Endophytic diversity in *Persea americana* (avocado) trees and their ability to display biocontrol activity against *Phytophthora cinnamomi*

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Abstract

Plants host a variety of endophytic microorganisms that can promote growth and protection against pathogens. Endophytes have been widely investigated in plants and used in biological control of plant pathogens. However, little is known about the diversity of endophytes in *Persea americana* Mill. (avocado) roots and their potential role in biocontrol of *Phytophthora cinnamomi* (*Pc*). This Oomycete is the causal agent of Phytophthora root rot, the most important disease in avocado producing countries worldwide. This study aimed to identify potential biocontrol agents from avocado roots and to use selected endophytes with biological activity against *P. cinnamomi in vivo*. The identification was based on morphological characteristics of the isolates as well as using ITS, β -tubulin, EF-1α and 16S ribosomal RNA gene sequencing. Twenty four different fungal species and 8 bacterial species were identified as endophytes from the roots of avocado plants from various locations in South Africa. Two fungal strains and 2 bacterial strains were selected on the basis of their *in vitro* antagonistic activity against *P. cinnamomi*. Clonal as well as endophyte-free tissue-cultured avocado plants were inoculated with each of the selected endophytes at 10^6 spores ml^{-1} for fungi and 10^8 CFU ml^{-1} for bacteria. After 4 weeks of inoculation, each plant received 10^5 *Pc* zoospores ml^{-1} directly sprayed onto roots, except for negative control plants. Positive control plants received no endophytes. Disease symptoms were assessed 21 days post infection and disease incidence was calculated. Avocado plants that received endophytes prior to *Pc*- infection showed a significant decrease in disease incidence with ratings from 2-40% compared to 94-100% for the positive control plants.

Resumen

Las plantas hospedan una gran variedad de microorganismos endófitos que pueden promover el crecimiento y la protección contra patógenos. Los endófitos han sido ampliamente investigados y usados como control biológico de fitopatógenos. Sin embargo, se conoce poco sobre la diversidad de endófitos en raíces de *Persea americana* Mill (aguacate) y su papel en el biocontrol de *Phytophtora cinnamomi* (*Pc*). E ste Oomycete es agente causal de la pudrición de la raíz, la enfermedad más importante alrededor del mundo en aguacate. Este estudio ayudó a identificar el potencial de los agentes biocontroladores de las raíces de aguacate y utilizar determinados endófitos con cierta actividad contra P. cinnamomi *in vitro*. La identificación fue basada en caracteres morfológicos de los aislamientos y la utilización de ITS, β-tubulin, EF-1α y secuenciación genética de 16S ARN ribosomal. 24 especies fúngicas diferentes y 8 especies de bacterias fueron identificadas como endófitos en raíces de plantas de aguacate en varios lugares de Sudáfrica. 2 cultivos de hongos y 2 de bacterias se seleccionaron con base en la actividad antagónica *in vitro* contra *P. cinnamomi*. También se inocularon tejidos de plantas con endófitos seleccionados en 106 e sporas ml-1 para hongos y 108 CFU ml-1 para bacterias. Después de 4 semanas de inoculación, cada planta recibió 105 zoosporas *Pc* ml-1 rociando directamente las raíces, excepto para el control negativo de las plantas. El control positivo no recibió endófitos. Los síntomas de la enfermedad fueron valorados después de 21 días de la infección y se calculó la posterior incidencia de la enfermedad. Las plantas de aguacate recibieron endófitos antes que *Pc* mostrara un aumento o incremento significativo de la enfermedad con rangos entre 2-40% comparado con 94% de las plantas controles positivos. La presencia de endófitos en raíces de aguacate juega un papel importante en la inhibición en el crecimiento de *Pc* y desarrollo de la enfermedad. Este estudio ha proporcionado un valioso dato para el uso de endófitos en la protección de las plantas.

Key words: Endophytes, Biocontrol, *Phytophthora cinnamomi*, *Persea americana*

1. Introduction

Phytophthora root rot, a plant disease caused by the oomycete *Phytophthora cinnamomi,* is the major biological factor that limits avocado production worldwide (Zentmyer 1984; Pegg *et al.,* 1982; Kotzé *et al.,* 1987). The disease is traditionally controlled with Phosphite trunk injections and tolerant rootstocks. However, Phosphite has been shown to be ineffective due to the resistance of *P.cinnamomi* to phosphates (Duvenhage 1994) and further has a negative impact on the environment and human health (Chet and Inbar, 1994; Harman and Kubicek, 1998). In order to reduce the use of chemical control agents, the use of natural antagonists such as endophytic microorganisms would be a sustainable biological alternative to control the pathogen (Bateman, 2002; Bonos *et al*., 2005; Clarke *et al*., 2006).

