# DISTRIBUTION, MOLECULAR IDENTIFICATION AND THE EFFECT OF BIOLOGICAL CONTROL OF *PHYTOPHTHORA CINNAMOMI* ON MACADAMIA IN THE LIMPOPO AND MPUMALANGA PROVINCES, SOUTH AFRICA.

By

**Nontokozo Sibahle Kunene**

Submitted in fulfillment of the requirements for the degree of

**MASTER OF SCIENCE**

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1. University of KwaZulu-Natal (UKZN), King Edward Avenue, Pietermaritzburg, Private Bag X01, Scottsville, 3209

And

2. Agricultural Research Council – Tropical and Subtropical Crops (ARC-TSC), Private Bag X11208, Mbombela, Mpumalanga, 1200

Email: KuneneN@arc.agric.za

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# DISSERTATION SUMMARY

Macadamia is a nut-bearing tree belonging to the family *Proteaceae. Macadamia tetraphylla*, and *Macadamia integrifolia* (also *M. ternifolia*), and their hybrids are of importance in commercial cultivation. Root rot and trunk canker are the most important diseases caused by *P. cinnamomi* that could cause 60% yield losses and an estimated 10% of the annual gross value of macadamia. The objective of this study was to determine the distribution, molecular identification and the effect of biological control of *P. cinnamomi* in the main macadamia growing areas of South Africa.

The first part of this study assesses disease incidence and severity as well as the distribution of *P. cinnamomi* in the main macadamia growing areas of South Africa. *Phytophthora cinnamomi* was recovered from soil samples by baiting and from plant tissues by plating on *P. cinnamomi* selective medium. Root rots and stem cankers were recorded in 52 % of the farms sampled. No significant ($P > 0.05$) differences were observed in disease incidence and severity between the sampled provinces. The highest disease incidence (64.2 %) was found in the Mpumalanga province. *Phytophthora cinnamomi* had a wide distribution in all the main macadamia growing areas.

The second part of the study investigated DNA detection of *P. cinnamomi* from soil samples. A nested PCR amplification protocol was optimised with both primary and nested PCR specific for *P. cinnamomi* detection. The protocol improved both the specificity and sensitivity of PCR amplification in comparison to the one-step PCR. The application of diagnostic nested PCR together with the DNA extracted using the baiting bioassay was verified by comparison with DNA extracted using a kit. The nested PCR using DNA extracted by baiting was found to be more sensitive.

The final part of the study examined two *Trichoderma* spp. and eight unknown *Bacillus* spp. as potential biological control agents (BCAs) for management of *P. cinnamomi.* The potential BCAs were evaluated for their *in vitro* growth inhibition of seven *P. cinnamomi* isolates. All the isolates were sensitive to the ten potential bio-control agents. The *Trichoderma* spp. and two best *Bacillus* spp. (B 41b and NB 4) caused *in vitro* growth inhibition of 22 – 90 % in the laboratory *in vitro* studies. Depending on the mode of action, these BCAs should be evaluated further for their potential use in the integrated management of root rots and stem canker of macadamia.

# Declaration

I declare that this thesis is my original work and has not been submitted for a degree at any other tertiary education institution. To the best of my knowledge, this dissertation contains no material or work performed by others, published or unpublished without due reference being made within the text.

**Signed…………………………………** **Date:……………………………**

**Nontokozo Sibahle Kunene (Candidate)**

**Signed…………………………………** **Date:……………………………**

**Dr. AEC Jooste (Supervisor)**

**Signed…………………………………** **Date:……………………………..**

**Prof A. Gubba (Co-supervisor)**

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# Dedication

{To iNdlovukazi, Jobe kaMatshane, Mthembu weGubazi… Gogo, this one is for you}

# Table of Contents


**DISSERTATION SUMMARY** ........ i

**Declaration** ........ ii

**Acknowledgements** ........ iii

**Dedication** ........ iv

**INTRODUCTION TO DISSERTATION** ........ 1

**CHAPTER 1** ........ 6

**LITERATURE REVIEW** ........ 6

1.1 Macadamia ........ 6

1.1.1 History and Distribution ........ 6

1.1.2 Botany ........ 7

1.1.3 Economic Importance ........ 9

1.2 The Macadamia Industry ........ 10

1.2.1 Global Macadamia Industry ........ 10

1.2.2 The South African Macadamia Industry ........ 12

1.3 Insect Pests and Diseases of Macadamia ........ 14

1.3.1 The Macadamia Stinkbug (*Nezara viridula*) ........ 15

1.3.2 The Nut Borer ........ 15

1.3.3 Raceme Blight ........ 15

1.3.4 Macadamia husk spot ........ 16

1.3.5 *Phytophthora* Blight ........ 16

1.4 The Genus *Phytophthora* ........ 16

1.5 *Phytophthora cinnamomi* ........ 18

1.5.1 Epidemiology and Aetiology ........ 20

1.5.2 Mechanism of Infection ........ 20

1.5.3 Disease Development on Macadamia ........ 22

1.5.4 Control ........ 24

1.5.4.1 Cultural ........ 25

1.5.4.2 Chemical ........ 25

1.5.4.3 Resistance ........ 26

1.5.4.4 Biological Control ........ 27

1.6 Detection techniques for *Phytophthora cinnamomi* ........ 28

1.6.1 Isolation ........ 28

1 1.6.2 Baiting 29
2 1.6.3 Immunological Detection 30
3 1.7 DNA Detection Methods 31
4 1.7.1 PCR Detection of *P. cinnamomi* 32
5 1.8 References 34
6 **CHAPTER 2** 55
7 **OPTIMISATION OF A NESTED PCR PROTOCOL FOR THE DETECTION OF**
8 ***PHYTOPHTHORA CINNAMOMI* ON MACADAMIA IN THE LIMPOPO AND MPUMALANGA**
9 **PROVINCES, SOUTH AFRICA** 55
10 Abstract 55
11 2.1 Introduction 56
12 2.2 Materials and Methods 57
13 2.2.1.1 *P. cinnamomi* isolates 57
14 2.2.1.2 *P. cinnamomi* isolates from soil samples 57
15 2.2.2 Maintenance of *P. cinnamomi* isolates 57
16 2.2.3 DNA extraction from mycelial cultures 58
17 2.2.4 DNA extraction from soil samples 59
18 2.2.5 Polymerase chain reaction (PCR) amplification 59
19 2.2.6 Gel electrophoresis 61
20 2.3 Results 61
21 2.3.1 Optimized PCR amplification with nested primers CIN3A/CINITS4 and CIN3B/CIN2R
22 61
23 2.3.2 PCR amplification with LPV3 primers 61
24 2.3.3 Comparison between DNA isolated with the soil kit and DNA isolated by the baiting
25 bioassay using nested PCR amplification 62
26 2.4 Discussion 64
27 2.5 References 65
28 **CHAPTER 3** 68
29 **THE INCIDENCE AND DISTRIBUTION OF *PHYTOPHTHORA CINNAMOMI* IN THE MAIN**
30 **MACADAMIA GROWING AREAS OF SOUTH AFRICA** 68
31 Abstract 68
32 3.1 Introduction 69
33 3.2 Materials and Methods 70
34 3.2.1 Disease surveys 70
35 3.2.2 Recovery of *P. cinnamomi* 70

1 3.2.3 Detection of *P. cinnamomi* ........ 71
2 3.2.4 Data analysis ........ 71
3 3.3 Results ........ 72
4 3.3.1 Disease surveys ........ 72
5 3.3.2 *P. cinnamomi* isolates recovered ........ 72
6 3.3.3 Detection of *P. cinnamomi* ........ 73
7 3.4 Discussion ........ 74
8 3.5 References ........ 76
9 **CHAPTER 4** ........ 80
10 ***IN VITRO* EVALUATION OF BIOLOGICAL CONTROL AGENTS AGAINST *PHYTOPHTHORA***
11 ***CINNAMOMI* ON MACADAMIA IN LIMPOPO AND MPUMALANGA, SOUTH AFRICA** ........ 80
12 Abstract ........ 80
13 4.1 Introduction ........ 81
14 4.2 Materials and Methods ........ 82
15 4.2.1 *Phytophthora cinnamomi* isolates ........ 82
16 4.2.2 Screening of *P. cinnamomi* isolates for pathogenicity and virulence on green apples 83
17 4.2.3 Selected potential biological control agents ........ 83
18 4.2.4 *In vitro* antagonistic bioassays ........ 84
19 4.2.5 Data Analysis ........ 85
20 4.3 Results ........ 85
21 4.3.1 Pathogenicity and virulence tests on green apples ........ 85
22 4.3.2 *In vitro* antagonistic bioassays of *Trichoderma* against *P. cinnamomi* isolates ........ 86
23 4.3.3 *In vitro* antagonistic bioassays of *Bacillus* against *P. cinnamomi* isolates ........ 87
24 4.3 Discussion ........ 91
25 4.5 References ........ 93
26 **CHAPTER 5** ........ 98
27 **GENERAL DISCUSSION** ........ 98
28 5.1 Overview of major outcomes ........ 98
29 5.2 Implications of outcomes ........ 98
30 5.3 Recommendations ........ 99
31 5.4 References ........ 100
32 **PROJECT OUTCOMES** ........ 101
33 **APPENDICES** ........ 102
34

# INTRODUCTION TO DISSERTATION

Macadamia is a nut-bearing tree that belongs to the family *Proteaceae* that consists of evergreen woody plants (Augstburger *et al*., 2002). The aboriginal tribes used the nuts as a staple food and as a base for medicines and cosmetics in Australia, where the nut originated from (de Villiers, 2003). Two different species of macadamia, namely *Macadamia integrifolia (*Maiden and Betche) (also *M. ternifolia*), and *Macadamia tetraphylla* (L.A.S. Johnson)*,* together with their hybrids, are of importance in commercial cultivation (Augstburger *et al*., 2002). Macadamia production is limited to the tropical and subtropical regions of the world, but the nut is exported worldwide. In South Africa, macadamia production is mainly confined to the Limpopo, Mpumalanga, and KwaZulu-Natal provinces; and to a lesser extent to the Eastern and Western Cape provinces (DAFF, 2015).

Plant pathogens (fungi, stramenopiles, bacteria, viruses, nematodes) are a major threat to plant production since they result in quality and quantity reduction of commercial crops worldwide (Bailey, 2010). There are major losses in agriculturally essential crops due to these pathogens, and they, therefore, remain important constraints in agricultural production (Bailey, 2010). A number of factors affect macadamia production, but those of great economic importance are the disease-causing and quality-reducing pathogens. The main and most severe diseases in macadamia are caused by *Phytophthora* spp*.* These are capable of reducing vigour, production, and may cause complete trunk death (Akinsanmi and Drenth, 2010). *Phytophthora cinnamomi* (Rands) has been the chief limiting cause to successful macadamia production in countries such as Hawaii (Ko, 2009), Australia (Rosengarten, 2004), California (Zentmyer, 1980), Kenya (Sikinyi, 1993) and South Africa (Manicom, 2003). It causes stem canker, root rot and quick decline in macadamia worldwide (Serfontein *et al*., 2007). Root rot and trunk canker are major diseases that could cause 60% yield losses and an estimated 10% of the annual gross value of macadamia (Muthoka *et al*., 2005).

*P. cinnamomi* is the most important and destructive oomycete of not only macadamia worldwide, but over 1000 plant types (Zentmyer, 1980), including avocados, eucalyptus, kiwi fruit, chestnut, peach, pineapple, pear, and many native Australian and

South Africa plants (Pegg *et al.*, 2002). The pathogen is of specific importance to the avocado (*Persea americana* Mill.) and macadamia industries because of its potential to destroy avocado and macadamia orchards in a short time frame. It infects and kills trees of all ages, from nursery trees to large fruit-bearing trees through the destruction of feeder roots (Bekker, 2007). *P. cinnamomi* is responsible for the widespread damage of macadamia trees worldwide. It infects the feeder roots. Infection occurring through the fine feeder roots results in root rots whereas infection occuring through wounded trunks of mature trees results in the development of trunk cankers (Mbaka, 2011). Infected trees die in three to five years; this, however, depends on the management of the orchard.

**Significance of Research**

More than 95 % of produced macadamia nuts are exported internationally to Europe, Japan and the United States of America (DAFF, 2015). The industry has the capability to enrich rural livelihoods of macadamia growers. However, root rot and stem cankers caused by *P. cinnamomi* are a notable macadamia production constraint in South Africa. To develop effective management strategies for root rot and trunk cankers, the distribution of *P. cinnamomi* in macadamia growing areas of the country needs to be established.

Early and reliable detection is fundamental to developing appropriate control strategies for plant diseases and limit their further spread. Routine methods that are currently used for the detection and identification of *P. cinnamomi* entail isolating the pathogen directly from soil samples onto antibiotic media (Anderson, 2006). The potential for improved control of this pathogen requires development of molecular detection techniques to confirm morphological identification of *P. cinnamomi*.

Chemical control is the most effective control measure for *P. cinnamomi*, and to this end, phosphate-based fungicides play the prime role (Bekker, 2007). Pathogens, however, have the potential to overcome chemicals by developing resistance. Biological control agents (BCAs) such as *Trichoderma* and *Bacillus* spp. have been reported to control several soil-borne diseases. Their effectiveness in control of *P. cinnamomi* induced root rots, and trunk cankers of macadamia need to be established. This will structure a foundation for their addition in the integrated management of the two diseases.

## Research Objectives

The objectives of this study were, therefore, to:

1. Determine the presence and severity of *P. cinnamomi* in the main macadamia producing areas of South Africa through surveys;
2. Detect *P. cinnamomi* from macadamia soil using nested PCR;
3. Evaluate the use of biological control agents (BCAs) to control *P. cinnamomi* on macadamia.

## Dissertation Structure

This dissertation is composed of five chapters. The first chapter is a review of literature which outlines the history and economic importance of macadamia and the industry, the *Phytophthora* genus, the epidemiology, pathology, and management of *P. cinnamomi* as well as the detection methods used for *P. cinnamomi*. The second chapter focuses on surveys conducted in macadamia farms within the two main macadamia growing provinces, Limpopo and Mpumalanga, as well as disease incidence and severity caused by *P. cinnamomi* on macadamia. The third chapter concentrates on the detection and characterization of *P. cinnamomi* from the soil using an optimized nested PCR. The fourth chapter zeros in on the *in vitro* evaluation of selected bio-control agents against *Phytophthora cinnamomi*. The dissertation ends with Chapter 5 that outlines the major outcomes of this study as well as suggestions for future research.

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# CHAPTER 1

# LITERATURE REVIEW

## 1.1 Macadamia

### 1.1.1 History and Distribution

Macadamia is an evergreen tree that is indigenous to Australia. Knowledge of its existence dates back to 1828 when its nuts were observed to make good food for pigs (Anonymous, 1998). This nut tree was originally found in the subtropical rainforests on the Australian East Coast, in Queensland and New South Wales (Rosengarten, 1984). The Aborigines gathered the nuts during autumn as it was an essential source of food, but did not commercially cultivate the plant. They also used the plant as a base for medicines as well as cosmetics for facial decoration (Anonymous, 1998). In 1858, botanist Baron Sir Ferdinand Jakob Heinrich von Mueller taxonomically classified *Macadamia ternifolia* to honour his good friend Dr. John Macadam, naming the plant macadamia, hence, establishing the genus *Macadamia,* which was endemic to Australia (Shigeura and Ooka, 1984). Before the public adopted the common name macadamia, the nut was also known as the Australian nuts, Queensland nuts, Bauple nuts, Bush nuts or the Australian hazelnut.

Macadamia is the only native Australian plant developed as a commercial food crop (Rosengarten, 1984). The macadamia nuts were domesticated for the first time in 1858 in Australia, and the first commercial plantation was established in 1888 (Rosengarten, 1984). In only 40 years, the Australian macadamia industry expanded from virtually nothing to the largest producer globally (Rosengarten, 1984). William Herbert Purvis then introduced macadamia to Hawaii in 1881 (Forbes, 1928). The Jordan brothers introduced the macadamia nut again in Hawaii in 1892 (Shigeura and Ooka, 1984); the seedlings of the top yielding macadamia cultivars during those years formed the backbone of the present day macadamia industry.  Macadamia was successfully cultivated in Hawaii in 1931, where the first processing factory was established (Shigeura and Ooka, 1984). Commercial cultivation of the nuts has since spread to many other subtropical counties throughout the world.

There is uncertainty as to when the first macadamia trees were introduced in South Africa, but the Durban Botanical Garden already had a tree in 1915 (de Villiers and Joubert,

2003). The tree was possibly established at least eight years earlier (Joubert, 1986). In 1931, the Agricultural Research Council - Tropical and Subtropical Crops (ARC-TSC) established the first seedling trees from imported seeds (Joubert and Thomas, 1963). The seedlings were planted at Soekmekaar in 1957, and by 1960, Reims nursery in KwaZulu-Natal (KZN) had sold over 60 000 seedlings (de Villiers, 2003). The first research on macadamia was conducted by the ARC-TSC in 1963 (Joubert, 1986). Vegetative propagation of suitable, locally selected cultivars, Nelmak 1, 2 and 26, was initiated during the 1970's and since then their production has increased. The first macadamia and pecan nut symposium were held at Polotisi in the Limpopo province in 1979. The interest generated during the symposium led to the formation of the South African Macadamia Growers' Association (SAMAC) (Anonymous, 1988) and the first grower's handbook was published in 1993 (de Villiers, 2003). Macadamia has since become the fastest growing tree crop industry in the country, and macadamias are now widely distributed throughout South Africa.

Macadamia is grown mainly in Australia, Brazil, China, Costa Rica, Guatemala, Hawaii, Kenya, Malawi, South Africa, and Zimbabwe (Wilkie, 2008). There are a number of countries that grow the crop on a small scale, such as Argentina, Colombia, Fiji, Israel, Jamaica, Mexico, New Zealand, Swaziland, Tanzania, United States of America, and Venezuela (Wasilwa *et al.*, 2003). The prices of the nuts are increasing continuously to this day due to the worldwide increase in familiarity and popularity of macadamia, leading to the continuous demand of the "gourmet nut."

### 1.1.2 Botany

Macadamia belongs to a family which consists of evergreen woody plants, the family Proteaceae. The trees can attain a height of up to 20 m and a width of 15 m (Fig. 1.1). They have an even but rough bark, with a brown exterior but dark-red internally. Around the parent root axis, the trees have compact clusters of short lateral rootlets in well-defined rows referred to as proteoid roots (Duke, 1989). The principal function of plant roots, other than to increase the surface area of the root system, which anchors the plant, is for maximum uptake of water and nutrient elements. The plant is thus supplied with growth hormones (Lovegrove and Hooley, 2000), and serves as storage for carbohydrate reserves (Wolstenholme, 1981).

**Figure 1.1:** Mature macadamia trees.

*Macadamia tetraphylla* L.A.S. Johnson and *Macadamia integrifolia* Maiden and Betche are two of the ten species that are commercially cultivated for their edible nuts (McHargue, 1996). The other eight species produce small, inedible and bitter nuts, which contain potentially poisonous cyanogenic glycosides (Joubert, 1986). *M. integrifolia* grows between latitudes 25.5° and 28.3°S; it has round nuts with a smooth shell, leaf margins without spines and three leaves at each node. *M tetraphylla* is characterised by rough-shelled nuts with a spindle shape and four leaves at each node with serrated spiny leaf margins; it is found more southerly, between 27.6° and 29°S (Nagao and Hirae, 1992; Gross, 1995). Macadamia trees produce bunch-like flower clusters that grow up to 30 cm long with 100-300 blossoms. *M. integrifolia* produces yellow-white blossoms whereas *M. tetraphylla* produces pink ones. These trees are hermaphrodites and are capable of self-pollination, but in practice, yields are much higher when two or more varieties are grown in

proximity (Schoeman, 2009). This explains why almost every plantation will cultivate a combination of varieties. It takes 6-11 months from blossom to mature fruit. The mature fruit consists of a green husk that surrounds a hard brownish shell which contains a cream white kernel (nut) (Bittenbender and Hirae, 1990; Yokoyama *et al.*, 1990).

Grafting is used to propagate macadamia. The tree takes about ten years to reach maturity and maximum nut yield, and can continue bearing for over 100 years (Nagao and Hirae, 1992). Macadamia growth is favoured by well-drained, fertile soils, and rainfall of 1000–2000 mm. The optimum growth temperature is 25 °C, although once established the trees can withstand light frosts (Hamilton and Fukunaga, 1959).

### 1.1.3 Economic Importance

The macadamia nut is of great economic importance and has achieved the status as the world's deluxe nut. The nut can be roasted or eaten raw, whole or chopped (Duke, 1989). It can be processed into bakery products, confectionery, ice-cream nut paste, and sauces (Sato and Waithaka, 1996; Yokoyama *et al.*, 1990). Macadamia nuts have 80% mono-saturated fatty acids while the nuts themselves contain 75% fat by weight (Hiraoka-Yamamoto *et al.*, 1994) allowing for edible oil extraction. The oil is a health food product and sustains low blood cholesterol levels, as it comprises no cholesterol (Onsongo, 2003). The fatty acids found in macadamia nuts reduce cardiovascular disease risk factors due to the high palmitoleic percentage, which lowers blood cholesterol levels (Nestel *et al.*, 1994; Curb *et al.*, 2000; Amy *et al.*, 2008; Mattham *et al.*, 2009). The high levels of palmitoleic acid in the oil make it a desirable element in soaps and cosmetics as well. Macadamia oil has a similar composition to olive oil (Cavaletto, 1980).

After oil extraction, the seed cake that contains 43.3 % free nitrogen extract remains, 33.4 % crude protein, 12.6 % oil, 8.1 % moisture, and 2.6 % crude fibre (Mueller, 1957). This seed cake is used as a substitute for fodder (Woodroof, 1967). The husk and shell are useful fuel sources and can be used as potting soil, mulching, and compost (Jenkins and Ebeling, 1985). The husk is used for manufacturing coke, roasting coffee and to dry the macadamia nuts (Augstburger *et al.*, 2002). It was suggested by Rumsey (1927) that the tree is used as an ornamental and as timber. *M. tetraphylla* and *M. integrifolia nuts* have

equal oil content (Saleeb *et al.*, 1973). The nut contains 691 calories, 15.1 - 15.9g total carbohydrates, 3.0 - 3.1g of water, 71.4 - 71.6g fat, 7.8 - 8.7g protein, 2.5g fibre, 1.7g ash, 48mg calcium, 161mg phosphorous, 20mg iron and 264mg potassium in 100g.

The steady and increasing demand for the “prime” edible nut, as well as the growth in world production results in the high market value for macadamia nuts. The need to expand sources of agricultural income and the constantly increasing value of macadamia nuts, especially in developing countries, has led to more plantings and production (Serfontein *et al.*, 2007; Wilkie, 2008).

## 1.2 The Macadamia Industry

### 1.2.1 Global Macadamia Industry

Macadamias have had significant growth of 78% in world production, from 2004 to 2015, achieving 46 399 metric tons (MT) (kernel basis) in 2015 (INC, 2015). Australia has been the largest macadamia nut producer since 2015. It produced 13 530 MT of macadamia, followed by South Africa (12 900 MT), Kenya (8 846 MT), The United States of America (USA) (3 600 MT), and Malawi (1 781 MT) (Fig. 1.2). These countries account for 88% of the world production as indicated in Figure 1.3 (INC, 2015).

Australia provides approximately 29 % of the world supply to more than 40 countries, leading South Africa and Hawaii, which supply 25 and 16 % of macadamia kernels, respectively (AMS, 2016). South Africa was the top exporter of shelled macadamias with the USA as the principal destination accounting for 43 % of all South African kernel exports. Australia has ranked number one in export since 2015 with Japan being their biggest export market, followed by Europe and North America (AMS, 2016). The AMS (2017) announced that in 2017 the Australian macadamia crop was forecast to reach 54 000 tons in shell at 10 % moisture and 50 500 tons at 3.5 % moisture; this represents the fourth consecutive year of steady growth for the Australian industry.

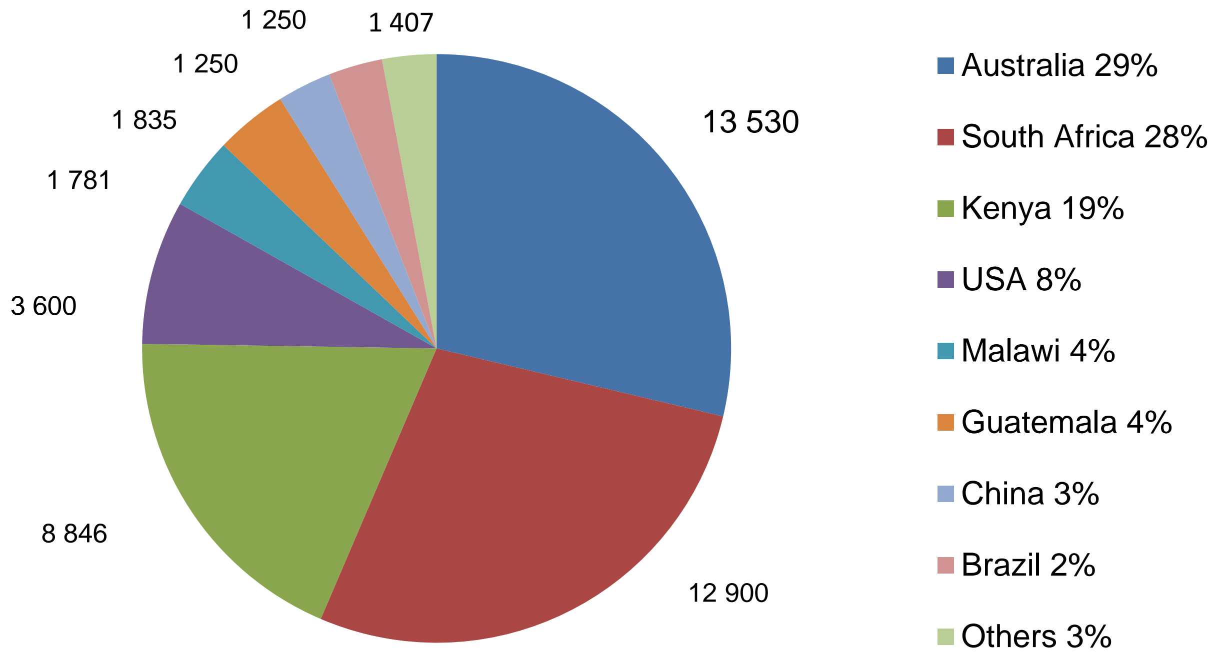



**Figure 1.2:** Estimated macadamia production in 2015 (INC, 2015).

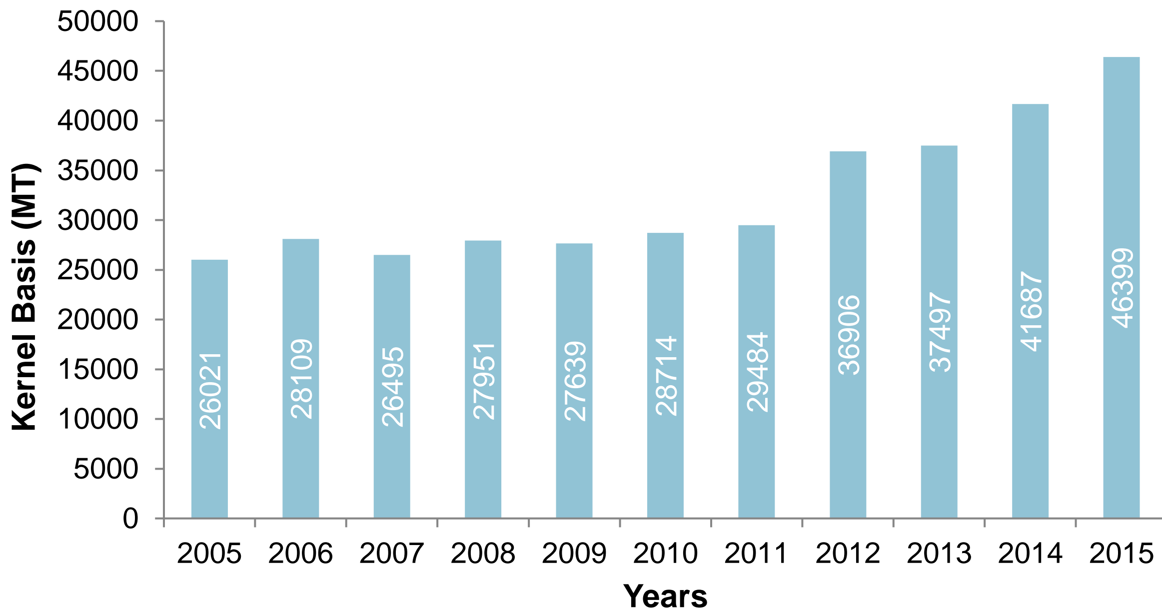



**Figure 1.3:** Macadamia global production from 2005 to 2015 (INC, 2015).

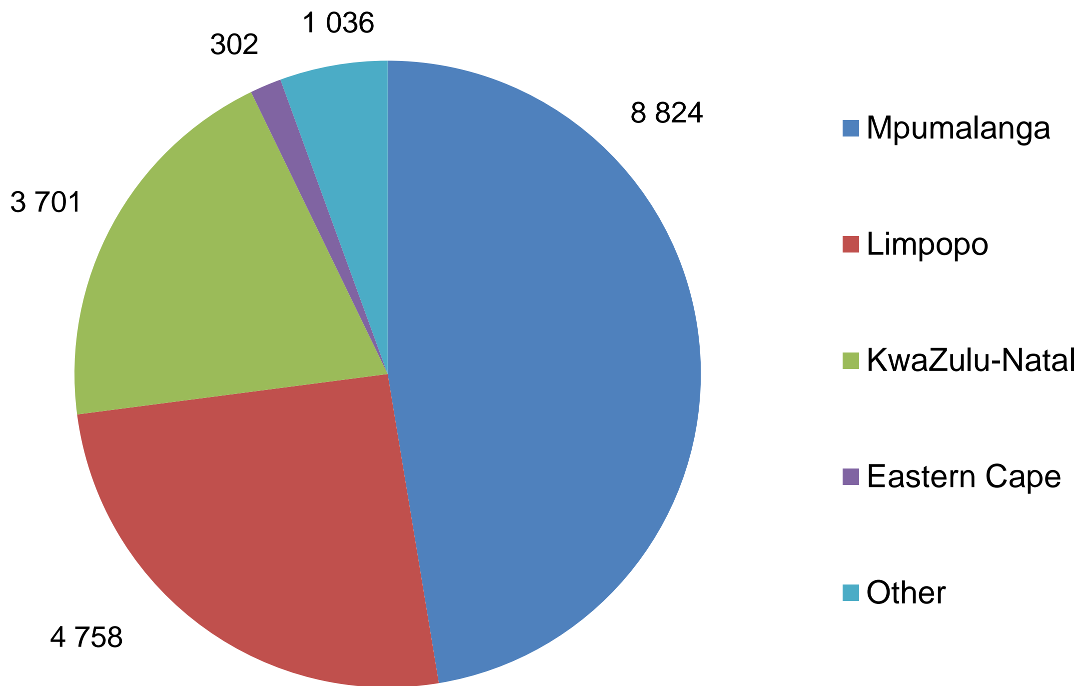
Macadamias currently account for less than 3% of the world tree nut market, therefore, marketing into new and existing markets, allows the unlimited potential for macadamia nuts. On average, South Africa exports 9 254 MT of shelled macadamia to other countries; that is 30% of the world production exported worldwide (INC, 2015). Australia and China export 5 647 (18%) and 3 927 (13%), respectively. These three countries are the highest in macadamia export worldwide (INC, 2015).

### 1.2.2 The South African Macadamia Industry

Macadamia nuts are one of four most broadly grown subtropical tree crops in South Africa together with avocado, litchi, and mango. Macadamias are the fastest growing tree crop industry in the country as they cover 44% of agricultural land used followed by avocados which cover 34% (Jaskiewicz, 2015).

The industry is formally organized through an industry body called the South African Macadamia Growers' Association (SAMAC), which is made up of nut growers, processors, nurseries, marketers, international members, handlers and service providers (SAMAC, 2016). SAMAC is a member of The International Nut and Dried Fruit Council (INC), which presents the opportunity to interact with international macadamia role players and other numerous significant role players within the international nut trade (SAMAC, 2016). South Africa is the largest producing country in Africa. According to SAMAC (2016), the area under macadamia tree is approximately 17 800 hectare with roughly 5.3 million macadamia nuts trees.

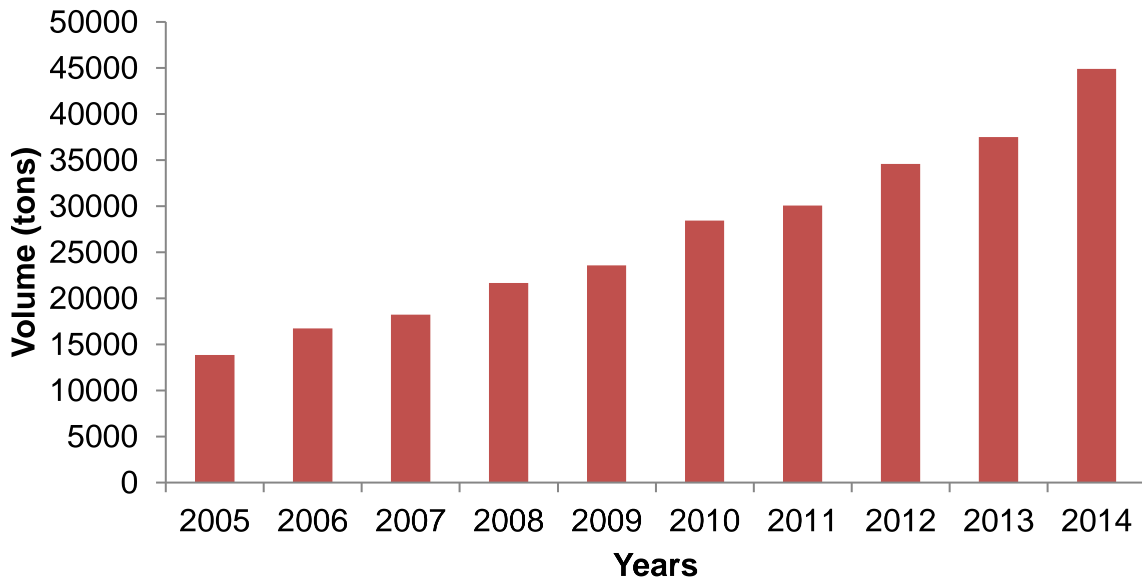
Mpumalanga, Limpopo, and KwaZulu-Natal are the main production areas of macadamias due to their subtropical climate; other minor production areas include the Eastern and Western Cape (Fig. 1.4). Mpumalanga has over 2.4 million number of trees followed by Limpopo with over 1.4 million, KwaZulu-Natal with approximately 1.1 million and Eastern Cape with 91 506 (DAFF, 2015). Macadamia production has steadily increased to more than triple (from 16 000 to 43 000 tons) over the last ten years, from 2005 to 2014 (Fig. 1.5) (DAFF, 2015).




