019).

*Root phosphite extraction and quantification*
Phosphite was extracted and quantified from roots as previously described (McLeod *et al*., 2018).

**Rootstock trials**
*Orchard selection and trial layout*
The rootstock trial was conducted in one orchard (GL) in the Tzaneen region which is known to have had a high PRR disease pressure and where phosphonates have not been applied. Each rootstock was replicated five times in a completely randomised block design, with each replicate containing five trees. One or two trees were randomly selected from each of the Dusa® and R0.06 rootstock replicates, thus resulting in a total of eight trees selected per rootstock. All rootstocks were grafted with Hass® and trees were approximately 4 years old. At the time of sampling, the trees had a disease severity score of 0 to 2, based on the Ciba-Geigy tree health rating scale. The Ciba-Geigy scale ranges from 0 (healthy tree) to 10 (dead tree).

*Root sampling and Pc quantification*
Roots were sampled from each tree over three sampling months (November 2017, March 2018 and May 2018). DNA extraction and qPCR quantification were conducted as described for the phosphonate trials.

**Statistical analysis**
For the phosphonate trial data, analyses of variance (ANOVA) was performed on the root phosphite concentrations, Pc root DNA concentrations and Pc soil DNA concentrations using the GLM (General Linear Models) Procedure of SAS statistical software (Version 9.4; SAS Institute Inc., Cary, USA). For the rootstock trial, ANOVA was also performed on the Pc root DNA concentrations. Pearson's correlation analyses and the significance of correlations were conducted on the root phosphite concentrations and Pc DNA concentrations (root and soil) using XLStat (Version 2014; Addinsoft, New York, USA).

**RESULTS**
**Phosphonate trials**
*Phytophthora cinnamomi root qPCR quantification*
Levene's test for homogeneity showed that there was a significant difference ($P$ = 0.0008) in variance between the data of the three orchards for the Log (x + 0.01) transformed Pc root DNA concentrations. Therefore, a weighted analysis was conducted. ANOVA analysis showed that the Pc root DNA concentrations did not differ significantly between the treatments ($P$ = 0.3361).

*Phytophthora cinnamomi soil qPCR quantification*
Levene's test for homogeneity showed that there was no significant difference ($P$ = 0.3337) in variance between the data of the three orchards, for the Log (x + 0.00001) transformed Pc soil DNA concentrations. Therefore, a weighted analysis was not required. ANOVA analysis showed the three treatments did not differ significantly from each other in Pc soil DNA concentrations ($P$ = 0.4422).

*Root phosphite quantification*
Levene's test for homogeneity showed that there was a significant difference ($P$ = 0.0011) in variance between the data of the three orchards for the Log (x + 1) transformed root phosphite concentration data. Therefore, a weighted analysis was conducted.

ANOVA analysis showed that there was a significant orchard x treatment x month interaction for the root phosphite concentrations ($P$ = 0.0492). This interaction was thus investigated further. For all three orchards and for both sampling months (May and October), the 2x trunk injection treatment yielded significantly higher root phosphite concentrations (19.06 to 57.11 µg/$g_{FW}$) than the untreated control (1.36 to 30.55 µg/$g_{FW}$) (Table 1). In contrast, the 1x trunk injection treatment, for two of the orchards (EL and FM), did not differ significantly from the untreated control at either sampling month. In the third orchard (BL), this was only true for the May sampling month and not for October. The root phosphite concentrations of the 2x trunk injection treatment did not differ significantly from the 1x trunk injection treatment (19.71 to 50.31 µg/$g_{FW}$) for either of the sampling months for the EL and FM orchards. However, for the BL orchard, for both sampling months, the 2x trunk injection treatment yielded significantly higher root phosphite concentrations than the 1x trunk injection treatment (Table 1).