Endophytic fungi and bacteria live asymptomatically within intra- and intercellular spaces of plant tissues (Wilson 1995; Stone *et al*., 2004; Hyde & Soytong 2008) interacting with plants in symbiotic, mutualistic and other types of relationships (Zilber-Rosenberg & Rosenberg 2008). The host produces amino acids for the endophytes while they in turn produce bioactive substances, specifically mycotoxins such as alkaloids that improve both plant resistance to pathogenic microorganisms (Bent & Chanway, 1998; Sharma *et al*., 1998; Hallmann *et al*., 1997), and the growth and development of plants (Lodewyckx *et al*., 2002; Barrow *et al*., 2008). Although some endophytes may become slightly pathogenic to the plant under adverse conditions, other endophytes are able to suppress those latent pathogens (Mahesh *et al*., 2005).

Several studies have been conducted for the control of root rot disease of avocado trees using antagonistic microorganisms isolated from the avocado rhizoplane. However, microorganisms from the soil might not be compatible with the plant roots or their efficiency may depend on environmental conditions of the area in which they are isolated. In this study, we identified endophytic bacteria and fungi isolated from avocado root tissue. These endophytes were then screened *in vitro* for their potential antagonism against *P. cinnamomi* growth and subsequently evaluated for inhibition of root rot of avocado plants under green-house conditions. This study is a contribution towards understanding the role of endophytes as a means to develop effective biocontrol agents of *P. cinnamomi* in avocado trees.

2. Materials and methods

2.1. Isolation and identification of endophytes

Endophytic fungi and bacteria were isolated from healthy feeder roots of avocado trees collected from various locations in South Africa where avocado is grown. All samples were immediately transferred to the laboratory, and the tissues were screened for endophytes following the modified method of Weber *et al.,* (2004). To eliminate epiphytic microorganisms, all the samples were initially surface sterilized. Samples were washed properly in running tap water and three times in distilled water before processing. Younger and healthy roots were cut into 0.5 cm-long segments and immersed in 70% ethanol for 1 min and then sterilized with 2% aqueous sodium hypochlorite for 2 min, rinsed in 70% ethanol for 1 min before finally rinsing three times in sterilized water for 2 min. Each sample was then dried under aseptic conditions. The effectiveness of the surface sterilization was tested by the method of Cao *et al*., (2004), and no growth was observed. Surface-sterilized samples were soaked in 5 ml sterile water and stirred for 1 min. Aliquots of 3 ml were then inoculated on potato dextrose agar (PDA) and Malt extract agar (MEA) plates containing 50 mg/l of chloramphenicol for fungi and on nutrient agar (NA) plates for bacteria and checked for microbial growth. After epiphytic sterilization, root samples were placed on the surface of the media, the Parafilm-sealed Petri dishes were then incubated for 21 days at 28^0C for fungal isolates and 2 days at 25^0C for bacterial isolates. The plates were checked on alternate days. Colonies of each bacteria plate were picked based on the colour and morphology. Bacterial single colonies were picked and stored in the appropriate Microbank solution at -70^0C for further experiments. Fungal hyphal tips of actively growing fungi were then subcultured, purified and transferred to Synthetic nutrient agar (SNA), Oat meal agar (OA) and *Fusarium* specific medium (FSM) for further characterization. Single cultures were morphologically characterized (Bell *et al*., 1995; Sturz & Christie, 1996) and the rest of the mycelia were cut into small blocks of agar and stored into 10 ml of sterile water at 4^oC for further experiments.

Fresh cultures were grown from stored material for DNA isolation from both fungi and bacteria. Single beads containing cryopreserved bacteria were used to inoculate new bacteriological medium and were grown overnight at 28^{o}C before extracting the DNA using a genomic DNA kit (ZymoResearch; Orange, CA, USA). Single blocks of fungal cultures were transferred to new agar plates and grown for 3 days at 25^{o}C. Fungal DNA was prepared according to a modified CTAB method (Wu *et al*., 2001). A total of 7 genes for fungi and one gene for bacteria were amplified and sequenced to identify the endophytes (Table 1). The PCR products were purified and sequenced. The sequences were identified by BlastN program (Zhang *et al.,* 2000) against the NCBI database (National Center for Biotechnology Information, http:// www.ncbi.nlm.nih.gov). Results from both molecular and morphological identification were compared.