1

2 **Figure 1.4:** Major macadamia production areas of South Africa in hectares (SAMAC, 2016).

3




4

5 **Figure 1.5:** South Africa's dry in shell macadamia production (DAFF, 2015).

There is an average of 20% increase in prices of nuts per kg in the macadamia nut sector every year due to the sector's rapid growth (DAFF, 2015). In 2015, the predicted average price was 16.17 USD per kilogram of kernel (Green Farms, 2015). No regulations are placed on macadamia nut prices by the South African government; the market forces of demand and supply determine the costs. The major purchasers of macadamia nuts in South Africa are Pick n Pay, Spar, and Woolworths; the nuts are also marketed directly to processing companies (DAFF, 2015); and over the last decade, about 3 500 new job opportunities have been created on macadamia farms.

The South African macadamia industry is almost entirely export based with more than 95% of annual production shipped to international markets, with South Africa being number one in the world in macadamia nut exports from 2011 to 2014, commanding 37.1% share of the world exports (DAFF, 2015). Of the world production, 12% is exported annually, and in 2015, 52% of nuts were distributed as nut in shell, and the rest were processed to kernel (DAFF, 2015). The primary markets of South African macadamia nuts are Asia (predominantly Hong Kong, China), Europe, and the USA (DAFF, 2015).

Raw macadamia kernel that has been vacuum packed, provided it conformed to all quality specifications, is stored in a cool (15°C to 25°C), dry and well-ventilated area (Cavaletto *et al*., 1966). The nuts should be dried to a moisture content of approximately 1.5%. If the nuts are stored immediately without being dried, fungal growth could occur (DAFF, 2015). The macadamia industry suffers a high incidence of local, organized theft and more importantly, it suffers from diseases caused by pests (mostly the stinkbug and nut borer) and pathogens that affect production and nut quality.

## 1.3 Insect Pests and Diseases of Macadamia

The stinkbug (*Nezara viridula* Linnaeus) and the false codling moth best known as the macadamia nut borer (*Cryptophlebia leucotreta* Meyr.) are the two major insect pests that attack macadamia nuts (Jones and Caprio, 1994; Golden *et al*., 2006). Cultural control methods that produce smoke to repel the insects such as immediately burning the husks after de-husking and burning trash under the trees are used to control the two insect pests (Schoeman, 2009). Termites (*Macrotenus* spp.), thrips (*Heliothrips haemorrhoides* Bouche),

and weevils (*Nematocerus* spp.), are minor insect pests that also attack macadamia nuts (Ironside, 1981).

### 1.3.1 The Macadamia Stinkbug (*Nezara viridula*)

The stinkbug feeds on kernels causing substantial fruit drop of small macadamia fruits as well as sunken lesions on kernels of mature nuts (van de Berg, 1995). It injects saliva which contains enzymes into the kernel by inserting its needle-like mouthpart into the nut. The enzymes liquefy the tissue around the tip of the mouthpart, and the bug consumes the liquid (van de Berg, 1995). Infected kernels are followed by secondary fungal infections causing the kernels to become spongy, sometimes with brown pit-like depressions (Mbaka, 2011). Nuts infested on the ground may also have white or brown discolouring. These kernels are not white but have a translucent appearance, are soft, shrivelled and inedible. The damage caused by macadamia stinkbug can be as high as 90% in uncontrolled, large orchards and higher temperature conditions (Wright *et al*., 2007).

### 1.3.2 The Nut Borer

Nut borers infect fallen nuts and stick-tights (nuts that fail to abscise after maturation). After hatching, the larvae of the nut borer eat and bore their way into the shell of macadamia nuts; they can also penetrate the shell, therefore, destroying the kernel (de Villiers, 1993). The fruit will drop, and larvae develop to maturity on the fallen fruit. Mature fruit damaged by the nut borer may weep and stain other fruit in the cluster or those hanging below (de Villiers, 1993). Actual losses caused by this pest may be underestimated because feeding by the nut borer introduces mould.

### 1.3.3 Raceme Blight

Raceme blight is a disease caused by the fungi *Cladosporium cladosporioides* (Fresen.) *and Botrytis cinerea* (Persoon) (Bittenbender *et al*., 1998; van de Berg *et al*., 2008). The fungi, causing flower abortion results in a reduction in yield of the nuts. It infects young racemes and the flowers of the macadamia tree. Flowers are susceptible until nuts set. The disease is, however, more severe in older orchards and in situations of high-density plantings where trees are shaded (Sikinyi, 1993).

### 1.3.4 Macadamia husk spot

This disease is caused by the fungus *Pseudocercospora macadamiae* sp. nov. (Drenth, 2007). Chlorotic spots appear on the husk of the nut; they become dark brown and are harder than the surrounding tissue. Husk spots start as yellow flecks and extend to 3-6 mm before turning brown in the centre (Drenth, 2007). Spots may produce a velvety grey carpet of spores under moist conditions. The fungus does not affect the shell and kernel, but infected nuts may drop prematurely. These nuts are usually not suitable for processing as they are immature and of low oil content, causing direct yield losses of up to 40% (Stephenson *et al*., 2003; Drenth, 2007; Miles *et al*., 2009).

### 1.3.5 *Phytophthora* Blight

*Phytophthora* blights destroy flowers and developing nuts and are characterised by blighting of immature racemes and nuts. The fungus *Phytophthora capsici* is responsible for this disease (Hunter *et al*., 1971; Kunimoto *et al*., 1975). The first point of infection occurs on the husk, where a brownish-black discolouration is observed (Kunimoto *et al*., 1975). Infection can occur before the hard nut shell is formed. This causes the fungus to rapidly penetrate and destroy the kernel (Hunter *et al*., 1971). A few days post infection, diseased nuts fall from the branches. This disease usually develops in rainy or foggy environments, with a lot of moisture (Drenth, 2007). Areas with low air circulation and high planting densities are more susceptible to *Phytophthora* blights; foliage flushes are negatively affected, and yields are reduced (Drenth, 2007).

Most fungal diseases that affect macadamia are not of major importance, except *Phytophthora cinnamomi*, which is amongst those of great economic importance (Augstburger *et al*., 2000). Stem cankers and root rots are the foremost macadamia production constraints. The rots of the feeder roots result in aerial tree symptoms (Zentmyer, 1984). In South Africa, *P. cinnamomi* is reported to cause quick decline, root rot, and trunk cankers in macadamia (Serfontein *et al*., 2007).

## 1.4 The Genus *Phytophthora*

*Phytophthora* is a diverse genus of Oomycete plant pathogens that belong in the Kingdom Stramenopila, and Phylum Oomycota (Erwin and Ribeiro, 1996). The Oomycetes includes four orders. The important plant pathogens are found in the Peronosporales and

the Saprolegniales; the remaining two encompasses small groups of mostly aquatic fungal-like organisms (Table 1.1). The family Pythiaceae is found within the Peronosporales, which contains the best-known genera which are *Phytophthora* and *Pythium* (van der Plaats-Niterink, 1981). The cell wall of oomycetes is composed of a mix of cellulosic compounds and glycan, and not of chitin which is found in true fungi (Money *et al*., 2004). *Phytophthora* species are water moulds and are favoured by free water in the soil and on foliage.

The name *Phytophthora* originated from the Greek and means plant (*phyto*) destroyer (*phthora*) (Erwin and Ribeiro 1996). This genus consists of some of the most destructive plant pathogens known; these plant pathogens affect many crops worldwide. One hundred and twenty-three species have been described in the genus *Phytophthora* (www.phytopthoradb.org/species.php*)*, most of which are plant pathogens that cause significant production losses in a wide range of host plants (Zentmyer, 1980). Some *Phytophthora* species are responsible for some of the world's most destructive plant diseases such as the 19th century's European potato famines caused by *P. infestans* (Bourke, 1964).

*Phytophthora* species are, therefore, of environmental and economic importance in various plant systems including agriculture, natural ecosystems, forestry and horticultural based industries in the tropical and temperate zones of the world (Hardy and Sivesithamparam, 1988). Due to the wide host range of *Phytophthora* species, numerous species are able to exist within a single system at the same time. For example, *P. megasperma*, *P. citricola*, *P. gonapodyoides*, *P. syringae*, *P. haveae*, *P. citrophthora*, *P. nicotiane*, *P. drechsleri*, *P. lateralis*, *P. cryptogea* and *P*. *cinnamomi* are root pathogens of woody ornamental tree species in nursery systems. There is a possibility of the pathogens have the spreading to wherever the plants are sold (Hardy and Sivesithamparam, 1988; Ferguson and Jeffers, 1999). Furthermore, the epidemic recognised as "Jarrah dieback" in the south-west of Western Australia may be caused by *P. cinnamomi*, *P. citricola*, *P. cryptogea*, *P. nicotianae* or *P. megasperma* var. *sojae* (Shearer *et al*., 1987).

The integration of structural, biochemical and DNA characteristics determines the taxonomic classification of *Phytophthora* species (Cooke and Duncan, 1997; Cooke *et al*., 2000; Hardham, 2005). According to the most recent taxonomic review, *P. cinnamomi* Rands is classified in the kingdom Stramenopila; Phylum Oomycota; order Peronosporales; family Peronosporaceae and genus *Phytophthora* (Hardham, 2005). This recent

classification acknowledges the genetic and biochemical discrepancy between *Phytophthora* and other fungi.

**Table 1.1 Classifications of the Oomycetes (Hawksworth *et al.*, 1995).**

| Kingdom | Class | Order | Family | Genus |
|---|---|---|---|---|
| Stramenopila | Oomycetes | Lagenidiales | | |
| | | Leptomitales | | |
| | | Saprolegniales | Saprolegniaceae | *Achlya*<br>*Saprolegnia* |
| | | Peronosporales | Pythiaceae | *Pythium*<br>***Phytophthora*** |
| | | | Peronosporaceae | *Bremia*<br>*Peronospora* |
| | | | Albuginaceae | *Albugo* |

## 1.5 *Phytophthora cinnamomi*

*Phytophthora cinnamomi* is a notorious soilborne "*pseudofungus"* (Hardy *et al.*, 2001) with a global distribution that affects numerous plants in agricultural, forest and horticultural ecosystems (Pérez-Jiménez, 2008). Rands was the first to describe this pathogen in 1922 (Erwin and Ribeiro, 1996) as the causal organism of a stem canker on *Cinnamomum burmannii* Blume (cinnamon tree) in Sumatra (Tucker, 1931). The origin of this pathogen is unclear, but phylogenetic studies suggest that *P. cinnamomi* originated from New Guinea-Malaysia-Celebes and was introduced to the various tropical and subtropical regions of the world where it has been documented (Linde *et al.*, 1999).

*Phytophthora cinnamomi* is one of the most universal and destructive plant pathogens. Since its discovery, *P. cinnamomi* has been recognised widely worldwide, being found in more than 75 countries (Pérez-Jiménez, 2008). Within the genus *Phytophthora*, *P. cinnamomi* is renowned as the species with the largest host range. More than 3 000 plants have been documented as susceptible to *P. cinnamomi* (Pérez-Jiménez, 2008). Thus the pathogen poses a threat to many economically important agricultural, ornamental and many native Australian and South African plants (Erwin and Ribeiro, 1996). These plants include: avocado, chestnut, pear, kiwi fruit, and eucalyptus, to name but a few (Pegg *et al*., 2002). The destruction of an approximated 202 500 ha of the jarrah forests (*Eucalyptus marginata Sm.*) of Western Australia between 1927 and 1986, amply demonstrated the destructive potential of *P. cinnamomi* (Podger, 1972).

The first record of *P. cinnamomi* in South Africa was on avocado in 1931 (Doidge and Bonomley, 1931; Wager, 1931). This led to diseases of major financial significance, such as root diseases of *Eucalyptus* and *Pinus* species (Wingfield and Knox-Davies, 1980; Linde *et al*., 1994) and root rot of avocado (Wager, 1942). The first report of this pathogen on macadamia was in October 1959, where stem cankers were seen on two macadamia trees on a commercial farm in Vista, California (Zentmyer, 1960). The macadamia trees were replanted in in an area where avocado trees had been removed because of *Phytophthora* root rot, caused by *P. cinnamomi*. *Phytophthora cinnamomi* was described as the causal agent of macadamia root rots and stem cankers in Hawaii, Australia, and California (Hine, 1961; Zentmyer, 1979; Rosengarten, 2004; Ko, 2009). In the early 1980s, there were cases of death of macadamia trees in South Africa, and the cause was identified as root rot caused by *P. cinnamomi* (Sikinyi, 1983). Evidence that *P. cinnamomi* may be indigenous to the South Western Cape Province of South Africa was presented by von Broembsen and Kruger (1985), as the pathogen was isolated from numerous local plants in undisturbed areas and also from rivers flowing from secluded mountain areas.

*P. cinnamomi* is one of the most important and damaging diseases of macadamia causing up to 60% in yield losses (Muthoka *et al*., 2005). The pathogen attacks trees of all ages, from nursery trees to large fruit-bearing trees, causing decay of the fine feeder roots resulting in leaves are smaller and light green to yellow rather than dark green. On mature macadamia trees, trunk cankers develop above the soil line (Mbaka *et al*., 2009). Dark,

sooty material emanates from the infected areas which become cracked or irregularly flattened due to death of the tree's cambium (Schroth *et al.*, 2000; Janick and Paul, 2008). Due to infection through the fine feeder roots, root rots occur resulting in chlorosis, die back and failure of new branch development leading to a substantial reduction in the incremental growth rate (Zentmeyer, 1960). Benson and von Broembsen (2001) noted that depending on soil nutrient and moisture balance, infected trees die in about three years or more.

### 1.5.1 Epidemiology and Aetiology

*P. cinnamomi* has a complex life cycle consisting of many forms, which enhances its persistence in the soil (Zentmyer, 1983). Dissimilar to many soil-borne plant pathogens, *P. cinnamomi* is polycyclic, meaning its inoculum can increase from low, often undetectable levels, to high levels in a short period of time, particularly in warm, moist and well-aerated soils (Zentmyer, 1980). The pathogen has a short generation time and an immense reproductive capacity, which promotes further escalation in disease potential.

Infection is escalated by high soil moisture due to increased sporangial production, resulting in zoospore release, motility, and movement to feeder roots. Zoospores, which are responsible for rapid colonisation witnessed during epidemics, occur soon after (Zentmyer and Mircetich, 1966). Zoospores are short-lived and only motile in soils for short periods of time (minutes to hours). In the course of unfavourable temperature and moisture conditions, *P. cinnamomi* mainly survives as chlamydospores and mycelium in root debris and soil (McCarren *et al.*, 2005). *P. cinnamomi* can persist in symptomless plants, debris, and topsoil for a few years (Kong *et al.*, 2003b). The rapid rebuilding of the population occurs under favourable conditions from these sources of inoculum. Weste and Vithanage (1978) stated that the prompt production of infective zoospores intensifies the dissemination and persistence of the pathogen to new hosts.

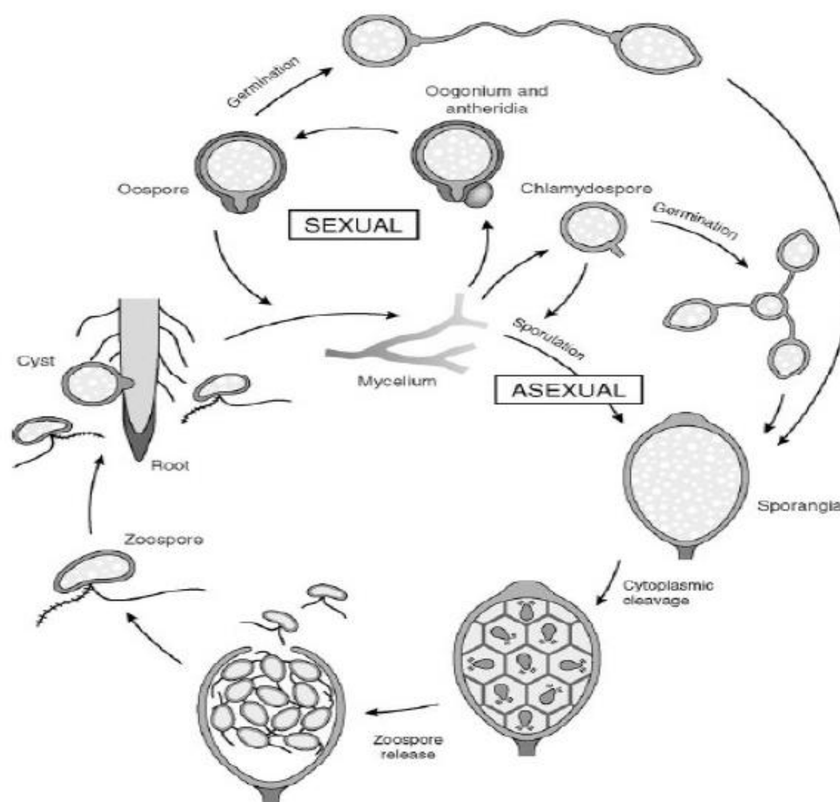
### 1.5.2 Mechanism of Infection

*P. cinnamomi* has two mating types, A1, which is geographically limited with fewer hosts, and A2, which has a global distribution; it is diploid and heterothallic (Galindo and Zentmyer, 1964). In South Africa, the A2 mating type has been coupled with cultivated

forests and agricultural crops (von Broembsen, 1984). *P. cinnamomi* reproduces in two phases: the asexual, which produces motile zoospores, non-motile chlamydospores and sporangia (Ribeiro, 1983); and the sexual, where hetero-gametangial contact takes place to produce non-motile, thick-walled, oospores (Elliot, 1983). The life cycle of *P. cinnamomi* is represented in Figure 1.6. During sexual reproduction, the oogonium penetrates the antheridium resulting in the development of an oospore, by fusion of gametangial nuclei (Ribeiro, 1978).

At an early development stage, the antheridium attaches firmly to the oogonium. The attachment can be either amphigynous (where oogonium grows through antheridium) or paragynous (where antheridium is attached on the side of the oogonium) (Pegg *et al.*, 2002). Following fertilisation, an oospore nearly filling the interior of the oogonium develops. Thick membranes envelop these oospores, as they are the most resistant structures produced and can survive in the soil for many years (Mckay, 1957; Duncan and Cowan, 1980). Oospores can germinate directly (by forming a germination tube) or indirectly (by releasing zoospores) depending on numerous factors. These factors include temperature (Klisiewicz, 1970), light (Ribeiro *et al.*, 1976), nutrition (Banihashemi and Mitchell, 1976) and enzymes (Ribeiro, 1983).

*P. cinnamomi* can also reproduce asexually by producing non-motile chlamydospores, which germinate by germ tubes, and motile zoospores (Ribeiro, 1983). Zoospores operate as the primary structures involved in the infection development (Erwin and Ribeiro, 1996). They form inside the sporangium before being released through the apex of the sporangium (Zentmyer, 1983). Upon release, zoospores swim towards and adhere to the root surface where they build up (Carlile, 1983). Once they reach the plant root, zoospores develop a cell wall and encyst (Erwin and Ribeiro, 1996), providing that highly unfavourable conditions do not cause lysis (Aveling and Rijkenberg, 1989). Zoospores become adhesive during the early stages of encystment allowing them to attach firmly to plant roots and invade them (Sing and Bartnicki-Garcia, 1975).




1
2 **Figure 1.6:** Generalised disease cycle of *Phytophthora cinnamomi* (EPPO, 2004).
3
4 Germ tubes of cysts located near macadamia roots emerge from the side nearest to
5 the root and grow toward it (Zentmyer, 1970). Encysted zoospores can either germinate
6 directly to produce additional zoospores (repeated emergence) or create vegetative hyphae.
7 They produce germ tubes that grow in the direction of the root tip elongation area (Carlile,
8 1983). The germ tubes either penetrate the roots directly and the hyphae grow into the host
9 tissues. Toxins that kill the host cell are produced by the pathogen, followed by host
10 invasion (Carlile, 1983; Guest and Brown, 1997), or they form appressoria-like swellings
11 before penetration (Carlile, 1975).
12
13 **1.5.3 Disease Development on Macadamia**
14 *P. cinnamomi* causes root rot where the macadamia feeder root system is invaded
15 through penetration of the epidermis and cortex, killing the underlying root tissues
16 (Zentmyer, 1980). As the disease progresses, the feeder roots blacken, become brittle,

shrivelled and necrotic, ultimately killing the tree (EPPO, 2004). Pegg *et al*. (2002) stated that larger roots could be infected as well, leading to the formation of brown lesions in the wood, resulting in the bark peeling and trunk wounds ultimately causing trunk canker. Trunk cankers are a result of *P. cinnamomi* entering root and trunk wounds. These appear as roughened, sunken, deeply furrowed lesions that expand to 2 m or more up the stem, ultimately girdling the stem and adjacent branches of the plant.

Water and nutrient uptake by the plant is reduced when the feeder roots are destroyed (Broadley, 1992; Erwin and Ribeiro, 1996); this can decrease nut set and contribute to early nut drop. The disease can promptly spread in younger plants causing sudden wilt (Erwin and Ribeiro, 1996). Metabolic alterations and phytotoxic metabolites produced by *P. cinnamomi* may indirectly be the cause for water shortage to the aboveground parts of the plant, leading to wilting and disease development on the crop. Additionally, the mycelium that grows through intercellular spaces within macadamia root tissues physically blocks the relocation of plant metabolites, nutrients and water (Keen and Yoshilawa, 1983). Unnatural distribution of nutrients in plant tissue and interference with nutrient uptake results in visible symptoms such as chlorosis, smaller leaves that wilt and drop prematurely, and limited new leaf growth. The canopy then becomes bare and may be reduced to a skeleton of focal branches (Fig. 1.7).

*P. cinnamomi* propagules are disseminated by soil movement, including irrigation ditches, debris or wind-blow, or by water flow and run-off in drainage (EPPO, 2004). Nursery stocks may be the main reason for the spread of *P. cinnamomi* to disease-free areas. This kind of distribution, however, has decreased due to the introduction of stringent hygiene procedures in the nursery industry (Hardy and Sivesithamparam, 1988). Control of this pathogen is difficult due to the expansive host range and the durability of propagules in the soil.

**Figure 1.7:** Macadamia tree showing aboveground symptoms of *P. cinnamomi*.

#### 1.5.4 Control

*P. cinnamomi* can persevere in plants, plant debris or soil for extensive periods without showing symptoms, making this pathogen challenging to manage (Kong *et al.*, 2003b). Control of this pathogen varies in the various systems (agricultural, nursery and native plants) where it exists. *P. cinnamomi* can be eradicated from small sites, and its spread can be controlled by the use of containment methods (Commonwealth of Australia, 2014). Further work is, however, required to minimize its dispersal to un-infested sites and to reduce its impact on infested sites (Dunstan *et al.*, 2011). There are currently no robust methods available to exterminate this pathogen from spot infestations or to inhibit its spread along an active disease front. The need to eradicate or contain the pathogen is now paramount to ensure macadamia and other threatened flora are protected for the long term. To limit or eradicate the pathogen, biological, cultural and chemical methods may be applied in controlled environments (Erwin and Ribeiro, 1996). A combination of these procedures could be used in integrated plant protection predominantly to prevent and, if infection takes place, to coexist with the pathogen, without eradication but controlling the

population, enhancing appropriate environmental conditions for root development and plant growth and increase production despite of the pathogen presence (Coffey, 1984).

#### 1.5.4.1 Cultural

Quarantine and hygiene are the first line of defence for *P. cinnamomi* and are important in the control of this pathogen. Hygienic procedures focus on averting the introduction of the pathogen through soil, water or tools and its spread from infected areas into nurseries and uninfected parts to secure pathogen-free macadamia plants (Hardy *et al.*, 2001). Weste (1983) found that improving aeration by increasing drainage, balanced mineral nutrition and alleviation of high soil moisture levels aids in the plant's tolerance of *P. cinnamomi* by enhancing its defence responses and ability to survive low levels of infection. Enhancement of soil organic content escalates the quantity and activity of soil microflora that can inhibit *P. cinnamomi* in some soils and may be prospective biocontrol agents (Menge *et al.*, 2001). Tsao and Oster (1981) stated that animal manures reduce *P. cinnamomi* populations. In South African macadamia orchards, composted pig and chicken manure reduced and suppressed *P. cinnamomi* in the soil (Aryantha *et al.*, 2000). Moreover, the use of resistant macadamia trees will inhibit the pathogen. Zentmyer (1979) reported that *M. tetraphylla* seedlings were more resistant to *P. cinnamomi* than those of *M. integrifolia*.

#### 1.5.4.2 Chemical

In 1977, Schwinn *et al.* (1977) identified phenylamides (acylalanines), which contains furalaxyl and metalaxyl as inorganic fungicides to control diseases caused by oomycetes. In that very same year, Bertrand *et al.* (1977) found phosphonates such as fosetyl-Na and fosetyl-Al could control oomycetes as well. These chemicals have proved to be most effective as curative and systemic fungicides for control of *P. cinnamomi* when applied as foliar sprays or soil drench (Aryantha and Guest, 2004; Nartavaranat *et al.*, 2004). However, accelerated biodegradation has caused limitations in the management of diseases caused by *Phytophthora* spp. (Gisi and Cohen, 1996). Phosphonates have been found to be more ecologically friendly for the management of *P. cinnamomi* infestation (Guest and Grant, 1991).

Phosphonate fungicides and their breakdown product, phosphorous acid ($H_3PO_3$), are highly mobile in plants (Guest *et al.*, 1995). Translocation in association with photo-assimilates, in a source-sink relationship by both phloem and xylem, leads to a direct relationship between phosphite concentration in plant tissue and application rate (Hardy *et al.,* 2001). Phosphonates control *P. cinnamomi* by stimulation of host defence mechanisms as well as by direct fungitoxic activity (Guest *et al.*, 1995; Wong *et al.*, 2009). Phosphites (salts of phosphonic acid, $H_3PO_3$), also have direct effects on controlling the pathogen, resulting in the reduction of zoospores in infected plants (Wilkinson *et al.*, 2001). The efficacy of phosphite differs with various *P. cinnamomi* isolates (Hardy *et al.*, 2001) and environment (Guest and Grant, 1991). Phytotoxic conditions in phosphate-deprived plants can be observed, where phytotoxicity symptoms show a linear relationship with phosphite application rate and are likely to occur in all instances where phosphite is applied, even at recommended rates (Hardy *et al.*, 2001). The fungicide does, however, not affect new growth. Different application methods of phosphites have been applied such as foliar sprays, soil drenches, trunk injections and trunk paints (Hardy *et al.*, 2001). Darvas *et al.* (1983, 1984) first reported the use of a trunk injection method by injecting 0.4g fosetyl-Al.$m^2$ canopy area and obtained “outstanding control” of *P. cinnamomi*. Trunk injections require a much lower chemical dosage than foliar sprays (Whiley *et al.*, 1995), are longer lasting (Hardy *et al.*, 2001), and are currently the preferred option.

*P. cinnamomi* strains can develop resistance to fosetyl-Al and $H_3PO_3$; this was confirmed *in vitro* when *P. cinnamomi* isolates obtained from trees treated with fosetyl-Al or $H_3PO_3$ were less affected by fosetyl-Al and $H_3PO_3$ compared to isolates acquired from untreated trees (Duvenhage, 1994). Duvenhage (1994) concluded that the possibility of resistance exists. However, the use of resistant or tolerant macadamia rootstocks against *P. cinnamomi* would be the best method for reducing the pathogen (Coffey, 1987).

#### 1.5.4.3 Resistance

One of the most efficient approaches of managing diseases caused by soil-borne pathogens is the use of plants expressing increased natural resistance. Prior knowledge of the presence of *P. cinnamomi* enables the selection of plant species or cultivars resistant or tolerant to *P. cinnamomi.* In this context, the approach to the control of *P. cinnamomi* on macadamia by resistance has to involve two aspects: the search for resistant macadamia

rootstocks and the search for resistant plants to use as replacement crops. Genetic diversity must be explored by macadamia breeders to breed favourable traits (McHargue, 1996). This can be done through a number of approaches, such as Marker-assisted selection (MAS) which can select a trait of interest such as disease resistance based on the marker linked to it (Semagn *et al*., 2006). Tissue culture techniques resulting in clonal plant material can also be used to induce resistance in macadamia trees by enriching the plantlets *in vitro* using endophytes to stimulate resistance against *P. cinnamomi* (Saikkonen et al., 2004). Another way would be through plant genetic transformation, which introduces foreign DNA sequences that result in transgenic plants. The shoots and roots are regenerated from cells that contain the foreign DNA through tissue culture techniques (McClean, 1998). Since none of these resistant macadamia trees have been developed and more work is still being done, a more sustainable approach to reducing *P. cinnamomi* is the use of biological control agents (BCAs).

#### 1.5.4.4 Biological Control

Biological control through alteration of soils with modifications or applying proficient bio-control agents (BCAs) shows promising results in decreasing root rot (Pegg *et al*., 2002). Components of the soil micro-flora subdue *P. cinnamomi* in some soils and are possible BCAs (Borst, 1983; Weste, 1983). A number of authors including Pegg (1977), Casale (1990) and Duvenhage and Kotze (1993) and studied biological methods to manage *P. cinnamomi*. As a result, specific antagonistic isolates of bacterial and fungal species that have shown antagonistic properties have been selected from suppressive soils using various approaches to examine their bio-control potential towards *P. cinnamomi*. This inhibition is via parasitism, antibiosis, nutrient competition, competitive exclusion and saprophytism (Korsten and De Jager, 1995). A reduction of more than 50% in *P. cinnamomi* populations was reported by McLeod *et al.* (1995) using *Trichoderma* isolates. *Angullospora pseudolongissma, Catenaria anguillae, Ceratomyces tessulatus, Epiccocum purpurscens, Hypochytrium catenoides, Humicola fuscoatra, Microspermacarbonaceae, Myrothecium roridum, Myrothecium verrucaria, Streptomyces griseoalbus, Streptomyces vioascens* and *Trichoderma harzianum* proved to be antagonistic against *P. cinnamomi* (Erwin and Ribeiro, 1996; Duvenhage and Köhne, 1997; Downer, 1998). There are currently some commercial

bio-control products available with *Gliocladium, Bacillus* or *Trichoderma* as the bio-control agents (Chambers and Scott, 1995).

## 1.6 Detection techniques for *Phytophthora cinnamomi*

Early and reliable detection is essential for the restriction of plant diseases, making it a primary weapon against plant pathogens (Eden *et al*., 2000). Highly specific, robust and sensitive detection techniques are required to allow implementation of management strategies and preventing further spread of the pathogen. Detection assays are especially necessary for diseases that show symptoms only when the pathogen is well established such as those caused by *P. cinnamomi* (Kong *et al*., 2003b). Early detection and diagnosis of *P. cinnamomi* is hindered by the fact that the pathogen can only be diagnosed once symptoms are visible on plant tissues above the ground (Aberton *et al*., 2001). Latent symptom expression and infection by secondary pathogens mean pathogen diagnosis based on symptom development may be too late for efficacious management to be undertaken (Anderson, 2006). Detection of the *P. cinnamomi* from soil samples is, therefore, paramount for disease management (Eden *et al*., 2000). To effectively deal with the needs of the modern commercial world, detection methods for *P. cinnamomi* need to ensure accurate diagnosis, enable surveys in areas with suspected infestations and provide answers rapidly (Judelson and Messenger-Routh, 1996).

### 1.6.1 Isolation

*P. cinnamomi* is a primary invader that attacks only living or freshly wounded tissue that has not been invaded by other pathogens (Drenth and Sendall, 2001). A current routine method for the detection and identification of *P. cinnamomi* involves isolation of the pathogen directly from soil samples and plant tissue onto selective antibiotic media.

*P. cinnamomi* isolation from infected macadamia roots is relatively simple and efficacious if the tissue is in an active stage of infection. It is intricate to isolate *Phytophthora* from necrotic tissue as it often contains many secondary pathogens, enabling the pathogen to be present with no visible symptoms (Erwin and Ribeiro, 1996). For successful *P. cinnamomi* isolation from plant tissue, the tissue must be selected from the edge of an

actively growing lesion and transferred onto PARPH medium (Drenth and Sendall, 2001). To isolate the pathogen from the roots, the roots are washed with water, cut into small segments, and then plated onto PARPH (EPPO, 2004). Plates are incubated for 2–6 days in the dark at 22–27°C. Following isolation, mycelia suspected to be *P. cinnamomi* is transferred to potato dextrose agar (PDA) for observations by a compound microscope. On PDA *P. cinnamomi* mycelium resembles rose petals or a camellia flower. It is easy to identify *P. cinnamomi* due to its unique morphological features: sessile terminal or lateral protuberances produced singly or in clusters, a distinctive coralloid mycelium with abundant hyphal swellings, and swollen vesicles (EPPO, 2004). Ribeiro (1978) reported that the presence of *Pythium* makes isolation of *Phytophthora* from roots and soil difficult. However, the use of a selective medium PARPH (Jeffers and Martin, 1986) containing hymexazol (a fungicide, 3-hydroxy-5-methyl isoxazole) provided a breakthrough against the contamination of *Phytophthora* isolation media by *Pythium* (Ribeiro, 1978).

*P. cinnamomi* is found in low densities in the soil making direct isolations difficult prior to the development of selective media (Tsao, 1983). Mitchell and Kannwischer-Mitchell (1992) stated that because *P. cinnamomi* survives as chlamydospores in the soil, it is easy to collect and germinate on selective media. Sprinkling soil crumbs on PVP agar can qualitatively detect *P. cinnamomi* (Zentmyer, 1980). As difficult as it is to isolate *P. cinnamomi* from the soil, baiting techniques increase the frequency of successful isolation even in severely infected soils.

