BIOCONTROL OF POSTHARVEST PATHOGENS INFECTING AVOCADO USING ENDOPHYTIC *TRICHODERMA* SPP.

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ABSTRACT

Pre-harvest fungal infections cause both pre- and postharvest avocado diseases. Agrochemicals are being lost to the avocado industry due to EU MRL levels being reduced. This study is on the use of endophytic biocontrol agents to replace agrochemicals to manage these pathogens. Therefore, the objective of this study is to isolate and screen endophytic strains of *Trichoderma* spp. to control key fungal pathogens such as *Colletotrichum, Pseudocercospora*, *Botryosphaeria, Cladosporium* and *Sphaceloma.* The key fungal pathogens were isolated, as well as 17 strains of *Trichoderma* spp. in or on avocado tissues. These were screened for endophytic properties. Ten of the *Trichoderma* isolates were selected as possible biological control agents, based on their endophytic properties. The isolates were tested *in vitro* and *in vivo* using various techniques. Seven of the *Trichoderma* isolates were able to control all the fungal pathogens during *in vitro* screening (at levels of between 70% and 100%). The best strains are now being tested for *in vivo* activity on avocado fruit. These trials are to confirm the potential of endophytic strains of *Trichoderma* spp. to control pre-harvest infections of avocado fruit by key fungal pathogens.

INTRODUCTION

Fungal pathogens are a major cause of plant diseases and result in significant crop losses (Alvarez, 2004). Pre-harvest fungal infections cause both pre- and postharvest avocado diseases (Anjiku *et al.,* 2020). Historically, agrochemicals have been used to manage most avocado plant diseases. However, public sentiment has turned against agrochemicals due to residues in food and the damage that they are perceived to cause to the environment (Majeed, 2018). Therefore, agrochemicals are being lost due to the minimum accepted residue levels (MRLs) being reduced in many countries, including those of the EU, a major market for South African fruit exports (Majeed, 2018). The use of biological control agents (BCAs), such as *Trichoderma* species, has been regarded as a promising and environmental friendly approach to controlling plant diseases (Butt *et al.,* 1999). *Trichoderma* spp. are opportunistic, avirulent plant symbionts and are used as BCAs against plant pathogens due to their multi-mode of action against pathogens, including antibiosis, mycoparasitism, production of antimicrobials, induction of plant defensive mechanisms and promotion of plant growth (Arikit *et al*., 2020). Furthermore, some strains of *Trichoderma* spp. have been found to be symbiotic endophytes, due to their ability to colonise internal plant tissues in leaves, roots and fruits. The combination of biocontrol activity and endophytic nature plays an important role in the defence that they can provide against several pathogens by releasing metabolites that act as antifungal compounds (Arikit *et al*., 2020). Fungal endophytes are found living within the intercellular or intracellular spaces of host plants, without causing apparent disease symptoms, in symbiotic relationships with their host plants (Bae *et al.,* 2019).

The aim of this study was to isolate endophytic strains of *Trichoderma* spp. and to screen them for their potential to control several fruit diseases of avocado caused by key fungal pathogens such as *Botryosphaeria, Cladosporium, Colletotrichum* and *Pseudocercospora*.

MATERIALS AND METHODS

Isolation of pathogens

Fifteen avocado fruits from supermarkets in Pietermaritzburg, KwaZulu-Natal Province, that displayed symptoms of some of the key fungal pathogens, were washed with tap water, surface-sterilised with 2% sodium hypochlorite for one minute, rinsed 3 times in distilled water and air-dried. Small pieces of symptomatic flesh were cut and placed on Potato Dextrose Agar (PDA) medium and incubated at 25 °C for 3 to 7 days. After that, fungal isolates

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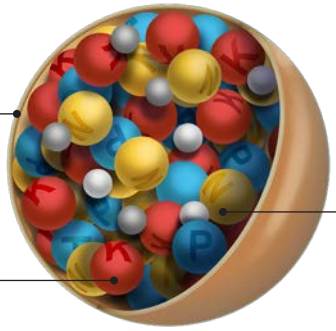

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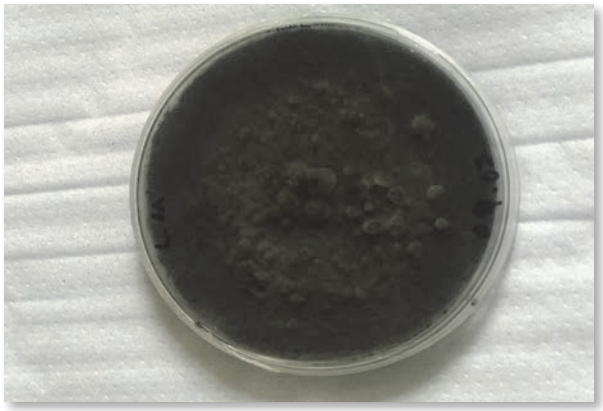

Figure 1: *Pseudocercospora purpurea* on PDA.