*Correlation analyses between Phytophthora cinnamomi quantities and root phosphite concentrations*
Only one significant correlation was observed between the three measured parameters (root phosphite, Pc root DNA and Pc soil DNA) in the phosphonate trials. A highly significant negative (r = -0.348; $P$ = 0.003) correlation was found between the root phosphite concentrations in May and the May Pc root DNA concentrations, indicating that as root phosphite increased, the Pc DNA in roots decreased. However, no significant correlation existed between the root phosphite concentrations in October, and the October Pc root DNA concentrations (r = -0.071; $P$ = 0.556). No significant correlations were found between Pc root DNA and Pc soil DNA concentrations for either sampling month; May (r = -0.034; $P$ = 0.780) and October (r = 0.129; $P$ = 0.280). There were likewise no significant ($P \geq 0.100$) other correlations between the three investigated parameters when compared in all possible combinations.

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**Table 1.** Effect of phosphonate treatments on root phosphite concentrations over two sampling months (May and October) in three avocado orchards.[a]

| | Orchard BL | | Orchard EL | | Orchard FM | |
|---|---|---|---|---|---|---|
| Treatment [b] | May'18 | October'18 | May'18 | October'18 | May'18 | October'18 |
| Control | 8.84 gh | 1.36 i | 19.06 def | 12.26 fgh | 30.55 bcd | 21.22 def |
| 1x trunk injection April'18 | 14.00 h | 12.47 h | 32.22 bcd | 19.71 def | 50.31 ab | 22.40 cde |
| 2x trunk injection Nov'17, Apr'18 | 21.38 de | 19.16 efg | 41.15 abc | 28.29 cde | 57.11 a | 41.20 abc |

[a] Values in columns and rows followed by the same letter do not differ significantly according to Fisher's least significant difference test ($P \geq 0.05$). Post-hoc analysis was conducted on Log (x + 1) transformed root phosphite data. The actual root phosphite concentrations ($\mu g/g_{FW}$ roots) are shown. Values represent the average of eight replicates per treatment, with each replicate consisting of one tree.

[b] The 1x trunk injection treatment involved the application of one phosphonate trunk injection in April 2018 (after the summer foliar flush had hardened off), whereas the 2x trunk injection treatment consisted of phosphonate trunk injections applied in November 2017 (after the spring foliar flush had hardened off) and in April 2018. Trunk injections were all applied at the preventative dosage of 0.3 g a.i./$m^2$.

**Rootstock trials**

*Phytophthora cinnamomi root qPCR quantification*

The May 2018 sampling month yielded little to no Pc root DNA concentrations for either rootstock (Dusa® [0.00917 ng/$mg_{DW}$] and R0.06 [0 ng/$mg_{DW}$]), and many replicates furthermore contained no Pc root DNA. Consequently, there was no significant difference ($P$ = 0.3506) between the two rootstocks for May. There was also no significant difference ($P$ = 0.3995) between the Pc root DNA concentrations obtained from the Dusa® (0.08325 ng/$mg_{DW}$) and R0.06 (0.05410 ng/$mg_{DW}$) rootstocks in the March 2018 sampling month. However, for the November 2017 sampling month, there was a significant difference ($P$ = 0.0793) at the 90% confidence interval between Pc root DNA concentrations. There was a tendency for the Dusa® (0.49580 ng/$mg_{DW}$) rootstock to yield higher Pc root DNA concentrations than the R0.06 (0.14003 ng/$mg_{DW}$) rootstock.

**DISCUSSION**

The current study showed that qPCR quantification of Pc in avocado roots and rhizosphere soil, using the *Ypt*1 and multiplex *Ypt*1/EIPC assays, respectively, were able to quantify the pathogen in avocado roots and soil. Although the methods were unable to reveal significant differences in the effect of management strategies (phosphonate and rootstock) in trees that were mainly asymptomatic, it might have potential to do so in symptomatic orchards. This is especially true for phosphonate trials, since a significant negative correlation was found between Pc root DNA quantities and root phosphite quantities.

