Microbial biomes of South African avocado fruit

F Viljoen[1], M Bill[1], Z van Rooyen[2], P Novela[3], L Chidamba[1] and L Korsten[1]

[1]Department of Plant and Soil Sciences, University of Pretoria
[2]Westfalia Research Technologies, Westfalia, Tzaneen
[3]Research and Development, ZZ2, Mooketsi

E-mail: lise.korsten@up.ac.za / francoisfviljoen@gmail.com

ABSTRACT

The fungal diversity associated with 'Hass' avocados from flowering to the ready-to-eat stage was investigated during the 2017/18 season at a farm in Limpopo, South Africa. The study aimed at understanding surface and endophyte microbiome dynamics with particular emphasis on the presence of the pathogenic genera *Colletotrichum* and *Botryosphaeria* spp.. Pyrosequencing analysis of fungal communities targeting the ITS1-4 region, detected sequences belonging to the genus *Colletotrichum* in two surface microbiome samples at the flowering stage i.e. *Colletotrichum gloeosporioides* (0.03%) and at fruit-onset as *Colletotrichum* sp. (0.23%), and one fruit endophyte sample after postharvest processing (0.11%) i.e. *Colletotrichum acutatum.* The genus *Botryosphaeria* was detected in all samples with abundances ranging from 0.07% to 0.43%. The highest abundances were detected after prochloraz treatment (*Botryosphaeria* sp. 1.57% and *Lasiodiplodia theobromae* 0.14%) and ready-to-eat fruit five weeks after harvest (*Botryosphaeria* sp. 1.38%). The other *Botryosphaeria* members detected in relatively higher abundances were *Spencermartinsia viticola*, observed in flower surface microbiome (0.11%) and endophytic samples from stem-end at harvest (0.10%) and fruit after two weeks storage at 5°C (0.04%). Postharvest processing appears to be the stage when significant fruit contamination occurs, although pesticides are used and it appears that a significant proportion of fungi may survive and re-establish as pesticide residue levels most likely dropped, something that should be confirmed in future.

INTRODUCTION

The avocado is a popular fruit, mostly due to its highly nutritious and tasty characteristics (Dreher and Davenport, 2013). Most of the avocado (60%) produced in South Africa is exported mainly to European countries which have stringent fruit quality standards. The postharvest quality of avocados is affected by pre-harvest fungal pathogens that result in postharvest development of fruit symptoms at the market end of the supply chain. Among the major postharvest diseases that cause serious losses during export, is anthracnose caused by *Colletotrichum gloeosporioides* Penz. and stem-end rot (SER) by *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. (Griffon, 1909), *Dothiorella aromatica* (Sacc.) Petr. and Syd. (Petrak, 1927), *Thyronectria pseudotrichia* (Schw.) Seeler (Seeler, 1940), and / or *Phomopsis perseae* Zerova (Schaffer *et al.*, 2013; Coates *et al.*, 2002; Zerova, 1940; Darvas and Kotze, 1987; Diskin *et al.*, 2017).

Limited control of anthracnose disease has been reported with monthly applications of copper oxychloride sprays (Dann *et al*., 2013). However, copper sprays have potential adverse effects on the environment, because of the risk of accumulation that may lead to arable soil deterioration (Rowell, 1983). In addition, the need for alternative strategies are driven by a complex regulatory environment, the ever widening spectrum of diseases and industry and consumer demand for commercial viable safe quality products (Wisniewski *et al.*, 2016). Pressure from global markets has renewed interest in the search for alternative control methods due to a growing consumer interest in safe food that is chemical free and produced in an environmentally safer and sustainable way (Naamani, 2011). Pesticide usage in orchards has also been linked to the development of fungicide resistant strains of postharvest pathogens (Droby and Wisniewski, 2018; Jamalizadeh *et al.*, 2011; Droby *et al.*, 2009; Sharma *et al.*, 2009).