### 1.6.2 Baiting

Baiting is commonly used for successful isolation and detection of *P. cinnamomi* from the soil. Soil samples should be taken from the moist soil, under the edge of the tree canopy near healthy roots, at least 5cm below the soil surface. Soil samples are flooded with water with susceptible pieces of plant material, referred to as baits, floating on the surface (Erwin and Ribeiro, 1996). Avocado, lemon leaves or pineapple leaf-base may be used as baits, which are left floating for about two to three days allowing the zoospores produced by the pathogen to infect them. The leaves are plated onto PARPH medium and incubated to enable *P. cinnamomi* isolates to grow and be isolated. Nechwatal *et al*. (2001) stated that the key problem with the baiting method is that fast-growing organisms such as

*Pythium* can inhibit the growth of *P. cinnamomi*; therefore, the formation of zoospores on the baits is not always adequate for a positive result. Tsao (1983) reported that many different chemical and biological inhibitors found in the soil inhibit the detection of *P. cinnamomi* when using the baiting method. When thin layers of soil are baited, that is when detection is most sensitive since detection of low levels of inoculum from thick layers of soil can be limited by the physical blocking of zoospore release from soil samples (Eden *et al.*, 2000).

Baiting assays are applied in the detection of *P. cinnamomi* since they do not involve extremely complex laboratory equipment; there is, however, variable detection and low levels of the pathogen cannot be detected leading to false negatives in detection analysis (Eden *et al.*, 2000). Wilson *et al*. 2000 stated that in 16% of cases where zoospores were detected in the water, the use of antibody tests showed that the results of the baiting were negative.

### 1.6.3 Immunological Detection

Immunological assays are quick and precise detection methods; however, they should not entirely substitute direct isolation (EPPO, 2004). *P. cinnamomi* can be detected by serological methods, using Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA). Cahill and Hardham (1994) described the precise identification of *P. cinnamomi* by means of a dipstick immunoassay with a monoclonal antibody, Cpa-3, that identified an antigen positioned on the cyst periphery. In a succeeding study, Wilson *et al*. (2000) revealed that the detection of *P cinnamomi* soil by baiting could be improved by use of the dipstick assay to test the bait water for the presence of zoospores. An ultraviolet light microscope can be used to detect *P. cinnamomi* using fluorescent antibodies (MacDonald and Duniway, 1979). Immunological assays are sensitive, and have shown to detect low levels of the target pathogen (down to 40 zoospores per millilitre of water) and can additionally be used to give a quantitative assessment of the pathogen in plant tissue or soil samples. These techniques, however, are limited due to detecting dead as well as living tissue, and cross-species reactivity (Cahill and Hardham, 1994). The reliance on ELISA as an initial screening tool is disputable due to the high rate of false negatives obtained. Culture-based and immunological detection methods lack reliability for routine pathogen detection and are inefficient (Coelho *et al.*, 1997). They are time-consuming, labour

intensive and have low throughputs, therefore, limiting *Phytophthora* infestation identification at an early phase; therefore, delaying the application of control measures beyond the point when they would be prominently effective (Kong *et al*., 2003b).

Immunological practices should be considered as being complementary to other diagnostic procedures. However, detection methods that are highly specific, rapid, sensitive and more reliable are needed and have been found through molecular identification (Weller-Alm *et al*., 2000). DNA detection methods overcome numerous difficulties associated with immunological based methods of detecting *P. cinnamomi* (Coelho *et al*., 1997).

## 1.7 DNA Detection Methods

DNA detection methods are now being widely used due to their specificity, sensitivity, rapidness and reliability (Weller-Alm *et al*., 2000; Bonants *et al*., 2003). DNA is detected straightforwardly, and it is present regardless of the pathogens developmental stage or environmental conditions. Moreover, processing large of samples for DNA analysis significantly reduces the cost of processing large numbers of samples and has a high throughput (Martin *et al*., 2000). Essentially all DNA detection methods include a polymerase chain reaction (PCR) amplification where a pair of oligonucleotide primers flank a region of interest.

Legay *et al*. (2000) stated that PCR is amongst the most convenient and efficient methods used for the detection of nucleic acids. It is a method that uses DNA polymerase to rapidly yield numerous copies of a restricted DNA segment using species-specific primer sequences (Campbell, 1996). The number of copies of the targeted DNA increases exponentially by undergoing 20-40 reaction cycles of synthesis (Campbell, 1996; Henson and French, 1993). This means that a single copy of target DNA is amplified to produce millions of copies of itself (Bohm *et al*., 1999); therefore, massively increasing the sensitivity of DNA based detection (Henson and French, 1993). The amplification products are then detected by gel electrophoresis. PCR assays have been developed for many species of *Phytophthora*. Most of these assays use internal transcribed spacers (ITS) derived primers, (Cooke *et al*., 1995a,b; Bonants *et al*., 1997; Tooley *et al*., 1997; Trout *et al*., 1997; Liew *et al*., 1998; Tooley *et al*., 1998; Schubert *et al*., 1999; Bonants *et al*., 2000; Judelson and Tooley, 2000; Winton and Hansen, 2001; Grote *et al*., 2002; Ippolito *et al*., 2002) or elicitin genes (Coelho *et al*., 1997; Lacourt and Duncan, 1997; Kong *et al*., 2003a).

### 1.7.1 PCR Detection of *P. cinnamomi*

A number of PCR methods targeting various regions in the *P. cinnamomi* genome have been developed (Table 1.2) (Coelho *et al*., 1997; Kong *et al*., 2003b; Anderson, 2006; Engelbrecht *et al*., 2013). PCR by Coelho *et al*. (1997) was combined with colourimetric hybridization; however, the assay was not applied directly to DNA extracts from infected soil samples. Kong *et al*. (2003b) studied the sensitivity of detection from artificially inoculated soilless medium. However, the sensitivity of detection in the presence of PCR inhibitors or detection of *P. cinnamomi* from naturally infested soils was not addressed. Engelbrecht *et al*. (2013) developed a nested qPCR using the LPV3 primers designed by Kong *et al*. (2003b), which investigated the sensitivity of the PCR assay in plant tissues, again, the issue of sensitivity in the presence of PCR inhibitors or detection from infested soils was not addressed. The above-mentioned procedures are valuable substitutes for identification of the pathogen from pure culture, but they need to undergo further optimization tests for specificity with non-*Phytophthora* species.

Most diagnostic primers for *Phytophthora* species were derived from the internal transcribed spacer regions (ITS), and PCR protocols with primers derived from the ITS regions have been reported for *P. cinnamomi* (Ippolito *et al*., 2002; Grote *et al*., 2002; Martin *et al*., 2004). DNA sequences of these regions are easily detected due to high copy numbers in the genome, increasing the concentration of target DNA (Hayden *et al*., 2004). The ITS1 and ITS2 regions together with the ribosomal DNA found in the ITS (Fig. 1.8) consists of conserved and unique DNA regions, making them ideal for PCR tests (Lindsley *et al*., 2001). Universal primers may target the highly conserved regions in the ribosomal genes to amplify a variety of related species. The distinction of specific species is, however, permitted by variable regions in the ITS region (White *et al*., 1990). The use of these regions or genes for species-specific detection has been enabled by the plethora of records available on the level of intraspecific sequence conservation (Hayden *et al*., 2004).

Anderson (2006) developed primers from the ITS region and adopted the nested protocol with both primary and nested PCR specific for *P. cinnamomi* detection. The PCR amplification of *P. cinnamomi* DNA isolated from infested soil was optimized.

A nested design is popular for diagnostic PCR of plant pathogens with low levels of target inoculum (Hayden *et al*., 2004). It is used regularly for the detection of soil-borne pathogens (Grote *et al*., 2002), and it increases the sensitivity of detection from soil and plant samples (Grote *et al*., 2002; Ippolito *et al*., 2002; Martin *et al*., 2004).

**Table 1.2 Diagnostic primer sets known to produce specific PCR products for *P. cinnamomi*.**

| Reference | Primer | Sequence | Amplicon Length |
|---|---|---|---|
| Coelho *et al*., 1997 | 95.422 | GCTCGTGAGTATCCTGTCCG | 349 bp |
| | 96.007 | CTCAGTAAATGGCTAGCCCGATAC | |
| Kong *et al*., 2003b | LPV3 | GTGCAGACTGTCGATGTG | 450 bp |
| | | GAACCACAACAGGCACGT | |
| Engelbrecht *et al*., 2013 | LPV3N | GTGCAGACTGTCGATGTG | 77 bp |
| | | GAGGTGAAGGCTGTTGAG | |
| Anderson, 2006 | CIN3A | CATTAGTTGGGGGCCTGCT | 783 bp |
| | CINITS4 | TGCCACCACAAGCACACA | |
| | CIN3B | ATTAGTTGGGGGCCTGCT | 396 bp |
| | CIN2R | CACCTCCATCCACCGACTAC | |

Nested PCR involves re-amplification of initial PCR product in a second round of PCR using a second pair of primers (nested primers) that lie inside the binding sites of the primary PCR amplicon (Grote *et al*., 2002). A number of studies on various *Phytophthora* species have testified that nested PCR significantly improves the sensitivity of PCR detection from 100 up to 1,000 fold compared to single round PCR (Grote *et al*., 2002; Hayden *et al*., 2004).

Even though suitable sets of primers have been developed and used, they have, however, not been studied for the sensitivity of detecting *P. cinnamomi* directly from soil and plant material. Therefore, more prompt and reliable practices are necessary for detection of *P. cinnamomi* directly from the soil. The efficiency of PCR amplification was investigated by optimization and application of a nested PCR assay for the detection of *P. cinnamomi*, as well as assessing for sensitivity in comparison to a one-step PCR assay.

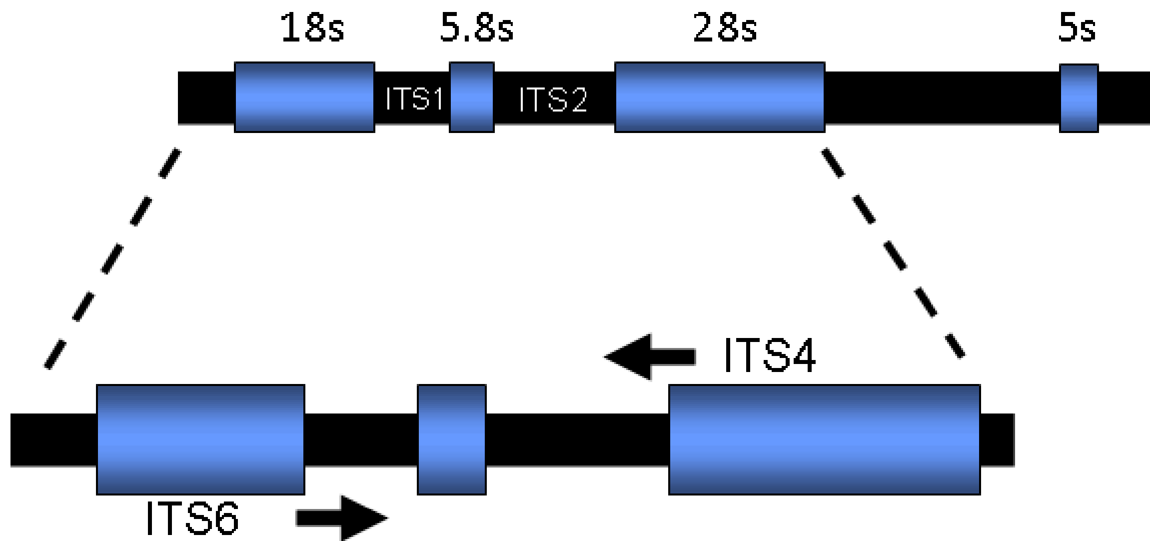



**Figure 1.8:** Diagrammatic presentation of nuclear ribosomal DNA regions (Grünwald *et al.*, 2011).

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# CHAPTER 2

# OPTIMISATION OF A NESTED PCR PROTOCOL FOR THE DETECTION OF *PHYTOPHTHORA CINNAMOMI* ON MACADAMIA IN THE LIMPOPO AND MPUMALANGA PROVINCES, SOUTH AFRICA


## Abstract

Accurate identification of plant pathogens is key to developing appropriate and sustainable control strategies to ensure economically viable agricultural production. The aim of this study was to optimize a nested PCR protocol to enable the specific amplification of *P. cinnamomi* DNA with the intention of applying the assay for diagnostic analysis of infected soil and plant material. Seven *P. cinnamomi* isolates and two isolates from soil samples were used for *P. cinnamomi* detection and cultures were preserved over the project period in sterile distilled water at room temperature in the dark. A boiling technique was used in the extraction of DNA from pure *P. cinnamomi* cultures, followed by the baiting bioassay technique to optimise the detection system. DNA was also extracted using a Powersoil® DNA isolation kit and the two DNA extraction methods were compared. *P. cinnamomi* specific primers from the 3' end of 18s gene, through the 5.8S gene to the 5' end of the 28S gene, together with a small multigene family, *Lpv,* were used for polymerase chain reaction (PCR) amplification. Detection of *P. cinnamomi* using nested PCR yielded the expected amplicon size of 396 bp while detection using one-step PCR was unsuccessful. Nested PCR using the DNA extraction method from cultures obtained with the baiting bioassay displayed the highest level of *P. cinnamomi* specificity and sensitivity in comparison to that of the soil extraction kit. Following the baiting bioassay, nested PCR proved to be the best detection method for *P. cinnamomi* and this assay will be applied as a preferred protocol for the detection of *P. cinnamomi* by macadamia growers.

## 2.1 Introduction

The genus *Phytophthora* is complex and occupies various terrestrial and aquatic habitats; it comprises about 70 described species (Erwin and Ribeiro, 1996). Root infecting *Phytophthora* species such as *Phytophthora cinnamomi* cause symptoms similar to those caused by other *Phytophthora* and *Pythium* species, which include yellowing and wilting of the above-ground foliage (Therman *et al*., 2002). *P. cinnamomi* causes root rot on a wide host range and is the main cause organism of macadamia root rots and trunk cankers in South Africa (Sikinyi, 1993). Accurate detection and identification of *P. cinnamomi* is, therefore, necessary to effectively manage/control such a widespread and destructive disease.

The design of primers and optimization of reaction conditions governs the specificity of any PCR reaction (Ekman, 1999). This makes the identification of microbial species by PCR dependent on specificity and sensitivity of the amplification (Romanowski *et al*., 1993). Small variations in amplification when optimizing PCR protocols has been widely recognized as necessary and having significant influence on the specificity and yield of PCR amplification (Ekman, 1999). In detection applications, the specificity of PCR amplification is the foundation of all successive analysis of species identity. Target sequences must be distinguished from all other DNA within the biological extract (Liew *et al*., 1998). A fine balance between target concentration, primer concentration and specificity, and amplification conditions is needed to achieve an efficient and specific PCR reaction (Legay *et al*., 2000; Grote *et al*., 2002), Additionally, optimized PCR cycle temperatures for the primer set and amplification conditions are also required (Coelho *et al*., 1997).

In diagnostic applications, it is crucial that molecular detection using PCR are executed under conditions that promote the amplification of target species while minimizing that of non-target species (Ekman, 1999). This takes into consideration that low levels of non-specific amplification occurs in most PCR as primers occasionally bind to non-target DNA sequences that are consequently amplified (Suzuki and Giovannoni, 1996). The optimization of PCR may limit such amplifications to levels below the thresholds detected by agarose gel electrophoresis (Ekman, 1999). A number of authors have employed a universal primary PCR amplification of the ITS regions of ribosomal DNA followed by a specific nested PCR for the detection of the target species (Grote *et al*., 2002; Ippolito *et al*., 2002). A nested PCR application can overcome the complication of amplifying DNA from

environmental samples that have limited target DNA within soil and plant samples by re-amplifying the primary product which may be undetectable by agarose gel electrophoresis (Cullen and Hirsch, 1998; Hayden et al., 2004). The amplification of DNA present in higher titres is reduced using specific primers in the primary and nested PCR cycles, therefore, intensifying specificity and allowing species-specific detection following both cycles (Grote *et al*., 2002).

Against this background, the aim of this study was to optimize a nested PCR protocol to enable the specific amplification of *P. cinnamomi* DNA with the intention to apply the assay for diagnostic analysis of infected soil and plant material.

## 2.2 Materials and Methods

### 2.2.1.1 *P. cinnamomi* isolates

Seven *P. cinnamomi* isolates were sourced from the Agricultural Research Council – Plant Protection and Research (ARC-PPR), University of Stellenbosch, Du Roi Laboratory Services and Barnard Farm, together with isolates from two soil samples obtained at ARC – Friendenheim (Table 2.1). To maintain cultures for DNA extraction, these were routinely sub-cultured on PDA in 90 mm diameter Petri dishes and incubated in the dark at 25 ± 2 °C for seven days.

### 2.2.1.2 *P. cinnamomi* isolates from soil samples

*Phytophthora cinnamomi* was recovered from soil samples using a baiting bioassay. This assay involved the flooding of soil samples with distilled water and pineapple leaf-base as host bait to trap the pathogen. After two days, the baits were plated on *P. cinnamomi* selective medium to allow the growth of the pathogen. This baiting bioassay is explained in detail in Chapter 3 under materials and methods.

### 2.2.2 Maintenance of *P. cinnamomi* isolates

*P. cinnamomi* isolates were preserved in sterile distilled water at room temperature in the dark for the duration of the study. Each isolate was grown on potato dextrose agar (PDA) for 5 days after which a few plugs (5mm diameter) were taken from the edge of each

colony and placed in sterilised Bijou bottles with 5 ml sterile distilled water (Gerretson-Cornell, 1983). To regenerate fresh cultures as required, a single plug from the water was plated on PDA and incubated at 25 ± 2 °C for seven days (Gerretson-Cornell, 1983). Multiple bottles and plugs were stored for each isolate to enable regeneration of cultures throughout the study period.

**Table 2.1 *P. cinnamomi* isolates used for PCR detection experiments**

| Isolate code | Source | Host |
|---|---|---|
| Bar 7 | ARC- PPR | Macadamia soil |
| Ph 333 | University of Stellenbosch | *Agathosma betulina* |
| Ph 336 | University of Stellenbosch | *Agathosma betulina* |
| Ph 347 | Du Roi Laboratory Services | Avocado roots |
| Ph 379 | Du Roi Laboratory Services | Avocado soil |
| Ph 580 | Barnard Farm | Macadamia soil |
| Ph 581 | Barnard Farm | Macadamia roots |
| S1 | Friedenheim | Macadamia soil |
| S2 | Friedenheim | Macadamia soil |

### 2.2.3 DNA extraction from mycelial cultures

A boiling technique (Kong *et al*., 2003b) was used in the extraction of DNA from pure *P. cinnamomi* cultures following the baiting bioassay. Mycelium was harvested from a week-old culture using a sterile surgical blade. The surgical blade was sterilized prior to use on a different culture by placing it in 70% ethanol and passing it through a Bunsen burner. The harvested mycelia were placed in 2 ml micro-centrifuge tubes containing 500 µl of 10 mm Tris-HCl (pH 7.5). A spatula was used to break any agar picked with mycelia inside the micro-centrifuge tube. The tubes were placed in a heat block for 20 minutes and vortexed

for three minutes to release DNA. The supernatant was collected and DNA concentrations were measured and recorded using a NanoDrop Lite Spectrophotometer (ThermoFisher Scientific Inc, Waltham, MA, USA). The DNA was used immediately or stored at – 20 °C until further use.

### 2.2.4 DNA extraction from soil samples

DNA was extracted from soil using the Powersoil® DNA isolation kit according to the manufacturer's instructions (MO BIO Laboratories, Inc, USA). 0.25 g of soil sample was added to the provided power bead tubes and vortexed to mix. 60 µl of solution C1 was added and briefly vortexed. The samples were homogenised at maximum speed for 10 mins in the Precellys24 homogeniser (Bertin Instruments, Montigny-le-Bretonneux, France) and spun in an Eppendorf centrifuge (Germany) at 10 000 x g (relative centrifugal force) for 30 seconds. The supernatant was transferred to a clean collection tube where 250 µl of solution C2 was added, briefly vortexed and incubated at 4 °C for 5 minutes before being centrifuged at 10 000 x g for 1 min. 600 µl of the supernatant was transferred to a clean collection tube where 200 µl of solution C3 was added, briefly vortexed and incubated at 4 °C for 5 mins followed by centrifugation at 10 000 x g for 1 minute. 750 µl of supernatant was collected in a clean collection tube and 1 200 µl of solution C4 was added and vortexed for 5 seconds. 675 µl was loaded onto a spin filter and centrifuged at 10 000 x g for 1 minute, discarding the flow through. This process was repeated for each sample until no supernatant was left. 500 µl of solution C5 was added, centrifuged at 10 000 x g for 30 seconds and discarded the flow through. Further spinning at 10 000 × g for 1 minute was done before carefully placing the spin filter in a clean collection tube. 100 µl of solution C6 was added to the centre of the spin filter and centrifuged at 10 000 x g for 30 seconds before discarding the spin filter. Total elution volume was 100 µl which is the recommended volume for optimal DNA yield according to the manufacturers.

### 2.2.5 Polymerase chain reaction (PCR) amplification

The region of the ribosomal repeat from the 3' end of 18s gene, through the 5.8S gene to the 5' end of the 28S gene, together with a small multigene family, *Lpv*, which

encode putative storage proteins in large peripheral vesicles in zoospores of *P. cinnamomi,* were defined by oligonucleotide primers described in Table 2.2. DNA samples of *P. cinnamomi* isolates sourced from descriptions in Table 2.1 were amplified with *P. cinnamomi* specific LPV3 (forward and reverse) primers (Kong *et al*., 2003b) and the nested primers CIN3A (forward), CINITS4 (reverse), CIN3B (forward), CIN2R (reverse) (Anderson, 2006).

**Table 2.2 Sequences of *P. cinnamomi* oligonucleotide primers used in this study**

| Primer | Sense | Sequence |
|---|---|---|
| CIN3A | Forward | CATTAGTTGGGGGCCTGCT |
| CINITS4 | Reverse | TGCCACCACAAGCACACA |
| CIN3B | Forward | ATTAGTTGGGGGCCTGCT |
| CIN2R | Reverse | CACCTCCATCCACCGACTAC |
| LPV3 | Forward | GTGCAGACTGTCGATGTG |
| | Reverse | GAACCACAACAGGCACGT |

Amplifications of DNA were carried out in a ProFlex PCR System (Applied Biosystems, Singapore). For the nested PCR, amplification was carried out in 25 µl reactions with 12.5 µl of the EmeraldAmp® Max HS PCR Master Mix (TAKARA BIO INC, Clonetech Laboratories, Inc., China), 2 µl each of 3 µM forward and reverse primers, 2 µl of 3 ng/µl DNA and 8.5 µl. 2 µl of PCR grade water was added to each negative control instead of DNA. PCR cycling, producing first round products, was optimized using the CIN3A/CINITS4 primer pair and the reaction started with an initial denaturation of 10 minutes at 94 °C, followed by 30 cycles of 30 seconds at 94 °C, 30 seconds at 60 °C and 30 seconds at 74 °C and a final extension of 74 °C at 10 minutes. The second round PCR cycling, using the CIN3B/CIN2R primer pair, was run under similar conditions with the only exceptions being 15 seconds for all intervals instead of 30 seconds and the use of 59 °C instead of 60 °C as the annealing temperature.

For the LPV3 primers, each 25 µl PCR reaction contained 2 µl of DNA templates, 12.5 µl of the EmeraldAmp® Max HS PCR Master Mix, 2.5 µl each of 10 µM forward and reverse primers, and 8.5 µl of PCR grade water. The reaction was programmed with initial denaturation at 96 °C for 2 minutes, followed by 39 cycles of 94 °C for 30 seconds, 60 °C for 45 seconds, 72 °C for 1 minute, and a final extension step at 72 °C for 10 minutes (Kong *et al*., 2003a).

### 2.2.6 Gel electrophoresis

A 10 µl aliquot of PCR product from each reaction was loaded into a 2% agarose gel, SeaKem® LE Agarose (Lonza, Rockland, ME, USA) in 1x TAE buffer (Tris-Acetate-EDTA). Gels were stained with 3 µl $ml^{-1}$ ethidium bromide for visualization under a UV transilluminator. Fragment sizes were determined by comparison with 6 µl of a 100 bp molecular weight marker, GeneRuler™ (Fermentas Inc., Maryland, USA). Gel plates were immersed in 1× TAE buffer in gel trays and electrophoresed at 110 V for 30 to 45 minutes. Images were captured using the Genesnap1 software.

## 2.3 Results

### 2.3.1 Optimized PCR amplification with nested primers CIN3A/CINITS4 and CIN3B/CIN2R

The *P. cinnamomi* specific CIN3A/CINITS4 and CIN3B/CIN2R nested primers were able to amplify DNA from all seven *P. cinnamomi* isolates and yielded the expected amplicon size of 396 bp (Fig. 2.1, Lanes 2-8). The presence of *P. cinnamomi* in two of the tested macadamia soil samples was also confirmed (Fig. 2.1, Lanes 9 & 8).

DNA obtained using the boiling technique was of adequate quality for template amplification as determined in a study using a ITS6 and ITS7 primer pair (Kong *et al*., 2003b).

### 2.3.2 PCR amplification with LPV3 primers

The LPV3 primers were unable to amplify the seven *P. cinnamomi* isolates and two soil samples and showed unspecific amplification. The PCR did not yield the expected band size of 450 bp (Fig. 2.2).

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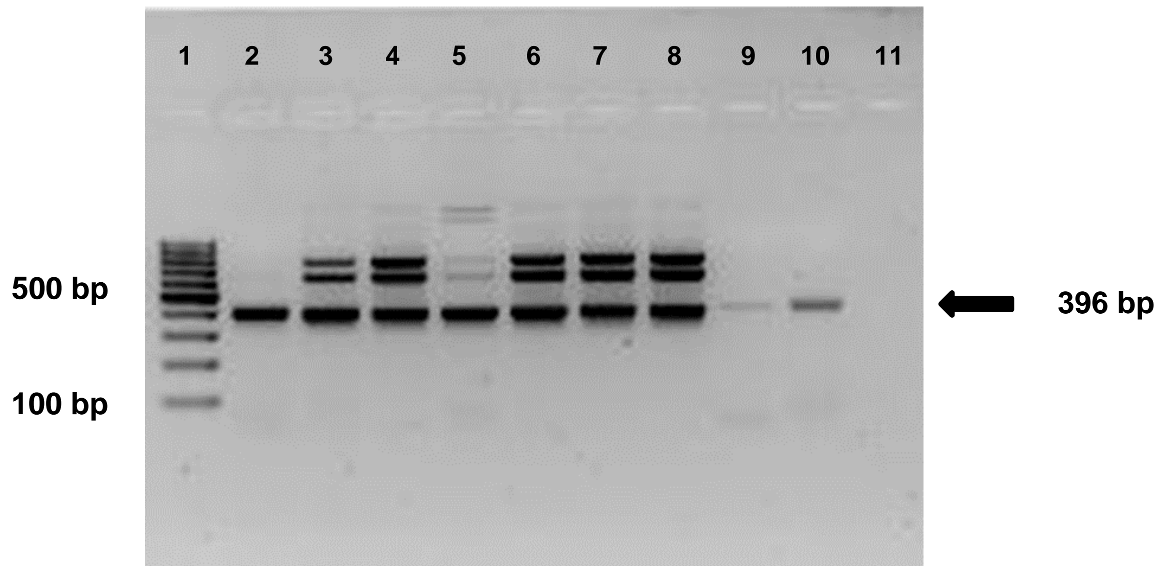


2

3 **Figure 2.1:** Agarose gel electrophoresis of nested PCR products of seven *P. cinnamomi*
4 isolates and two soil samples using primers designed by Anderson (2006). Lane 1: 100 bp
5 DNA ladder; Lane 2: *P. cinnamomi* Bar 7; Lane 3: *P. cinnamomi* Ph 333; Lane 4: *P.*
6 *cinnamomi* Ph 336; Lane 5: *P. cinnamomi* Ph 347; Lane 6: *P. cinnamomi* Ph 379; Lane 7:
7 *P. cinnamomi* Ph 580; Lane 8: *P. cinnamomi* Ph 581; Lane 9: Sample 1 from macadamia
8 soil; Lane 10: Sample 2 from macadamia soil; Lane 11: Negative control.

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10 **2.3.3 Comparison between DNA isolated with the soil kit and DNA isolated by the**
11 **baiting bioassay using nested PCR amplification**

12 PCR was used to determine the efficacy of the two DNA extraction methods that
13 were employed in the study. PCR was able to amplify *P. cinnamomi* DNA extracted using
14 either technique (Fig. 2.3).Of the two methods, the baiting bioassay displayed the highest
15 level of *P. cinnamomi* specificity and sensitivity (Fig. 2.3A).

16

17

**Figure 2.2:** Agarose gel electrophoresis of nested PCR products of seven *P. cinnamomi* isolates and two soil samples using primers designed by Kong *et al.*, (2003b). Lane 1: 100 bp DNA ladder; Lane 2: *P. cinnamomi* Bar 7; Lane 3: *P. cinnamomi* Ph 333; Lane 4: *P. cinnamomi* Ph 336; Lane 5: *P. cinnamomi* Ph 347; Lane 6: *P. cinnamomi* Ph 379; Lane 7: *P. cinnamomi* Ph 580; Lane 8: *P. cinnamomi* Ph 581; Lane 9: Sample 1 from macadamia soil; Lane 10: Sample 2 from macadamia soil; Lane 11: Negative control.

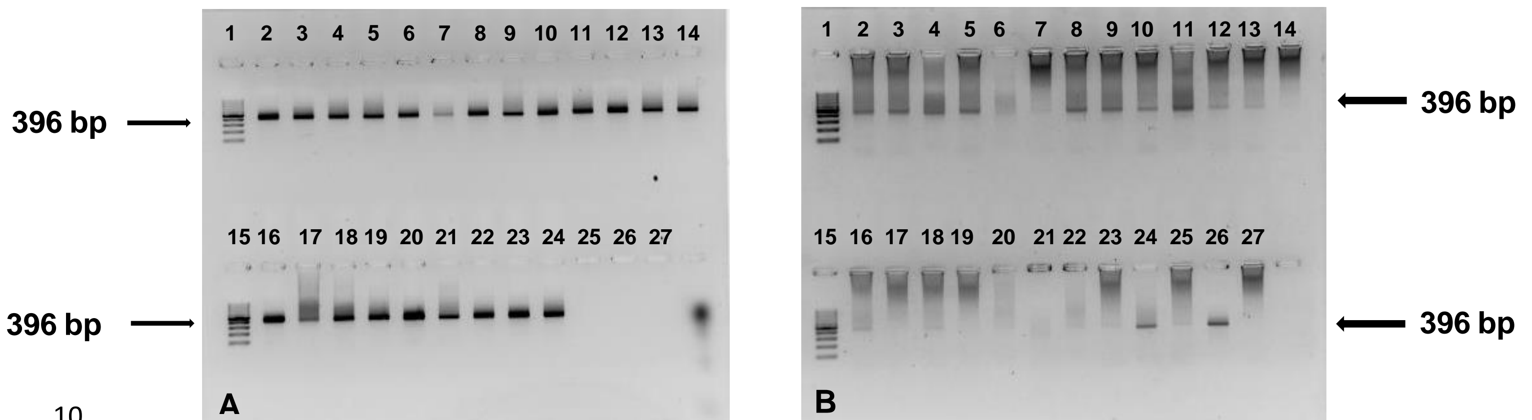


**Figure 2.3:** Comparison of agarose gel electrophoresis of nested PCR products of DNA isolated using the A) baiting bioassay and B) soil extraction kit. Lane 1 and 15: 100 bp DNA ladder; Lane 2 and 16: *P. cinnamomi* isolate (Bar 7) positive control; Lanes 3 to 14 and lanes 17 to Lane 27: Samples from macadamia soil; Lane 28: Negative control.

## 2.4 Discussion

*P. cinnamomi* was successfully detected using an optimised nested PCR protocol with the primer pairs described earlier (Anderson, 2006) (Fig. 2.1). The sensitivity of PCR is an important factor in detecting plant pathogens from soil and plant samples (Judelson and Tooley, 2000). To that end, nested PCR was chosen for the detection of *P. cinnamomi* from macadamia soils samples. In other studies (Grote *et al*., 2002; Martin *et al*., 2004) where other species of *Phytophthora* were detected, nested PCR showed a 1000-fold increase in sensitivity. Anderson (2006) mentioned that the selection of four specific primers for a nested PCR assay has the benefit of specific amplification of *P. cinnamomi* in the primary and secondary PCR. This assay permits species-specific detection by both rounds of PCR.

The one-step PCR using the LPV3 primers was unsuccessful in amplifying *P. cinnamomi* (Fig. 2.2). The specificity and sensitivity of PCR amplification are key factors in PCR detection applications (Romanowski *et al*., 1993). False negatives can occur in the detection assay if the conditions for the reaction are too stringent; equally so, false positives could occur if the parameters are not sufficiently stringent (Ekman, 1999). Specificity and sensitivity need to be balanced as increasing stringency frequently decreases amplification of product with a subsequent loss of sensitivity (Ekman, 1999). The unsuccessful amplification of the pathogen DNA using LPV3 primers could be due to the conditions of the reaction being too stringent as the PCR was not optimised leading to the reaction not being specific. Kong *et al*. (2003a) reported that even though PCR with the LPV3 primers was the most specific for *P. cinnamomi*, two isolates *P. sojae* and one out of six isolates of *P. capsici* produced amplicons with sizes similar to that of *P. cinnamomi,* implying that the primers were not specific for *P. cinnamomi* only. This is a case of non-specific binding by the primers. There is need to optimize the PCR reaction conditions to avoid this problem.

Amplification of *P. cinnamomi* through nested PCR was successful for both DNA extraction methods using the soil kit extraction and baiting bioassay. The PCR reaction for DNA obtained using the baiting bioassay showed higher specificity and sensitivity than the DNA extracted using the kit (Fig. 2.3). Baiting is commonly used for successful isolation and detection of *P. cinnamomi* from the soil (Erwin and Ribeiro, 1996) and detection is most sensitive when thin layers of soil are baited (Eden *et al*., 2000). The baiting method displayed higher specificity and sensitivity due to a large amount of soil being used to trap the pathogen as compared to using the kit which uses a small amount of soil which

increases the probability of taking soil where the pathogen was not present. In this study, *P. cinnamomi* detected from the baiting bioassay was confirmed using molecular techniques. As proven by a number of studies, serological or molecular diagnostic methods are more sensitive than just using soil baiting (Pettit *et al*., 2002; Hayden *et al*., 2004; Davison and Tay, 2005).