Table 1. Sequences of primers used in the experiments.

Primer name	Primer sequence 5'-3'
T1	-GCG TAC TAG CGT ACC ACG TGT CGA CT-
Bt2b	-ACC CTC AGT GTA GTG ACC CTT GGC-
LROR	-ACC CGC TGA ACT TAA GC-
LR5	-TCC TGA GGG AAA CTT CG-
EF1-728F	-CAT CGA GAA GTT CGA GAA GG-
EF1-986R	-TAC TTG AAG GAA CCC TTA CC-
ITS1	-TCC GTA GGT GAA CCT GCG G-
ITS 2	-ATG CTT AAA TTT AGG GGG TAG TC-
CalF	-CTG ACC ATG ATG GCC AGA AA-
CalR	-GTT AGC TTC TCC CCA GCT T-
BT1a	-TTC CCC CGT CTC CAC TTC TTC ATG-
BT1b	-GAC GAG ATC GTT CAT GTT GAA CTC-
ITS4	-TCC TCC GCT TAT TGA TAT GC-
pA	-AGA GTT TGA TCC TGG CTC AG-
pH	-AAG GAG GTG ATC CAG CCG CA-

2.2. *In vitro* antagonistic assays

Dual cultures were produced to evaluate the *in vitro* antagonistic activity of identified endophytes against *P. cinnamomi* growth in order to select the prospective endophytes that will be used for *in vivo* screening trials. Hyphal plugs of *P. cinnamomi* isolate were placed 2 cm from one edge of 90 mm Petri dishes containing V8 agar culture medium. Two days later, selected endophytic isolates were placed 4 cm apart in the same plate. The plates were incubated at 25^{o}C and the evaluation of interactions began 3 days after bacterial endophytes and seven days after fungal endophytes were placed into assay plates. Each plate was duplicated. Antagonism towards *P.cinnamomi* was scored using the Badalyan (2002) rating scale: A = deadlock with mycelia contact, B = deadlock at a distance, C = replacement, overgrowth without initial deadlock. The Antagonism Index (AI) expressed in percentage was calculated considering the ray of *P. cinnamomi* colony towards the antagonist (rm) and the average of the three rays of the colony in the radial directions in a Petri dish (RM): AI (%) = [(RM-rm)/RM]x100. For bacterial antagonists, total growth diameter (TGD), *P. cinnamomi* plug inoculum diameter (PcPID) and radial growth (RG), where RG = (TGD-PcPID)/2, were taken into consideration to determine the level of antibiosis produced by bacterial endophytes. Antagonism (%) = (RG bacteria/RG *Pc* mycelia) x 100.

2.3. *In vivo* inhibition of *Phytophthora cinnamomi*

2.3.1. Preparation of inoculum of endophytes

For this, two fungi and two bacteria were selected based on *in vitro* assays as antagonistic endophytes to *P. cinnamomi* growth. The inoculum was prepared following the modified method of Chin-A-Woeng *et al*., 1998. These fungal isolates were cultured in Water-agar plates containing sterile pine needles and the plates were incubated at 25^{o}C under UV light. After 10 days of growth the plates were checked under the microscope for sporulation and all the sporulated cultures were filtered through sterile glass wool to remove mycelia. Conidia were harvested by centrifugation of the suspension at 10,000xg for 10 min, resuspended in sterile water and the spore suspension was adjusted to 10^6 spores ml^{-1} using the haemocytometer. Bacterial single colonies were grown on Nutrient agar at 25^{o}C for 2 days and transferred to 500ml Erlenmeyer flasks containing Nutrient broth medium. Flasks were incubated at 28^{o}C on a 125 rpm shaker for 5 days. Bacterial cell suspension was adjusted to 10^8 CFU ml^{-1} using the haemocytometer.

2.4. Plant materials

Two types of avocado plants were used for the *in vivo* experiments: (i) 9-month old clonal avocado rootstock plants and (ii) Tissue-cultured avocado plants produced *in vitro* using tissue culture techniques (Pliego Alfaro, 1988) with the aim of obtaining endophyte-free plants (López Herrera *et al*., 1992). After 8 weeks, tissue-cultured plants were transferred into clean *ex-vitro* conditions for acclimatization for 12 weeks under 24^{o}C, 70% humidity, and 16:8 Light:Dark photoperiod in order to harden their roots. Random roots were sampled from each plant to confirm that roots were endophyte-free before inoculations.