The focus for future avocado production, given the changing global regulations with regards to pesticide use, weighs heavily on the need to develop more holistic approaches in agricultural research. Investigating the microbiomes in agricultural production systems present new opportunities to approach disease control in a more holistic way. The first observations made on microbiome significance were based on the link between plant health and degree of production that reflect the interaction with microbial communities in the soil and their complex diversities (Hartmann *et al.*, 2008). These interactions influence the outcomes of the host plant's defence responses, health (Droby and Wisniewski, 2018; Vandenkoornhuyse *et al.*, 2015) and plant productivity (Lebeis *et al.*, 2012; Weyens *et al.*, 2009).

The composition and resilience of these communities are dependent on variables such as climate, region, site as well as host species or plant cultivar (Chou *et al.*, 2018).

The majority of the plant microbiome and its contribution to the host phenotype are not yet clearly understood (Turner *et al.*, 2013). It has been hypothesised that the plant microbiomes are greatly influenced by the host genome and these communities should be considered an extension of the plant genome or "pan-genome" (Turner *et al.,* 2013). Studies on other tree crops, such as mango (Diskin *et al.*, 2017), have made direct links between the endophytic communities and disease incidence.

The advent of modern technologies, including next generation and associated microbe identification platforms, has opened research domains in microbial ecology, rapid disease detection and ecological approaches to disease control. The current popular trend is to study the whole plant microbial biome, to unravel the pathogenic components in the population and the associate interactions. The aim of this study was to characterise microbial communities associated with avocado fruit developmental stages and to follow the postharvest supply chain up to the ready-to-eat stage with particular emphasis on pathogens causing anthracnose and stem-end rot. Findings from the study will be beneficial in formulating new innovative disease control strategies.

METHODS AND MATERIALS

Study site, sample collection and processing

The experiment was conducted at an avocado farm in Limpopo Province during the 2017/18 production season. A total of 27 'Hass' avocado trees were selected in a randomized block design. In total 81 fruit from 27 avocado trees were collected. This consisted of three fruit per tree sampled at the top, middle and lower regions of the tree canopy. The samples were collected at different stages of production i.e. at the flowering (full bloom), fruit set, after the first copper spray, at harvest, after prochloraz dip, after two weeks cold storage (5.5°C), four weeks cold storage and after seven days room temperature (~20°C). The trial was designed to be a first stage scoping study to map the avocado microbiome from flowering to the postharvest stages.

Sample processing and DNA extraction

For epiphytic microflora, the collected samples were weighed in triplicates and each of the 25 g samples suspended in sterile peptone buffered water (225 ml), before stomaching. The micro-floral wash was concentrated through cellulose nitrate filters (0.45 mm pore size; Sartorius, Gottingen, Germany) from which DNA was later extracted. Samples (1 ml) of the micro-floral surface wash were also preserved for later use. To process endophyte samples, the whole fruit, stem-ends and stems were processed separately according to Diskin *et al*. (2017). Briefly, the fruit were surface disinfected with 70% ethanol for five minutes and then rinsed three times with sterile water to remove surface microorganisms. The surface disinfected fruit were aseptically peeled at the stem-end with a disinfected peeler and the internal portion of the stem-end portion of the fruit, sampled for analysis. The stems of the same avocado fruit were cut into smaller pieces using a sterile scalpel blade. The sampled material i.e. stem-ends and stems, were immediately snap-frozen in liquid nitrogen and ground with a coffee grinder and stored at -80°C for DNA extractions. The DNA extraction of the total community from fruit pulp or surface washings was carried out with the use of fungal/bacterial Zymo Research kit (ZymoResearch, USA), following the manufacturer's instructions, before storing at -20°C.

Amplification and sequencing

DNA samples were sent for sequencing at MR DNA (Shallowater, TX, USA) and the sequence data from illumina platform were processed using MR DNA analysis pipeline (Shallowater, TX, USA) as previously described (Carmichael *et al.,* 2017). Diversity indices (Shannon, Choa1 and Observed richness) for each sample were calculated on rarefied OTU table using the "Visualization and Analysis of Microbial Populations Structure (VAMPS)" (http://vamps.mbl.edu) online pipeline.