Figure 2: *Cladosporium* sp. on PDA.

Figure 3: *Colletotrichum* sp. on PDA.

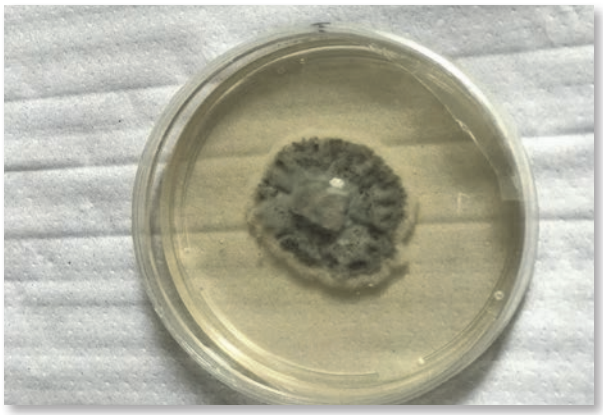

Figure 4: Unidentified species on PDA.

were purified on PDA, depending on their colony colour and conidial morphology.

Isolation of *Trichoderma* spp.

Avocado leaves were sampled from five avocado trees of the cultivar Fuerte, growing at Ukulinga Farm, UKZN, Pietermaritzburg. Twelve leaves (four young leaves, four old leaves and four diseased leaves) were sampled from each tree, resulting in a total of sixty leaves. Small fragments of the leaves were placed onto *Trichoderma* Selective Media (TSM) in Petri dishes, sealed using Parafilm and incubated at 25 °C for 7 days. Pure cultures were purified on PDA and monitored every second day to record the growth.

Endophytic screening

Fifty-two avocado seedlings of the cultivar Edranol were transplanted into pots and sprayed with benomyl to kill off any natural *Trichoderma* spp. growing endophytically. After spraying benomyl, there was a waiting period of two weeks. *Trichoderma* strains isolated at the laboratory were used to prepare suspensions with a concentration of $1x10^6$ conidia per ml. The *Trichoderma* suspensions were sprayed on 48 seedlings (the other 4 seedlings were used as controls), with each strain being sprayed onto 4 trees. After two weeks the seedling leaves were sampled randomly, rinsed with tap water, surface-sterilised with 2% sodium hypochlorite for one minute, rinsed 3 times in distilled water and air-dried. The leaves were cut into fragments and placed on TSM plates to check for the presence of *Trichoderma*.

Inhibition analysis

The extent of the anti-fungal activity of endophytic strains of *Trichoderma* spp. was evaluated against four postharvest pathogens of avocado. Screening for *in vitro* biocontrol activity was carried out using a Dual Culture Method, or Bell Test. Small squares of the fungal pathogens (one in each plate) and *Trichoderma* were placed at opposite sides of PDA plates, sealed with parafilm and incubated at 25 °C for seven days. Inhibition of radial growth of the pathogen was measured and recorded.

RESULTS AND DISCUSSION

Isolation of pathogens

In this study, from fifteen samples of avocado fruits displaying symptoms of the key fungal pathogens, ten isolates were identified based on their mycelial colony and microscopic features (Cheesbrough, 2006) into 6 fungal species belonging to 4 genera (Table 1). *Colletotrichum* spp. (Fig. 3) were the most frequently isolated affecting avocado fruits, followed by *Pseudocercospora* spp. (Fig. 1). The rest of the fungal species were isolated rarely (e.g., *Cladosporium* spp., Fig. 2) and one unidentified genus (Fig. 4), which is in the process of being identified using genomic analysis. The fungal pathogens were designated as Cs (*Colletotrichum* strain 1, 2, 3 and 4), Pp1 (*Pseudocercospora purpurea* strain 1) and Cc1 (*Cladosporium cladosporioides* strain 1).

Table 1: Colonial and morphological characteristics of fungal pathogens

Isolates no.	Characteristics of colony	Structural morphology	Identification of genera
Cs1 Cs2 Cs3 Cs4	White to dark grey White, pink to orange Cream to pale grey	Aerial mycelium, simple conidiophores with ovoid to oblong conidia.	*Colletotrichum* spp.
Pp1	Greyish to blackish-brown	Conidia are long cylindrical, obclavate, multi-septate. Conidiophores are hyaline to dark brown.	*Pseudocercospora* spp.
Cc1	Dark green to black	Conidiophores arise from hyphae, bearing branched ramoconidia. Chains oval-spherical conidia	*Cladosporium* spp.
-	Light brownish olive	Dark olive conidial masses hyaline conidiophores	Unidentified

Isolation of *Trichoderma* spp.

