The temporal nature of *Phytophthora cinnamomi* colonisation in avocado roots – Preliminary report

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ABSTRACT

The devastating effects of avocado root rot, caused by *Phytophthora cinnamomi*, are well known. However, the temporal nature of pathogen activity in roots is still poorly understood. This has led to an inability to accurately quantify the pathogen within asymptomatic avocado orchards in order to assess root infection levels. The aim of this study was to determine the time of year in which *P. cinnamomi* root colonisation was most prevalent. Two different sampling strategies (one group of 20 trees per orchard, versus four groups of five trees per orchard) and two different DNA extraction scales (small versus large) were evaluated to determine the best method for reliable quantification. *Phytophthora cinnamomi* was quantified from roots using quantitative real-time PCR in six asymptomatic orchards located in two climatically different regions (Mooketsi and Letaba) at four time points (March, May, August and November 2017). Three orchards (A, E and F) had low pathogen root DNA concentrations in March, which increased in May, persisted or decreased in August, and then decreased further in November. Orchards B and D followed a similar pattern, except that in orchard B pathogen root DNA concentrations peaked in August, while in orchard D pathogen root DNA concentrations peaked in November. Orchard C had a somewhat different temporal nature in pathogen root colonisation, since its highest pathogen root DNA concentration was in March, with an increase occurring in November. Irrespective of differences in the temporal nature of pathogen colonisation among the six orchards, May was identified as the best time of year to sample and quantify *P. cinnamomi* in both of the climatic regions, since two orchards peaked in pathogen root DNA concentration in May, while the rest of the orchards had their second highest pathogen root DNA concentration in May. The small scale root DNA extraction (50 mg dry weight) yielded slightly higher root DNA concentration of *P. cinnamomi* than the large scale root DNA extraction (2.5 g dry weight); however, results must still be analysed statistically to determine if these differences will be significant. Evaluation of the two orchard sampling strategies showed that they were comparable, i.e. the one group of 20 trees did not consistently yield lower or higher pathogen root DNA concentrations than when four groups of trees (each containing five trees) were analysed separately.

INTRODUCTION

Avocado root rot, caused by *Phytophthora cinnamomi*, is a highly destructive disease that affects avocado production worldwide. The highly destructive nature of *P. cinnamomi* can mainly be attributed to its asexual life cycle (i.e. zoospore production), which has a short generation time and high reproductive capacity (Shea and Broadbent, 1983). Little is known about the temporal nature of zoospore production and infections, colonisation of roots by *P. cinnamomi* and release of pathogen inoculum into the rhizosphere soil in avocado orchards. The aforementioned aspects have been studied to some extent by Zentmyer (1981), who showed that in California, *P. cinnamomi* can infect avocado roots for at least nine months of the year, but that infection and colonisation were highest in summer and autumn.

A few environmental soil conditions and host factors are known to influence *P. cinnamomi* infections and colonisation (Zentmyer, 1981). Optimal soil conditions for pathogen infection include high moisture levels (i.e. over irrigation or poor drainage) and cool to warmer temperatures of 15-27 ºC, with temperatures between 21-27 ºC being most optimal

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(Zentmyer, 1980; 1981). *Phytophthora cinnamomi* can survive and infect at temperatures between 27-33 ºC, although these are not optimal (Roth and Kuhlman, 1966). It has been shown that increases in soil temperature above 27 ºC, which favours host plant development, may result in an improved tolerance of the host to pathogen infections (Zentmyer, 1980); therefore, fewer pathogen infections would be expected during the summer season. Another host plant factor that influences infection is the occurrence of root growth, since *P. cinnamomi* zoospore root attractions and infections are most prevalent at vigorously growing and absorbing roots. Zoospores mostly infect at the root elongation zone (i.e. root tips) (Zentmyer, 1980). This suggests that avocado root flush windows would also have an influence on pathogen behavioural patterns, provided that soil moisture and temperature conditions are favourable.