2.5. Plant inoculation with endophytes

Avocado plants were subdivided into 3 treatments: (B) treatment with bacterial endophytes, (F) treatment with fungal endophytes, and (B + F) treatment with bacterial and fungal endophytes combined. Two inoculation methods were used: root collar injection, and soil drench. For each selected isolate a stock suspension was adjusted to 10^6 fungal spores ml^{-1} and 10^8 CFU ml^{-1} for bacteria. The root collar injection method was performed using a sterile disposable needle to facilitate injection of 5 µl of each suspension with a syringe. For the drench inoculation method, 20ml of each suspension was applied to the substrate in each pot Seedlings were removed from the soil and soaked into the suspension for two hours before transferring them to the new perlite-vermiculite drenched with 10 ml of the suspension per pot. Control plants received sterile water in the same way as mentioned above. The plant inoculation with antagonistic endophytes was repeated every 4 weeks to maintain sufficient inoculum in the substrate. There were three treatments for both, clonal plants and tissue-cultured plants. The inoculation consisted of (i) two fungal isolates, (ii) two bacterial isolates, (iii) fungal and bacterial isolates combined.

2.6. Root infection

Eight weeks after plant inoculation with endophytes, plants were grouped into 5 treatments (Tables 6&7): *Pc: P. cinnamomi* as positive controls; B+*Pc*; F+Pc; B+F+Pc; and B+F as negative controls. Plants were carefully removed from pots and 5 ml of a suspension of 10^5 *Pc* zoospores ml^{-1} was directly sprayed onto each plant's roots. Negative control plants were sprayed with water. Aerial symptoms of the infection (figure 3) were assessed every 7 days to calculate the disease incidence (DI) as described by Cazorla *et al*., 2006 in the following formula ; where the letters a, b, c and d corresponding to the number of plants showing disease values of 0, (no symptom of the disease); 1, (yellowing and wilting of the aerial parts); 2, (overall dying of the tree); 3, (completely dead) and n, (the total number of plants tested) (Table 7).

$$DI = 100 \text{ x } \frac{(\text{ax0}) + (\text{bx1}) + (\text{cx2}) + (\text{dx3})}{3\text{xn}} \quad (1)$$

3. Results and Discussion

3.1. Identification of endophytes

A total of 24 species of fungi (Table 2) and 8 species of bacteria (Table 3) were identified as endophytes from avocado root tissues. *Cylindrocarpon sp.* was isolated most frequently, with 21%of the isolates belonging to this species. *Neonectria sp.* constituted 10.5% and *Fusarium oxysporum* 9.3% of the total number of endophytes isolated from avocado roots. Only 6.5% of the isolates were identified as *Trichoderma sp*. A number of endophytic fungi have previously been identified as latent pathogens in other plants. *Cylindrocarpon obtusisporum* causes Black-foot disease of grapevine (Scheck *et al*., 1998). *Trichoderma harzianum* and *Trichoderma hamatum* have previously demonstrated biological activities against phytopathogens (Wells, 1988) and have shown growth-promoting capacities that may be integral to biological control (Lumsden, 1998).

Table 2. Fungal endophytes isolated from avocado roots

Endophyte	Abundance (%)	Endophyte	Abundance (%)
Cylindrocarpon sp.	21.0	*Cladosporium sp*	2.6
Neonectria sp.	10.5	*Lasiodiplodia sp.*	2.6
Fusarium oxysporum	9.2	*Fusarium solani*	2.6
Hypocrea lixii	6.5	*Neonectria macrodydima*	2.6
Trichoderma hamatum	6.5	*Glionectria tenuis*	2.6
Fusarium sp.	6.5	*Diaporthe sp.*	1.3
Botryosphaeria parva	5.2	*Penicillium sp.*	1.3
Ascomycota sp.	5.2	*Penicillium crustosum*	1.3
Pyronema domesticum	3.9	*Pestalothiopsis sp.*	1.3
Ascomycete sp.	3.9	*Penicillium commune*	1.3
Glomerella sp.	2.6	*Alternaria sp.*	1.3