RESULTS

Sequencing results and pathogenic genus abundancies

Sequencing of samples representing different avocado production stages, from flowering to postharvest handling, generated a total of 1374325 ITS sequences from 12 samples after paired-end alignments, quality filtering and deletion of chimeric sequences and singletons. Sequence abundances per sample ranged from 48 176 to 181 838 with averages of 114527. ITS sequences belonging to the genus *Colletotrichum* were detected only in two surface microbiome samples, identified as *Colletotrichum gloeosporioides* at flower (0.03%) and *Colletotrichum* sp. at fruit-onset stage (0.23%). *Colletotrichum acutatum* (0.11%) was detected in one fruit endophyte sample collected after postharvest processing (Table 1).

The genus *Botryosphaeria* was detected in all samples with abundances ranging from 0.07% to 0.43%. Sequences belonging to the genus *Botryosphaeria* were detected in their highest abundance after prochloraz treatment (*Botryosphaeria* sp. 1.57% and *Lasiodiplodia theobromae* 0.14%) and ready-to-eat fruit, five weeks after harvest (*Botryosphaeria* sp. 1.38%). The other Botryosphaeria members detected in relatively higher abundances were *Spencermartinsia viticola* observed in flower surface microbiome (0.11%) and endophytic samples from stem-end at harvest (0.10%) and fruit after two weeks storage at 5°C (0.04%). The remainder of the species observed had prevalence under 0.01% and included *Sphaeropsis sapinea* detected in flower surface microbiome (0.02%) and fruit endophyte at harvest (0.05%), *Auerswaldia dothiorella* (0.01-0.09) and *Phaeobotryosphaeria eucalypti* in stem-end at harvest (0.07%).

Detection of *Botryosphaeria* sp. in endophytic samples showed a gradual decline from 0.43% at harvest to 0.07% in ready-to-eat fruit after five weeks postharvest storage. This represented a relatively lower abundance compared to surface communities. Interestingly, endophyte abundances had about four times (0.43%) higher concentrations before harvest compared to the surface populations, which ranged from 0.01-0.09% (Fig. 1).

The sequences generated from the avocado fungal communities characterising the different developmental stages could be assigned to 873 OUT. Generally surface microbiomes had 72.1% to 78.6% more sequences abundance than endophyte microbiomes, with the exception of ready-to-eat avocados (165 032) and samples collected at harvest (170 803). The highest surface fungal abundances were observed in flower samples (181 838) and ready-to-eat (142 874).

Although the stem-end sample had one of the lowest sequence abundancies observed (48798), they had the highest number of OTUs observed and diversity with Shannon-Weaver diversity indices of 3.73. Surface microbial community diversity were highest at flower (2.34) and fruit onset, but significantly declined with copper spraying and were lowest at harvest. Although postharvest processing enriched surface microbiome diversity communities, they declined after two weeks storage at 5°C, maybe due to residual pesticide effect and cold conditions.

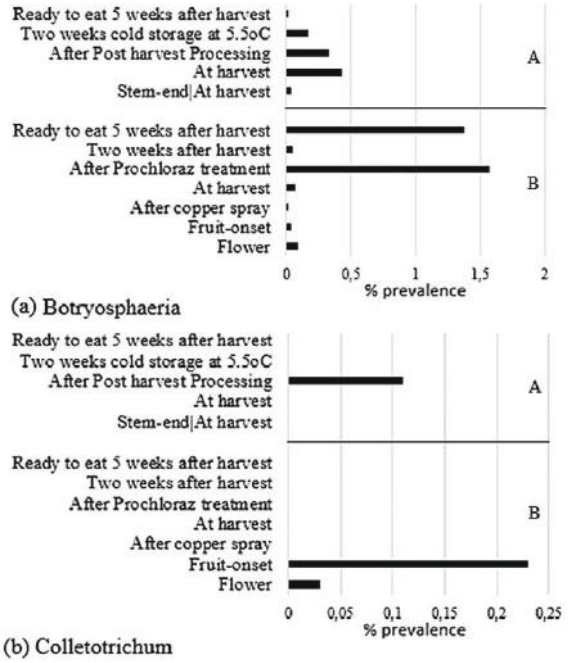

Figure 1. Prevalence of known fungal pathogenic groups in endophyte (A) and surface (B) microbial communities in different avocado fruit development and production stages.