Phytophthora cinnamomi can be quantified in avocado roots through root DNA extractions and subsequent quantitative real-time PCR (qPCR) analyses (Engelbrecht *et al*., 2013). qPCR is beneficial in that it is very sensitive, species-specific, quantitative and less labour intensive than conventional isolation studies (Martin *et al.*, 2000; Li *et al.*, 2008). As of yet, no study has used this approach to study the temporal nature of *P. cinnamomi* colonisation in avocado roots under orchard conditions. Engelbrecht *et al.* (2013) conducted a temporal root infection study of two avocado rootstocks under laboratory conditions, but their qPCR assay was later reported as not being specific towards *P. cinnamomi* (Kunadiya *et al.*, 2017). Aside from the importance of using a *P. cinnamomi* specific qPCR assay, it is also important to determine the weight of roots that is optimal for pathogen quantification, i.e. whether a small scale root DNA extraction of 50 mg dry root weight can represent pathogen colonisation levels within avocado orchards as accurately as a large scale root DNA extraction that uses 2.5 g dry root weight. There is also a lack of cost-effective and accurate orchard sampling strategies available to farmers that could allow the monitoring of pathogen root colonisation levels.

The aim of this study, funded by SAAGA, was to determine the time of year in which *P. cinnamomi* root colonisation was most prevalent in asymptomatic orchards. This will assist in determining when the best time of year will be to test orchards for the presence of the pathogen. Furthermore, knowledge on when the pathogen is most active in roots can also help to optimise management strategies. For example, when using phosphonates for managing the disease, optimal root phosphite (active compound of phosponates in plants) concentrations will have to be maintained during periods of the year when the pathogen is most active. qPCR analyses of roots were used to assess pathogen colonization in the current study. Two different orchard sampling strategies (one group of 20 trees per orchard versus four groups of five trees each per orchard) and two DNA extraction scales (small versus large) were also evaluated to determine the best method for reliable quantification.

MATERIALS AND METHODS

Orchards sampled and sampling strategy

Six asymptomatic orchards were selected in January 2017 from two climatically different regions. Three orchards were located in the warmer and drier Mooketsi region (A, C and F). The other three orchards were located in the cooler and wetter Letaba region (B, D and E). Both of the regions have a summer rainfall. All orchards were not treated with phosphonates for the duration of the sampling. Orchards A, D, E and F were established on Duke 7® rootstocks, while orchards C and B were established on Dusa® rootstocks. Twenty trees were randomly selected from each orchard at a rate of 20 trees/ha, thus resulting in a total selection of 120 trees across the six orchards. Feeder roots, a mixture of white and suberised roots, were sampled from the same 20 trees per orchard at four different time points, i.e. March, May, August and November 2017; each sampling occurring within the first two weeks of each month. For each orchard, approximately 250 ml of roots were sampled from each of the 20 trees, which were pooled using two different approaches. In the first approach, the roots of five trees were pooled into one sample, resulting in four pooled samples per orchard, hereafter referred to as the 4x5 sampling group. In the second approach the roots of all 20 trees were pooled into one sample, hereafter referred to as the 1x20 sampling group.

Root DNA extraction and qPCR analyses

DNA was extracted from roots using the NucleoSpin PLANT II kit (Macherey-Nagel Gmbh and Ko, Duren, Germany) with slight modifications to the manufacturer's instructions. A small DNA extraction (50 mg dry weight) was performed on the 4x5 sampling group for each orchard at each time point. The August time point was selected to compare small DNA extractions (50 mg dry weight) to large DNA extractions (2.5 g dry weight) for the 4x5 sampling group of each orchard. The same time point was also selected to compare the 4x5 and 1x20 sampling groups of each orchard using large DNA extractions (2.5 g dry weight).

Pathogen DNA was quantified from the extracted root DNA using an optimised qPCR assay targeting the *Ypt*1 gene region. The primers and probes targeting the *Ypt*1 region were designed in a previous study and were shown to be specific to *P. cinnamomi* (Masikane, 2017). Each qPCR test included a positive control (known *P. cinnamomi* concentration) and negative control (dH_2O). PCR reaction conditions were as described by Masikane (2017). Amplification conditions consisted of a hot-start of 5 min at 95 ºC for one cycle, followed by cycling at 95 ºC for 10 sec and then annealing for 60 ºC with the extension of 40 sec. Pathogen root DNA concentrations (ng/reaction) were calculated by importing a previously established *P. cinnamomi* standard curve.