Table 3. Bacterial endophytes isolated from avocado roots

Endophyte	Abundance (%)
Bacillus cereus	35.7
Bacillus subtilis	21.3
Bacillus anthracis	7.5
Bacillus fusiformis	7.1
Bacillaceae bacterium	7.1
Lysinibacillus sp.	7.0
Paenibacillus polymyxa	6.8
Enterobacter sp.	6.1

Bacillus spp. were the most abundant bacterial species (76.1%) isolated from avocado roots. Others including *Bacillaceae bacterium*, *Lysinibacillus sp*. *Paenibacillus polymyxa* and *Enterobacter sp*. are least abundant inavocado roots. *Bacillus cereus* and *Paenibacillus polymyxa* have previously demonstrated biological activities against phytopathogens.

3.2. *In vitro* activity of fungal endophytes against *P. cinnamomi*

Dual plate assays were conducted to evaluate the *in vitro* antagonistic activity of fungal and bacterial endophytes against *P. cinnamomi*. Not every identified endophyte was tested against the pathogen due to their ability to become pathogenic. *Trichoderma harzianum*, *Fusarium oxysporum* and *Trichoderma hamatum* have had high inhibitory effect on mycelia growth of *P. cinnamomi* with AI=36.8; 23.3 and 17.6 respectively (Table 4). *Neonectria macrodydima* expressed lower inhibition of *P. cinnamomi* growth with AI=2.0. *Trichoderma harzianum* and *Trichoderma hamatum* have previously been used as biological agents and showed high levels of inhibition of Pc and were therefore selected for *in vivo* assays.

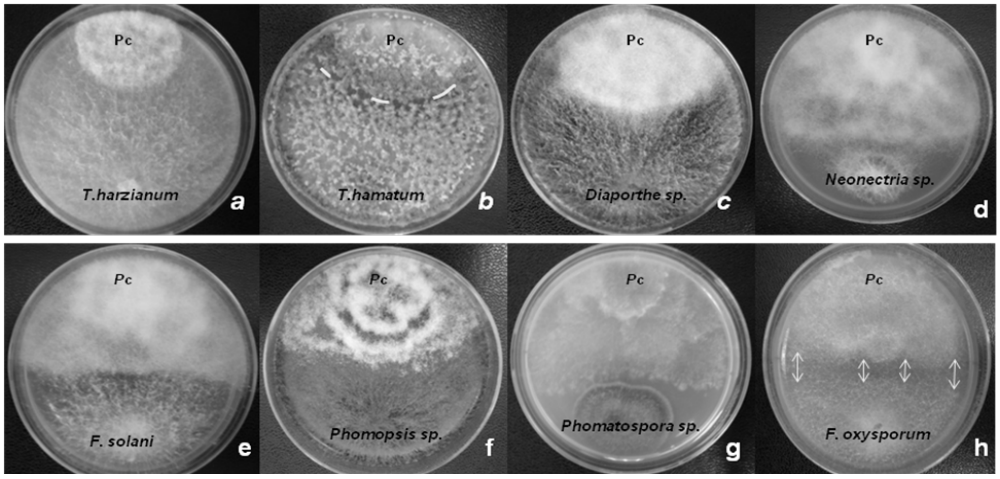

Figure 1. Competitive interactions between fungal endophytes and *Phytophthora cinnamomi* on V8 agar at 25 ^{0}C after 7 days. *a, c ,f*: Deadlock with mycelia contact; *b*: Replacement; *d, e, g, h*: Deadlock at a distance.

Table 4. *In vitro* antagonistic activity of fungal endophytes

Fungal isolate	Mycelia growth of *Pc* (%)	Mycelia diameter (cm) of *Pc* towards the antagonist	Antagonist index (AI)
Trichoderma harzianum	30.0^{AC}	1.2	36.8
Fusarium oxysporum	57.5^{BC}	2.3	23.3
Trichoderma hamatum	50.0^{A}	2.0	17.6
Fusarium solani	27.5^{A}	1.1	17.2
Diaporthe sp.	40.0^{A}	1.6	13.9
Phomopsis sp.	50^{A}	2.0	13.0
Neonectria macrodydima	70.0^{B}	2.8	2.0

3.3. Antagonistic bacteria

Eight endophytic bacterial isolates were tested against *P.cinnamomi.* All the isolates demonstrated the ability to inhibit the growth of *P.cinnamomi in vitro* (Table 5). *Bacillus cereus* and *Paenibacillus polymyxa.* were selected for the *in vivo* assays based on the fact that, not only did they inhibited the

growth of the pathogen but they have also been used against *P.cinnamomi* in many other plants. *Bacillus cereus*, the best inhibitor was the most commonly isolated in avocado roots.

