Chapter Two

Die-back of cold-tolerant *Eucalyptus* associated with *P. nicotianae* and *P. cinnamomi* in South Africa

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ABSTRACT

Phytophthora root and collar rot is a widespread disease associated with cold-tolerant *Eucalyptus* spp. in South Africa. Various soil borne pathogens, including *P. cinnamomi* have been reported as the causal agents of this disease. In recent years, *P. nicotianae* rather than *P. cinnamomi* has consistently been isolated from the rhizosphere and from infected root collars of cold-tolerant *Eucalyptus* spp. During 2000–2003, sampling was undertaken in several *Eucalyptus* plantations in the Mpumalanga and KwaZulu-Natal Provinces of South Africa to determine the primary cause of root disease on these trees. The aim of this study was to identify the species responsible for the disease outbreaks and assess the potential threat posed by *P. nicotianae* to cold-tolerant *Eucalyptus* spp. A large number of *P. nicotianae* and *P. cinnamomi* isolates, from diseased trees as well as from the soil surrounding their roots were obtained and identified based on morphology, mating type and sequence data for the ITS region of the ribosomal DNA operon. The pathogenicity of isolates of these species was determined by inoculating one-year old *Eucalyptus smithii* in the field. Results obtained in this study showed that *P. nicotianae* and *P. cinnamomi* are commonly associated with symptoms on dying trees in the sampled areas. The former species was more frequently isolated from soil and diseased trees than *P. cinnamomi*. Pathogenicity tests showed that *P. nicotianae* isolates were less aggressive than those of *P. cinnamomi.* However, overall results suggest that *P. nicotianae* was the more common causal agent of the die-back outbreaks that occurred during the period under investigation.

Keywords: *Phytophthora* die-back, disease tolerance, stems inoculation

1. Introduction

Cold-tolerant *Eucalyptus* spp. were first introduced into South Africa more than a century ago for the production of mine timber props (Poynton 1979). Planting the *Eucalyptus* spp. has increased steadily over the years, mainly due to the expansion of the forestry industry and to an increased demand for raw material for pulpwood (Darrow 1984a, 1984b). Cold-tolerant *Eucalyptus* spp. are planted in high altitude (above 1000 m), areas in Mpumalanga and KwaZulu-Natal, which are prone to frost and thus unsuitable for the commonly grown *E. grandis* (Schönau *et al*. 1994).

One of the factors that impacts negatively on the propagation of cold-tolerant *Eucalyptus* in South Africa is root and collar rot (Wingfield *et al*. 2001). A primary disease symptom is death of the root cambium which results in a lesion that commonly extends up into the collar region (Fig 1). Advanced disease symptoms on affected trees include chlorosis and wilting of leaves, gum exudation from cankers at the tree collar and eventually death. In the past, this disease problem resulted in large-scale losses in forest nurseries and plantations in South Africa (Lundquist & Baxter 1985, Wingfield & Roux 2000). During the 1980s, breeding programmes that used highly susceptible species such as *E. fastigata* and *E. elata* were abandoned due to root rot related disease problems (Herbert 1994). Other species reported to be susceptible to collar and root rot include *E. fraxinoides* and *E. smithii* (Linde *et al.* 1994).

In South Africa, root and collar rot of *Eucalyptus* has been associated with species of *Phytophthora* and *Pythium* (Wingfield *et al*. 2001). Amongst the *Phytophthora* species reported to affect this host, *P. cinnamomi* is the most destructive causing die-back of cold-tolerant *Eucalyptus* (Linde *et al*. 1999a). In a previous study, Linde *et al* (1994) obtained a small number of isolates of *P. nicotianae* from *Eucalyptus* forest soil. These isolates were amongst the most aggressive when inoculated on *E. grandis* and *E. fastigata.*

Other than a brief preliminary report (Maseko *et al.* 2001),very little is known about the effect of *P. nicotianae* on cold-tolerant *Eucalyptus* spp. in South Africa. The aim of this study was to investigate the distribution of *Phytophthora* spp. in *Eucalyptus* plantations in Mpumalanga and KwaZulu-Natal Provinces of South Africa. *Eucalyptus* plantations were tested for the presence of *Phytophthora* spp. which were then identified using molecular technology. Pathogenicity tests using selected *P. nicotianae* and *P. cinnamomi* isolates were conducted on *E. smithii* to assess their pathogenicity and the potential threat they pose to cold-tolerant *Eucalyptus* spp. in plantations

2. Materials and Methods

2.1 Sampling

During 2000 and 2003, 66 commercial *Eucalyptus* stands located in 31 sites in the Mpumalanga and KwaZulu-Natal Provinces of South Africa were selected for investigation (Fig 2). Stands in commercial plantations as well as small private holdings were chosen. Sampling was carried out from August to April when the soil temperature and moisture are reported conducive to the isolation of *Phytophthora* spp. (Weste & Vithanage 1979).