Seventeen different *Trichoderma* strains were isolated during this investigation. Colour difference and radial growth measurements were two characteristics that were used to differentiate the strains of *Trichoderma* spp. (Fig. 5). Table 2 shows the different radial growth of the colonies of each strain by Day 3 and Day 5 on PDA. The colonies of *Trichoderma* grow rapidly, woolly and became compact in time. In the beginning the colour of all colonies was white, but as conidia were formed, orange-green or yellow-green patches become visible.

Biocontrol activity of endophytic strains of *Trichoderma* against fungal pathogens

During this study ten strains of *Trichoderma* spp. were found to be endophytic. During the *in vitro* screening for inhibition by endophytic strains of *Trichoderma* spp. against all key fungal pathogens on plates (Table 2), seven of the endophytic strains of *Trichoderma* spp. controlled colony growth of all fungal pathogens at levels of between 70% and 100% (Fig. 6). This confirmed the potential of some endophytic strains of *Trichoderma* spp. to act as biocontrol agents against several pathogens (Arikit *et al*., 2020).

Future research

Ongoing research is focused on *in vivo* screening for pathogenicity of fungal pathogens on the avocado fruit using multiple infection techniques. *In vivo* screening of seven endophytic strains of *Trichoderma* spp. against the key fungal pathogen is being conducted on healthy avocado fruit. *Botrysphaeria* spp. were added to the list of key fungal pathogens and are currently begin tested *in vitro* and *in vivo* for susceptibility to the seven selected endophytic strains of *Trichoderma* spp..

Acknowledgements

We would like to thank the South African Avocado Growers' Association (SAAGA) and Postharvest Health Innovation (PHI) for funding this project; and University of KwaZulu-Natal staff and students for their contribution.

Figure 5: *Trichoderma* spp. on PDA.

Table 2: Fungal growth of *Trichoderma* spp. on PDA after day 3 and day 5

Strains	Day 3 (cm)	Day 5 (cm)
1C	6	8.5
1E	5	8.5
2B	6.8	8.3
2C	4.1	7.9
2E	4.8	8.1
2F	5.2	8.4
2G	6.5	8.5
2H	4.5	7.6
3A	4	7
3B	4.4	7.3
3E	5	8.1
3F	4.9	8.5
3G	6.2	8.5
4C	4.5	7.7
4G	5.8	8.1
5A	6	8.5
5C	6.1	8.5

Table 3: *In vitro* inhibition screening of endophytic strains of *Trichoderma* spp. against key fungal pathogens

Tricho-derma isolates	Pp1	Cc1	Cs1	Cs2	Cs3	Cs4	Unidenti-fied
UK1C	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled
UK1E	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled
UK2B	Controlled	Controlled	Co-existed	Controlled	Controlled	Co-existed	Controlled
UK2G	Controlled	Co-existed	Controlled	Controlled	Controlled	Controlled	Controlled
UK3E	Co-existed	Co-existed	Did not control	Did not control	Controlled	Co-existed	Controlled
UK3G	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled	Controlled
UK4C	Did not control	Co-existed	Did not control	Co-existed	Co-existed	Controlled	Controlled
UK4G	Co-existed	Co- excited	Controlled	Controlled	Controlled	Did not con-trol	Co-existed
UK5A	Controlled	Controlled	Co-existed	Controlled	Controlled	Co-existed	Controlled
UK5C	Controlled	Controlled	Did not control	Controlled	Controlled	Controlled	Co-existed

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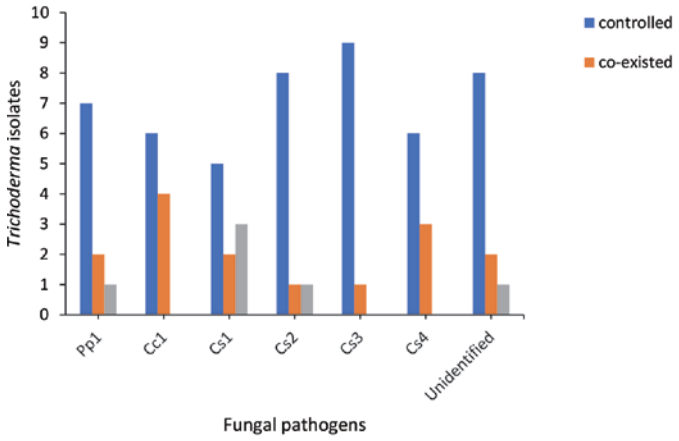

Figure 6: Inhibition by endophytic strains of *Trichoderma* spp. against fungal pathogens *in vitro* screening on plates.

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