In conclusion, nested PCR together with the baiting bioassay proved to be the best detection method for *P. cinnamomi*. To our knowledge, this is the first comparison study conducted in South Africa to test optimal detection *P. cinnamomi* from macadamia soils, as other research have detected it from avocado soils (Engelbrecht and van den Berg, 2013). This assay will, therefore, be practically applied as a protocol for the detection of *P. cinnamomi* from macadamia soils.

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# CHAPTER 3

# THE INCIDENCE AND DISTRIBUTION OF *PHYTOPHTHORA CINNAMOMI* IN THE MAIN MACADAMIA GROWING AREAS OF SOUTH AFRICA


## Abstract

Limpopo and Mpumalanga are the main macadamia growing provinces in South Africa. 95 % of macadamia nuts produced in the country are exported worldwide. Root rots and stem cankers, caused by *Phytophthora cinnamomi* Rands, are one of the major production constraints of macadamia worldwide. Against this background, the aim of this study was to establish the incidence, distribution and severity of *P. cinnamomi* in macadamia growing areas of South Africa. Surveys were conducted on macadamia farms and commercial orchards to establish the incidence and the distribution of *P. cinnamomi* in the main macadamia growing provinces of South Africa. A baiting bioassay using pineapple leaf-base pieces as host baits was used to recover *P. cinnamomi* from the soil by plating onto a *Phytophthora* selective medium (PARPH). *P. cinnamomi* was identified based on colony, mycelia, and sporangia morphology, and zoospore release using a Nikon Eclipse Ni microscope at various levels of magnification (10 x, 40 x, and 1000 x in oil emersion). Descriptive survey data analysis was done by use of MS Excel 2007 and GenStat for Windows 18$^{th}$ Edition. The root rot incidence and severity on macadamia trees were not significantly different ($P > 0.05$) between the provinces. The highest root rot incidence and were observed in Mpumalanga (46.6 ± 4.8 %). Mpumalanga had a 5% higher root rot severity (67.78 ± 12.5 %) when compared to Limpopo (62.0 ± 11.8 %). *P. cinnamomi* was characterized by dense mycelia that grows like rose petals with a creamy white colour as well as by characteristic coralloid hyphae and profuse chlamydospores distinctive of *P. cinnamomi*. A total of 107 out of 205 samples collected from four different locations in the two provinces tested positive for *P. cinnamomi*. 52 % of *P. cinnamomi* was detected from macadamia soils during these surveys. The study established that *P. cinnamomi* occurs in the main macadamia growing areas of South Africa. The high *P. cinnamomi* incidence requires a closer look at control strategies and assessment of their effectiveness.

## 3.1 Introduction

*Phytophthora cinnamomi* has a universal distribution and a wide host range, infecting over 3000 plant species (Hardman, 2005). It is the most destructive soil-borne pathogen worldwide causing roots rots and truck cankers of eucalyptus, avocado, pineapple, peaches and according to the Australian Government's Department of the Environment and Energy (2017), *P. cinnamomi* threatens several plants with extinction. This pathogen is the only soil-borne pathogen to have such an enormous impact globally and over a great range of plant hosts (Hüberli *et al*., 2001).

In South Africa (SA), root rots and stem cankers have been observed in many macadamia orchards in the country, especially those in the main macadamia-growing provinces of Limpopo and Mpumalanga. In other African countries such as Kenya, these diseases were described as economically important causing 60 % yield losses in some macadamia orchards (Sikinyi, 1993; Muthoka *et al*., 2005; Mbaka *et al*., 2009). Since SA is the second largest macadamia nut producer in the world, after Australia (DAFF, 2016), diseases caused by *P. cinnamomi* on macadamia need to be properly managed for the economic production of the crop. Due to the increase in SA domestic production, macadamia nut exports have concomitantly increased by 2.5 % and the country continues to lead in macadamia nut exports. To this end, SA was the number one country in exports from 2011 to 2015, with more than 95 % of macadamia being exported worldwide (DAFF, 2016).These percentages emphasize the importance of the production of high-quality nuts to stay competitive globally.

As a member of both the World Trade Organisation Agreement on the application of Sanitary and Phytosanitary Measures and the International Plant Protection Convention, SA has the responsibility to maintain high-quality phytosanitary measures to protect the country on scientific data and international standards. It is imperative to have reliable information transfer to trading partners about the presence and distribution of plant pests within South Africa (Carstens *et al*., 2012).

*P. cinnamomi* is identified to be a pathogen of economic importance on macadamia in SA but there are no current records of its incidence, distribution and severity on the crop. Additionally, there are no effective management strategies for stem cankers and root rot in the macadamia agro-ecosystems of SA currently. In order to develop effective disease

management strategies, it is imperative to determine the incidence and distribution of the pathogen in the major macadamia growing areas in the country. The aim of this study, therefore, was to establish the incidence, distribution and severity of *P. cinnamomi* in macadamia growing areas of South Africa.

## 3.2 Materials and Methods

### 3.2.1 Disease surveys

Surveys were conducted on macadamia farms and commercial orchards located in the two main macadamia growing provinces of Mpumalanga and Limpopo. Identification of farms was done in collaboration with the South African Macadamia Growers' Association (SAMAC). Macadamia trees were examined for typical *P. cinnamomi* symptoms, such as stem cankers above the soil line. A disease rating score was used where root rot was observed. The root rot severity was calculated from the total number of infected trees in each province and it was expressed as a percentage. The root rot incidence was recorded as the percentage of the total number of macadamia trees that showed a visible decline and/ or trunk cankers above the soil line, described above. From each field, 10 to 20 macadamia trees were selected randomly for sample collection, depending on the size of the farm. About 300 – 500 g of soil samples were collected under the tree canopy to a depth of 30 cm after removing the litter from the soil surface. Samples were placed in sterile plastic bags, which were then sealed and labelled before being stored in an insulated foam box until analysis.

### 3.2.2 Recovery of *P. cinnamomi*

*P.* cinnamomi was recovered from soil samples using the baiting bioassay, a method that has been validated (Ferguson and Jeffers, 1999) and is routinely used in the Agricultural Research Council - Tropical and Subtropical Crops (ARC-TSC) laboratory. Soil samples were mixed thoroughly and 300 g of soil was placed in a 1000 ml plastic container and flooded with 400 ml of distilled water. Six pineapple (*Ananas comosus* L. Merr.) leaf-base discs (5 mm in diameter) were floated on the surface of the water as baits and incubated at room temperature (22-25 °C) for 3 days. On the third day, leaf discs were blot dried under a laminar flow cabinet and plated on PARPH selective medium to detect *P.*

*cinnamomi*. PARPH was prepared according to a modified protocol of Kannwischer and Mitchell (1978). The chemicals 0.2g pentachloronitrobenzene (PCNB), 0.34g ampicillin and 0.02g rifampicin were dissolved in 10 ml of 95 % ethanol, then 0.08 g pimaricin and 0.1g hymexazol were suspended in 10 ml sterile, distilled water. These mixtures of chemicals were then added to 39 g $L^{-1}$ of basal medium potato dextrose agar (PDA).

The PARPH medium was poured in 90 mm diameter Petri dishes, allowed to cool overnight under a laminar flow bench and stored at 4 °C in the dark to avoid degradation of the antibiotics. The PARPH plates with leaf discs were sealed with parafilm and incubated in the dark at 25 ± 2 °C for 3 days. 5 mm agar blocks were cut from the edge of the growing *P. cinnamomi* mycelia and placed in Petri dishes containing PDA. The plates were sealed and incubated for seven days, after which pure *P. cinnamomi* cultures were easily recognized based on typical morphological features observed (Erwin and Ribeiro, 1996; Zentmyer, 1980). Morphological identification was confirmed using a Nikon Eclipse Ni microscope at various levels of magnification (10 x, 40 x, and 1000 x in oil emersion).

### 3.2.3 Detection of *P. cinnamomi*

The presence of *P. cinnamomi* in the collected field samples was detected using nested Polymerase Chain Reaction (PCR), as described in Chapter 1. Following the baiting bioassay, the mycelia were subjected to a boiling method to release DNA which was used in nested PCR (refer to Chapter 2 method’s section).

### 3.2.4 Data analysis

Descriptive statistics of the incidence of root rot and severity in two provinces of South Africa data was done by the use of MS Excel 2007 and GenStat for Windows 18$^{th}$ Edition. Descriptive statistics of the nested PCR results was carried out using the Statistica software versions (13.0, StatSoft Inc., USA). The analysis of variance (ANOVA) was used to test for significant differences between provinces. Means were separated using the Least Significant Difference (LSD) test.

## 3.3 Results

### 3.3.1 Disease surveys

The survey areas are in the North-Eastern part of South Africa between latitudes 22° S and $25^0$ S (Table 3.1). Annual rainfall is high (> 1000), but there are some orchards in semi-arid regions with rainfall of ± 400 mm per annum. These are the avocado zones according to SAAGA (2007) (Fig. 3.1). Due to confidentially, the specific farm names are not indicated.

**Table 3.1 Location of survey sites for the distribution of *Phytophthora cinnamomi* associated with root rot of macadamia in South Africa**

| Province | Location | Co-ordinates | Number of Farms | Number of samples |
|---|---|---|---|---|
| Limpopo | Levubu | 23.085$^0$S, 30.284$^0$E | 6 | 83 |
| Mpumalanga | Mbombela | 25.465$^0$S, 30.985$^0$E | 2 | 30 |
| | Kiepersol | 25.063$^0$S, 31.039$^0$E | 5 | 69 |
| | Bushbuckridge | 24.838$^0$S, 31.073$^0$E | 2 | 23 |
| Total | | | 15 | 205 |

Root rot was observed in all the main macadamia growing areas of South Africa. The root rot incidence and severity on macadamia trees were not significantly different ($P > 0.05$) between the provinces (Table 3.2). The highest root rot incidence was recorded in Mpumalanga (64.2 ± 4.8 %). Mpumalanga had a 5% higher root rot severity (67.78 ± 12.5 %) when compared to Limpopo (62.0 ± 11.8 %).

### 3.3.2 *P. cinnamomi* isolates recovered

Colony morphology on PDA was used to characterize isolates recovered from the soil samples. *P. cinnamomi* was characterized by dense mycelia that grew like rose petals with a creamy white colour (Fig. 3.2A) as well as coralloid hyphae and profuse

chlamydospores distinctive of *P. cinnamomi* (Erwin and Ribeiro, 1996), which were observed microscopically (Fig. 3.2B).

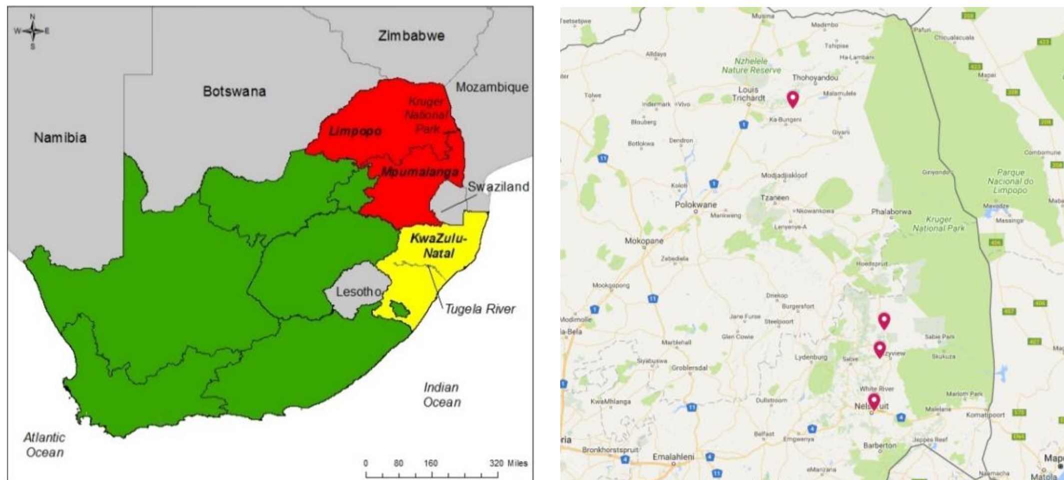


**Figure 3.1:** The avocado growing areas and location of survey sites for the distribution of *Phytophthora cinnamomi* on macadamia in the Mpumalanga and Limpopo Provinces indicated in red.

**Table 3.2 Incidence of root rot and severity in two provinces of South Africa**

| **Province** | **Root rot incidence** | **Root rot severity** |
|---|---|---|
| Limpopo | 56.05 ± 10.8 | 62.0 ± 11.8 |
| Mpumalanga | 64.16 ± 8.8 | 67.78 ± 12.5 |

* ANOVA, refer to Appendix 2.

### 3.3.3 Detection of *P. cinnamomi*

A total of 107 out of 205 samples collected from four different locations in the two provinces tested positive for *P. cinnamomi* using specific primers for the pathogen in nested PCR (Table 3.3). The number of samples positive for *P. cinnamomi* in Mpumalanga was double (66 %) that of Limpopo. A mean of 52 % for *P. cinnamomi* was detected between the two provinces. There were significant differences ($P < 0.05$) in the presence of *P. cinnamomi* in each province. Mpumalanga showed high levels of *P. cinnamomi* (0.5 ± 0.45) compared to Limpopo (0.32 ± 0.42) (Table 3.3).

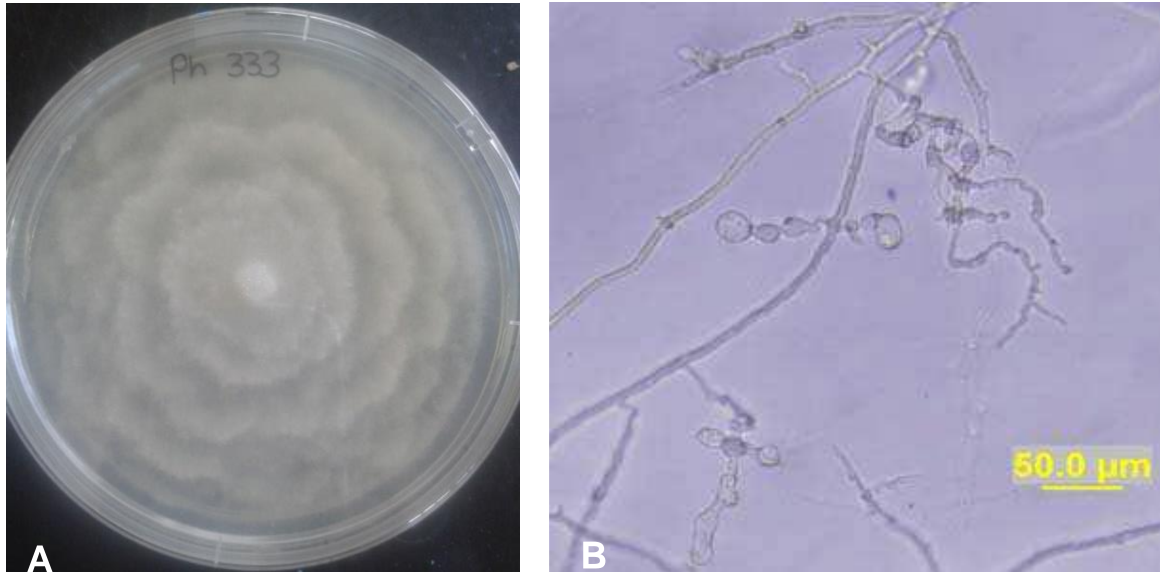


**Figure 3.2:** Colony morphology of *P. cinnamomi*. A: *P. cinnamomi* mycelia on PDA. B: Characteristic coralloid hyphae and profuse production of chlamydospores distinctive of *P. cinnamomi.*

**Table 3.3 Nested PCR results for the detection of *P. cinnamomi* in soil samples collected**

| Province | Samples tested | Positive samples | (%) | Mean PCR results |
|---|---|---|---|---|
| Limpopo | 83 | 27 | 33 | 0.32 ± 0.42a |
| Mpumalanga | 122 | 80 | 66 | 0.5 ± 0.45b |
| Total | 205 | 107 | 52 | 0.52 ± 0.5 |

* ANOVA, refer to Appendix 1.

## 3.4 Discussion

The surveys were done in the provinces of Limpopo and Mpumalanga, in which are the main macadamia growing areas in South Africa. The crop is also grown to a lesser extent in KwaZulu-Natal (DAFF, 2016). The results showed a high incidence (64.2 %) of macadamia root rot in the Mpumalanga province. This could be due to the presence of other crop and tree species such as avocado that are hosts of *P. cinnamomi*. This is in agreement with a report by Muthoka *et al*. (2005), which states that the presence of plants

that are hosts of *P. cinnamomi* in the macadamia orchards will increase the root rot incidence in that area. A lower root rot incidence (56.05 %) was observed in Limpopo; this could be because a number of farmers reported the use of organic manure in growing macadamia. Several researchers have reported that organic matter in the form of pine bark with a C: N ratio between 25: 1 and 100: 1, yard trimmings, alfalfa straw, wheat straw, sorghum stubble, and maize stubble was inhibitory to avocado root rot caused by *P. cinnamomi* (Broadbent and Baker, 1974; Pegg *et al.*, 1982; Borst, 1983; Downer, 1988; Turney and Menge, 1994; Wolstenholme *et al.*, 1996).

*P. cinnamomi* was broadly distributed in the Mpumalanga province. Out of the 122 samples, 80 tested positive for *P. cinnamomi*, which amounts to 66% of the samples. In Limpopo, only 33 % of the samples tested positive. A high mean disease severity was observed in both provinces. Mpumalanga had a higher root rot severity (67.78 ± 12.5 %) and high levels of *P. cinnamomi* (0.5 ± 0.45) compared to Limpopo's severity (62.0 ± 11.8 %) and levels of *P. cinnamomi* (0.32 ± 0.42). This indicates that *P. cinnamomi* is widely distributed in both provinces and is severe as the severity indicates that more than 60 % of the trees surveyed were infected by *P. cinnamomi*. The low percentage of *P. cinnamomi* from samples collected in the Limpopo province could be due to the fact that the Limpopo regions are drier and more arid compared to those of Mpumalanga, which is hotter and more humid (South Africa Online, 2017). This is in agreement with Zentmyer (1980) who reported that *P. cinnamomi* thrives in warm and wet conditions, which explains the high levels of *P. cinnamomi* detection in the Mpumalanga province. A mean of 52 % for *P. cinnamomi* was detected between the two provinces. The wide distribution and high disease incidence and severity observed in this study, like other previous studies (Linde *et al.*, 1997; Manicom, 2003), confirms the existence of *P. cinnamomi* and the threat it poses to macadamia production in Africa and South Africa.

Various host baits for detection of *Phytophthora* species from soil have been used. Ideally, a bait used in a bioassay for *Phytophthora* must have the following characteristics; susceptibility to *Phytophthora* species, seasonal nature, high sensitivity, ease of use, and substrate availability (Dance *et al.*, 1975). Due to their possession of the above-mentioned characteristics, pineapple leaf-bases were used in this study. The detection and isolation of *P. cinnamomi* from the soil has been successful using selective media containing antibacterial and antifungal agents (Ribeiro *et al.*, 1976; Masago *et al.*, 1977; Tsao and Guy,

1977; Zentmyer, 1980). Plant tissues have been used to detect *P. cinnamomi* but once the tissue dies, the pathogen also dies, therefore, making it possible that root and bark samples could die from infection prior to collection.

In conclusion, the study established that *P. cinnamomi* occurs in the two prominent macadamia growing areas of South Africa, namely Limpopo and Mpumalanga provinces, where it is associated with root rots and trunk cankers. The use of organic manure could reduce the severity and incidence of root rot, as the manure increases soil fertility as well as soil micro-organisms antagonistic to *P. cinnamomi*. The baiting bioassay was successful in detecting *P. cinnamomi* from soil samples and pineapple leaf-bases were effective as baits for *P. cinnamomi*. This is a simple and affordable method that can be used for qualitative detection of *P .cinnamomi* in soils. The wide distribution and high *P. cinnamomi* incidence and severity necessitate a closer look at control strategies and assessment of their effectiveness.

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# CHAPTER 4

# *IN VITRO* EVALUATION OF BIOLOGICAL CONTROL AGENTS AGAINST *PHYTOPHTHORA CINNAMOMI* ON MACADAMIA IN LIMPOPO AND MPUMALANGA, SOUTH AFRICA


## Abstract

Biological control is an effective tool used for the management of disease-causing pathogens. The use of biological control agents (BCAs) is important in the face of the indiscriminate use of pesticides that negatively affects the environment and has resulted in the development of resistant strains of pathogens. Against this background, the aim of the study was to determine the *in vitro* inhibitory effect of selected BCAs against *P. cinnamomi*. To this end, six *P. cinnamomi* isolates were first screened for their pathogenicity and virulence on green apples. *Trichoderma hamatum*, *Trichoderma harzianum*, and eight unknown *Bacillus* spp. were evaluated for their biocontrol activity against *P. cinnamomi* growth *in vitro* using the dual culturing technique. Data generated was analysed using a factorial analysis of variance (ANOVA). All six *P. cinnamomi* isolates examined in this study were pathogenic to green apples. Both *Trichoderma* spp. were able to inhibit all six isolates of *P. cinnamomi*. *T. hamatum* inhibited *P. cinnamomi* through deadlock with mycelial contact, whereas *T. harzianum* replaced and overgrew the pathogen. There was no significant difference ($P > 0.05$) in *in vitro* growth inhibition by the two *Trichoderma* spp. among all *P. cinnamomi* isolates. All the *Bacillus* species had the ability to inhibit the growth of *P. cinnamomi in vitro*; B 41b and NB 4 caused high levels of growth inhibition. There were significant differences ($P < 0.05$) in *in vitro* growth inhibition by different *Bacillus* spp. and in the sensitivity of *P. cinnamomi* isolates to the different *Bacillus* spp. The study showed that the pathogenicity test could be used to determine the presence of *P. cinnamomi* inoculum in soils and to evaluate the efficacy of soil treatment for control of *P. cinnamomi*. Additionally, it was shown that the fungal and bacterial BCAs had inhibitory effects on the mycelial growth of *P. cinnamomi in vitro*, demonstrating their potential to be used in the management/control root rots and trunk cankers of macadamia under field conditions.

## 4.1 Introduction

*Phytophthora cinnamomi* has an extensive host range and is found globally in countries that commercially grow avocados and macadamia (López-Hèrrèra and Pèrèz-Jimènèz, 1995). A broad variation in pathogenicity of *P. cinamomi* isolates has been observed, which is not related to mating type (Dudzisnki *et al*., 1993). Rands (1922) revealed that *P. cinnamomi* isolates varied in pathogenicity when he first described the pathogen. The pathogen's resistance to control measures and its endurance in hostile environments may be enhanced by the occurrence of variation among isolates. When there is a high level of variety, there is a possibility for the pathogen to evolve and adjust to its environment as selection pressure creates circumstances where the pathogen is compelled to change to survive (Mbaka, 2011). The environment suffers through exploitive agriculture worldwide, and the indiscriminate use of pesticides resulted in the development of resistant strains of pathogens (Nakkeeran *et al*., 2005). Biological control is thus a solution to the efficient management of disease-causing pathogens.

*Phytophthora* does not rely on sterol synthesis, which inhibits true fungal pathogens, therefore, a lot fungicides do not have the ability to control of this genus (Bartinicki-Garcia and Wang, 1983). However, a few classes of compounds do inhibit *Phytophthora* growth and plant infection (Griffith *et al*., 1992; Erwin and Ribeiro, 1996; Schwinn and Staub, 1995). Chemicals that caused inhibition of *P. cinnamomi in vitro* and *in vivo* studies are the phenylamides (Gisi and Cohen, 1996) and the phosphonates (Hardy *et al*., 2001), which are systemic inhibitors. Resistance to the phenylamide, metalaxyl, is one of the main restrictions in the management of *Phytophthora* diseases as it has been witnessed in many agricultural systems in which this fungicide has been applied (Coffey and Bower, 1984). Phosphonates such as phosphite are a valued inhibitor. On the other hand, its efficiency differs with various *P. cinnamomi* isolates (Hardy *et al*., 2001; Wilkinson *et al*., 2001). However, it may become ineffective due to *P. cinnamomi* developing resistance (Duvenhage, 1994). Due to the development of resistance, control measures should be focused on the use of biocontrol agents (BCAs), which reduce the disease, with a high level of safety and minimal environmental impact (Osman *et al*., 2011).

Weste (1983) isolated some soil micro-flora that may be potential BCAs as they could subdue *P.cinnamomi* in some soils. Inhibition of *P. cinnamomi* via competition, antibiosis or saprophytism has been shown by a number soil microorganisms. These

include; *Ceratomyces tessulatus* (Cooke), *Streptomyces vioascens* (Preobrazhenskaya and Sveshnikova), *Trichoderma hamatum* (Bonorden), and *Trichoderma harzianum* (Rifai) (Downer, 1998; Duvenhage and Kotzè, 1993; Duvenhage and Köhne, 1995; Erwin and Ribeiro, 1996). Bell *et al*., (1982) revealed that *Trichoderma* spp are potential candidates for the management of plant diseases as they antagonise and contest with plant pathogens by overpowering pathogenicity enzymes of the pathogens and competing for space and nutrients with the pathogen. They also stimulate plant resistance against disease and aid roots in the absorption of soil nutrients (Yedidia *et al*., 1999). *Trichoderma* spp. formulations are, hence, used as wound dressings in infected trees (Neri *et al*., 2008), as soil drenches, soil and seed treatments, and as foliar sprays (Otieno *et al*., 2003; Onsando and Waudo, 1994). Numerous bacterial species have displayed antagonistic activity towards fungi (Kotze *et al*., 2011). Bacteria belonging to the genera *Bacillus* are considered to be safe and beneficial microorganisms for agronomical industries (Stein, 2005). Some of these bacilli are endophytes (Sneath, 1986; McSpadden-Gardener, 2004). The effectiveness of using *Bacillus* spp. as BCAs applied to soil has been reported (Pérez-García *et al*., 2011).

To date, no single control measure has been reported to be effective against *P. cinnamomi* which causes stem cankers and root rots of macadamia (Aryantha *et al*., 2000). There are currently no recommended control strategies for the management of these diseases, and there is a need for the development of an integrated management strategy for macadamia root rots and stem cankers in South Africa. The aim of the study, therefore, was to determine the *in vitro* inhibitory effect of selected BCAs against *P. cinnamomi*.

## 4.2 Materials and Methods

### 4.2.1 *Phytophthora cinnamomi* isolates

Six of the seven *P. cinnamomi* isolates listed in Table 2.1 were used in this study. Ph 379 could not be revived, and was, therefore, omitted from the study. Agar plugs of the isolates were transferred from the Bijou bottles in storage and plated on PDA in 90 mm Petri dishes, sealed with Parafilm® and incubated at 25 ± 2 °C to allow regrowth for five days.

### 4.2.2 Screening of *P. cinnamomi* isolates for pathogenicity and virulence on green apples

The six *P. cinnamomi* isolates were screened for their pathogenicity and virulence on green apples. Green apples (*Malus domestica* Mill. × *M. sylvestris* Borkh.) cultivar Granny Smith were sourced from a local supermarket, and only those with no spots were used for the test.

Ethanol (70%) was used to surface sterilise the apples. A sterile scalpel was used to cut a 10 × 10 mm incision in the apple and a 5 mm *P. cinnamomi* plug of each isolate grown on PDA for five days was inserted (mycelia face down) into the incision and sealed with Parafilm®. The apple tissue in contact with the inoculum disc will therein be referred to as the site of inoculation. Controls were inoculated with sterile PDA agar discs. Five replications were used for each isolate and control treatments. The fruits were incubated in disinfected plastic trays at 25 ± 2 °C in the dark and were arranged in a complete randomized design (CRD). Eight days post inoculation (dpi), the total length of each externally visible lesion extending from the site of inoculation was measured. The daily lesion extension ($mm^{d-1}$) was calculated by subtraction of the distance from the centre of point of inoculation to beginning of lesion on the apple fruit from the lesion length (mm) 8 dpi then dividing by 8. Two apples were randomly selected per isolate and control measures for re-isolation on PARPH medium to confirm the presence of *P. cinnamomi*. Pathogenicity was determined by the presence of necrosis, however small. The size of lesion extension per day was taken as a measure of virulence of the pathogenic isolates.

### 4.2.3 Selected potential biological control agents

Two *Trichoderma* spp., namely; *Trichoderma hamatum* and *Trichoderma harzianum*, and eight unknown *Bacillus* spp. were sourced from the Agricultural Research Council – Plant Protection and Research (ARC-PPR), Pretoria. They were selected based on their proven antagonistic ability against *Phytophthora* and as a means of controlling the pathogen *in vitro* (Table 4.1).

**Table 4.1. Selected BCAs evaluated for their *in vitro* effect on isolates of *P. cinnamomi***

| Type | Genus | BCA code |
|---|---|---|
| Fungi | *Trichoderma* | *T. hamatum* strain 382 |
| Fungi | *Trichoderma* | *T. harzianum* strain SQR-T037 |
| Bacteria | *Bacillus* | B 31a |
| Bacteria | *Bacillus* | B 31b |
| Bacteria | *Bacillus* | B 41b |
| Bacteria | *Bacillus* | B 616 |
| Bacteria | *Bacillus* | BV 1C |
| Bacteria | *Bacillus* | NB 4 |
| Bacteria | *Bacillus* | NB 51b |
| Bacteria | *Bacillus* | NB 616 |

#### 4.2.4 *In vitro* antagonistic bioassays

A dual culture technique was used to evaluate the *in vitro* biocontrol activity of selected BCAs against *P. cinnamomi* growth. Hyphal plugs (5 mm) of *P. cinnamomi* isolates were placed 2 cm from one edge of 90 mm Petri dishes containing PDA. Two days later, a 5 mm mycelial plug from the margin of a four day old *Trichoderma* culture or a two day old *Bacillus* culture was placed on the opposite side on the same plate. Trials for each pathogen were set up in five replications, with five Petri dishes for the controls and dual culture plates. The plates were incubated at 25 ± 2 °C, and the evaluation of interactions was evaluated seven days after they were placed into assay plates. Antagonism towards *P.cinnamomi* was scored using the Badalyan (2002) rating scale where A = deadlock with mycelia contact, B = deadlock at a distance, C = replacement, overgrowth without initial deadlock. For fungal antagonists, the Antagonism Index (AI) was calculated considering the ray of *P. cinnamomi* mycelial colony towards the antagonist (rm) and the average of the three rays of the colony in the radial directions in a Petri dish (RM), it was expressed as a percentage: AI (%) = [(RM-rm)/RM]x100 (Hakizimana *et al.*, 2000). 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 (Hakizimana *et al.*, 2000).

### 4.2.5 Data Analysis

The generated data was subjected to analysis of variance (ANOVA). Student's t-LSD (Least significant difference) was calculated at a 5% significance level (P=0.05) to compare means of significant source effects (Snedecor and Cochran, 1967). Laboratory experiments were performed as a randomized complete block design. All the data were analysed using GenStat for Windows 18$^{th}$ Edition (GenStat, 2015).

## 4.3 Results

### 4.3.1 Pathogenicity and virulence tests on green apples

All six *P. cinnamomi* isolates examined in this study were pathogenic to green apples. Lesions were observed eight dpi and extended rapidly up to 16 dpi (Fig. 4.1A). Re-isolation of *P. cinnamomi* was done using tissue from the lesions and from the site of inoculation where no lesions developed. Control apples had no lesions, and *P. cinnamomi* was not recovered from the fruits (Fig. 4.1B). There was no significant difference (P>0.05) in virulence among the isolates (Table 4.2). The lesion extension lengths varied between 1.46 and 2.43 mm$^{d-1}$.

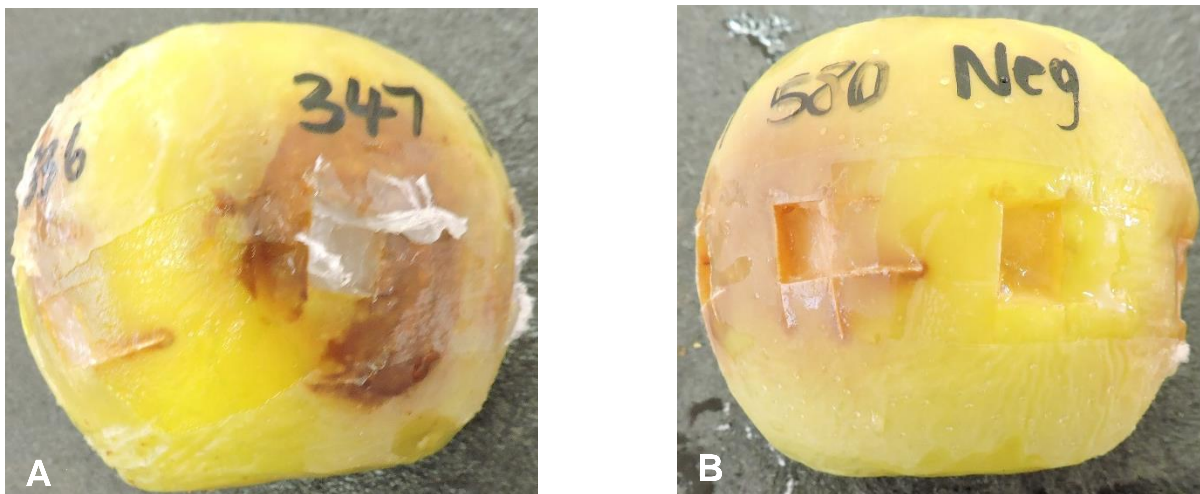


**Figure 4.1:** Lesions on *Malus domestica.* A: Lesion observed eight days post inoculation. B: Lesion 8 days after inoculation and negative control showing no lesion.

**Table 4.2 Means ± standard deviations for mean lesion extension rate ($mm^{d-1}$) on green apples after inoculation with *P. cinnamomi* isolates**

| Isolate | Mean lesion extension rate ($mm^{d-1}$) |
|---|---|
| 7 | 1.93 ± 0.66 |
| 333 | 1.98 ± 0.70 |
| 336 | 2.28 ± 0.77 |
| 347 | 2.43 ± 0.40 |
| 580 | 1.78 ± 0.16 |
| 581 | 1.46 ± 0.52 |
| P – value | 0.144 |

* ANOVA, refer to Appendix 3.