RESULTS

Root DNA extraction and qPCR analyses

Orchard A, E and F had low initial pathogen root DNA concentrations in March and increases in pathogen root DNA concentrations in May. Thereafter pathogen root DNA concentrations either persisted or decreased in August and then decreased in November (Fig. 1). Orchard B and D followed a similar pattern to orchards A, E and F, except that orchards B and D had pathogen root DNA concentrations peaking in August and November, respectively (Fig. 1). Orchard C had a pathogen colonisation pattern that differed the most from the other five orchards, since its highest pathogen root DNA concentration was in March with an increase in pathogen root DNA concentration again in November. However, its May and August pathogen root DNA concentrations were comparable to those of orchards A, D, E and F (Fig. 1). Although orchards A and E were the only two orchards with their highest pathogen root DNA concentrations in May, orchards B, C, D and F all had their second highest pathogen root DNA concentrations in May (Fig. 1). There were no obvious differences between orchards in their temporal pathogen colonisation patterns when comparing the two climatically different regions or two rootstocks (Fig. 1).

Little variation was found between the *P. cinnamomi* root DNA concentrations obtained from the small and large scale root DNA extractions, which were evaluated using the 4x5 sampling strategy for each orchard in August 2017 (Fig. 2). However, the small root DNA extraction performed slightly better than the large root DNA extraction in that it detected a higher root pathogen DNA concentration in four out of the six orchards. The results must still be analysed statistically to determine whether these differences are significant. Considering the incidence, i.e. how many of the four tree groups within an orchard contained the pathogen, there were also no obvious differences between the large and small scale root DNA extractions. The small root DNA extraction in two orchards had a higher incidence than the large extraction, whereas for the two other orchards the opposite was true. The remaining two orchards had similar pathogen incidences among the four tree groups for the large and small root DNA extractions (Fig. 2).

A comparison of the two orchard sampling strategies, i.e. 4x5 and 1x20 sampling groups, did not reveal a consistent trend in that the one approach was better than the other in quantifying *P. cinnamomi* in roots (Fig. 3). *Phytophthora cinnamomi* root DNA concentrations using the

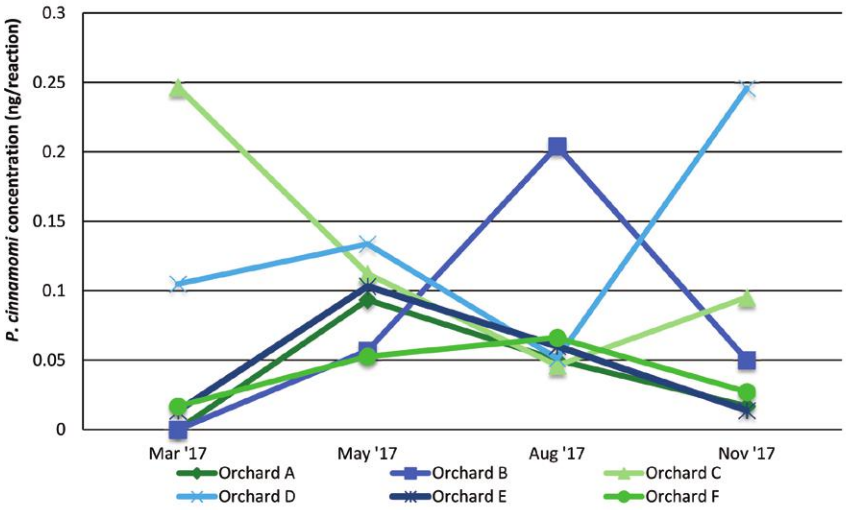

Figure 1: *Phytophthora cinnamomi* root DNA concentrations in six asymptomatic avocado orchards over four time points (March, May, August and November 2017). Each line graph point represents the average of 20 trees, which were analysed as four separate groups, each containing five trees (4x5 sampling group). From each tree group, DNA was extracted using a small scale root DNA extraction method (50 mg dry weight), followed by real-time PCR amplification with a *P. cinnamomi* specific assay. Orchards that were situated in the Letaba region are represented by lines that are shades of blue, and those in the Mooketsi region by lines that are shades of green.