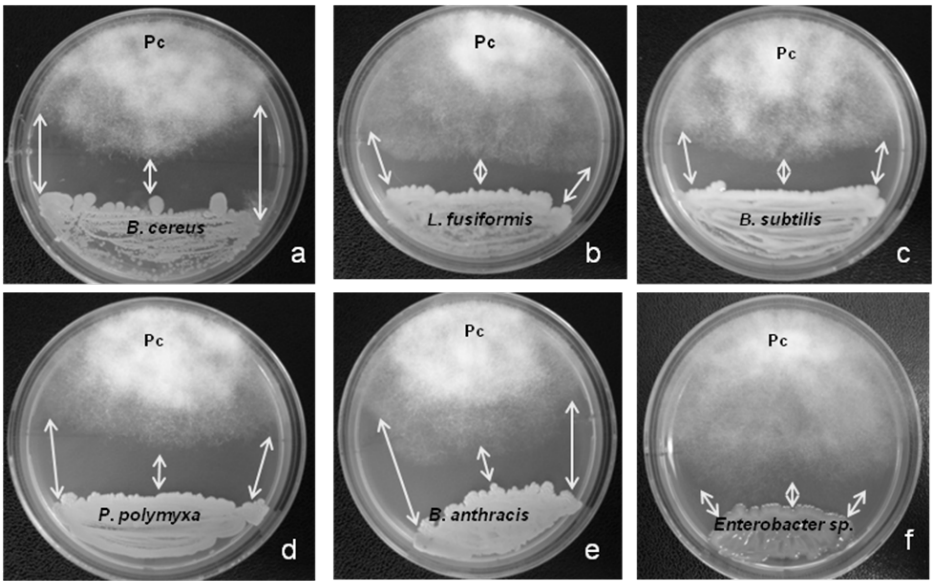

Figure 2. Antagonism between bacterial endophytes *and P. cinnamomi* on V8 agar at 24°C after 7 days.

Table 5. *In vitro* antagonism of bacteria against *P. cinnamomi*

Isolate	**Inhibition zone (cm)**	***Pc* growth inhibition (%)**
Bacillus cereus	1.28	28.4
Bacillus anthracis	1.22	27.1
Paenibacillus polymyxa	1.20	26.6
Bacillus subtilis	1.17	26.0
Lysinibacillus fusiformis	0.62	13.7
Enterobacter sp.	0.60	13.3

Bacillus spp. and *Paenibacillus polymyxa* inhibited *P. cinnamomi* with a reduction in the range of 26.0-28.4% growth. *P. polymyxa* has been used to control plant diseases such as crown rot disease in peanut (Haggag *et al*., 2007)

3.4. *In vivo* assays

3.4.1. Endophytes inoculation and root infection

Varying degrees of inhibition of *P. cinnamomi in vivo* were observed. Plants inoculated with endophytes demonstrated the inhibition of Phytophthora root rot with DI in the range of 2-40 % compared to infected control with DI= 94-100% (Table 6).