### 4.3.2 *In vitro* antagonistic bioassays of *Trichoderma* against *P. cinnamomi* isolates

Dual plate assays were conducted to evaluate the *in vitro* antagonistic activity of *T. hamatum* and *T. harzianum* against *P. cinnamomi*. Both *Trichoderma* spp. were able to inhibit all six isolates of *P. cinnamomi* even though the pathogen was allowed to grow before the BCAs were placed on the same plates. *T. hamatum* suppressed *P. cinnamomi* through deadlock with mycelial contact, whereas *T. harzianum* replaced and overgrew *P. cinnamomi* (Fig. 4.2). This shows that the tested strains of *T. harzianum* and *T. hamatum* have a high inhibitory effect on myceliae growth of *P. cinnamomi*.

There were significant differences ($P < 0.05$) in percentage growth and the diameter of the *P. cinnamomi* isolates towards the antagonist (Table 4.3). The growth percentages and the diameter of the *P. cinnamomi* isolates towards the antagonist range from 37.1 – 50.9% and 30.4 – 43.4 mm, respectively. This indicates that *T. harzianum* and *T. hamatum* were able to inhibit the pathogen before it could reach more than 50% growth. Significant differences ($P < 0.05$) were observed between the *Trichoderma* strains. This shows tested strains of *T. harzianum* and *T. hamatum* inhibit *P. cinnamomi* using different mechanisms.

There were no interactions between the *Trichoderma* spp. and *P. cinnamomi* isolates, as there was no significant difference (P > 0.05) (Appendix 4).

**Table 4.3 Means ± standard deviations for mean percentage growth and diameter (mm) of *P. cinnamomi* isolates**

| Isolate | Growth (%) | Diameter towards antagonist (mm) |
|---|---|---|
| 7 | 50.9 ± 3.48a | 43.4 ± 4.18a |
| 347 | 46.3 ± 5.71ab | 38.0 ± 4.69b |
| 333 | 46.2 ± 6.86ab | 37.9 ± 5.63b |
| 581 | 42.1 ± 3.88bc | 34.5 ± 3.17bc |
| 580 | 40.4 ± 8.55c | 33.1 ± 6.99c |
| 336 | 37.1 ± 4.67c | 30.4 ± 3.82c |

* Means followed by the same letter are not significantly different according to the LSD test at P ≥ 0.05.
* Refer to Appendix 4 for ANOVA.

### 4.3.3 *In vitro* antagonistic bioassays of *Bacillus* against *P. cinnamomi* isolates

Out of the eight unknown *Bacillus* species tested against *P. cinnamomi*, B 41b and NB 4 caused high levels of growth inhibition (Fig. 4.3). All the strains demonstrated the ability to inhibit the growth of *P. cinnamomi in vitro*, although some showed poor inhibition. *Bacillus* spp. B31a and NB51b caused the lowest level of inhibition. The two strains only managed to inhibit 1 % of the fast-growing *P. cinnamomi* isolate, 7. The other strains of *Bacillus* spp. also caused low inhibition to the *P. cinnamomi* 7, ranging between 2.32 to 3.82 %, excluding the two best strains of *Bacillus* spp.

There were significant differences (P < 0.05) in mean inhibition zones caused by the tested strains of *Bacillus* spp. against *P. cinnamomi* isolates (Table 4.4). Significant differences (P < 0.05) were also observed in *in vitro* growth inhibition by different *Bacillus*

spp. (Table 4.5). The sensitivity of P. cinnamomi isolates to the different Bacillus spp. was significantly different according to the LSD ($P < 0.05$) test.

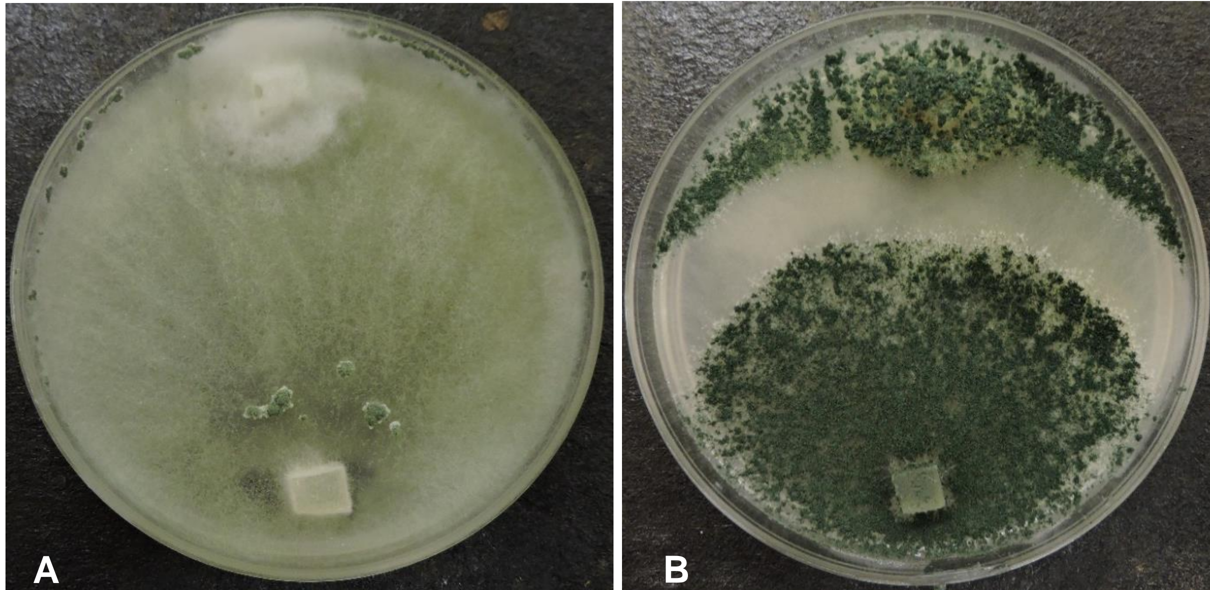


**Figure 4.2:** Competitive interactions between *Trichoderma* and *Phytophthora cinnamomi* on PDA at 25 °C after seven days. A: *T. hamatum* inhibiting *P. cinnamomi* via deadlock with myceliae contact. B: *T. harzianum* inhibiting *P. cinnamomi* through replacement.

*Bacillus* B 41b and NB 4 caused the highest *in vitro* growth inhibition of 35.6 to 57.5 % as well as the highest inhibition zone of 15.1 to 22.7 mm on six of the seven *P. cinnamomi* isolates. These were followed by *Bacillus* B 31 b, BV 1C and B 616 that caused growth inhibition between 9.6 and 49.7 % and had inhibition zones between 5.7 to 37.2 mm in six of the seven *P. cinnamomi* isolates. *Bacillus* NB 616, B 31a and NB 51b caused the lowest *in vitro* growth inhibition of 2.8 to 36.9 % and inhibition zones between 2.1 to 21.2 mm on six of the seven P. cinnamomi isolates.

1 **Table 4.4 Inhibition zone caused by eight strains of *Bacillus* species against six isolates of *P. cinnamomi***

| 2 ***Bacillus* spp** | ***Phytophthora cinnamomi* isolates (Inhibition zone (mm)) *** | | | | | |
|---|---|---|---|---|---|---|
| 3 | 7 | 333 | 336 | 347 | 580 | 581 |
| 4 B31a | 2.66 ± 1.25pq | 7.28 ± 4.49n-p | 17.6 ± 6.54b-i | 6.28 ± 2.93o-p | 10.64 ± 7.49j-o | 14.04 ± 4.74g-m |
| 5 B31b | 1.06 ± 0.13q | 17.6 ± 6.19b-i | 37.24 ± 5.28a | 10.63 ± 8.17j-o | 16.18 ± 7.42d-j | 23.34 ± 7.99b |
| 6 B41b | 10.66 ± 1.41j-o | 15.1 ± 4.0f-m | 22.66 ± 4.8bc | 22.72 ± 2.96b | 20.02 ± 6.8b-g | 19.32 ± 5.17b-h |
| 7 B616 | 1.84 ± 1.09pq | 9.7 ± 7.56l-o | 19.2 ± 6.19b-h | 10.16 ± 7.19k-o | 12.46 ± 8.19i-n | 9.32 ± 5.79m-o |
| 8 BV1C | 1.68 ± 0.95pq | 5.72 ± 1.67o-q | 21.92 ± 4.12b-d | 6.62 ± 1.31n-q | 15.72 ± 4.33e-k | 15.58 ± 7.55e-l |
| 9 NB4 | 13.7 ± 4.13h-m | 16.72 ± 5.39c-i | 21.14 ± 4.65b-e | 18.34 ± 4.98b-i | 19.0 ± 3.87b-h | 20.1 ± 2.73b-f |
| 10 NB51b | 1.10 ± 0.14q | 6.40 ± 2.39o-q | 21.24 ± 5.38b-e | 2.14 ± 0.31p-q | 10.58 ± 4.27j-o | 13.38 ± 7.09h-m |
| 11 NB616 | 6.32 ± 8.98o-q | 7.16 ± 6.95n-p | 17.72 ± 2.18b-i | 7.34 ± 5.32n-p | 9.28 ± 4.75m-o | 10.54 ± 5.07j-o |

12 * Means in the same column followed by the same letter are not significantly different according to the LSD test at P ≥ 0.05.

13 * ANOVA, refer to Appendix 5.

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**Table 4.5 Percentage *in vitro* growth inhibitions of isolates of *P. cinnamomi* by eight strains of *Bacillus* species**

| ***Bacillus* spp** | ***Phytophthora cinnamomi* isolates (percentage growth inhibition) *** | | | | | |
|---|---|---|---|---|---|---|
| | 7 | 333 | 336 | 347 | 580 | 581 |
| B31a | 3.82 ± 1.9p-s | 13.06 ± 9.4n-s | 34.64 ± 14.5e-j | 12.45 ± 6.2o-s | 18.56 ± 13.8k-p | 27.90 ± 11.4g-n |
| B31b | 1.24 ± 0.3s | 27.70 ± 11.3g-n | 72.86 ± 17.5a | 16.53 ± 12.9l-r | 28.24 ± 13.4g-m | 49.68 ± 21.1b-d |
| B41b | 22.54 ± 4.4h-o | 35.58 ± 14.2d-l | 55.62 ± 14.3bc | 57.52 ± 11.3b | 45.76 ± 22.6b-f | 46.08 ± 19.2b-f |
| B616 | 2.36 ± 1.8rs | 18.66 ± 18.0k-p | 41.76 ± 18.5c-g | 22.35 ± 18.4h-o | 22.66 ± 15.7h-o | 16.18 ± 12.2l-r |
| BV1C | 2.32 ± 1.7rs | 9.62 ± 3.5o-s | 47.72 ± 12.6b-f | 10.92 ± 2.8o-s | 33.10 ± 12.5f-k | 36.74 ± 21.5d-h |
| NB4 | 28.72 ± 12.5g-l | 36.88 ± 15.9d-h | 53.64 ± 15.8bc | 44.28 ± 16.6b-f | 44.72 ± 11.0 b-f | 49.40 ± 9.1b-e |
| NB51b | 1.14 ± 0.1s | 10.02 ± 5.1o-s | 36.88 ± 9.6d-h | 2.82 ± 0.4q-s | 17.62 ± 8.0l-q | 21.86 ± 10.1i-o |
| NB616 | 13.14 ± 21.7n-s | 13.48 ± 14.9m-s | 34.88 ± 6.6d-j | 15.42 ± 14.9l-s | 16.38 ± 9.1l-r | 20.32 ± 11.5j-o |

* Means in the same column followed by the same letter are not significantly different according to the LSD test at P ≥ 0.05.

* ANOVA, refer to Appendix 5.

Isolate 7 displayed a remarkably low sensitivity to all the *Bacillus* spp. tested in this study with growth inhibition ranging from 1.14 to 28.7 % and inhibition zones between 1.1 to 13.7 mm. There an interaction between the tested strains of *Bacillus* and *P. cinnamomi* ($P < 0.05$) in inhibition zones and *in vitro* growth inhibition (Appendix 5).

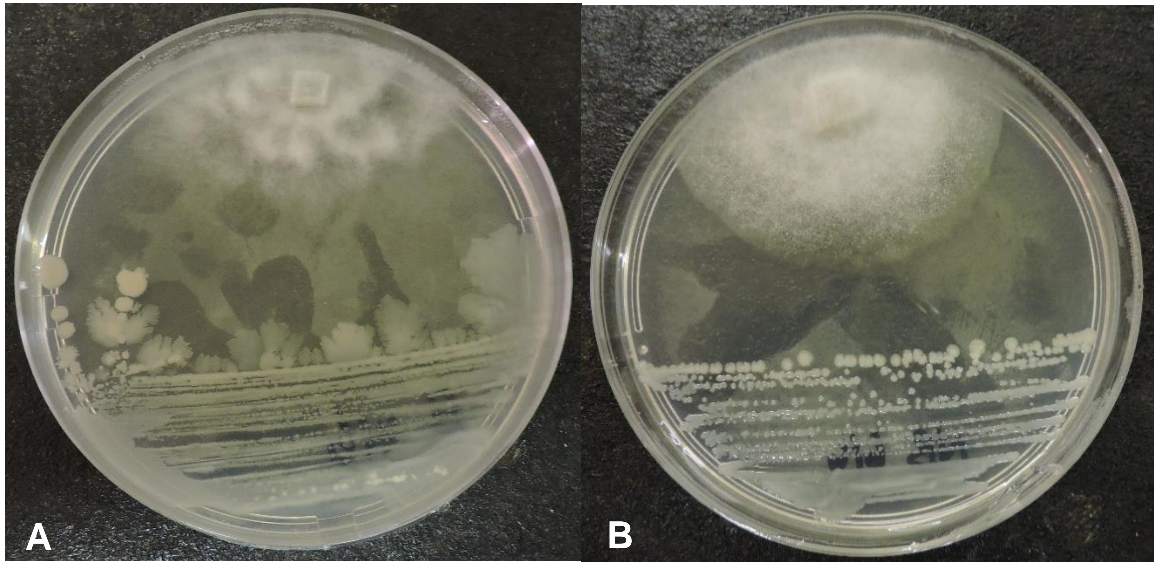


**Figure 4.3:** Highest growth inhibition of *Phytophthora cinnamomi* by *Bacillus* spp. on PDA at 25 °C after seven days. A: Growth inhibition of *P. cinnamomi* by B 41b. B: Growth inhibition of *P. cinnamomi* by NB 4.

## 4.3 Discussion

A number of pathogenicity studies have been conducted on *P. cinnamomi* globally; the challenge. However, there has been the absence of specificity in *P. cinnamomi* isolates of Australian (Dudzisnki *et al*., 1993), French (Robin and Desprez-Loustau, 1998) and South African (Linde *et al*., 1997) origin. In the late 2000's, Serfontein *et al*. (2007) established an accurate pathogenicity test, using apples. The test proved to be precise due to the distinctive brown hard rots that appeared on the apples caused by *P. cinnamomi*. The test has since been used to separate pathogenic from non-pathogenic *P. cinnamomi* isolates (Mbaka, 2011). In this study, six of six *P. cinnamomi* isolates were pathogenic to green apples.

The inhibitory effect of the test strains of *T. hamatum* and *T. harzianum* on all the *P. cinnamomi* isolates indicates that the test strains had a direct effect on suppression of

mycelial growth. This was in agreement with Yedidia *et al*., (1999), who found that the mode of antagonism of *Trichoderma* spp. on *P. cinnamomi* included competition for nutrients on agar media, which explained the inhibitory effect the BCAs displayed on the *P. cinnamomi* isolates *in vitro*. *Trichoderma* spp. inhibit the pathogenic fungi through the non-volatile and volatile metabolites (Küçük and Kivan, 2003). Isolates of *Trichoderma* spp. produce lytic enzymes and antifungal antibiotics which suppress the pathogens (Benitez *et al*., 2004; Harman, 2000). Shalini *et al*., (2006) described the different mechanisms active in mycoparasitic activity.

The eight strains of unknown *Bacillus* spp. were able to suppress the growth of *P. cinnamomi in vitro*. This proved what Shoda (2000) stated, that bacteria produce cell wall-degrading enzymes and secondary metabolites to inhibit the growth of other microorganisms. *Bacillus* B 41b and NB 4 caused the highest *in vitro* growth inhibition of *P. cinnamomi*. This could be because, as some researchers have observed, *Bacillus* spp. can intensively colonise *Phytophthora* spp. hyphae (Broadbent and Baker, 1974a, 1974b; Nesbitt *et al*., 1981a; Malajczuk, 1988). The interaction (Appendix 5) of the eight unknown strains of *Bacillus* spp. and the six *P. cinnamomi* isolates ishowed that Bacillus species are able to inhibit P. cinnamomi. This was proved by Sneh *et al*. (1977) who stated that the inoculum of *P. cinnamomi* in the soil is reduced by antagonistic bacteria that attack the sporangia, mycelium or the more resistant oospores and chlamydospores. Another possibility would be the process of feeding on exudates; the bacteria may produce metabolites that degrade *P. cinnamomi* (Nesbitt *et al*., 1981b).

Isolate 7 had the lowest sensitivity to the test bio-control agents. These results were contrary to the findings of Coffey and Bower, (1984), who stated that in spite of *P. cinnamomi*'s broad host range, it had a comparatively narrow spectrum of sensitivities.

In conclusion, researchers can use the pathogenicity test to determine the presence of *P. cinnamomi* inoculum in soils. The test can also be used to evaluate the efficacy of soil treatment (drenching with chemicals, fumigation, and solarisation) for control of *P. cinnamomi*. The fungal and bacterial BCAs tested in this study had inhibitory effects on the mycelial growth of *P. cinnamomi in vitro*, demonstrating the potential for use to manage root rots and trunk cankers of macadamia under field conditions. It is, however, necessary to compare *in vitro* with *in vivo* inhibitions to see whether they are parallel. The *Bacillus* spp. used in this study need to be characterised further with molecular comparisons.

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# CHAPTER 5

# GENERAL DISCUSSION

## 5.1 Overview of major outcomes

This study, along with other *P. cinnamomi* studies in South Africa (Linde *et al.*, 1997; Manicom, 2003), confirms the existence of *P. cinnamomi* and the threat it poses to macadamia production in Africa and South Africa. In Chapter 2, based on an optimised nested PCR, it was confirmed that there is a widespread distribution of macadamia root rots and trunk cankers in the main macadamia growing areas of South Africa. The optimised nested PCR was more sensitive in the detection of *P. cinnamomi* in comparison to the one-step PCR. This was shown in previous studies where other species of *Phytophthora* were detected with nested PCR showing a 1000-fold increase in sensitivity (Grote *et al.*, 2002; Martin *et al.*, 2004). This assay permits species-specific detection by both rounds of PCR.

Results reported in Chapter 3 highlighted that macadamia root rot, and trunk cankers are caused by the oomycete *Phytophthora cinnamomi*. This was in agreement with Mbaka (2011) who reported that *P. cinnamomi* was the causal organism for macadamia root rot and trunk cankers in Kenya. A high mean disease incidence of 60.9% and severity of 65.5% in the core macadamia growing areas of South Africa was reported. This high *P. cinnamomi* incidence and severity requires a closer look at control strategies and assessment of their effectiveness.

The outcomes of Chapter 4 emphasized the ability of some strains of *Trichoderma* spp. and *Bacillus* spp. to inhibit *P. cinnamomi in vitro*. No single method has been found to be efficacious for the control of *P. cinnamomi* (Aryantha *et al.*, 2000). To this end, an integrated management strategy that combines chemical, cultural and biological control is needed.

## 5.2 Implications of outcomes

The distribution of *P. cinnamomi* in macadamia growing areas has a negative impact on farming. *P. cinnamomi* reduces vigour, production and may cause complete trunk death of macadamia trees, resulting in a loss in the annual gross value of macadamia. Nested PCR and the baiting bioassay will find practical application as

protocols for the detection of *P. cinnamomi*. The bio-control agents evaluated in this study can be included in an integrated management strategy for the control of macadamia root rots and trunk cankers depending on their mode of action. They could be applied as foliar sprays, root dips, soil drenches, trunk injections and wound treatments.

## 5.3 Recommendations

*Phytophthora cinnamomi* has an expansive host range and wide distribution. It is, therefore, recommended to undertake studies that will find susceptible crops and tree species in macadamia growing regions of South Africa. Cross pathogenicity of *P. cinnamomi* isolates from diverse hosts should be established so as to be able to select crops that should be used in macadamia orchards when intercropping.

All macadamia growing areas of South Africa need to be surveyed to determine the distribution of *P. cinnamomi*. To reduce the incidence of macadamia stem cankers and root rots, susceptible crop hosts such as *Eucalyptus* species, *Grevillea*, and avocado should be avoided when intercropping macadamia.

The optimized nested PCR established in this study and the baiting bioassay should be used in combination for the detection of *P. cinnamomi*. To conduct successful disease surveys, DNA based strategies for isolation of the pathogen from plant tissues and plagued soils should be developed as customary techniques for isolation, culturing and identification of *P. cinnamomi* are dreary and require skill and labour. The strategies will be pertinent to macadamia and other crops. Farmers and researchers in poor environments need to be taught about these molecular procedures.

The BCAs that were identified in this study should be evaluated for their effectiveness in the management of *P. cinnamomi* induced macadamia root rots *in vivo* and field trials. If these BCAs are then successful in inhibiting *P. cinnamomi*, they could be used as an active ingredient in agrochemicals.

Tolerant or resistant macadamia cultivars need to be established and used in macadamia propagation. There is an urgent need to distinguish macadamia genotypes and assess them for tolerance or resistance to *P. cinnamomi* infections. A vital aspect of disease management is the planting of disease-free plant material. Macadamia nurseries and field operators should be taught about *P. cinnamomi* affecting macadamia and how to identify and manage the pathogen.

### 5.4 References


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# PROJECT OUTCOMES

The research was presented at the following conferences:

NS Kunene, MD Ncango, M Schoeman, B Christie, A Gubba and G Tlou. A rapid diagnostic tool for *Phytophthora cinnamomi* on Macadamia using fluorescence-based qualitative real-time polymerase chain reaction (qPCR), a reveiw. 7th International Macadamia Symposium. 11-13 August, Kruger National Park, Skukuza, South Africa.

NS Kunene, MD Ncango, M Schoeman, B Christie, A Gubba and G Tlou. Resistant rootstocks as disease management options against *Phytophthora cinnamomi* on Macadamia, a reveiw. 7th International Macadamia Symposium. 11-13 August, Kruger National Park, Skukuza, South Africa.

N. Kunene, G. Gubba and A.E.C. Jooste. 2016. Let your soil speak for you….are you *Phytophthora* free? SAMAC Research Symposium. 24 August 2016, Emnotweni Arena, Riverside, Mbombela, South Africa.

Kunene, N.S., Jooste, A.E.C., Gubba, A. Molecular Identification and the effect of Biological Control of *Phytophthora cinnamomi* on Macadamia in South Africa. 50th Anniversary congress of the Southern African society for Plant Pathology. 15th-18th January 2017, Champagne Sports Resort, Drakensberg, South Africa.

Kunene, N.S., Gubba, A., Jooste, A.E.C., Distribution, molecular identification and the effect of biological control of *Phytophthora cinnamomi* on macadamia in the Limpopo and Mpumalanga provinces. Professional Development Programme Conference. 4-6 September 2017, ARC-VOP, Pretoria, South Africa.

# APPENDICES

**Appendix 1:** PCR results data

| Effect | Univariate Results for Each DV (PCR results) Sigma-restricted parameterization Effective hypothesis decomposition | | | | |
|---|---|---|---|---|---|
| | Degr. of Freedom | PCR Results SS | PCR Results MS | PCR Results F | PCR Results p |
| Intercept | 1 | 52.45672 | 52.45672 | 254.5426 | 0.000000 |
| Province | 14 | 11.99559 | 0.85683 | 4.1577 | 0.000002 |
| Error | 190 | 39.15563 | 0.20608 | | |
| Total | 204 | 51.15122 | | | |

| Effect | Descriptive Statistics (Root incidence and severity + PCR results) | | | | |
|---|---|---|---|---|---|
| | Level of Factor | N | PCR Results Mean | PCR Results Std.Dev. | PCR Results Std.Err |
| Total | | 205 | 0.521951 | 0.500741 | 0.034973 |
| Province | Mpumalanga1 | 20 | 0.900000 | 0.307794 | 0.068825 |
| Province | Mpumalanga2 | 11 | 0.818182 | 0.404520 | 0.121967 |
| Province | Mpumalanga3 | 17 | 0.235294 | 0.437237 | 0.106046 |
| Province | Mpumalanga4 | 15 | 0.466667 | 0.516398 | 0.133333 |
| Province | Mpumalanga5 | 15 | 0.800000 | 0.414039 | 0.106904 |
| Province | Mpumalanga6 | 11 | 0.545455 | 0.522233 | 0.157459 |
| Province | Mpumalanga7 | 10 | 0.800000 | 0.421637 | 0.133333 |
| Province | Mpumalanga8 | 10 | 0.700000 | 0.483046 | 0.152753 |
| Province | Mpumalanga9 | 13 | 0.615385 | 0.506370 | 0.140442 |
| Province | Limpopo1 | 12 | 0.333333 | 0.492366 | 0.142134 |
| Province | Limpopo2 | 10 | 0.400000 | 0.516398 | 0.163299 |
| Province | Limpopo3 | 19 | 0.315789 | 0.477567 | 0.109561 |
| Province | Limpopo4 | 10 | 0.000000 | 0.000000 | 0.000000 |
| Province | Limpopo5 | 15 | 0.466667 | 0.516398 | 0.133333 |
| Province | Limpopo6 | 17 | 0.411765 | 0.507300 | 0.123038 |

**Appendix 2:** Root rot incidence and severity data

File name is Root rot incidence and severity.GEN
Compare root rot incidence and severtity between provinces
November 2017

*Message: You have input sufficient data, READ terminated.*

| Identifier | Minimum | Mean | Maximum | Values | Missing |
|---|---|---|---|---|---|
| %Severity | 46.00 | 65.47 | 80.00 | 15 | 0 |
| %Rootrot | 41.70 | 60.91 | 80.00 | 15 | 0 |

| Identifier | Values | Missing | Levels |
|---|---|---|---|
| PROVINCE | 15 | 0 | 2 |

# Analysis of variance

Variate: %Severity

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| PROVINCE | 1 | 120.2 | 120.2 | 0.80 | 0.388 |
| Residual | 13 | 1955.6 | 150.4 | | |
| Total | 14 | 2075.7 | | | |

# Tables of means

Variate: %Severity

Grand mean 65.5

| PROVINCE | Limpopo | Mpumalanga |
|---|---|---|
| | 62.0 | 67.8 |
| rep. | 6 | 9 |

## Standard errors of means

| Table | PROVINCE | |
|---|---|---|
| rep. | unequal | |
| d.f. | 13 | |
| e.s.e. | 5.01 | min.rep |
| | 4.09 | max.rep |

# Stratum standard errors and coefficients of variation

Variate: %Severity

| d.f. | s.e. | cv% |
|---|---|---|
| 13 | 12.26 | 18.7 |

=========== Summary of original data =============

| PROVINCE | Mean | Variance | s.d. |
|---|---|---|---|
| Limpopo | 62.00 | 140.0 | 11.83 |
| Mpumalanga | 67.78 | 156.9 | 12.53 |

| PROVINCE | %Severity | FITTED | RESIDUAL |
|---|---|---|---|
| Mpumalanga | 75.00 | 67.78 | 7.22 |
| Mpumalanga | 50.00 | 67.78 | -17.78 |
| Mpumalanga | 3.000 | 67.78 | -7.78 |
| Mpumalanga | 3.000 | 67.78 | -2.78 |
| Mpumalanga | 2.000 | 67.78 | -19.78 |
| Mpumalanga | 3.000 | 67.78 | 9.22 |
| Mpumalanga | 3.000 | 67.78 | 11.22 |
| Mpumalanga | 3.000 | 67.78 | 12.22 |
| Mpumalanga | 3.000 | 67.78 | 8.22 |
| Limpopo | 2.000 | 62.00 | -16.00 |
| Limpopo | 3.000 | 62.00 | 9.00 |
| Limpopo | 3.000 | 62.00 | 7.00 |
| Limpopo | 3.000 | 62.00 | 13.00 |
| Limpopo | 2.000 | 62.00 | -12.00 |
| Limpopo | 3.000 | 62.00 | -1.00 |

## Analysis of variance

Variate: %Rootrot

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| PROVINCE | 1 | 236.52 | 236.52 | 2.53 | 0.136 |
| Residual | 13 | 1217.62 | 93.66 | | |
| Total | 14 | 1454.14 | | | |

## Tables of means

Variate: %Rootrot

Grand mean 60.9

| PROVINCE | Limpopo | Mpumalanga |
|---|---|---|
| | 56.0 | 64.2 |
| rep. | 6 | 9 |

## Standard errors of means

| Table | PROVINCE | |
|---|---|---|
| rep. | unequal | |
| d.f. | 13 | |
| e.s.e. | 3.95 | min.rep |
| | 3.23 | max.rep |

## Stratum standard errors and coefficients of variation

Variate: %Rootrot

| d.f. | s.e. | cv% |
|---|---|---|
| 13 | 9.68 | 15.9 |

=========== Summary of original data =============

| PROVINCE | Mean | Variance | s.d. |
|---|---|---|---|
| Limpopo | 56.05 | 118.71 | 10.896 |
| Mpumalanga | 64.16 | 78.01 | 8.832 |

| PROVINCE | %Rootrot | FITTED | RESIDUAL |
|---|---|---|---|
| Mpumalanga | 63.60 | 64.16 | -0.556 |
| Mpumalanga | 60.00 | 64.16 | -4.156 |
| Mpumalanga | 55.60 | 64.16 | -8.556 |
| Mpumalanga | 66.70 | 64.16 | 2.544 |
| Mpumalanga | 50.00 | 64.16 | -14.156 |
| Mpumalanga | 70.00 | 64.16 | 5.844 |
| Mpumalanga | 80.00 | 64.16 | 15.844 |
| Mpumalanga | 70.00 | 64.16 | 5.844 |
| Mpumalanga | 61.50 | 64.16 | -2.656 |
| Limpopo | 41.70 | 56.05 | -14.350 |
| Limpopo | 60.00 | 56.05 | 3.950 |
| Limpopo | 65.00 | 56.05 | 8.950 |
| Limpopo | 70.00 | 56.05 | 13.950 |
| Limpopo | 46.70 | 56.05 | -9.350 |
| Limpopo | 52.90 | 56.05 | -3.150 |

End of Nontokozo Kunene - TSC - Project no: P03000100. Current data space: 1 block, peak usage 1% at line 44.

Genstat 64-bit Release 18.2 ( PC/Windows 8) 13 November 2017 11:52:52
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Registered to: ARC

**Appendix 3:** Pathogenicity test green data

File name is Pathogenicity test green apples.GEN
P. cinnamomi screened for pathogenicity and virulence on green apples
Pathogenicity tests
October 2017

*Message: You have input sufficient data, READ terminated.*

| Identifier | Minimum | Mean | Maximum | Values | Missing |
|---|---|---|---|---|---|
| Lesion | 1.125 | 1.972 | 3.000 | 30 | 0 |

| Identifier | Values | Missing | Levels |
|---|---|---|---|
| REP | 30 | 0 | 5 |
| P_cinnamoni | 30 | 0 | 6 |

# Analysis of variance

Variate: Lesion

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| P_cinnamoni | 5 | 3.0267 | 0.6053 | 1.83 | 0.144 |
| Residual | 24 | 7.9218 | 0.3301 | | |
| Total | 29 | 10.9484 | | | |

# Tables of means

Variate: Lesion

Grand mean 1.97

| P_cinnamoni | 7 | 333 | 336 | 347 | 580 | 581 |
|---|---|---|---|---|---|---|
| | 1.93 | 1.98 | 2.27 | 2.42 | 1.77 | 1.46 |

# Standard errors of means

| Table | P_cinnamoni |
|---|---|
| rep. | 5 |
| d.f. | 24 |
| e.s.e. | 0.257 |

# Stratum standard errors and coefficients of variation

Variate: Lesion

| d.f. | s.e. | cv% |
|---|---|---|
| 24 | 0.575 | 29.1 |

=========== Summary of original data ============

| | Mean | Variance | s.d. |
|---|---|---|---|
| P_cinnamoni | | | |
| 7 | 1.925 | 0.4344 | 0.6591 |
| 333 | 1.975 | 0.4953 | 0.7038 |
| 336 | 2.275 | 0.5891 | 0.7675 |
| 347 | 2.425 | 0.1609 | 0.4012 |
| 580 | 1.775 | 0.0266 | 0.1630 |
| 581 | 1.455 | 0.2742 | 0.5236 |

| REP | P_cinnamoni | Lesion | FITTED | RESIDUAL |
|---|---|---|---|---|
| 1 | 347 | 2.125 | 2.425 | -0.3000 |
| 2 | 347 | 2.750 | 2.425 | 0.3250 |
| 3 | 347 | 1.875 | 2.425 | -0.5500 |
| 4 | 347 | 2.625 | 2.425 | 0.2000 |
| 5 | 347 | 2.750 | 2.425 | 0.3250 |
| 1 | 580 | 2.000 | 1.775 | 0.2250 |
| 2 | 580 | 1.625 | 1.775 | -0.1500 |
| 3 | 580 | 1.750 | 1.775 | -0.0250 |
| 4 | 580 | 1.625 | 1.775 | -0.1500 |
| 5 | 580 | 1.875 | 1.775 | 0.1000 |
| 1 | 336 | 3.000 | 2.275 | 0.7250 |
| 2 | 336 | 2.625 | 2.275 | 0.3500 |
| 3 | 336 | 2.750 | 2.275 | 0.4750 |
| 4 | 336 | 1.875 | 2.275 | -0.4000 |
| 5 | 336 | 1.125 | 2.275 | -1.1500 |
| 1 | 581 | 1.250 | 1.455 | -0.2050 |
| 2 | 581 | 1.125 | 1.455 | -0.3300 |
| 3 | 581 | 1.375 | 1.455 | -0.0800 |
| 4 | 581 | 2.375 | 1.455 | 0.9200 |
| 5 | 581 | 1.150 | 1.455 | -0.3050 |
| 1 | 7 | 1.625 | 1.925 | -0.3000 |
| 2 | 7 | 1.250 | 1.925 | -0.6750 |
| 3 | 7 | 1.500 | 1.925 | -0.4250 |
| 4 | 7 | 2.500 | 1.925 | 0.5750 |
| 5 | 7 | 2.750 | 1.925 | 0.8250 |
| 1 | 333 | 2.125 | 1.975 | 0.1500 |
| 2 | 333 | 2.500 | 1.975 | 0.5250 |
| 3 | 333 | 2.750 | 1.975 | 0.7750 |
| 4 | 333 | 1.125 | 1.975 | -0.8500 |
| 5 | 333 | 1.375 | 1.975 | -0.6000 |

End of Nontokozo Kunene - TSC - Project no: P03000100. Current data space: 2 blocks, peak usage 1% at line 60.