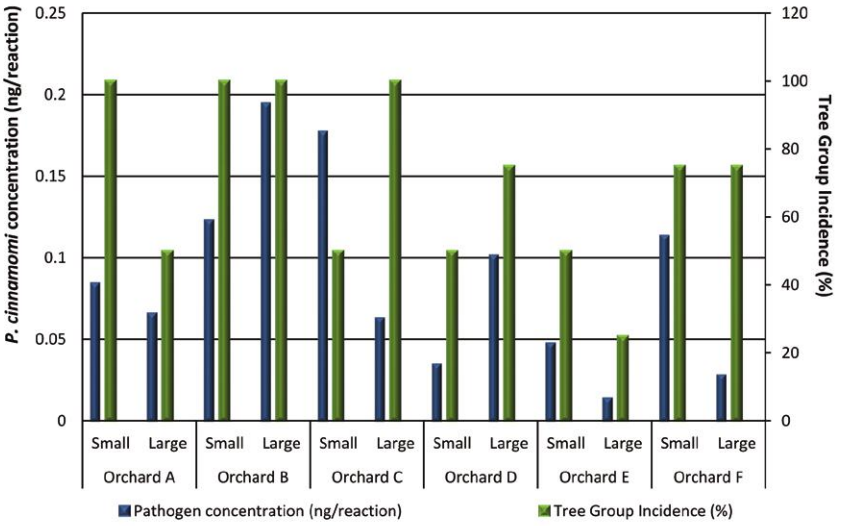

Figure 2: *Phytophthora cinnamomi* root DNA concentrations when comparing small (50 mg dry weight) versus large (2.5 g dry weight) root DNA extractions using the 4x5 sampling group strategy in six asymptomatic avocado orchards that were sampled in August 2017. The tree group incidence represents the percentage of the four tree groups (five trees each) within an orchard that tested positive for *P. cinnamomi* in real-time PCR assays. The *P. cinnamomi* concentrations are the average of the four tree groups per orchard.

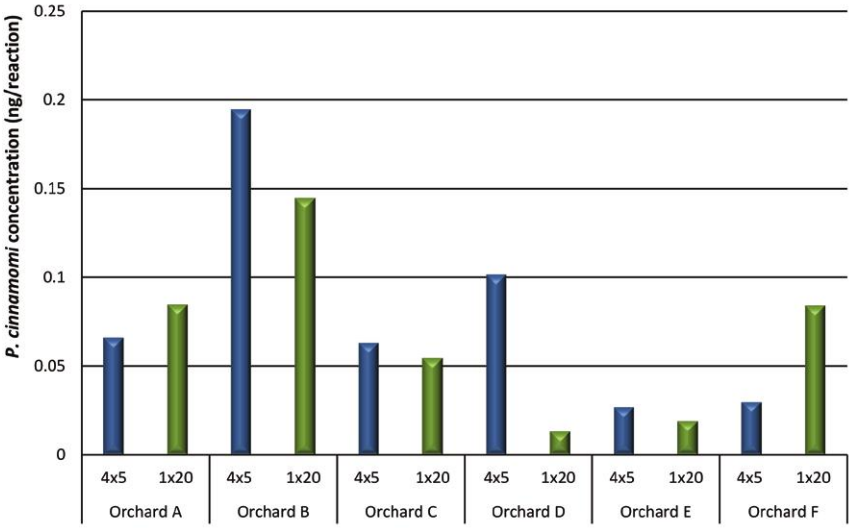

Figure 3: *Phytophthora cinnamomi* root DNA concentrations when comparing two orchard sampling strategies that consisted of analysing 20 trees per orchard either as four groups of trees (each containing five trees) (4x5 sampling group) or as one group of 20 trees (1x20 sampling group) in six asymptomatic orchards in August 2017. *Phytophthora cinnamomi* concentrations for the 4x5 sampling group represents the average of the four groups.

two approaches were comparable for orchards A, B, D and E. However, orchards D and F showed considerable differences between the 4x5 sampling and 1x20 sampling group, i.e. differences greater than double the lowest DNA concentration.