Table 1. Total sequences, OUTs and pathogenic genera abundancies in surface and endophyte microbial communities from different avocado fruit development and production stages.

	Surface microbiome							Endophyte microbiome				
	Flower	Fruit-Set	After copper spray	At harvest	After post-harvest processing	Two weeks after harvest	Ready-to-eat 5 weeks after harvest	Stem-end at harvest	At harvest	After post-harvest processing	Two weeks cold storage at 5.5°C	Ready-to-eat 5 weeks after harvest
Total observed ITS sequences	181838	125557	117102	114650	107393	98238	142847	48798	170803	53891	48176	165032
Total OTU	192	164	85	77	136	65	132	243	96	162	140	62
Botryosperaceae spp. (% prevalence in samples)												
Botryosphaeria sp.	0.09%	0.03%	0.01%	0.07%	1.57%	0.04%	1.38%	0.03%	0.43%	0.33%	0.16%	0.07%
Sphaeropsis sapinea	0.02%	-	-	-	-	-	-	-	0.05%	-	-	-
Auerswaldia dothiorella	0.01%	-	-	-	-	-	-	0.01%	-	-	0.09%	-
Lasiodiplodia theobromae	0.01%	0.01%	-	0.03%	0.14%	-	-	0.26%	0.05%	-	-	-
Spencermartinsia viticola	0.11%	-	-	-	-	-	-	0.10%	-	-	0.04%	-
Phaeobotryosphaeria eucalypti	-	-	-	-	-	-	-	0.07%	-	-	-	-
Colletotrichum spp (% prevalence in samples)												
Colletotrichum gloeosporioides	0.03%	-	-	-	-	-	-	-	-	-	-	-
Colletotrichum sp.	-	-	-	-	-	-	-	-	-	0.11%	-	-
Glomerella acutata	-	0.23%	-	-	-	-	-	-	-	-	-	-

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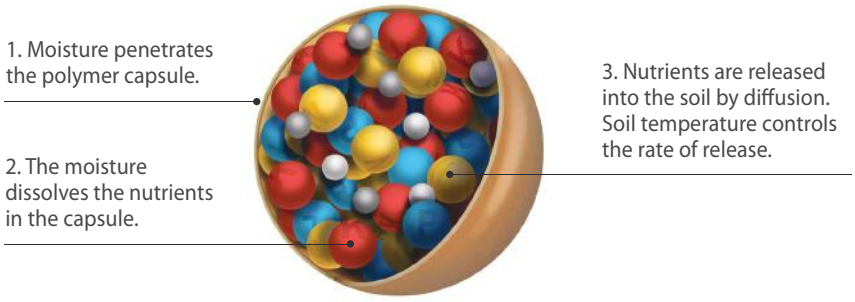

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Microbial community dynamics

Evaluation of the prevalence of different fungal communities showed the phylum Ascomycota to be the most dominant in both surface and endophytic populations of avocado fruit (Fig. 2). The most dominant Ascomycota class on surfaces fungal population was *Dothideomycetes* and constituted more than 50% of the total population during flower (77.05%) and fruit onset (79.3%) stages. Prochloraz treatment reduced the *Dothideomycetes* population from 41.2% to 11.5% when comparing communities after postharvest treatment and two weeks after harvest, although it recovered to above 50% five weeks after harvest on ready-to-eat fruit. In all endophyte samples this class was observed lowest at 50.5% after prochloraz treatment, otherwise it ranged from 82.9% to 96.7% in fruit and was observed at 62% in the only stem-end sample. Fungicide treatment appeared to promote the abundance of the basidiomycetes, *tremellomycetes* and *agaricomycetes* (Fig. 2). The yeast class *Saccharomycetes* was noted at 11.8% after postharvest processing and declined in abundance with storage from two weeks (4.9%) to ready-to-eat fruit at five weeks (3.6%). The Ascomycota, Eurotiomycetes class increased in abundance from flowering (1.79%) and was not affected by prochloraz treatment (25.5%), but declined with storage, going down to (5%) in ready-to-eat fruit after five weeks. Among endophytes, Eurotiomycetes were only abundant in the stem-end sample (8.3%) with lower abundances 2.65% to 0.16% observed in surface microbiomes with increasing storage.