Our study showed that in the phosphonate trials, based on Pc DNA concentrations obtained from roots in two different months (May and October), 1x or 2x preventative phosphonate trunk injections (0.3 g a.i./$m^2$) were unable to suppress the pathogen. *Phytophthora cinnamomi* root DNA concentrations from trees that received 1x trunk injection (after the summer foliar flush had hardened off) or 2x trunk injections (after the summer and spring foliar flushes had hardened off) did not differ significantly from the untreated control. This was surprising, since the 2x trunk injection treatment yielded significantly higher root phosphite concentrations than the untreated control and would thus have been expected to reduce Pc root colonisation. The importance of root phosphite concentrations as being indicative of Pc suppression was also suggested by the significant negative correlation that was found between root phosphite concentrations and Pc root DNA concentrations for the month of May. The 2x trunk injection treatment (0.3 g a.i./$m^2$) was expected to be effective since it is registered in South Africa (Fighter® and Avoguard®) for PRR control. In contrast, the 1x trunk injection treatment was not expected to be effective (reduce Pc DNA concentrations), since it was not applied according to label recommendations and it generally yielded root phosphite concentrations that did not differ significantly from the untreated control.

The effect of phosphonate treatments on Pc soil population levels was also investigated in the current study to determine whether phosphonate treatments can reduce soil inoculum build-up. Since a high variability in PCR inhibitors and the efficacy of DNA extractions can occur between different soil samples (Daniell *et al.*, 2012), a multiplex qPCR assay was developed to allow for the relative quantification of Pc from soil samples. In the current study, the quantification of Pc from soil samples using the multiplex *Ypt*1/EIPC qPCR assay was unsuccessful, since very low concentrations were detected, including samples taken from the untreated control trees. The difficulty in quantifying Pc from soil is likely due to the erratic nature of Pc distribution within soil (Pryce *et al.*, 2002), its naturally low occurring soil population levels (Hendrix and Kuhlman, 1965; Eden *et al.*, 2000) as well as the small quantities of soil that can be analysed with commercial soil DNA extraction kits (Sena *et al.*, 2018). The poor performance of Pc soil DNA extractions has likewise been reported in another study (Sena *et al.*, 2018). Sena *et al.* (2018) were unable to detect Pc soil propagules using DNA-based methods, despite positive detections being reported for soil baiting culture methods.

The roots of the Dusa® rootstock tended to yield higher Pc root DNA quantities than the R0.06 rootstock in November 2017. This can be expected, since a reduction in Pc root colonisation has previously been associated for PRR-tolerant rootstocks (Engelbrecht *et al.*, 2013) and it has been suggested that R0.06 is more PRR-tolerant than Dusa® (Van Rooyen, 2017). Data from the current study thus supports the theory that the R0.06 rootstock may have a greater PRR tolerance than Dusa®. However, lower Pc root colonisation levels do not always equal a greater PRR tolerance, since rootstocks with a high root regenerative ability can compensate for root damage caused by Pc (Kellam and Coffey, 1985). The lower Pc root colonisation levels associated with the R0.06 rootstock may be due to a reduction and/or delay in zoospore germination (i.e. decreased pathogen infection rate). In addition, during early stages of infection, strong activation of β-1,3-glucanase and deposition of impermeable callose at the site of host plant cell penetration can occur (Van den Berg *et al.*, 2018).

In conclusion, qPCR analyses of avocado root- and soil samples may have potential for assessing the efficacy of management strategies in PRR symptomatic orchards. From a disease management strategy perspective, investigating soil inoculum is important for orchard replant situations and further work would thus be required to investigate useful soil quantification techniques. It will also be important to conduct long-term studies on the effect of phosphonate treatments on soil inoculum, since this might only become evident after several seasons. From the current study, it was noticeable that the Pc DNA concentrations were highly variable in both root and soil samples. Therefore, it might be useful for future studies to use a trial design where a larger number of trees are assessed within replicates, in order to compensate for this high variability observed in qPCR pathogen quantification datasets. Analysis should also be conducted in PRR symptomatic orchards to determine whether the current failure of qPCR analyses to differentiate treatment effects is due to Pc inoculum levels being too low in the investigated orchards.

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