Genstat 64-bit Release 18.2 ( PC/Windows 8) 25 October 2017 12:28:19
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Registered to: ARC

**Appendix 4:** *Trichoderma* spp. biocontrol data

File name is Bell test results Fungal.GEN
In vivo evaluation of different biocontrol agents against different isolates of P. cinnamomi
Fungal isolates
October 2017

*Message: You have input sufficient data, READ terminated.*

| Identifier | Minimum | Mean | Maximum | Values | Missing |
|---|---|---|---|---|---|
| %Growth | 25.00 | 43.83 | 62.80 | 60 | 0 |
| Diameter | 20.50 | 36.21 | 59.20 | 60 | 0 |
| AI | 4.400 | 20.92 | 32.30 | 60 | 0 |

| Identifier | Values | Missing | Levels |
|---|---|---|---|
| REP | 60 | 0 | 5 |
| FUNGAL | 60 | 0 | 2 |
| P_cinnamoni | 60 | 0 | 6 |

# Analysis of variance

Variate: %Growth

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| REP stratum | 4 | 122.04 | 30.51 | 0.86 | |
| REP.*Units* stratum | | | | | |
| FUNGAL | 1 | 1873.77 | 1873.77 | 52.93 | <.001 |
| P_cinnamoni | 5 | 1221.80 | 244.36 | 6.90 | <.001 |
| FUNGAL.P_cinnamoni | 5 | 172.72 | 34.54 | 0.98 | 0.443 |
| Residual | 44 | 1557.66 | 35.40 | | |
| Total | 59 | 4947.99 | | | |

# Tables of means

Variate: %Growth

Grand mean 43.82

| FUNGAL | Trichoderma_hamatum | Trichoderma_harzanium |
|---|---|---|
| | 49.41 | 38.24 |

| P_cinnamoni | 7 | 333 | 336 | 347 | 580 | 581 |
|---|---|---|---|---|---|---|
| | 50.86 | 46.21 | 37.05 | 46.34 | 40.39 | 42.10 |

| FUNGAL | P_cinnamoni | 7 | 333 | 336 | 347 | 580 |
|---|---|---|---|---|---|---|
| Trichoderma_hamatum | | 59.64 | 49.52 | 43.42 | 51.46 | 45.76 |
| Trichoderma_harzanium | | 42.08 | 42.90 | 30.68 | 41.22 | 35.02 |

| FUNGAL | P_cinnamoni | 581 |
|---|---|---|
| Trichoderma_hamatum | | 46.68 |
| Trichoderma_harzanium | | 37.52 |

# Standard errors of means

| Table | FUNGAL | P_cinnamoni | FUNGAL P_cinnamoni |
|---|---|---|---|
| rep. | 30 | 10 | 5 |
| d.f. | 44 | 44 | 44 |
| e.s.e. | 1.086 | 1.882 | 2.661 |

## Least significant differences of means (5% level)

| Table | FUNGAL | P_cinnamoni | FUNGAL P_cinnamoni |
|---|---|---|---|
| rep. | 30 | 10 | 5 |
| d.f. | 44 | 44 | 44 |
| l.s.d. | 3.096 | 5.363 | 7.584 |

## Stratum standard errors and coefficients of variation

Variate: %Growth

| Stratum | d.f. | s.e. | cv% |
|---|---|---|---|
| REP | 4 | 1.595 | 3.6 |
| REP.*Units* | 44 | 5.950 | 13.6 |

## Fisher's protected least significant difference test

### FUNGAL

*Warning 231, code UF 2, statement 180 in procedure AMCOMPARISON*

The number of MEANS must be greater than 2.

### P_cinnamoni

| | Mean | |
|---|---|---|
| 7 | 50.86 | a |
| 347 | 46.34 | ab |
| 333 | 46.21 | ab |
| 581 | 42.10 | bc |
| 580 | 40.39 | c |
| 336 | 37.05 | c |

### FUNGAL.P_cinnamoni

*Warning 232, code UF 2, statement 194 in procedure AMCOMPARISON*

Fisher's protected LSD is not calculated as variance ratio for FUNGAL.P_cinnamoni is not significant.

=========== Summary of original data =============

| P_cinnamoni | 7 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 59.64 | 11.73 | 3.425 |
| Trichoderma_harzanium | 42.08 | 12.51 | 3.537 |
| Margin | 50.86 | 96.43 | 9.820 |

| P_cinnamoni | 333 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 49.52 | 82.75 | 9.097 |
| Trichoderma_harzanium | 42.90 | 21.32 | 4.618 |
| Margin | 46.21 | 58.43 | 7.644 |

| P_cinnamoni | 336 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 43.42 | 22.88 | 4.783 |
| Trichoderma_harzanium | 30.68 | 20.68 | 4.547 |
| Margin | 37.05 | 64.44 | 8.028 |

| P_cinnamoni | 347 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 51.46 | 27.58 | 5.252 |
| Trichoderma_harzanium | 41.22 | 38.10 | 6.173 |
| Margin | 46.34 | 58.32 | 7.637 |

| P_cinnamoni | 580 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 45.76 | 104.19 | 10.207 |
| Trichoderma_harzanium | 35.02 | 47.42 | 6.886 |
| Margin | 40.39 | 99.42 | 9.971 |

| P_cinnamoni | 581 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 46.68 | 19.93 | 4.464 |
| Trichoderma_harzanium | 37.52 | 10.84 | 3.292 |
| Margin | 42.10 | 36.98 | 6.081 |

| P_cinnamoni | Margin | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 49.41 | 65.65 | 8.102 |
| Trichoderma_harzanium | 38.24 | 40.36 | 6.353 |
| Margin | 43.83 | 83.86 | 9.158 |

1

| 2 | REP | FUNGAL | P_cinnamoni | %Growth | FITTED | RESIDUAL |
|---|---|---|---|---|---|---|
| 3 | 1 | Trichoderma_hamatum | 347 | 58.50 | 53.36 | 5.140 |
| 4 | 2 | Trichoderma_hamatum | 347 | 50.00 | 49.63 | 0.373 |
| 5 | 3 | Trichoderma_hamatum | 347 | 44.50 | 50.13 | -5.635 |
| 6 | 4 | Trichoderma_hamatum | 347 | 50.00 | 52.66 | -2.660 |
| 7 | 5 | Trichoderma_hamatum | 347 | 54.30 | 51.52 | 2.782 |
| 8 | 1 | Trichoderma_hamatum | 581 | 40.20 | 48.58 | -8.380 |
| 9 | 2 | Trichoderma_hamatum | 581 | 51.20 | 44.85 | 6.353 |
| 10 | 3 | Trichoderma_hamatum | 581 | 50.60 | 45.36 | 5.245 |
| 11 | 4 | Trichoderma_hamatum | 581 | 45.70 | 47.88 | -2.180 |
| 12 | 5 | Trichoderma_hamatum | 581 | 45.70 | 46.74 | -1.038 |
| 13 | 1 | Trichoderma_hamatum | 333 | 62.80 | 51.42 | 11.380 |
| 14 | 2 | Trichoderma_hamatum | 333 | 45.80 | 47.69 | -1.887 |
| 15 | 3 | Trichoderma_hamatum | 333 | 39.60 | 48.20 | -8.595 |
| 16 | 4 | Trichoderma_hamatum | 333 | 45.10 | 50.72 | -5.620 |
| 17 | 5 | Trichoderma_hamatum | 333 | 54.30 | 49.58 | 4.722 |
| 18 | 1 | Trichoderma_hamatum | 580 | 54.30 | 47.66 | 6.640 |
| 19 | 2 | Trichoderma_hamatum | 580 | 29.90 | 43.93 | -14.027 |
| 20 | 3 | Trichoderma_hamatum | 580 | 54.30 | 44.44 | 9.865 |
| 21 | 4 | Trichoderma_hamatum | 580 | 48.20 | 46.96 | 1.240 |
| 22 | 5 | Trichoderma_hamatum | 580 | 42.10 | 45.82 | -3.718 |
| 23 | 1 | Trichoderma_hamatum | 336 | 50.00 | 45.32 | 4.680 |
| 24 | 2 | Trichoderma_hamatum | 336 | 36.60 | 41.59 | -4.987 |
| 25 | 3 | Trichoderma_hamatum | 336 | 44.50 | 42.09 | 2.405 |
| 26 | 4 | Trichoderma_hamatum | 336 | 42.70 | 44.62 | -1.920 |
| 27 | 5 | Trichoderma_hamatum | 336 | 43.30 | 43.48 | -0.178 |
| 28 | 1 | Trichoderma_hamatum | 7 | 58.50 | 61.54 | -3.040 |
| 29 | 2 | Trichoderma_hamatum | 7 | 62.20 | 57.81 | 4.393 |
| 30 | 3 | Trichoderma_hamatum | 7 | 54.30 | 58.31 | -4.015 |
| 31 | 4 | Trichoderma_hamatum | 7 | 62.80 | 60.84 | 1.960 |
| 32 | 5 | Trichoderma_hamatum | 7 | 60.40 | 59.70 | 0.702 |
| 33 | 1 | Trichoderma_harzanium | 336 | 27.40 | 32.58 | -5.180 |
| 34 | 2 | Trichoderma_harzanium | 336 | 36.60 | 28.85 | 7.753 |
| 35 | 3 | Trichoderma_harzanium | 336 | 25.00 | 29.36 | -4.355 |
| 36 | 4 | Trichoderma_harzanium | 336 | 32.30 | 31.88 | 0.420 |
| 37 | 5 | Trichoderma_harzanium | 336 | 32.10 | 30.74 | 1.362 |
| 38 | 1 | Trichoderma_harzanium | 7 | 45.10 | 43.98 | 1.120 |
| 39 | 2 | Trichoderma_harzanium | 7 | 36.60 | 40.25 | -3.647 |
| 40 | 3 | Trichoderma_harzanium | 7 | 45.10 | 40.76 | 4.345 |
| 41 | 4 | Trichoderma_harzanium | 7 | 42.70 | 43.28 | -0.580 |
| 42 | 5 | Trichoderma_harzanium | 7 | 40.90 | 42.14 | -1.238 |
| 43 | 1 | Trichoderma_harzanium | 580 | 42.10 | 36.92 | 5.180 |
| 44 | 2 | Trichoderma_harzanium | 580 | 29.90 | 33.19 | -3.287 |
| 45 | 3 | Trichoderma_harzanium | 580 | 26.20 | 33.70 | -7.495 |
| 46 | 4 | Trichoderma_harzanium | 580 | 40.90 | 36.22 | 4.680 |
| 47 | 5 | Trichoderma_harzanium | 580 | 36.00 | 35.08 | 0.922 |
| 48 | 1 | Trichoderma_harzanium | 581 | 35.40 | 39.42 | -4.020 |
| 49 | 2 | Trichoderma_harzanium | 581 | 42.10 | 35.69 | 6.413 |
| 50 | 3 | Trichoderma_harzanium | 581 | 33.50 | 36.20 | -2.695 |
| 51 | 4 | Trichoderma_harzanium | 581 | 37.80 | 38.72 | -0.920 |
| 52 | 5 | Trichoderma_harzanium | 581 | 38.80 | 37.58 | 1.222 |
| 53 | 1 | Trichoderma_harzanium | 333 | 37.20 | 44.80 | -7.600 |
| 54 | 2 | Trichoderma_harzanium | 333 | 45.80 | 41.07 | 4.733 |
| 55 | 3 | Trichoderma_harzanium | 333 | 49.10 | 41.58 | 7.525 |
| 56 | 4 | Trichoderma_harzanium | 333 | 40.90 | 44.10 | -3.200 |
| 57 | 5 | Trichoderma_harzanium | 333 | 41.50 | 42.96 | -1.458 |
| 58 | 1 | Trichoderma_harzanium | 347 | 37.20 | 43.12 | -5.920 |
| 59 | 2 | Trichoderma_harzanium | 347 | 37.20 | 39.39 | -2.187 |
| 60 | 3 | Trichoderma_harzanium | 347 | 43.30 | 39.90 | 3.405 |

| | | | | | |
|---|---|---|---|---|---|
| 4 | Trichoderma_harzanium | 347 | 51.20 | 42.42 | 8.780 |
| 5 | Trichoderma_harzanium | 347 | 37.20 | 41.28 | -4.078 |

## Analysis of variance

Variate: Diameter

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| REP stratum | 4 | 126.43 | 31.61 | 1.28 | |
| REP.*Units* stratum | | | | | |
| FUNGAL | 1 | 1420.09 | 1420.09 | 57.37 | <.001 |
| P_cinnamoni | 5 | 1038.85 | 207.77 | 8.39 | <.001 |
| FUNGAL.P_cinnamoni | 5 | 227.05 | 45.41 | 1.83 | 0.126 |
| Residual | 44 | 1089.16 | 24.75 | | |
| Total | 59 | 3901.58 | | | |

## Tables of means

Variate: Diameter

Grand mean 36.21

| FUNGAL | Trichoderma_hamatum | Trichoderma_harzanium |
|---|---|---|
| | 41.08 | 31.35 |

| P_cinnamoni | 7 | 333 | 336 | 347 | 580 | 581 |
|---|---|---|---|---|---|---|
| | 43.38 | 37.88 | 30.38 | 38.00 | 33.10 | 34.53 |

| FUNGAL | P_cinnamoni | 7 | 333 | 336 | 347 | 580 |
|---|---|---|---|---|---|---|
| Trichoderma_hamatum | | 52.26 | 40.60 | 35.60 | 42.20 | 37.50 |
| Trichoderma_harzanium | | 34.50 | 35.16 | 25.16 | 33.80 | 28.70 |

| FUNGAL | P_cinnamoni | 581 |
|---|---|---|
| Trichoderma_hamatum | | 38.30 |
| Trichoderma_harzanium | | 30.76 |

## Standard errors of means

| Table | FUNGAL | P_cinnamoni | FUNGAL<br>P_cinnamoni |
|---|---|---|---|
| rep. | 30 | 10 | 5 |
| d.f. | 44 | 44 | 44 |
| e.s.e. | 0.908 | 1.573 | 2.225 |

## Least significant differences of means (5% level)

| Table | FUNGAL | P_cinnamoni | FUNGAL<br>P_cinnamoni |
|---|---|---|---|
| rep. | 30 | 10 | 5 |
| d.f. | 44 | 44 | 44 |
| l.s.d. | 2.589 | 4.484 | 6.342 |

1
2
3

## Stratum standard errors and coefficients of variation

Variate: Diameter

| Stratum | d.f. | s.e. | cv% |
|---|---|---|---|
| REP | 4 | 1.623 | 4.5 |
| REP.*Units* | 44 | 4.975 | 13.7 |

## Fisher's protected least significant difference test

### FUNGAL

*Warning 233, code UF 2, statement 180 in procedure AMCOMPARISON*

The number of MEANS must be greater than 2.

### P_cinnamoni

| | Mean | |
|---|---|---|
| 7 | 43.38 | a |
| 347 | 38.00 | b |
| 333 | 37.88 | b |
| 581 | 34.53 | bc |
| 580 | 33.10 | c |
| 336 | 30.38 | c |

### FUNGAL.P_cinnamoni

*Warning 234, code UF 2, statement 194 in procedure AMCOMPARISON*

Fisher's protected LSD is not calculated as variance ratio for FUNGAL.P_cinnamoni is not significant.

=========== Summary of original data ============

| P_cinnamoni | 7 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 52.26 | 29.65 | 5.445 |
| Trichoderma_harzanium | 34.50 | 8.50 | 2.915 |
| Margin | 43.38 | 104.57 | 10.226 |

| P_cinnamoni | 333 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 40.60 | 55.55 | 7.453 |

| | | | |
|---|---|---|---|
| Trichoderma_harzanium | 35.16 | 14.43 | 3.798 |
| Margin | 37.88 | 39.32 | 6.271 |

| P_cinnamoni | 336 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 35.60 | 15.43 | 3.927 |
| Trichoderma_harzanium | 25.16 | 13.83 | 3.719 |
| Margin | 30.38 | 43.28 | 6.579 |

| P_cinnamoni | 347 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 42.20 | 18.58 | 4.310 |
| Trichoderma_harzanium | 33.80 | 25.70 | 5.070 |
| Margin | 38.00 | 39.28 | 6.267 |

| P_cinnamoni | 580 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 37.50 | 70.00 | 8.367 |
| Trichoderma_harzanium | 28.70 | 31.70 | 5.630 |
| Margin | 33.10 | 66.71 | 8.168 |

| P_cinnamoni | 581 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 38.30 | 13.33 | 3.650 |
| Trichoderma_harzanium | 30.76 | 7.21 | 2.686 |
| Margin | 34.53 | 24.92 | 4.992 |

| P_cinnamoni | Margin | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 41.08 | 58.46 | 7.646 |
| Trichoderma_harzanium | 31.35 | 27.11 | 5.206 |
| Margin | 36.21 | 66.13 | 8.132 |

| REP | FUNGAL | P_cinnamoni | Diameter | FITTED | RESIDUAL |
|---|---|---|---|---|---|
| 1 | Trichoderma_hamatum | 347 | 48.00 | 44.42 | 3.578 |
| 2 | Trichoderma_hamatum | 347 | 41.00 | 40.41 | 0.595 |
| 3 | Trichoderma_hamatum | 347 | 36.50 | 40.85 | -4.347 |
| 4 | Trichoderma_hamatum | 347 | 41.00 | 42.91 | -1.905 |
| 5 | Trichoderma_hamatum | 347 | 44.50 | 42.42 | 2.078 |
| 1 | Trichoderma_hamatum | 581 | 33.00 | 40.52 | -7.522 |
| 2 | Trichoderma_hamatum | 581 | 42.00 | 36.51 | 5.495 |
| 3 | Trichoderma_hamatum | 581 | 41.50 | 36.95 | 4.553 |
| 4 | Trichoderma_hamatum | 581 | 37.50 | 39.01 | -1.505 |
| 5 | Trichoderma_hamatum | 581 | 37.50 | 38.52 | -1.022 |
| 1 | Trichoderma_hamatum | 333 | 51.50 | 42.82 | 8.678 |
| 2 | Trichoderma_hamatum | 333 | 37.50 | 38.80 | -1.305 |
| 3 | Trichoderma_hamatum | 333 | 32.50 | 39.25 | -6.747 |
| 4 | Trichoderma_hamatum | 333 | 37.00 | 41.30 | -4.305 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 5 | Trichoderma_hamatum | 333 | 44.50 | 40.82 | 3.678 |
| 2 | 1 | Trichoderma_hamatum | 580 | 44.50 | 39.72 | 4.778 |
| 3 | 2 | Trichoderma_hamatum | 580 | 24.50 | 35.70 | -11.205 |
| 4 | 3 | Trichoderma_hamatum | 580 | 44.50 | 36.15 | 8.353 |
| 5 | 4 | Trichoderma_hamatum | 580 | 39.50 | 38.20 | 1.295 |
| 6 | 5 | Trichoderma_hamatum | 580 | 34.50 | 37.72 | -3.222 |
| 7 | 1 | Trichoderma_hamatum | 336 | 41.00 | 37.82 | 3.178 |
| 8 | 2 | Trichoderma_hamatum | 336 | 30.00 | 33.80 | -3.805 |
| 9 | 3 | Trichoderma_hamatum | 336 | 36.50 | 34.25 | 2.253 |
| 10 | 4 | Trichoderma_hamatum | 336 | 35.00 | 36.30 | -1.305 |
| 11 | 5 | Trichoderma_hamatum | 336 | 35.50 | 35.82 | -0.322 |
| 12 | 1 | Trichoderma_hamatum | 7 | 59.20 | 54.48 | 4.718 |
| 13 | 2 | Trichoderma_hamatum | 7 | 51.00 | 50.47 | 0.535 |
| 14 | 3 | Trichoderma_hamatum | 7 | 44.50 | 50.91 | -6.407 |
| 15 | 4 | Trichoderma_hamatum | 7 | 51.50 | 52.97 | -1.465 |
| 16 | 5 | Trichoderma_hamatum | 7 | 55.10 | 52.48 | 2.618 |
| 17 | 1 | Trichoderma_harzanium | 336 | 22.50 | 27.38 | -4.882 |
| 18 | 2 | Trichoderma_harzanium | 336 | 30.00 | 23.37 | 6.635 |
| 19 | 3 | Trichoderma_harzanium | 336 | 20.50 | 23.81 | -3.307 |
| 20 | 4 | Trichoderma_harzanium | 336 | 26.50 | 25.86 | 0.635 |
| 21 | 5 | Trichoderma_harzanium | 336 | 26.30 | 25.38 | 0.918 |
| 22 | 1 | Trichoderma_harzanium | 7 | 37.00 | 36.72 | 0.278 |
| 23 | 2 | Trichoderma_harzanium | 7 | 30.00 | 32.70 | -2.705 |
| 24 | 3 | Trichoderma_harzanium | 7 | 37.00 | 33.15 | 3.853 |
| 25 | 4 | Trichoderma_harzanium | 7 | 35.00 | 35.20 | -0.205 |
| 26 | 5 | Trichoderma_harzanium | 7 | 33.50 | 34.72 | -1.222 |
| 27 | 1 | Trichoderma_harzanium | 580 | 34.50 | 30.92 | 3.578 |
| 28 | 2 | Trichoderma_harzanium | 580 | 24.50 | 26.91 | -2.405 |
| 29 | 3 | Trichoderma_harzanium | 580 | 21.50 | 27.35 | -5.847 |
| 30 | 4 | Trichoderma_harzanium | 580 | 33.50 | 29.41 | 4.095 |
| 31 | 5 | Trichoderma_harzanium | 580 | 29.50 | 28.92 | 0.578 |
| 32 | 1 | Trichoderma_harzanium | 581 | 29.00 | 32.98 | -3.982 |
| 33 | 2 | Trichoderma_harzanium | 581 | 34.50 | 28.96 | 5.535 |
| 34 | 3 | Trichoderma_harzanium | 581 | 27.50 | 29.41 | -1.907 |
| 35 | 4 | Trichoderma_harzanium | 581 | 31.00 | 31.46 | -0.465 |
| 36 | 5 | Trichoderma_harzanium | 581 | 31.80 | 30.98 | 0.818 |
| 37 | 1 | Trichoderma_harzanium | 333 | 30.50 | 37.38 | -6.882 |
| 38 | 2 | Trichoderma_harzanium | 333 | 37.50 | 33.37 | 4.135 |
| 39 | 3 | Trichoderma_harzanium | 333 | 40.30 | 33.81 | 6.493 |
| 40 | 4 | Trichoderma_harzanium | 333 | 33.50 | 35.87 | -2.365 |
| 41 | 5 | Trichoderma_harzanium | 333 | 34.00 | 35.38 | -1.382 |
| 42 | 1 | Trichoderma_harzanium | 347 | 30.50 | 36.02 | -5.522 |
| 43 | 2 | Trichoderma_harzanium | 347 | 30.50 | 32.01 | -1.505 |
| 44 | 3 | Trichoderma_harzanium | 347 | 35.50 | 32.45 | 3.053 |
| 45 | 4 | Trichoderma_harzanium | 347 | 42.00 | 34.51 | 7.495 |
| 46 | 5 | Trichoderma_harzanium | 347 | 30.50 | 34.02 | -3.522 |

## Analysis of variance

Variate: AI

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|
| REP stratum | 4 | 191.85 | 47.96 | 1.74 | |
| REP.*Units* stratum | | | | | |
| FUNGAL | 1 | 81.43 | 81.43 | 2.95 | 0.093 |
| P_cinnamoni | 5 | 221.08 | 44.22 | 1.60 | 0.179 |
| FUNGAL.P_cinnamoni | 5 | 59.53 | 11.91 | 0.43 | 0.824 |
| Residual | 44 | 1213.47 | 27.58 | | |

| Total | 59 | 1767.35 |
|---|---|---|

## Tables of means

Variate: AI

Grand mean 20.93

| FUNGAL | Trichoderma_hamatum | Trichoderma_harzanium |
|---|---|---|
| | 22.09 | 19.76 |

| P_cinnamoni | 7 | 333 | 336 | 347 | 580 | 581 |
|---|---|---|---|---|---|---|
| | 22.14 | 19.54 | 23.73 | 20.57 | 17.77 | 21.80 |

| FUNGAL | P_cinnamoni | 7 | 333 | 336 | 347 | 580 |
|---|---|---|---|---|---|---|
| Trichoderma_hamatum | | 21.60 | 20.46 | 24.60 | 21.72 | 20.48 |
| Trichoderma_harzanium | | 22.68 | 18.62 | 22.86 | 19.42 | 15.06 |

| FUNGAL | P_cinnamoni | 581 |
|---|---|---|
| Trichoderma_hamatum | | 23.68 |
| Trichoderma_harzanium | | 19.92 |

## Standard errors of means

| Table | FUNGAL | P_cinnamoni | FUNGAL P_cinnamoni |
|---|---|---|---|
| rep. | 30 | 10 | 5 |
| d.f. | 44 | 44 | 44 |
| e.s.e. | 0.959 | 1.661 | 2.349 |

## Stratum standard errors and coefficients of variation

Variate: AI

| Stratum | d.f. | s.e. | cv% |
|---|---|---|---|
| REP | 4 | 1.999 | 9.6 |
| REP.*Units* | 44 | 5.252 | 25.1 |

## Fisher's protected least significant difference test

## FUNGAL

*Warning 235, code UF 2, statement 180 in procedure AMCOMPARISON*

The number of MEANS must be greater than 2.

## P_cinnamoni

*Warning 236, code UF 2, statement 194 in procedure AMCOMPARISON*

1
2 Fisher's protected LSD is not calculated as variance ratio for P_cinnamoni is not significant.
3
4
5 FUNGAL.P_cinnamoni
6
7 *Warning 237, code UF 2, statement 194 in procedure AMCOMPARISON*
8
9 Fisher's protected LSD is not calculated as variance ratio for FUNGAL.P_cinnamoni is not significant.
10
11
12
13 =========== Summary of original data =============
14
15

| P_cinnamoni | 7 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 21.60 | 6.49 | 2.548 |
| Trichoderma_harzanium | 22.68 | 25.82 | 5.081 |
| Margin | 22.14 | 14.68 | 3.832 |

| P_cinnamoni | 333 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 20.46 | 60.50 | 7.778 |
| Trichoderma_harzanium | 18.62 | 40.03 | 6.327 |
| Margin | 19.54 | 45.62 | 6.754 |

| P_cinnamoni | 336 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 24.60 | 18.86 | 4.343 |
| Trichoderma_harzanium | 22.86 | 43.51 | 6.596 |
| Margin | 23.73 | 28.56 | 5.344 |

| P_cinnamoni | 347 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 21.72 | 27.08 | 5.204 |
| Trichoderma_harzanium | 19.42 | 19.15 | 4.376 |
| Margin | 20.57 | 22.02 | 4.692 |

| P_cinnamoni | 580 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 20.48 | 9.57 | 3.093 |
| Trichoderma_harzanium | 15.06 | 55.44 | 7.446 |
| Margin | 17.77 | 37.05 | 6.087 |

| P_cinnamoni | 581 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| FUNGAL | | | |
| Trichoderma_hamatum | 23.68 | 22.10 | 4.701 |
| Trichoderma_harzanium | 19.92 | 22.78 | 4.773 |

| 1 | Margin | 21.80 | 23.87 | 4.886 |
|---|---|---|---|---|
| 2 | | | | |
| 3 | | | | |
| 4 | P_cinnamoni | Margin | | |
| 5 | | Mean | Variance | s.d. |
| 6 | FUNGAL | | | |
| 7 | Trichoderma_hamatum | 22.09 | 22.44 | 4.737 |
| 8 | Trichoderma_harzanium | 19.76 | 35.70 | 5.975 |
| 9 | Margin | 20.93 | 29.96 | 5.473 |
| 10 | | | | |
| 11 | | | | |
| 12 | | | | |

| 13 | REP | FUNGAL | P_cinnamoni | AI | FITTED | RESIDUAL |
|---|---|---|---|---|---|---|
| 14 | 1 | Trichoderma_hamatum | 347 | 22.10 | 23.41 | -1.312 |
| 15 | 2 | Trichoderma_hamatum | 347 | 23.70 | 19.07 | 4.630 |
| 16 | 3 | Trichoderma_hamatum | 347 | 26.90 | 21.54 | 5.363 |
| 17 | 4 | Trichoderma_hamatum | 347 | 13.00 | 23.94 | -10.937 |
| 18 | 5 | Trichoderma_hamatum | 347 | 22.90 | 20.64 | 2.255 |
| 19 | 1 | Trichoderma_hamatum | 581 | 31.30 | 25.37 | 5.928 |
| 20 | 2 | Trichoderma_hamatum | 581 | 25.20 | 21.03 | 4.170 |
| 21 | 3 | Trichoderma_hamatum | 581 | 20.30 | 23.50 | -3.197 |
| 22 | 4 | Trichoderma_hamatum | 581 | 20.80 | 25.90 | -5.097 |
| 23 | 5 | Trichoderma_hamatum | 581 | 20.80 | 22.61 | -1.805 |
| 24 | 1 | Trichoderma_hamatum | 333 | 24.30 | 22.15 | 2.148 |
| 25 | 2 | Trichoderma_hamatum | 333 | 10.20 | 17.81 | -7.610 |
| 26 | 3 | Trichoderma_hamatum | 333 | 31.00 | 20.28 | 10.723 |
| 27 | 4 | Trichoderma_hamatum | 333 | 19.50 | 22.68 | -3.177 |
| 28 | 5 | Trichoderma_hamatum | 333 | 17.30 | 19.39 | -2.085 |
| 29 | 1 | Trichoderma_hamatum | 580 | 16.50 | 22.17 | -5.672 |
| 30 | 2 | Trichoderma_hamatum | 580 | 21.00 | 17.83 | 3.170 |
| 31 | 3 | Trichoderma_hamatum | 580 | 21.30 | 20.30 | 1.003 |
| 32 | 4 | Trichoderma_hamatum | 580 | 24.80 | 22.70 | 2.103 |
| 33 | 5 | Trichoderma_hamatum | 580 | 18.80 | 19.41 | -0.605 |
| 34 | 1 | Trichoderma_hamatum | 336 | 25.50 | 26.29 | -0.792 |
| 35 | 2 | Trichoderma_hamatum | 336 | 18.70 | 21.95 | -3.250 |
| 36 | 3 | Trichoderma_hamatum | 336 | 26.70 | 24.42 | 2.283 |
| 37 | 4 | Trichoderma_hamatum | 336 | 30.00 | 26.82 | 3.183 |
| 38 | 5 | Trichoderma_hamatum | 336 | 22.10 | 23.52 | -1.425 |
| 39 | 1 | Trichoderma_hamatum | 7 | 21.30 | 23.29 | -1.992 |
| 40 | 2 | Trichoderma_hamatum | 7 | 18.70 | 18.95 | -0.250 |
| 41 | 3 | Trichoderma_hamatum | 7 | 25.30 | 21.42 | 3.883 |
| 42 | 4 | Trichoderma_hamatum | 7 | 22.70 | 23.82 | -1.117 |
| 43 | 5 | Trichoderma_hamatum | 7 | 20.00 | 20.52 | -0.525 |
| 44 | 1 | Trichoderma_harzanium | 336 | 25.80 | 24.55 | 1.248 |
| 45 | 2 | Trichoderma_harzanium | 336 | 18.70 | 20.21 | -1.510 |
| 46 | 3 | Trichoderma_harzanium | 336 | 15.20 | 22.68 | -7.477 |
| 47 | 4 | Trichoderma_harzanium | 336 | 32.30 | 25.08 | 7.223 |
| 48 | 5 | Trichoderma_harzanium | 336 | 22.30 | 21.79 | 0.515 |
| 49 | 1 | Trichoderma_harzanium | 7 | 25.70 | 24.37 | 1.328 |
| 50 | 2 | Trichoderma_harzanium | 7 | 16.20 | 20.03 | -3.830 |
| 51 | 3 | Trichoderma_harzanium | 7 | 21.00 | 22.50 | -1.497 |
| 52 | 4 | Trichoderma_harzanium | 7 | 29.50 | 24.90 | 4.603 |
| 53 | 5 | Trichoderma_harzanium | 7 | 21.00 | 21.61 | -0.605 |
| 54 | 1 | Trichoderma_harzanium | 580 | 11.20 | 16.75 | -5.552 |
| 55 | 2 | Trichoderma_harzanium | 580 | 21.00 | 12.41 | 8.590 |
| 56 | 3 | Trichoderma_harzanium | 580 | 4.40 | 14.88 | -10.477 |
| 57 | 4 | Trichoderma_harzanium | 580 | 22.60 | 17.28 | 5.323 |
| 58 | 5 | Trichoderma_harzanium | 580 | 16.10 | 13.98 | 2.115 |
| 59 | 1 | Trichoderma_harzanium | 581 | 20.30 | 21.61 | -1.312 |
| 60 | 2 | Trichoderma_harzanium | 581 | 22.40 | 17.27 | 5.130 |

| | | | | | |
|---|---|---|---|---|---|
| 3 | Trichoderma_harzanium | 581 | 11.70 | 19.74 | -8.037 |
| 4 | Trichoderma_harzanium | 581 | 23.80 | 22.14 | 1.663 |
| 5 | Trichoderma_harzanium | 581 | 21.40 | 18.84 | 2.555 |
| 1 | Trichoderma_harzanium | 333 | 22.10 | 20.31 | 1.788 |
| 2 | Trichoderma_harzanium | 333 | 10.20 | 15.97 | -5.770 |
| 3 | Trichoderma_harzanium | 333 | 27.00 | 18.44 | 8.563 |
| 4 | Trichoderma_harzanium | 333 | 17.60 | 20.84 | -3.237 |
| 5 | Trichoderma_harzanium | 333 | 16.20 | 17.55 | -1.345 |
| 1 | Trichoderma_harzanium | 347 | 25.30 | 21.11 | 4.188 |
| 2 | Trichoderma_harzanium | 347 | 13.30 | 16.77 | -3.470 |
| 3 | Trichoderma_harzanium | 347 | 18.10 | 19.24 | -1.137 |
| 4 | Trichoderma_harzanium | 347 | 21.10 | 21.64 | -0.537 |
| 5 | Trichoderma_harzanium | 347 | 19.30 | 18.35 | 0.955 |

End of Nontokozo Kunene - TSC - Project no: P03000100. Current data space: 2 blocks, peak usage 38% at line 93.