DISCUSSION

Three of the studied orchards had a similar pattern in the temporal variation of *P. cinnamomi* root DNA concentrations. This involved the highest or the second highest pathogen root DNA concentrations being in May and not in March. This was unexpected, since February to April follows the high summer rainfall period and also hosts one of the root flush windows that is present for most of the cultivars grown in the two analysed production regions (Letaba and Mooketsi). Pathogen concentrations were therefore expected to be the highest during March. However, it is possible that the initial *P. cinnamomi* infections in March would only be reflected as very low pathogen root DNA concentrations, with concentrations increasing from March to May as the pathogen colonised the roots following the initial infections. Alternatively, high soil temperatures experienced during the summer months may have hindered pathogen activity leading up to March. Although May falls under the lower rainfall season, irrigation practices ensure that water is not a limiting factor for *P. cinnamomi* activity (Zentmyer and Richards, 1952). Therefore, sufficient soil moisture along with cooler soil temperatures leading up to May may have created a more conducive environment for pathogen infection and colonisation (post root flush infection). It is furthermore likely that as fruit matures on trees and harvest occurs from March onwards, trees become more stressed, which makes the host more susceptible to pathogen infection and colonisation. Altogether, these factors could explain the higher overall pathogen root DNA concentrations found in the two orchards in May, and the other four orchards having their second highest pathogen root DNA concentrations in May.

The temporal analyses of *P. cinnamomi* colonisation in avocado roots in the current study, suggests that May is the best month for quantification of the pathogen in the Letaba and Mooketsi region in asymptomatic orchards. This is due to the fact that orchards A and E had their peak pathogen concentrations in May, while orchards B, C, D and F all had their second highest pathogen concentration in May.

A general decrease in pathogen root DNA concentrations was observed in August and November. For the August quantifications, this might be due to soil temperatures leading up to August being too cold for pathogen activity (i.e. July being coldest month), thus causing a hindrance in further pathogen development. Roots previously infected with *P. cinnamomi* may have also become brittle and started breaking away from the root system (Zentmyer, 1980) so that the pathogen could no longer be detected in roots attached to the trees that were sampled for DNA quantification in August. Although November hosts the second root flush window as well as higher rainfalls that all favour pathogen infection and colonisation, warmer temperatures leading up to sampling may have hindered pathogen activity. Also, similar to March, the initial November infections would have resulted in low root DNA concentrations being detected in roots. A previous study showed that infested soils had their highest concentrations of *P. cinnamomi* during the summer season (Marks *et al.*, 1973). This supports the idea that disease progression and root loss may be at its highest in November, resulting in the release of inoculum back into the soil, and thus resulting in the reduction of root DNA pathogen concentrations in roots still attached to trees.

It was interesting to note that orchard C had the highest recorded pathogen concentration of 0.2467 ng/reaction, despite having been established on the more tolerant Dusa® rootstock. This however was expected, since this was the only orchard where trees started showing visible above ground foliar symptoms due to root rot in September 2017. This may also explain why the temporal nature of *P. cinnamomi* in tree roots in this orchard differed substantially from the other five orchards that remained asymptomatic above ground.

The small scale root DNA extraction performed slightly better than the large scale root DNA extraction. However, there was a

lack of evidence suggesting that one root DNA extraction scale was more advantageous over the other. This indicates that a small root DNA extraction of 50 mg dry weight can still accurately represent pathogen colonisation within an orchard while remaining cost effective and having a higher throughput than large extractions.

The two sampling strategies, 4x5 sampling group and 1x20 sampling group, yielded comparable *P. cinnamomi* root DNA concentrations for four of the orchards. The reason why the two sampling strategies yielded markedly different results for the remaining two orchards is unknown. Since there was a general trend that the 1x20 sampling group yielded similar or higher pathogen root DNA concentrations than the 4x5 sampling group, the 1x20 sampling group could preferentially be used as it is more cost effective and less labour-intensive. This will, however, require a large root DNA extraction scale of 2.5 mg of roots.

Future research will involve obtaining a second year of data for 2018 in order to make reliable conclusions as to when *P. cinnamomi* colonisation is most prevalent in avocado orchard roots. Soil probe temperature and moisture data will also be analysed to determine whether certain fluctuations in soil moisture levels and soil temperatures during the year can be linked with pathogen concentrations found in the roots of each orchard. Root baiting followed by the plating of baits onto semi-selective media will also be included as another method for monitoring pathogen colonisation levels. This quantification approach will reduce the cost of pathogen quantification, and make the testing of orchards feasible for laboratories that do not have qPCR facilities. Ultimately the information obtained in this study will help to determine (i) which time of the year will be best for testing asymptomatic orchards for the presence of the pathogen, and (ii) whether there is a specific time of the year when it will be very important to ensure that root phosphite concentrations must be above the critical value required for suppression of the pathogen.

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