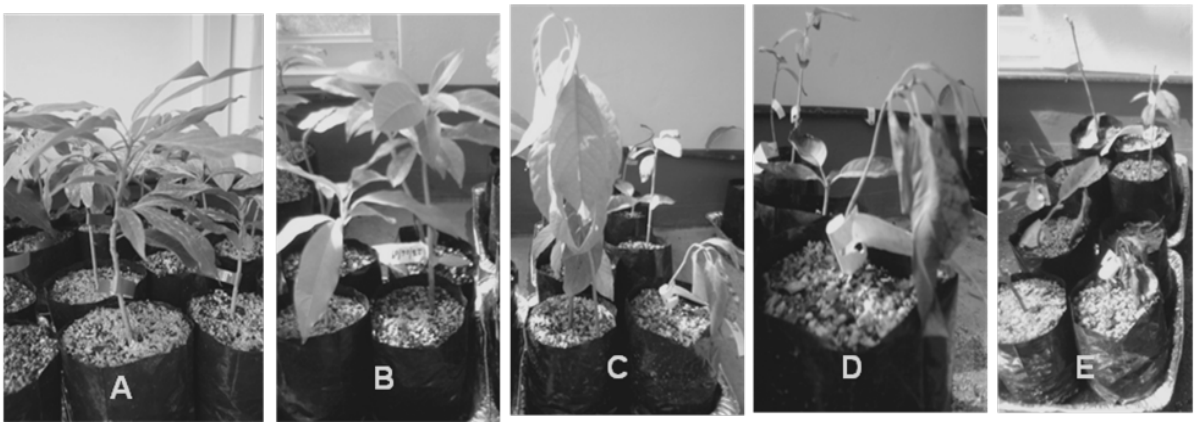

Figure 3 Avocado plants; A: Healthy, B: Yellowing, C: Wilting, D: Overall dying, E: Dead 15 days post-infection.

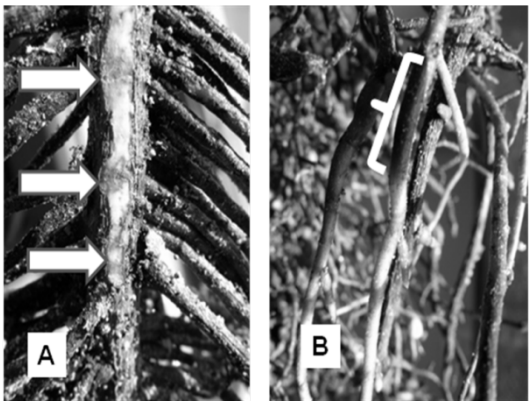

Figure 4. Root rot symptoms on control infected tree *A*. Root rot symptoms inside avocado roots infected with *P.cinnamomi*. *B*. Endophytes have the ability to reduce damage caused by the pathogen and to promote plant regeneration.

Table 6. Aerial symptoms of the infection on various rootstock plants (14 days post infection)

	Rootstock 1						Rootstock 2						Rootstock 3					
Trial	**n**	**a**	**b**	**c**	**d**	**DI**	**n**	**a**	**b**	**c**	**d**	**DI**	**n**	**a**	**b**	**c**	**d**	**DI**
B+Pc	12	7	2	1	2	28	11	9	1	0	1	12	12	7	1	1	3	33
F+Pc	10	7	2	1	0	40	12	6	4	0	0	10	13	11	2	0	0	5
B+F+Pc	24	19	3	1	1	11	19	17	2	0	0	3	17	16	1	0	0	2
Pc	8	0	0	0	8	100	6	0	0	1	5	94	6	0	0	0	6	100
B+F	16	16	0	0	0	0	12	12	0	0	0	0	12	12	0	0	0	0

Plants inoculated with a combination of fungal and bacterial isolates demonstrated the highest inhibition of *P. cinnamomi* infection with DI= 2-11 compared to plants inoculated with either fungal or bacterial with DI range of 5-40%. This is the result of the combination of biocontrol mechanisms involved by both bacterial and fungal isolates.

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Table 7. Aerial symptoms of infection with *Pc* on Tissue culture plants (14 days post infection)

Trial	n	a	b	c	d	DI
B+Pc	70	58	8	4	0	8
F+Pc	70	63	6	1	0	4
B+F+Pc	100	82	17	1	0	6
Pc	30	0	0	0	30	100
B+F	60	60	0	0	0	0

Very low disease incidence (4-8%) was observed in experiments on plants produced from embryo cultured plants when compared to clonal plants. Plants which had been pre-inoculated with endophytes showed only slight yellowing and wilting of the leaves but no tree death when compared to the control infected plants which all died. This may be due to the fact that tissue culture plants were endophyte-free before inoculating them with selected antagonistic isolates allowing our isolates to colonize the tissues without any competition from other microorganisms.

4. Conclusion

Isolation of endophytes from avocado roots resulted in the isolation of a variety of fungi and bacteria living inside avocado roots. In this study, *in vitro* assays to select and inoculate *P. cinnamomi* antagonists and good competitors such as *Trichoderma* species, antibiotics exhibitors and plant growth promoters such as *P. polymyxa* and *B. cereus* was successful. Regular inoculation of selected fungal and bacterial endophytes into the roots and in the substrate resulted in significant inhibition of the pathogen's growth. This study suggests that the production of endophyte-free plants using tissue culture techniques may be a way of providing good starting materials to be inoculated with endophytes before transferring them to the field. We have shown that endophytes isolated from the avocado roots were successful in inhibiting *P. cinnamomi* under greenhouse conditions, but we suggest that the work be followed by field trials.

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