Genstat 64-bit Release 18.2 ( PC/Windows 8) 25 October 2017 12:06:16
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**Appendix 5:** *Bacillus* spp. biocontrol data

File name is Bell test results Bacteria.GEN
In vivo evaluation of different biocontrol agents against different isolates of P. cinnamomi
Bacterial isolates
October 2017

*Message: You have input sufficient data, READ terminated.*

| Identifier | Minimum | Mean | Maximum | Values | Missing |
|---|---|---|---|---|---|
| InhZone | 0.9000 | 13.09 | 41.00 | 240 | 3 |
| %Inh | 0.7000 | 27.04 | 84.50 | 240 | 3 |

| Identifier | Values | Missing | Levels |
|---|---|---|---|
| Rep | 240 | 0 | 5 |
| Bacteria | 240 | 0 | 8 |
| P_cinnamoni | 240 | 0 | 6 |

## Analysis of variance

Variate: InhZone

| Source of variation | d.f. | (m.v.) | s.s. | m.s. | v.r. | F pr. |
|---|---|---|---|---|---|---|
| Rep stratum | 4 | | 842.65 | 210.66 | 9.15 | |
| Rep.*Units* stratum | | | | | | |
| Bacteria | 7 | | 3711.87 | 530.27 | 23.03 | <.001 |
| P_cinnamoni | 5 | | 6934.51 | 1386.90 | 60.25 | <.001 |
| Bacteria.P_cinnamoni | 35 | | 2403.08 | 68.66 | 2.98 | <.001 |
| Residual | 185 | (3) | 4258.76 | 23.02 | | |
| Total | 236 | (3) | 18131.26 | | | |

## Tables of means

Variate: InhZone

Grand mean 13.07

| Bacteria | B31a | B31b | B41b | B616 | BV1C | NB4 | NB51b |
|---|---|---|---|---|---|---|---|
| | 9.75 | 17.68 | 18.41 | 10.45 | 11.21 | 18.17 | 9.14 |

| Bacteria | NB616 |
|---|---|
| | 9.73 |

| P_cinnamoni | 7 | 333 | 336 | 347 | 580 | 581 |
|---|---|---|---|---|---|---|
| | 4.88 | 10.71 | 22.34 | 10.53 | 14.24 | 15.70 |

| Bacteria | P_cinnamoni | 7 | 333 | 336 | 347 | 580 | 581 |
|---|---|---|---|---|---|---|---|
| B31a | | 2.66 | 7.28 | 17.60 | 6.28 | 10.64 | 14.04 |
| B31b | | 1.06 | 17.60 | 37.24 | 10.63 | 16.18 | 23.34 |
| B41b | | 10.66 | 15.10 | 22.66 | 22.72 | 20.02 | 19.32 |
| B616 | | 1.84 | 9.70 | 19.20 | 10.16 | 12.46 | 9.32 |
| BV1C | | 1.68 | 5.72 | 21.92 | 6.62 | 15.72 | 15.58 |
| NB4 | | 13.70 | 16.72 | 21.14 | 18.34 | 19.00 | 20.10 |
| NB51b | | 1.10 | 6.40 | 21.24 | 2.14 | 10.58 | 13.38 |

| NB616 | 6.32 | 7.16 | 17.72 | 7.34 | 9.28 | 10.54 |
|---|---|---|---|---|---|---|

## Standard errors of means

| Table | Bacteria | P_cinnamoni | Bacteria P_cinnamoni |
|---|---|---|---|
| rep. | 30 | 40 | 5 |
| d.f. | 185 | 185 | 185 |
| e.s.e. | 0.876 | 0.759 | 2.146 |

(Not adjusted for missing values)

## Least significant differences of means (5% level)

| Table | Bacteria | P_cinnamoni | Bacteria P_cinnamoni |
|---|---|---|---|
| rep. | 30 | 40 | 5 |
| d.f. | 185 | 185 | 185 |
| l.s.d. | 2.444 | 2.117 | 5.987 |

(Not adjusted for missing values)

## Stratum standard errors and coefficients of variation

Variate: InhZone

| Stratum | d.f. | s.e. | cv% |
|---|---|---|---|
| Rep | 4 | 2.095 | 16.0 |
| Rep.*Units* | 185 | 4.798 | 36.7 |

## Fisher's protected least significant difference test

## Bacteria

| | Mean | |
|---|---|---|
| B41b | 18.41 | a |
| NB4 | 18.17 | a |
| B31b | 17.68 | a |
| BV1C | 11.21 | b |
| B616 | 10.45 | b |
| B31a | 9.75 | b |
| NB616 | 9.73 | b |
| NB51b | 9.14 | b |

## P_cinnamoni

| | Mean | |
|---|---|---|
| 336 | 22.34 | a |
| 581 | 15.70 | b |

1 580 14.24 b
2 333 10.71 c
3 347 10.53 c
4 7 4.88 d
5
6

7 Bacteria.P_cinnamoni

8
9

| Line | | Mean | |
|---|---|---|---|
| 10 | | Mean | |
| 11 | B31b 336 | 37.24 | a |
| 12 | B31b 581 | 23.34 | b |
| 13 | B41b 347 | 22.72 | b |
| 14 | B41b 336 | 22.66 | bc |
| 15 | BV1C 336 | 21.92 | bcd |
| 16 | NB51b 336 | 21.24 | bcde |
| 17 | NB4 336 | 21.14 | bcde |
| 18 | NB4 581 | 20.10 | bcdef |
| 19 | B41b 580 | 20.02 | bcdefg |
| 20 | B41b 581 | 19.32 | bcdefgh |
| 21 | B616 336 | 19.20 | bcdefgh |
| 22 | NB4 580 | 19.00 | bcdefgh |
| 23 | NB4 347 | 18.34 | bcdefghi |
| 24 | NB616 336 | 17.72 | bcdefghi |
| 25 | B31b 333 | 17.60 | bcdefghi |
| 26 | B31a 336 | 17.60 | bcdefghi |
| 27 | NB4 333 | 16.72 | cdefghi |
| 28 | B31b 580 | 16.18 | defghij |
| 29 | BV1C 580 | 15.72 | efghijk |
| 30 | BV1C 581 | 15.58 | efghijkl |
| 31 | B41b 333 | 15.10 | fghijklm |
| 32 | B31a 581 | 14.04 | ghijklm |
| 33 | NB4 7 | 13.70 | hijklm |
| 34 | NB51b 581 | 13.38 | hijklm |
| 35 | B616 580 | 12.46 | ijklmn |
| 36 | B41b 7 | 10.66 | jklmno |
| 37 | B31a 580 | 10.64 | jklmno |
| 38 | B31b 347 | 10.63 | jklmno |
| 39 | NB51b 580 | 10.58 | jklmno |
| 40 | NB616 581 | 10.54 | jklmno |
| 41 | B616 347 | 10.16 | klmno |
| 42 | B616 333 | 9.70 | lmno |
| 43 | B616 581 | 9.32 | mno |
| 44 | NB616 580 | 9.28 | mno |
| 45 | NB616 347 | 7.34 | nop |
| 46 | B31a 333 | 7.28 | nop |
| 47 | NB616 333 | 7.16 | nop |
| 48 | BV1C 347 | 6.62 | nopq |
| 49 | NB51b 333 | 6.40 | opq |
| 50 | NB616 7 | 6.32 | opq |
| 51 | B31a 347 | 6.28 | opq |
| 52 | BV1C 333 | 5.72 | opq |
| 53 | B31a 7 | 2.66 | pq |
| 54 | NB51b 347 | 2.14 | pq |
| 55 | B616 7 | 1.84 | pq |
| 56 | BV1C 7 | 1.68 | pq |
| 57 | NB51b 7 | 1.10 | q |
| 58 | B31b 7 | 1.06 | q |

59
60

=========== Summary of original data =============

| P_cinnamoni | 7 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| Bacteria | | | |
| B31a | 2.66 | 1.57 | 1.254 |
| B31b | 1.06 | 0.02 | 0.134 |
| B41b | 10.66 | 1.98 | 1.408 |
| B616 | 1.84 | 1.20 | 1.095 |
| BV1C | 1.68 | 0.91 | 0.952 |
| NB4 | 13.70 | 17.08 | 4.133 |
| NB51b | 1.10 | 0.02 | 0.141 |
| NB616 | 6.32 | 80.62 | 8.979 |
| Margin | 4.88 | 31.96 | 5.653 |

| P_cinnamoni | 333 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| Bacteria | | | |
| B31a | 7.28 | 20.17 | 4.491 |
| B31b | 17.60 | 38.26 | 6.186 |
| B41b | 15.10 | 16.01 | 4.001 |
| B616 | 9.70 | 57.17 | 7.561 |
| BV1C | 5.72 | 2.77 | 1.665 |
| NB4 | 16.72 | 29.15 | 5.399 |
| NB51b | 6.40 | 5.73 | 2.395 |
| NB616 | 7.16 | 48.34 | 6.953 |
| Margin | 10.71 | 44.34 | 6.658 |

| P_cinnamoni | 336 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| Bacteria | | | |
| B31a | 17.60 | 42.72 | 6.536 |
| B31b | 37.24 | 27.86 | 5.279 |
| B41b | 22.66 | 23.06 | 4.802 |
| B616 | 19.20 | 38.39 | 6.196 |
| BV1C | 21.92 | 16.96 | 4.118 |
| NB4 | 21.14 | 21.61 | 4.649 |
| NB51b | 21.24 | 28.98 | 5.383 |
| NB616 | 17.72 | 4.74 | 2.176 |
| Margin | 22.34 | 56.68 | 7.528 |

| P_cinnamoni | 347 | | |
|---|---|---|---|
| | Mean | Variance | s.d. |
| Bacteria | | | |
| B31a | 5.67 | 8.56 | 2.926 |
| B31b | 10.18 | 66.75 | 8.170 |
| B41b | 22.72 | 8.74 | 2.956 |
| B616 | 9.55 | 51.80 | 7.197 |
| BV1C | 6.62 | 1.71 | 1.308 |
| NB4 | 18.34 | 24.81 | 4.981 |
| NB51b | 2.14 | 0.10 | 0.313 |
| NB616 | 7.34 | 28.32 | 5.322 |
| Margin | 10.47 | 62.83 | 7.926 |

| | | | | |
|---|---|---|---|---|
| 1 | P_cinnamoni | 580 | | |
| 2 | | Mean | Variance | s.d. |
| 3 | Bacteria | | | |
| 4 | B31a | 10.64 | 56.21 | 7.498 |
| 5 | B31b | 16.18 | 55.06 | 7.420 |
| 6 | B41b | 20.02 | 46.29 | 6.803 |
| 7 | B616 | 12.46 | 67.07 | 8.190 |
| 8 | BV1C | 15.72 | 18.72 | 4.326 |
| 9 | NB4 | 19.00 | 14.96 | 3.868 |
| 10 | NB51b | 10.58 | 18.26 | 4.273 |
| 11 | NB616 | 9.28 | 22.59 | 4.753 |
| 12 | Margin | 14.23 | 45.57 | 6.751 |
| 13 | | | | |
| 14 | | | | |
| 15 | P_cinnamoni | 581 | | |
| 16 | | Mean | Variance | s.d. |
| 17 | Bacteria | | | |
| 18 | B31a | 14.04 | 22.43 | 4.736 |
| 19 | B31b | 23.34 | 63.97 | 7.998 |
| 20 | B41b | 19.32 | 26.75 | 5.172 |
| 21 | B616 | 9.32 | 33.48 | 5.786 |
| 22 | BV1C | 15.58 | 57.01 | 7.550 |
| 23 | NB4 | 20.10 | 7.44 | 2.728 |
| 24 | NB51b | 13.38 | 50.34 | 7.095 |
| 25 | NB616 | 10.54 | 25.69 | 5.068 |
| 26 | Margin | 15.70 | 50.77 | 7.125 |
| 27 | | | | |
| 28 | | | | |
| 29 | P_cinnamoni | Margin | | |
| 30 | | Mean | Variance | s.d. |
| 31 | Bacteria | | | |
| 32 | B31a | 9.79 | 48.23 | 6.945 |
| 33 | B31b | 17.86 | 165.39 | 12.860 |
| 34 | B41b | 18.41 | 36.09 | 6.008 |
| 35 | B616 | 10.37 | 61.81 | 7.862 |
| 36 | BV1C | 11.21 | 64.59 | 8.037 |
| 37 | NB4 | 18.17 | 21.97 | 4.687 |
| 38 | NB51b | 9.14 | 63.85 | 7.991 |
| 39 | NB616 | 9.73 | 44.29 | 6.655 |
| 40 | Margin | 13.09 | 76.83 | 8.765 |
| 41 | | | | |
| 42 | | | | |
| 43 | | | | |

| 44 | Rep | Bacteria | P_cinnamoni | InhZone | FITTED | RESIDUAL |
|---|---|---|---|---|---|---|
| 45 | 1 | NB4 | 7 | 12.70 | 11.76 | 0.945 |
| 46 | 2 | NB4 | 7 | 20.30 | 11.58 | 8.720 |
| 47 | 3 | NB4 | 7 | 14.70 | 15.52 | -0.821 |
| 48 | 4 | NB4 | 7 | 9.70 | 16.13 | -6.435 |
| 49 | 5 | NB4 | 7 | 11.10 | 13.51 | -2.409 |
| 50 | 1 | NB4 | 336 | 23.80 | 19.20 | 4.605 |
| 51 | 2 | NB4 | 336 | 13.50 | 19.02 | -5.520 |
| 52 | 3 | NB4 | 336 | 22.40 | 22.96 | -0.561 |
| 53 | 4 | NB4 | 336 | 25.50 | 23.57 | 1.925 |
| 54 | 5 | NB4 | 336 | 20.50 | 20.95 | -0.449 |
| 55 | 1 | NB4 | 580 | 16.70 | 17.06 | -0.355 |
| 56 | 2 | NB4 | 580 | 16.80 | 16.88 | -0.080 |
| 57 | 3 | NB4 | 580 | 22.00 | 20.82 | 1.179 |
| 58 | 4 | NB4 | 580 | 24.20 | 21.43 | 2.765 |
| 59 | 5 | NB4 | 580 | 15.30 | 18.81 | -3.509 |
| 60 | 1 | NB4 | 581 | 22.50 | 18.16 | 4.345 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 2 | NB4 | 581 | 15.70 | 17.98 | -2.280 |
| 2 | 3 | NB4 | 581 | 21.70 | 21.92 | -0.221 |
| 3 | 4 | NB4 | 581 | 19.30 | 22.53 | -3.235 |
| 4 | 5 | NB4 | 581 | 21.30 | 19.91 | 1.391 |
| 5 | 1 | NB4 | 347 | 12.00 | 16.40 | -4.395 |
| 6 | 2 | NB4 | 347 | 23.80 | 16.22 | 7.580 |
| 7 | 3 | NB4 | 347 | 14.70 | 20.16 | -5.461 |
| 8 | 4 | NB4 | 347 | 22.30 | 20.77 | 1.525 |
| 9 | 5 | NB4 | 347 | 18.90 | 18.15 | 0.751 |
| 10 | 1 | NB4 | 333 | 12.50 | 14.78 | -2.275 |
| 11 | 2 | NB4 | 333 | 12.90 | 14.60 | -1.700 |
| 12 | 3 | NB4 | 333 | 18.20 | 18.54 | -0.341 |
| 13 | 4 | NB4 | 333 | 25.50 | 19.15 | 6.345 |
| 14 | 5 | NB4 | 333 | 14.50 | 16.53 | -2.029 |
| 15 | 1 | BV1C | 581 | 20.00 | 13.64 | 6.365 |
| 16 | 2 | BV1C | 581 | 6.30 | 13.46 | -7.160 |
| 17 | 3 | BV1C | 581 | 25.70 | 17.40 | 8.299 |
| 18 | 4 | BV1C | 581 | 11.20 | 18.01 | -6.815 |
| 19 | 5 | BV1C | 581 | 14.70 | 15.39 | -0.689 |
| 20 | 1 | BV1C | 347 | 7.20 | 4.68 | 2.525 |
| 21 | 2 | BV1C | 347 | 5.30 | 4.50 | 0.800 |
| 22 | 3 | BV1C | 347 | 6.20 | 8.44 | -2.241 |
| 23 | 4 | BV1C | 347 | 5.80 | 9.05 | -3.255 |
| 24 | 5 | BV1C | 347 | 8.60 | 6.43 | 2.171 |
| 25 | 1 | BV1C | 333 | 4.80 | 3.78 | 1.025 |
| 26 | 2 | BV1C | 333 | 3.30 | 3.60 | -0.300 |
| 27 | 3 | BV1C | 333 | 6.50 | 7.54 | -1.041 |
| 28 | 4 | BV1C | 333 | 6.50 | 8.15 | -1.655 |
| 29 | 5 | BV1C | 333 | 7.50 | 5.53 | 1.971 |
| 30 | 1 | BV1C | 580 | 14.80 | 13.78 | 1.025 |
| 31 | 2 | BV1C | 580 | 12.30 | 13.60 | -1.300 |
| 32 | 3 | BV1C | 580 | 13.30 | 17.54 | -4.241 |
| 33 | 4 | BV1C | 580 | 23.20 | 18.15 | 5.045 |
| 34 | 5 | BV1C | 580 | 15.00 | 15.53 | -0.529 |
| 35 | 1 | BV1C | 336 | 24.80 | 19.98 | 4.825 |
| 36 | 2 | BV1C | 336 | 18.70 | 19.80 | -1.100 |
| 37 | 3 | BV1C | 336 | 27.70 | 23.74 | 3.959 |
| 38 | 4 | BV1C | 336 | 19.90 | 24.35 | -4.455 |
| 39 | 5 | BV1C | 336 | 18.50 | 21.73 | -3.229 |
| 40 | 1 | BV1C | 7 | 1.00 | -0.26 | 1.265 |
| 41 | 2 | BV1C | 7 | 3.30 | -0.44 | 3.740 |
| 42 | 3 | BV1C | 7 | 1.70 | 3.50 | -1.801 |
| 43 | 4 | BV1C | 7 | 1.00 | 4.11 | -3.115 |
| 44 | 5 | BV1C | 7 | 1.40 | 1.49 | -0.089 |
| 45 | 1 | B31b | 7 | 1.00 | -0.88 | 1.885 |
| 46 | 2 | B31b | 7 | 1.00 | -1.06 | 2.060 |
| 47 | 3 | B31b | 7 | 1.00 | 2.88 | -1.881 |
| 48 | 4 | B31b | 7 | 1.30 | 3.49 | -2.195 |
| 49 | 5 | B31b | 7 | 1.00 | 0.87 | 0.131 |
| 50 | 1 | B31b | 581 | 32.30 | 21.40 | 10.905 |
| 51 | 2 | B31b | 581 | 10.30 | 21.22 | -10.920 |
| 52 | 3 | B31b | 581 | 24.80 | 25.16 | -0.361 |
| 53 | 4 | B31b | 581 | 24.70 | 25.77 | -1.075 |
| 54 | 5 | B31b | 581 | 24.60 | 23.15 | 1.451 |
| 55 | 1 | B31b | 336 | 38.50 | 35.30 | 3.205 |
| 56 | 2 | B31b | 336 | 28.00 | 35.12 | -7.120 |
| 57 | 3 | B31b | 336 | 41.00 | 39.06 | 1.939 |
| 58 | 4 | B31b | 336 | 38.50 | 39.67 | -1.175 |
| 59 | 5 | B31b | 336 | 40.20 | 37.05 | 3.151 |
| 60 | 1 | B31b | 580 | 13.50 | 14.24 | -0.735 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 2 | B31b | 580 | 4.40 | 14.06 | -9.660 |
| 2 | 3 | B31b | 580 | 21.50 | 18.00 | 3.499 |
| 3 | 4 | B31b | 580 | 22.20 | 18.61 | 3.585 |
| 4 | 5 | B31b | 580 | 19.30 | 15.99 | 3.311 |
| 5 | 1 | B31b | 333 | 11.00 | 15.66 | -4.655 |
| 6 | 2 | B31b | 333 | 16.00 | 15.48 | 0.520 |
| 7 | 3 | B31b | 333 | 27.70 | 19.42 | 8.279 |
| 8 | 4 | B31b | 333 | 17.90 | 20.03 | -2.135 |
| 9 | 5 | B31b | 333 | 15.40 | 17.41 | -2.009 |
| 10 | 1 | B31b | 347 | 21.30 | 8.69 | 12.615 |
| 11 | 2 | B31b | 347 | 3.20 | 8.51 | -5.310 |
| 12 | 3 | B31b | 347 | * | 12.45 | * |
| 13 | 4 | B31b | 347 | 5.00 | 13.06 | -8.065 |
| 14 | 5 | B31b | 347 | 11.20 | 10.44 | 0.761 |
| 15 | 1 | NB51b | 333 | 6.30 | 4.46 | 1.845 |
| 16 | 2 | NB51b | 333 | 3.00 | 4.28 | -1.280 |
| 17 | 3 | NB51b | 333 | 8.30 | 8.22 | 0.079 |
| 18 | 4 | NB51b | 333 | 9.00 | 8.83 | 0.165 |
| 19 | 5 | NB51b | 333 | 5.40 | 6.21 | -0.809 |
| 20 | 1 | NB51b | 336 | 26.70 | 19.30 | 7.405 |
| 21 | 2 | NB51b | 336 | 15.80 | 19.12 | -3.320 |
| 22 | 3 | NB51b | 336 | 15.70 | 23.06 | -7.361 |
| 23 | 4 | NB51b | 336 | 26.30 | 23.67 | 2.625 |
| 24 | 5 | NB51b | 336 | 21.70 | 21.05 | 0.651 |
| 25 | 1 | NB51b | 347 | 2.70 | 0.20 | 2.505 |
| 26 | 2 | NB51b | 347 | 2.00 | 0.02 | 1.980 |
| 27 | 3 | NB51b | 347 | 2.00 | 3.96 | -1.961 |
| 28 | 4 | NB51b | 347 | 2.00 | 4.57 | -2.575 |
| 29 | 5 | NB51b | 347 | 2.00 | 1.95 | 0.051 |
| 30 | 1 | NB51b | 580 | 6.30 | 8.64 | -2.335 |
| 31 | 2 | NB51b | 580 | 8.20 | 8.46 | -0.260 |
| 32 | 3 | NB51b | 580 | 9.70 | 12.40 | -2.701 |
| 33 | 4 | NB51b | 580 | 17.50 | 13.01 | 4.485 |
| 34 | 5 | NB51b | 580 | 11.20 | 10.39 | 0.811 |
| 35 | 1 | NB51b | 7 | 1.20 | -0.84 | 2.045 |
| 36 | 2 | NB51b | 7 | 1.00 | -1.02 | 2.020 |
| 37 | 3 | NB51b | 7 | 1.00 | 2.92 | -1.921 |
| 38 | 4 | NB51b | 7 | 1.00 | 3.53 | -2.535 |
| 39 | 5 | NB51b | 7 | 1.30 | 0.91 | 0.391 |
| 40 | 1 | NB51b | 581 | 21.30 | 11.44 | 9.865 |
| 41 | 2 | NB51b | 581 | 20.80 | 11.26 | 9.540 |
| 42 | 3 | NB51b | 581 | 9.90 | 15.20 | -5.301 |
| 43 | 4 | NB51b | 581 | 6.70 | 15.81 | -9.115 |
| 44 | 5 | NB51b | 581 | 8.20 | 13.19 | -4.989 |
| 45 | 1 | B41b | 347 | 24.20 | 20.78 | 3.425 |
| 46 | 2 | B41b | 347 | 20.70 | 20.60 | 0.100 |
| 47 | 3 | B41b | 347 | 21.40 | 24.54 | -3.141 |
| 48 | 4 | B41b | 347 | 27.20 | 25.15 | 2.045 |
| 49 | 5 | B41b | 347 | 20.10 | 22.53 | -2.429 |
| 50 | 1 | B41b | 581 | 13.70 | 17.38 | -3.675 |
| 51 | 2 | B41b | 581 | 19.50 | 17.20 | 2.300 |
| 52 | 3 | B41b | 581 | 17.20 | 21.14 | -3.941 |
| 53 | 4 | B41b | 581 | 27.70 | 21.75 | 5.945 |
| 54 | 5 | B41b | 581 | 18.50 | 19.13 | -0.629 |
| 55 | 1 | B41b | 333 | 8.80 | 13.16 | -4.355 |
| 56 | 2 | B41b | 333 | 19.40 | 12.98 | 6.420 |
| 57 | 3 | B41b | 333 | 15.00 | 16.92 | -1.921 |
| 58 | 4 | B41b | 333 | 17.50 | 17.53 | -0.035 |
| 59 | 5 | B41b | 333 | 14.80 | 14.91 | -0.109 |
| 60 | 1 | B41b | 580 | 11.00 | 18.08 | -7.075 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 2 | B41b | 580 | 19.00 | 17.90 | 1.100 |
| 2 | 3 | B41b | 580 | 29.90 | 21.84 | 8.059 |
| 3 | 4 | B41b | 580 | 21.70 | 22.45 | -0.755 |
| 4 | 5 | B41b | 580 | 18.50 | 19.83 | -1.329 |
| 5 | 1 | B41b | 336 | 21.30 | 20.72 | 0.585 |
| 6 | 2 | B41b | 336 | 18.30 | 20.54 | -2.240 |
| 7 | 3 | B41b | 336 | 30.80 | 24.48 | 6.319 |
| 8 | 4 | B41b | 336 | 20.40 | 25.09 | -4.695 |
| 9 | 5 | B41b | 336 | 22.50 | 22.47 | 0.031 |
| 10 | 1 | B41b | 7 | 10.70 | 8.72 | 1.985 |
| 11 | 2 | B41b | 7 | 9.20 | 8.54 | 0.660 |
| 12 | 3 | B41b | 7 | 12.70 | 12.48 | 0.219 |
| 13 | 4 | B41b | 7 | 9.50 | 13.09 | -3.595 |
| 14 | 5 | B41b | 7 | 11.20 | 10.47 | 0.731 |
| 15 | 1 | B616 | 336 | 10.80 | 17.26 | -6.455 |
| 16 | 2 | B616 | 336 | 18.70 | 17.08 | 1.620 |
| 17 | 3 | B616 | 336 | 27.30 | 21.02 | 6.279 |
| 18 | 4 | B616 | 336 | 22.50 | 21.63 | 0.865 |
| 19 | 5 | B616 | 336 | 16.70 | 19.01 | -2.309 |
| 20 | 1 | B616 | 7 | 0.90 | -0.10 | 1.005 |
| 21 | 2 | B616 | 7 | 1.70 | -0.28 | 1.980 |
| 22 | 3 | B616 | 7 | 3.70 | 3.66 | 0.039 |
| 23 | 4 | B616 | 7 | 1.70 | 4.27 | -2.575 |
| 24 | 5 | B616 | 7 | 1.20 | 1.65 | -0.449 |
| 25 | 1 | B616 | 580 | 4.00 | 10.52 | -6.515 |
| 26 | 2 | B616 | 580 | 3.00 | 10.34 | -7.340 |
| 27 | 3 | B616 | 580 | 18.50 | 14.28 | 4.219 |
| 28 | 4 | B616 | 580 | 18.70 | 14.89 | 3.805 |
| 29 | 5 | B616 | 580 | 18.10 | 12.27 | 5.831 |
| 30 | 1 | B616 | 581 | 6.20 | 7.38 | -1.175 |
| 31 | 2 | B616 | 581 | 5.50 | 7.20 | -1.700 |
| 32 | 3 | B616 | 581 | 19.50 | 11.14 | 8.359 |
| 33 | 4 | B616 | 581 | 8.30 | 11.75 | -3.455 |
| 34 | 5 | B616 | 581 | 7.10 | 9.13 | -2.029 |
| 35 | 1 | B616 | 347 | 2.70 | 8.21 | -5.514 |
| 36 | 2 | B616 | 347 | 19.70 | 8.04 | 11.661 |
| 37 | 3 | B616 | 347 | 8.00 | 11.98 | -3.979 |
| 38 | 4 | B616 | 347 | * | 12.59 | * |
| 39 | 5 | B616 | 347 | 7.80 | 9.97 | -2.168 |
| 40 | 1 | B616 | 333 | 1.70 | 7.76 | -6.055 |
| 41 | 2 | B616 | 333 | 22.20 | 7.58 | 14.620 |
| 42 | 3 | B616 | 333 | 7.20 | 11.52 | -4.321 |
| 43 | 4 | B616 | 333 | 9.00 | 12.13 | -3.135 |
| 44 | 5 | B616 | 333 | 8.40 | 9.51 | -1.109 |
| 45 | 1 | NB616 | 333 | 3.30 | 5.22 | -1.915 |
| 46 | 2 | NB616 | 333 | 3.30 | 5.04 | -1.740 |
| 47 | 3 | NB616 | 333 | 4.30 | 8.98 | -4.681 |
| 48 | 4 | NB616 | 333 | 19.50 | 9.59 | 9.905 |
| 49 | 5 | NB616 | 333 | 5.40 | 6.97 | -1.569 |
| 50 | 1 | NB616 | 347 | 4.30 | 5.40 | -1.095 |
| 51 | 2 | NB616 | 347 | 4.00 | 5.22 | -1.220 |
| 52 | 3 | NB616 | 347 | 5.20 | 9.16 | -3.961 |
| 53 | 4 | NB616 | 347 | 16.70 | 9.77 | 6.925 |
| 54 | 5 | NB616 | 347 | 6.50 | 7.15 | -0.649 |
| 55 | 1 | NB616 | 580 | 4.40 | 7.34 | -2.935 |
| 56 | 2 | NB616 | 580 | 4.00 | 7.16 | -3.160 |
| 57 | 3 | NB616 | 580 | 13.70 | 11.10 | 2.599 |
| 58 | 4 | NB616 | 580 | 11.00 | 11.71 | -0.715 |
| 59 | 5 | NB616 | 580 | 13.30 | 9.09 | 4.211 |
| 60 | 1 | NB616 | 581 | 4.80 | 8.60 | -3.795 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 2 | NB616 | 581 | 5.30 | 8.42 | -3.120 |
| 2 | 3 | NB616 | 581 | 13.00 | 12.36 | 0.639 |
| 3 | 4 | NB616 | 581 | 14.80 | 12.97 | 1.825 |
| 4 | 5 | NB616 | 581 | 14.80 | 10.35 | 4.451 |
| 5 | 1 | NB616 | 7 | 1.00 | 4.38 | -3.375 |
| 6 | 2 | NB616 | 7 | 3.30 | 4.20 | -0.900 |
| 7 | 3 | NB616 | 7 | 3.00 | 8.14 | -5.141 |
| 8 | 4 | NB616 | 7 | 22.30 | 8.75 | 13.545 |
| 9 | 5 | NB616 | 7 | 2.00 | 6.13 | -4.129 |
| 10 | 1 | NB616 | 336 | 17.80 | 15.78 | 2.025 |
| 11 | 2 | NB616 | 336 | 16.00 | 15.60 | 0.400 |
| 12 | 3 | NB616 | 336 | 20.00 | 19.54 | 0.459 |
| 13 | 4 | NB616 | 336 | 19.70 | 20.15 | -0.455 |
| 14 | 5 | NB616 | 336 | 15.10 | 17.53 | -2.429 |
| 15 | 1 | B31a | 581 | 6.30 | 12.10 | -5.795 |
| 16 | 2 | B31a | 581 | 19.30 | 11.92 | 7.380 |
| 17 | 3 | B31a | 581 | 14.70 | 15.86 | -1.161 |
| 18 | 4 | B31a | 581 | 15.10 | 16.47 | -1.375 |
| 19 | 5 | B31a | 581 | 14.80 | 13.85 | 0.951 |
| 20 | 1 | B31a | 347 | 2.30 | 4.34 | -2.039 |
| 21 | 2 | B31a | 347 | 4.30 | 4.16 | 0.136 |
| 22 | 3 | B31a | 347 | 7.30 | 8.10 | -0.805 |
| 23 | 4 | B31a | 347 | * | 8.72 | * |
| 24 | 5 | B31a | 347 | 8.80 | 6.09 | 2.707 |
| 25 | 1 | B31a | 333 | 2.30 | 5.34 | -3.035 |
| 26 | 2 | B31a | 333 | 8.70 | 5.16 | 3.540 |
| 27 | 3 | B31a | 333 | 5.20 | 9.10 | -3.901 |
| 28 | 4 | B31a | 333 | 14.20 | 9.71 | 4.485 |
| 29 | 5 | B31a | 333 | 6.00 | 7.09 | -1.089 |
| 30 | 1 | B31a | 580 | 1.00 | 8.70 | -7.695 |
| 31 | 2 | B31a | 580 | 4.70 | 8.52 | -3.820 |
| 32 | 3 | B31a | 580 | 18.90 | 12.46 | 6.439 |
| 33 | 4 | B31a | 580 | 15.20 | 13.07 | 2.125 |
| 34 | 5 | B31a | 580 | 13.40 | 10.45 | 2.951 |
| 35 | 1 | B31a | 336 | 14.40 | 15.66 | -1.255 |
| 36 | 2 | B31a | 336 | 8.00 | 15.48 | -7.480 |
| 37 | 3 | B31a | 336 | 24.90 | 19.42 | 5.479 |
| 38 | 4 | B31a | 336 | 20.50 | 20.03 | 0.465 |
| 39 | 5 | B31a | 336 | 20.20 | 17.41 | 2.791 |
| 40 | 1 | B31a | 7 | 1.00 | 0.72 | 0.285 |
| 41 | 2 | B31a | 7 | 1.70 | 0.54 | 1.160 |
| 42 | 3 | B31a | 7 | 3.30 | 4.48 | -1.181 |
| 43 | 4 | B31a | 7 | 3.30 | 5.09 | -1.795 |
| 44 | 5 | B31a | 7 | 4.00 | 2.47 | 1.531 |

45

46 
## Analysis of variance

47

48 Variate: %Inh

49

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 50 | Source of variation | d.f. | (m.v.) | s.s. | m.s. | v.r. | F pr. |
| 51 | | | | | | | |
| 52 | Rep stratum | 4 | | 5866.8 | 1466.7 | 10.35 | |
| 53 | | | | | | | |
| 54 | Rep.*Units* stratum | | | | | | |
| 55 | Bacteria | 7 | | 27156.5 | 3879.5 | 27.37 | <.001 |
| 56 | P_cinnamoni | 5 | | 32891.3 | 6578.3 | 46.41 | <.001 |
| 57 | Bacteria.P_cinnamoni | 35 | | 11035.4 | 315.3 | 2.22 | <.001 |
| 58 | Residual | 185 | (3) | 26224.7 | 141.8 | | |
| 59 | | | | | | | |
| 60 | Total | 236 | (3) | 103075.2 | | | |