DISCUSSION

The presence of pathogenic signatures was evaluated based on the observed abundance of OTUs belonging to fungi responsible for postharvest diseases of avocado fruit, including *C. acutatum, C. gloeosporioides, Botryosphaeria parva, B. dothidea* and *Phomopsis* (Hartill, 1991). Members of the Botryosphaeriaceae family can cause cankers and fruit rot, and can survive as saprophytes or parasites, with certain species capable of surviving in symptomless tissue as latent infections (endophytes) (Twizeyimana *et al*., 2013). Botryosphaeria signatures were observed in their highest abundance after prochloraz treatment (1.6%) and ready-to-eat fruit five weeks after harvest (1.4%). Although at two weeks after harvest low abundances (0.04%) were observed, and could be attributed to the fungicide treatment or cold storage, the Botryosphaeria population had recovered by the fifth week after harvest and when kept at room temperature for one week.

Detection of the *Botryosphaeria* sp. as endophytes showed a gradual decline from 0.43% at harvest to 0.07% in ready-to-eat fruit after five weeks postharvest storage simulated, which is a relatively lower abundance compared to surface communities. Immediately after postharvest treatment, pesticide concentrations will be prevalent on the fruit surface but will gradually decline as it break down or diffuses into the fruit (Shiea *et al.*, 2015), which explains surface population recovery and the gradual decline in endophytic Botryosphaeria. Interestingly, endophyte abundances had about four times (0.43%) higher concentration before harvest, compared to the sur-

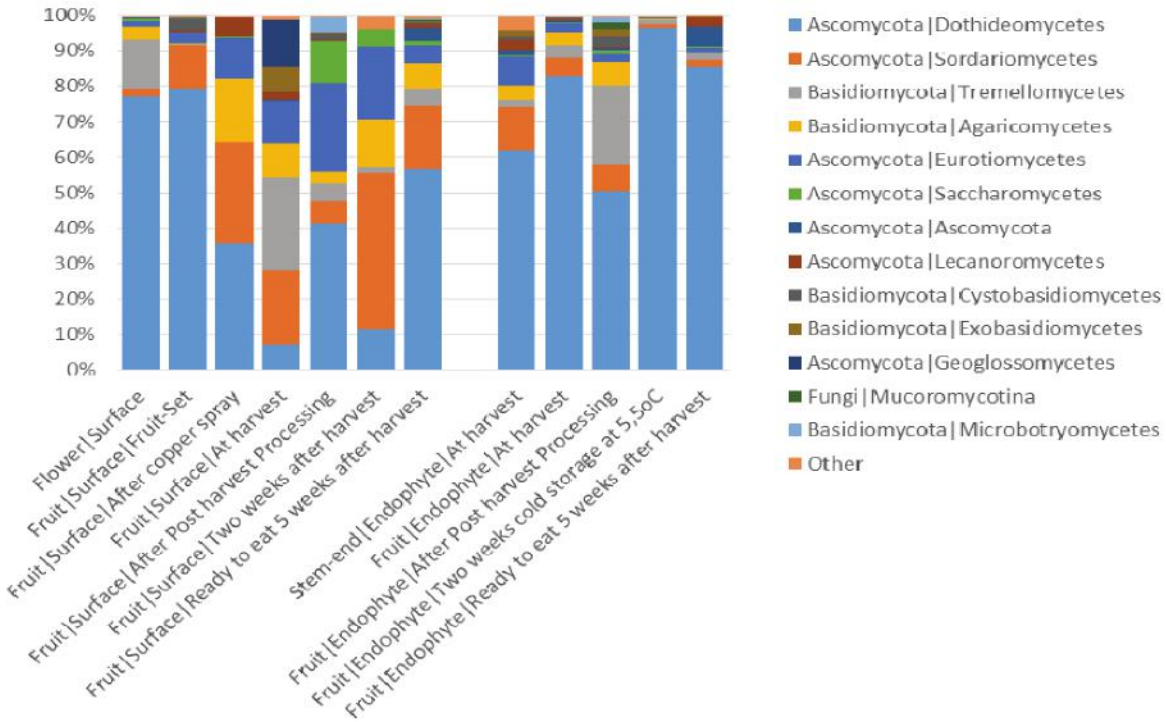

Figure 2. Prevalence of different phylum and their respective classes in endophyte and surface microbial communities in different avocado fruit development and production stages.

face populations which ranged from 0.01% to 0.09%. Although the species have been reported not to damage healthy avocado trees, they are considered as opportunistic pathogens, and their effects can be devastating in fruit where they can quickly recover from pesticide sprays to cause stem-end rot (Slippers & Wingfield, 2007; Pérez-Jiménez, 2008).

Colletotrichum gloeosporioides has previously been reported as the cause of anthracnose in South Africa (Sanders and Korsten, 2003) and Israel (Freeman *et al*., 2000). This pathogen was detected in the flower microbial community at 0.03% abundance in this study. *Colletotrichum gloeosporioides* is a species complex that has been shown to be comprised of 42 accepted species, which cause anthracnose, the most serious postharvest disease in avocados (Weir *et al*., 2012; Damm *et al.,* 2012; Jayawardena *et al*., 2016; Diao *et al.,* 2017; Guarnaccia *et al.,* 2017). Other *Colletotrichum* species detected included *Glomerella acutata* at fruit onset (0.23%) and unidentified *Colletotrichum* sp. in endophyte samples collected after postharvest processing (0.11%). Species reported to cause anthracnose in other avocado-producing countries include *C. aenigma* in Israel, *C. fructicola, C. queenslandicum* and *C. fioriniae* in Australia, *C. alienum* in Australia and New Zealand, *C. kahawae* subsp. *ciggaro* in New Zealand, and *C. siamense* in Australia and South Africa (Weir *et al.,* 2012).

Colletotrichum acutatum (Glomerella acutata) has mostly been reported in Australia and New Zealand where it has been isolated from both stem-end rots and anthracnose of 'Hass' avocados (Hartill, 1991). *Colletotrichum acutatum* spores are thought to infect avocado fruit while hanging on the tree, as they are released from infected dead twigs and fruit in the canopy or orchard floor (Damm *et al*., 2012). The observed abundance at fruit onset and non-detection throughout the development in this study may suggest flowering and fruit onset stages as critical points of control for this pathogen. *Colletotrichum acutatum* has been suggested to be a wound pathogen, and its chances for successful infection maybe linked to damage due to mechanical handling and postharvest processing (Hartill and Everett, 2002).

In conclusion, results of the present study provide a preliminary picture of the fungal diversity associated with avocado fruit development and postharvest handling. The majority of the sequences detected were identified up to genus and/or species level. It was therefore possible to make assumptions regarding their role in avocado production. In particular was the identification of known pathogen sequences and their prevalences during the production cycle. These findings reinforce the importance of investigating fungal biodiversity associated with avocado production and highlight the need for more detailed analyses in this field of study. Moreover, it will be beneficial to explore the presence of natural antagonists of the important avocado pathogens and develop ecological friendly biocontrol approaches based on microbial dynamics and dominance. This data further provides information that is critical in the implementation of future management strategies to combat diseases in avocado production systems.

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