# Tables of means

Variate: %Inh

Grand mean 27.00

| Bacteria | B31a | B31b | B41b | B616 | BV1C | NB4 | NB51b | NB616 |
|---|---|---|---|---|---|---|---|---|
| | 18.41 | 32.71 | 43.85 | 20.66 | 23.40 | 42.94 | 15.06 | 18.94 |

| P_cinnamoni | 7 | 333 | 336 | 347 | 580 | 581 |
|---|---|---|---|---|---|---|
| | 9.41 | 20.62 | 47.25 | 22.79 | 28.38 | 33.52 |

| Bacteria | P_cinnamoni 7 | 333 | 336 | 347 | 580 | 581 |
|---|---|---|---|---|---|---|
| B31a | 3.82 | 13.06 | 34.64 | 12.45 | 18.56 | 27.90 |
| B31b | 1.24 | 27.70 | 72.86 | 16.53 | 28.24 | 49.68 |
| B41b | 22.54 | 35.58 | 55.62 | 57.52 | 45.76 | 46.08 |
| B616 | 2.36 | 18.66 | 41.76 | 22.35 | 22.66 | 16.18 |
| BV1C | 2.32 | 9.62 | 47.72 | 10.92 | 33.10 | 36.74 |
| NB4 | 28.72 | 36.88 | 53.64 | 44.28 | 44.72 | 49.40 |
| NB51b | 1.14 | 10.02 | 36.88 | 2.82 | 17.62 | 21.86 |
| NB616 | 13.14 | 13.48 | 34.88 | 15.42 | 16.38 | 20.32 |

# Standard errors of means

| Table | Bacteria | P_cinnamoni | Bacteria P_cinnamoni |
|---|---|---|---|
| rep. | 30 | 40 | 5 |
| d.f. | 185 | 185 | 185 |
| e.s.e. | 2.174 | 1.883 | 5.325 |

(Not adjusted for missing values)

# Least significant differences of means (5% level)

| Table | Bacteria | P_cinnamoni | Bacteria P_cinnamoni |
|---|---|---|---|
| rep. | 30 | 40 | 5 |
| d.f. | 185 | 185 | 185 |
| l.s.d. | 6.065 | 5.252 | 14.856 |

(Not adjusted for missing values)

# Stratum standard errors and coefficients of variation

Variate: %Inh

| Stratum | d.f. | s.e. | cv% |
|---|---|---|---|
| Rep | 4 | 5.528 | 20.5 |
| Rep.*Units* | 185 | 11.906 | 44.1 |

1

2 # Fisher's protected least significant difference test

3

4

5 ## Bacteria

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| Line | | Mean | |
|---|---|---|---|
| 9 | B41b | 43.85 | a |
| 10 | NB4 | 42.94 | a |
| 11 | B31b | 32.71 | b |
| 12 | BV1C | 23.40 | c |
| 13 | B616 | 20.66 | cd |
| 14 | NB616 | 18.94 | cd |
| 15 | B31a | 18.41 | cd |
| 16 | NB51b | 15.06 | d |

17

18

19 ## P_cinnamoni

20

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22

| Line | | Mean | |
|---|---|---|---|
| 23 | 336 | 47.25 | a |
| 24 | 581 | 33.52 | b |
| 25 | 580 | 28.38 | b |
| 26 | 347 | 22.79 | c |
| 27 | 333 | 20.62 | c |
| 28 | 7 | 9.41 | d |

29

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31 ## Bacteria.P_cinnamoni

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34

| Line | | Mean | |
|---|---|---|---|
| 35 | B31b 336 | 72.86 | a |
| 36 | B41b 347 | 57.52 | b |
| 37 | B41b 336 | 55.62 | bc |
| 38 | NB4 336 | 53.64 | bc |
| 39 | B31b 581 | 49.68 | bcd |
| 40 | NB4 581 | 49.40 | bcde |
| 41 | BV1C 336 | 47.72 | bcdef |
| 42 | B41b 581 | 46.08 | bcdef |
| 43 | B41b 580 | 45.76 | bcdef |
| 44 | NB4 580 | 44.72 | bcdef |
| 45 | NB4 347 | 44.28 | bcdef |
| 46 | B616 336 | 41.76 | cdefg |
| 47 | NB4 333 | 36.88 | defgh |
| 48 | NB51b 336 | 36.88 | defgh |
| 49 | BV1C 581 | 36.74 | defgh |
| 50 | B41b 333 | 35.58 | defghi |
| 51 | NB616 336 | 34.88 | defghij |
| 52 | B31a 336 | 34.64 | efghij |
| 53 | BV1C 580 | 33.10 | fghijk |
| 54 | NB4 7 | 28.72 | ghijkl |
| 55 | B31b 580 | 28.24 | ghijklm |
| 56 | B31a 581 | 27.90 | ghijklmn |
| 57 | B31b 333 | 27.70 | ghijklmn |
| 58 | B616 580 | 22.66 | hijklmno |

| | | | |
|---|---|---|---|
| 1 | B41b 7 | 22.54 | hijklmno |
| 2 | B616 347 | 22.35 | hijklmno |
| 3 | NB51b 581 | 21.86 | ijklmno |
| 4 | NB616 581 | 20.32 | jklmno |
| 5 | B616 333 | 18.66 | klmnop |
| 6 | B31a 580 | 18.56 | klmnop |
| 7 | NB51b 580 | 17.62 | lmnopq |
| 8 | B31b 347 | 16.53 | lmnopqr |
| 9 | NB616 580 | 16.38 | lmnopqr |
| 10 | B616 581 | 16.18 | lmnopqr |
| 11 | NB616 347 | 15.42 | lmnopqrs |
| 12 | NB616 333 | 13.48 | mnopqrs |
| 13 | NB616 7 | 13.14 | nopqrs |
| 14 | B31a 333 | 13.06 | nopqrs |
| 15 | B31a 347 | 12.45 | opqrs |
| 16 | BV1C 347 | 10.92 | opqrs |
| 17 | NB51b 333 | 10.02 | opqrs |
| 18 | BV1C 333 | 9.62 | opqrs |
| 19 | B31a 7 | 3.82 | pqrs |
| 20 | NB51b 347 | 2.82 | qrs |
| 21 | B616 7 | 2.36 | rs |
| 22 | BV1C 7 | 2.32 | rs |
| 23 | B31b 7 | 1.24 | s |
| 24 | NB51b 7 | 1.14 | s |

25
26
27
28 =========== Summary of original data =============
29
30

| | | | | |
|---|---|---|---|---|
| 31 | P_cinnamoni | 7 | | |
| 32 | | Mean | Variance | s.d. |
| 33 | Bacteria | | | |
| 34 | B31a | 3.82 | 3.7 | 1.93 |
| 35 | B31b | 1.24 | 0.1 | 0.27 |
| 36 | B41b | 22.54 | 19.1 | 4.37 |
| 37 | B616 | 2.36 | 3.3 | 1.83 |
| 38 | BV1C | 2.32 | 2.8 | 1.68 |
| 39 | NB4 | 28.72 | 155.6 | 12.47 |
| 40 | NB51b | 1.14 | 0.0 | 0.09 |
| 41 | NB616 | 13.14 | 471.5 | 21.71 |
| 42 | Margin | 9.41 | 173.1 | 13.16 |

43
44

| | | | | |
|---|---|---|---|---|
| 45 | P_cinnamoni | 333 | | |
| 46 | | Mean | Variance | s.d. |
| 47 | Bacteria | | | |
| 48 | B31a | 13.06 | 88.4 | 9.40 |
| 49 | B31b | 27.70 | 128.2 | 11.32 |
| 50 | B41b | 35.58 | 200.9 | 14.17 |
| 51 | B616 | 18.66 | 324.0 | 18.00 |
| 52 | BV1C | 9.62 | 11.9 | 3.45 |
| 53 | NB4 | 36.88 | 255.2 | 15.98 |
| 54 | NB51b | 10.02 | 26.0 | 5.10 |
| 55 | NB616 | 13.48 | 223.6 | 14.95 |
| 56 | Margin | 20.62 | 242.3 | 15.57 |

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58

| | | | | |
|---|---|---|---|---|
| 59 | P_cinnamoni | 336 | | |
| 60 | | Mean | Variance | s.d. |

| | | | | |
|---|---|---|---|---|
| 1 | Bacteria | | | |
| 2 | B31a | 34.64 | 211.0 | 14.53 |
| 3 | B31b | 72.86 | 306.2 | 17.50 |
| 4 | B41b | 55.62 | 204.4 | 14.30 |
| 5 | B616 | 41.76 | 343.3 | 18.53 |
| 6 | BV1C | 47.72 | 158.2 | 12.58 |
| 7 | NB4 | 53.64 | 248.2 | 15.76 |
| 8 | NB51b | 36.88 | 92.2 | 9.60 |
| 9 | NB616 | 34.88 | 42.9 | 6.55 |
| 10 | Margin | 47.25 | 320.8 | 17.91 |
| 11 | | | | |
| 12 | | | | |
| 13 | P_cinnamoni | 347 | | |
| 14 | | Mean | Variance | s.d. |
| 15 | Bacteria | | | |
| 16 | B31a | 10.72 | 37.9 | 6.15 |
| 17 | B31b | 15.40 | 168.5 | 12.98 |
| 18 | B41b | 57.52 | 128.5 | 11.34 |
| 19 | B616 | 20.62 | 339.2 | 18.42 |
| 20 | BV1C | 10.92 | 7.6 | 2.75 |
| 21 | NB4 | 44.28 | 275.3 | 16.59 |
| 22 | NB51b | 2.82 | 0.1 | 0.38 |
| 23 | NB616 | 15.42 | 222.1 | 14.90 |
| 24 | Margin | 22.75 | 452.8 | 21.28 |
| 25 | | | | |
| 26 | | | | |
| 27 | P_cinnamoni | 580 | | |
| 28 | | Mean | Variance | s.d. |
| 29 | Bacteria | | | |
| 30 | B31a | 18.56 | 190.8 | 13.81 |
| 31 | B31b | 28.24 | 179.6 | 13.40 |
| 32 | B41b | 45.76 | 509.4 | 22.57 |
| 33 | B616 | 22.66 | 244.9 | 15.65 |
| 34 | BV1C | 33.10 | 157.1 | 12.53 |
| 35 | NB4 | 44.72 | 121.9 | 11.04 |
| 36 | NB51b | 17.62 | 64.6 | 8.04 |
| 37 | NB616 | 16.38 | 82.9 | 9.10 |
| 38 | Margin | 28.38 | 284.8 | 16.88 |
| 39 | | | | |
| 40 | | | | |
| 41 | P_cinnamoni | 581 | | |
| 42 | | Mean | Variance | s.d. |
| 43 | Bacteria | | | |
| 44 | B31a | 27.90 | 130.7 | 11.43 |
| 45 | B31b | 49.68 | 445.1 | 21.10 |
| 46 | B41b | 46.08 | 367.5 | 19.17 |
| 47 | B616 | 16.18 | 148.4 | 12.18 |
| 48 | BV1C | 36.74 | 462.6 | 21.51 |
| 49 | NB4 | 49.40 | 83.5 | 9.14 |
| 50 | NB51b | 21.86 | 101.4 | 10.07 |
| 51 | NB616 | 20.32 | 132.6 | 11.52 |
| 52 | Margin | 33.52 | 361.7 | 19.02 |
| 53 | | | | |
| 54 | | | | |
| 55 | P_cinnamoni | Margin | | |
| 56 | | Mean | Variance | s.d. |
| 57 | Bacteria | | | |
| 58 | B31a | 18.37 | 208.0 | 14.42 |
| 59 | B31b | 33.11 | 736.2 | 27.13 |
| 60 | B41b | 43.85 | 344.9 | 18.57 |

|  |  |  |  |  |
|---|---|---|---|---|
| 1 | B616 | 20.37 | 332.6 | 18.24 |
| 2 | BV1C | 23.40 | 395.5 | 19.89 |
| 3 | NB4 | 42.94 | 226.2 | 15.04 |
| 4 | NB51b | 15.06 | 194.0 | 13.93 |
| 5 | NB616 | 18.94 | 220.5 | 14.85 |
| 6 | Margin | 27.04 | 436.8 | 20.90 |

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| 10 | Rep | Bacteria | P_cinnamoni | %Inh | FITTED | RESIDUAL |
|---|---|---|---|---|---|---|
| 11 | 1 | NB4 | 7 | 22.10 | 23.68 | -1.583 |
| 12 | 2 | NB4 | 7 | 49.50 | 23.46 | 26.038 |
| 13 | 3 | NB4 | 7 | 31.30 | 33.22 | -1.921 |
| 14 | 4 | NB4 | 7 | 20.50 | 35.63 | -15.127 |
| 15 | 5 | NB4 | 7 | 20.20 | 27.61 | -7.406 |
| 16 | 1 | NB4 | 336 | 63.10 | 48.60 | 14.497 |
| 17 | 2 | NB4 | 336 | 27.30 | 48.38 | -21.082 |
| 18 | 3 | NB4 | 336 | 55.30 | 58.14 | -2.841 |
| 19 | 4 | NB4 | 336 | 68.00 | 60.55 | 7.453 |
| 20 | 5 | NB4 | 336 | 54.50 | 52.53 | 1.974 |
| 21 | 1 | NB4 | 580 | 37.10 | 39.68 | -2.583 |
| 22 | 2 | NB4 | 580 | 37.30 | 39.46 | -2.162 |
| 23 | 3 | NB4 | 580 | 53.00 | 49.22 | 3.779 |
| 24 | 4 | NB4 | 580 | 60.00 | 51.63 | 8.373 |
| 25 | 5 | NB4 | 580 | 36.20 | 43.61 | -7.406 |
| 26 | 1 | NB4 | 581 | 55.10 | 44.36 | 10.737 |
| 27 | 2 | NB4 | 581 | 34.10 | 44.14 | -10.042 |
| 28 | 3 | NB4 | 581 | 55.60 | 53.90 | 1.699 |
| 29 | 4 | NB4 | 581 | 47.70 | 56.31 | -8.607 |
| 30 | 5 | NB4 | 581 | 54.50 | 48.29 | 6.214 |
| 31 | 1 | NB4 | 347 | 21.00 | 39.24 | -18.243 |
| 32 | 2 | NB4 | 347 | 63.50 | 39.02 | 24.478 |
| 33 | 3 | NB4 | 347 | 35.90 | 48.78 | -12.881 |
| 34 | 4 | NB4 | 347 | 55.10 | 51.19 | 3.913 |
| 35 | 5 | NB4 | 347 | 45.90 | 43.17 | 2.734 |
| 36 | 1 | NB4 | 333 | 21.80 | 31.84 | -10.043 |
| 37 | 2 | NB4 | 333 | 26.90 | 31.62 | -4.722 |
| 38 | 3 | NB4 | 333 | 38.70 | 41.38 | -2.681 |
| 39 | 4 | NB4 | 333 | 63.00 | 43.79 | 19.213 |
| 40 | 5 | NB4 | 333 | 34.00 | 35.77 | -1.766 |
| 41 | 1 | BV1C | 581 | 54.30 | 31.70 | 22.597 |
| 42 | 2 | BV1C | 581 | 11.30 | 31.48 | -20.182 |
| 43 | 3 | BV1C | 581 | 61.90 | 41.24 | 20.659 |
| 44 | 4 | BV1C | 581 | 20.60 | 43.65 | -23.047 |
| 45 | 5 | BV1C | 581 | 35.60 | 35.63 | -0.026 |
| 46 | 1 | BV1C | 347 | 11.80 | 5.88 | 5.917 |
| 47 | 2 | BV1C | 347 | 8.00 | 5.66 | 2.338 |
| 48 | 3 | BV1C | 347 | 10.10 | 15.42 | -5.321 |
| 49 | 4 | BV1C | 347 | 9.50 | 17.83 | -8.327 |
| 50 | 5 | BV1C | 347 | 15.20 | 9.81 | 5.394 |
| 51 | 1 | BV1C | 333 | 7.70 | 4.58 | 3.117 |
| 52 | 2 | BV1C | 333 | 4.80 | 4.36 | 0.438 |
| 53 | 3 | BV1C | 333 | 10.70 | 14.12 | -3.421 |
| 54 | 4 | BV1C | 333 | 11.10 | 16.53 | -5.427 |
| 55 | 5 | BV1C | 333 | 13.80 | 8.51 | 5.294 |
| 56 | 1 | BV1C | 580 | 30.20 | 28.06 | 2.137 |
| 57 | 2 | BV1C | 580 | 21.60 | 27.84 | -6.242 |
| 58 | 3 | BV1C | 580 | 28.00 | 37.60 | -9.601 |
| 59 | 4 | BV1C | 580 | 54.50 | 40.01 | 14.493 |
| 60 | 5 | BV1C | 580 | 31.20 | 31.99 | -0.786 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 1 | BV1C | 336 | 52.80 | 42.68 | 10.117 |
| 2 | 2 | BV1C | 336 | 35.90 | 42.46 | -6.562 |
| 3 | 3 | BV1C | 336 | 66.70 | 52.22 | 14.479 |
| 4 | 4 | BV1C | 336 | 45.60 | 54.63 | -9.027 |
| 5 | 5 | BV1C | 336 | 37.60 | 46.61 | -9.006 |
| 6 | 1 | BV1C | 7 | 1.20 | -2.72 | 3.917 |
| 7 | 2 | BV1C | 7 | 5.20 | -2.94 | 8.138 |
| 8 | 3 | BV1C | 7 | 2.20 | 6.82 | -4.621 |
| 9 | 4 | BV1C | 7 | 1.10 | 9.23 | -8.127 |
| 10 | 5 | BV1C | 7 | 1.90 | 1.21 | 0.694 |
| 11 | 1 | B31b | 7 | 1.20 | -3.80 | 4.997 |
| 12 | 2 | B31b | 7 | 1.10 | -4.02 | 5.118 |
| 13 | 3 | B31b | 7 | 1.00 | 5.74 | -4.741 |
| 14 | 4 | B31b | 7 | 1.70 | 8.15 | -6.447 |
| 15 | 5 | B31b | 7 | 1.20 | 0.13 | 1.074 |
| 16 | 1 | B31b | 581 | 73.70 | 44.64 | 29.057 |
| 17 | 2 | B31b | 581 | 15.50 | 44.42 | -28.922 |
| 18 | 3 | B31b | 581 | 52.90 | 54.18 | -1.281 |
| 19 | 4 | B31b | 581 | 53.70 | 56.59 | -2.887 |
| 20 | 5 | B31b | 581 | 52.60 | 48.57 | 4.034 |
| 21 | 1 | B31b | 336 | 80.60 | 67.82 | 12.777 |
| 22 | 2 | B31b | 336 | 42.10 | 67.60 | -25.502 |
| 23 | 3 | B31b | 336 | 84.50 | 77.36 | 7.139 |
| 24 | 4 | B31b | 336 | 75.50 | 79.77 | -4.267 |
| 25 | 5 | B31b | 336 | 81.60 | 71.75 | 9.854 |
| 26 | 1 | B31b | 580 | 27.00 | 23.20 | 3.797 |
| 27 | 2 | B31b | 580 | 5.90 | 22.98 | -17.082 |
| 28 | 3 | B31b | 580 | 39.80 | 32.74 | 7.059 |
| 29 | 4 | B31b | 580 | 36.70 | 35.15 | 1.553 |
| 30 | 5 | B31b | 580 | 31.80 | 27.13 | 4.674 |
| 31 | 1 | B31b | 333 | 15.40 | 22.66 | -7.263 |
| 32 | 2 | B31b | 333 | 25.60 | 22.44 | 3.158 |
| 33 | 3 | B31b | 333 | 46.10 | 32.20 | 13.899 |
| 34 | 4 | B31b | 333 | 28.00 | 34.61 | -6.607 |
| 35 | 5 | B31b | 333 | 23.40 | 26.59 | -3.186 |
| 36 | 1 | B31b | 347 | 33.30 | 11.49 | 21.812 |
| 37 | 2 | B31b | 347 | 4.30 | 11.27 | -6.968 |
| 38 | 3 | B31b | 347 | * | 21.03 | * |
| 39 | 4 | B31b | 347 | 7.60 | 23.43 | -15.833 |
| 40 | 5 | B31b | 347 | 16.40 | 15.41 | 0.989 |
| 41 | 1 | NB51b | 333 | 8.30 | 4.98 | 3.317 |
| 42 | 2 | NB51b | 333 | 4.20 | 4.76 | -0.562 |
| 43 | 3 | NB51b | 333 | 12.80 | 14.52 | -1.721 |
| 44 | 4 | NB51b | 333 | 17.30 | 16.93 | 0.373 |
| 45 | 5 | NB51b | 333 | 7.50 | 8.91 | -1.406 |
| 46 | 1 | NB51b | 336 | 44.10 | 31.84 | 12.257 |
| 47 | 2 | NB51b | 336 | 23.80 | 31.62 | -7.822 |
| 48 | 3 | NB51b | 336 | 30.20 | 41.38 | -11.181 |
| 49 | 4 | NB51b | 336 | 46.50 | 43.79 | 2.713 |
| 50 | 5 | NB51b | 336 | 39.80 | 35.77 | 4.034 |
| 51 | 1 | NB51b | 347 | 3.50 | -2.22 | 5.717 |
| 52 | 2 | NB51b | 347 | 2.70 | -2.44 | 5.138 |
| 53 | 3 | NB51b | 347 | 2.60 | 7.32 | -4.721 |
| 54 | 4 | NB51b | 347 | 2.70 | 9.73 | -7.027 |
| 55 | 5 | NB51b | 347 | 2.60 | 1.71 | 0.894 |
| 56 | 1 | NB51b | 580 | 8.20 | 12.58 | -4.383 |
| 57 | 2 | NB51b | 580 | 12.10 | 12.36 | -0.262 |
| 58 | 3 | NB51b | 580 | 17.80 | 22.12 | -4.321 |
| 59 | 4 | NB51b | 580 | 28.90 | 24.53 | 4.373 |
| 60 | 5 | NB51b | 580 | 21.10 | 16.51 | 4.594 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 1 | NB51b | 7 | 1.20 | -3.90 | 5.097 |
| 2 | 2 | NB51b | 7 | 1.10 | -4.12 | 5.218 |
| 3 | 3 | NB51b | 7 | 1.20 | 5.64 | -4.441 |
| 4 | 4 | NB51b | 7 | 1.00 | 8.05 | -7.047 |
| 5 | 5 | NB51b | 7 | 1.20 | 0.03 | 1.174 |
| 6 | 1 | NB51b | 581 | 33.30 | 16.82 | 16.477 |
| 7 | 2 | NB51b | 581 | 32.00 | 16.60 | 15.398 |
| 8 | 3 | NB51b | 581 | 17.10 | 26.36 | -9.261 |
| 9 | 4 | NB51b | 581 | 11.50 | 28.77 | -17.267 |
| 10 | 5 | NB51b | 581 | 15.40 | 20.75 | -5.346 |
| 11 | 1 | B41b | 347 | 53.20 | 52.48 | 0.717 |
| 12 | 2 | B41b | 347 | 50.50 | 52.26 | -1.762 |
| 13 | 3 | B41b | 347 | 61.10 | 62.02 | -0.921 |
| 14 | 4 | B41b | 347 | 75.60 | 64.43 | 11.173 |
| 15 | 5 | B41b | 347 | 47.20 | 56.41 | -9.206 |
| 16 | 1 | B41b | 581 | 27.10 | 41.04 | -13.943 |
| 17 | 2 | B41b | 581 | 45.30 | 40.82 | 4.478 |
| 18 | 3 | B41b | 581 | 36.60 | 50.58 | -13.981 |
| 19 | 4 | B41b | 581 | 77.90 | 52.99 | 24.913 |
| 20 | 5 | B41b | 581 | 43.50 | 44.97 | -1.466 |
| 21 | 1 | B41b | 333 | 15.30 | 30.54 | -15.243 |
| 22 | 2 | B41b | 333 | 51.70 | 30.32 | 21.378 |
| 23 | 3 | B41b | 333 | 33.30 | 40.08 | -6.781 |
| 24 | 4 | B41b | 333 | 46.10 | 42.49 | 3.613 |
| 25 | 5 | B41b | 333 | 31.50 | 34.47 | -2.966 |
| 26 | 1 | B41b | 580 | 20.00 | 40.72 | -20.723 |
| 27 | 2 | B41b | 580 | 38.80 | 40.50 | -1.702 |
| 28 | 3 | B41b | 580 | 77.70 | 50.26 | 27.439 |
| 29 | 4 | B41b | 580 | 58.50 | 52.67 | 5.833 |
| 30 | 5 | B41b | 580 | 33.80 | 44.65 | -10.846 |
| 31 | 1 | B41b | 336 | 56.10 | 50.58 | 5.517 |
| 32 | 2 | B41b | 336 | 42.60 | 50.36 | -7.762 |
| 33 | 3 | B41b | 336 | 79.00 | 60.12 | 18.879 |
| 34 | 4 | B41b | 336 | 54.80 | 62.53 | -7.727 |
| 35 | 5 | B41b | 336 | 45.60 | 54.51 | -8.906 |
| 36 | 1 | B41b | 7 | 21.80 | 17.50 | 4.297 |
| 37 | 2 | B41b | 7 | 17.70 | 17.28 | 0.418 |
| 38 | 3 | B41b | 7 | 27.90 | 27.04 | 0.859 |
| 39 | 4 | B41b | 7 | 19.20 | 29.45 | -10.247 |
| 40 | 5 | B41b | 7 | 26.10 | 21.43 | 4.674 |
| 41 | 1 | B616 | 336 | 19.10 | 36.72 | -17.623 |
| 42 | 2 | B616 | 336 | 40.20 | 36.50 | 3.698 |
| 43 | 3 | B616 | 336 | 69.80 | 46.26 | 23.539 |
| 44 | 4 | B616 | 336 | 45.50 | 48.67 | -3.167 |
| 45 | 5 | B616 | 336 | 34.20 | 40.65 | -6.446 |
| 46 | 1 | B616 | 7 | 0.70 | -2.68 | 3.377 |
| 47 | 2 | B616 | 7 | 2.30 | -2.90 | 5.198 |
| 48 | 3 | B616 | 7 | 5.40 | 6.86 | -1.461 |
| 49 | 4 | B616 | 7 | 2.20 | 9.27 | -7.067 |
| 50 | 5 | B616 | 7 | 1.20 | 1.25 | -0.046 |
| 51 | 1 | B616 | 580 | 6.60 | 17.62 | -11.023 |
| 52 | 2 | B616 | 580 | 4.80 | 17.40 | -12.602 |
| 53 | 3 | B616 | 580 | 34.60 | 27.16 | 7.439 |
| 54 | 4 | B616 | 580 | 36.70 | 29.57 | 7.133 |
| 55 | 5 | B616 | 580 | 30.60 | 21.55 | 9.054 |
| 56 | 1 | B616 | 581 | 9.70 | 11.14 | -1.443 |
| 57 | 2 | B616 | 581 | 9.20 | 10.92 | -1.722 |
| 58 | 3 | B616 | 581 | 37.50 | 20.68 | 16.819 |
| 59 | 4 | B616 | 581 | 15.20 | 23.09 | -7.887 |
| 60 | 5 | B616 | 581 | 9.30 | 15.07 | -5.766 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 1 | B616 | 347 | 4.40 | 17.31 | -12.915 |
| 2 | 2 | B616 | 347 | 46.90 | 17.09 | 29.806 |
| 3 | 3 | B616 | 347 | 13.00 | 26.85 | -13.853 |
| 4 | 4 | B616 | 347 | * | 29.26 | * |
| 5 | 5 | B616 | 347 | 18.20 | 21.24 | -3.038 |
| 6 | 1 | B616 | 333 | 2.30 | 13.62 | -11.323 |
| 7 | 2 | B616 | 333 | 49.30 | 13.40 | 35.898 |
| 8 | 3 | B616 | 333 | 12.20 | 23.16 | -10.961 |
| 9 | 4 | B616 | 333 | 17.70 | 25.57 | -7.867 |
| 10 | 5 | B616 | 333 | 11.80 | 17.55 | -5.746 |
| 11 | 1 | NB616 | 333 | 5.20 | 8.44 | -3.243 |
| 12 | 2 | NB616 | 333 | 4.70 | 8.22 | -3.522 |
| 13 | 3 | NB616 | 333 | 8.20 | 17.98 | -9.781 |
| 14 | 4 | NB616 | 333 | 40.00 | 20.39 | 19.613 |
| 15 | 5 | NB616 | 333 | 9.30 | 12.37 | -3.066 |
| 16 | 1 | NB616 | 347 | 6.70 | 10.38 | -3.683 |
| 17 | 2 | NB616 | 347 | 7.50 | 10.16 | -2.662 |
| 18 | 3 | NB616 | 347 | 8.10 | 19.92 | -11.821 |
| 19 | 4 | NB616 | 347 | 41.70 | 22.33 | 19.373 |
| 20 | 5 | NB616 | 347 | 13.10 | 14.31 | -1.206 |
| 21 | 1 | NB616 | 580 | 6.70 | 11.34 | -4.643 |
| 22 | 2 | NB616 | 580 | 6.50 | 11.12 | -4.622 |
| 23 | 3 | NB616 | 580 | 25.80 | 20.88 | 4.919 |
| 24 | 4 | NB616 | 580 | 21.20 | 23.29 | -2.087 |
| 25 | 5 | NB616 | 580 | 21.70 | 15.27 | 6.434 |
| 26 | 1 | NB616 | 581 | 7.70 | 15.28 | -7.583 |
| 27 | 2 | NB616 | 581 | 8.30 | 15.06 | -6.762 |
| 28 | 3 | NB616 | 581 | 24.50 | 24.82 | -0.321 |
| 29 | 4 | NB616 | 581 | 30.50 | 27.23 | 3.273 |
| 30 | 5 | NB616 | 581 | 30.60 | 19.21 | 11.394 |
| 31 | 1 | NB616 | 7 | 1.20 | 8.10 | -6.903 |
| 32 | 2 | NB616 | 7 | 4.90 | 7.88 | -2.982 |
| 33 | 3 | NB616 | 7 | 4.30 | 17.64 | -13.341 |
| 34 | 4 | NB616 | 7 | 51.90 | 20.05 | 31.853 |
| 35 | 5 | NB616 | 7 | 3.40 | 12.03 | -8.626 |
| 36 | 1 | NB616 | 336 | 34.90 | 29.84 | 5.057 |
| 37 | 2 | NB616 | 336 | 26.00 | 29.62 | -3.622 |
| 38 | 3 | NB616 | 336 | 41.70 | 39.38 | 2.319 |
| 39 | 4 | NB616 | 336 | 40.60 | 41.79 | -1.187 |
| 40 | 5 | NB616 | 336 | 31.20 | 33.77 | -2.566 |
| 41 | 1 | B31a | 581 | 10.40 | 22.86 | -12.463 |
| 42 | 2 | B31a | 581 | 42.40 | 22.64 | 19.758 |
| 43 | 3 | B31a | 581 | 27.70 | 32.40 | -4.701 |
| 44 | 4 | B31a | 581 | 30.20 | 34.81 | -4.607 |
| 45 | 5 | B31a | 581 | 28.80 | 26.79 | 2.014 |
| 46 | 1 | B31a | 347 | 5.40 | 7.42 | -2.016 |
| 47 | 2 | B31a | 347 | 6.50 | 7.19 | -0.695 |
| 48 | 3 | B31a | 347 | 12.20 | 16.95 | -4.754 |
| 49 | 4 | B31a | 347 | * | 19.36 | * |
| 50 | 5 | B31a | 347 | 18.80 | 11.34 | 7.462 |
| 51 | 1 | B31a | 333 | 3.20 | 8.02 | -4.823 |
| 52 | 2 | B31a | 333 | 17.80 | 7.80 | 9.998 |
| 53 | 3 | B31a | 333 | 8.00 | 17.56 | -9.561 |
| 54 | 4 | B31a | 333 | 27.00 | 19.97 | 7.033 |
| 55 | 5 | B31a | 333 | 9.30 | 11.95 | -2.646 |
| 56 | 1 | B31a | 580 | 1.50 | 13.52 | -12.023 |
| 57 | 2 | B31a | 580 | 7.70 | 13.30 | -5.602 |
| 58 | 3 | B31a | 580 | 34.10 | 23.06 | 11.039 |
| 59 | 4 | B31a | 580 | 29.00 | 25.47 | 3.533 |
| 60 | 5 | B31a | 580 | 20.50 | 17.45 | 3.054 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1 | 1 | B31a | 336 | 25.50 | 29.60 | -4.103 |
| 2 | 2 | B31a | 336 | 13.70 | 29.38 | -15.682 |
| 3 | 3 | B31a | 336 | 47.90 | 39.14 | 8.759 |
| 4 | 4 | B31a | 336 | 44.60 | 41.55 | 3.053 |
| 5 | 5 | B31a | 336 | 41.50 | 33.53 | 7.974 |
| 6 | 1 | B31a | 7 | 1.30 | -1.22 | 2.517 |
| 7 | 2 | B31a | 7 | 2.30 | -1.44 | 3.738 |
| 8 | 3 | B31a | 7 | 4.80 | 8.32 | -3.521 |
| 9 | 4 | B31a | 7 | 4.80 | 10.73 | -5.927 |
| 10 | 5 | B31a | 7 | 5.90 | 2.71 | 3.194 |

End of Nontokozo Kunene - TSC - Project no: P03000100. Current data space: 2 blocks, peak usage 99% at line 272.

Genstat 64-bit Release 18.2 ( PC/Windows 8) 25 October 2017 12:03:53
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1 **Appendix 6:** Composition of artificial media used in this study

2 ***Phytophthora* Selective Medium (PARPH)**

| | | |
|---|---|---|
| 3 | Potato Dextrose Agar as basal medium | 1000 ml |
| 4 | Ampicillin | 0.34 g |
| 5 | Rifampicin | 0.02 g |
| 6 | Pentachloronitrobenzene (PCNB) | 0.2 g |
| 7 | Pimaricin | 0.08 g |
| 8 | Hymexazol | 0.1 g |

9 **Potato Dextrose Agar (PDA)**

| | | |
|---|---|---|
| 10 | Potato Dextrose Agar (Oxoid No.3) | 39.0g |
| 11 | Distilled water | 1000 ml |