Four of the most common and important cold-tolerant *Eucalyptus* spp. were considered in this study. These included *E. nitens*, *E. macarthurii*, *E. dunnii* and *E. smithii.* However, a greater number of *E. smithii* stands were sampled because of the economic importance of this host and its high susceptibility to *Phytophthora* root rot. During the course of this study, diseased plant material was also received by the diagnostic clinic of the Tree Protection Cooperative Programme, University of Pretoria, and isolates of *Phytophthora* spp. from these samples were included in this work.

Trees displaying symptoms of root and collar rot were tested for the presence of *Phytophthora* species. In total, 564 diseased trees and 320 soil samples were collected and tested for the presence of *Phytophthora* spp. Isolations were made from trees showing symptoms of root and collar rot and foliage chlorosis, whenever possible. *Phytophthora*-affected areas were characterised by patches of dead or dying plants. Soil and fine root samples were taken from the root zone of dying tress. In stands with healthy trees, soil samples with fine roots were taken from the rhizosphere of randomly selected visually healthy trees. At each sampling site, four randomly selected soil samples from a depth of about 10 cm were collected, mixed and pooled. Symptomatic plants were uprooted and placed in plastic bags for later diagnosis. Soil and plant samples were assayed within 48 hours of collection. The remaining samples were stored in a cool room at 5 °C for baiting, especially in cases where plants showed die-back symptoms but no *Phytophthora* spp. were retrieved.

Soil in each sample was divided into two equal portions (100 g) and placed into labelled 350 ml plastic containers. Dry soil samples were pre-moistened for two days to induce the growth of dormant spores. Soil which contained fine root was flooded with deionised water and then baited with citrus leaf pieces and *E. sieberi* cotyledons as described by Grimm & Alexander (1973) and Marks & Kassaby (1974) and incubated at room temperature in the dark for 3–4 days. Leaf discs that become infected developed blackened areas, whereas cotyledons that become infected turned chlorotic. Infected baits and bleached *E. sieberi* cotyledons were removed from the water, blotted dry and plated onto NARPH culture growth medium selective for *Phytophthora*. NARPH is made up of Corn Meal Agar (CMA) 17

g^{-1} amended with 50 µg/ml^{-1} nystatin, 200 µg ml^{-1} ampicillin, 10 µg ml^{-1} rifampicin, 25 µgml^{-1} pentacloronitrobenzene (PCNB), and 50 µg ml^{-1} hymexazol (Hüberli *et al*. 2000).

Bark from basal cankers adjacent to the root collar region was removed using a scalpel and cut into smaller pieces with diameter ≤ 5 mm and placed onto the surface of NARPH growth medium in Petri dishes. Petri dishes were then incubated in the dark at room temperature and examined using a compound microscope, daily after two days. All isolates with typical *Phytophthora* growth characteristics were sub-cultured on 10 % V8 agar plates for identification (Erwin & Riberio 1996). Cultures were stored in sterile distilled water at room temperature (Ko 2003) and are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI). Details of all isolates used in this study are presented in (Table. 1).

2.2 Identification of isolates

2.2.1 Morphology

All isolates sub-cultured on to V8 agar were separated into groups based on culture morphology since most *P. cinnamomi* and *P. nicotianae* have distinct rosaceous or stoloniferous colony growth, respectively. Zoosporangia were produced using a modification of the method of Byrt & Grant (1979). Single spore cultures from each sampling site were obtained using the method described by Wang-Ching & Wen-Hsiung (1997). All isolates were identified with the aid of taxonomic keys (Stamps *et al.* 1990). The characters used to identify *P. cinnamomi* included coralloid hyphae, grapelike hyphal swellings, numerous chlamydospores, and absence of zoospores in solid media or oospores in single cultures. Features used to identify *P. nicotianae* isolates included papillate zoosporangia with various shapes and their unique ability to survive at 35 °C. A sub-set of six isolates of *P. nicotianae* and *P. cinnamomi* were used for morphological examination. Dimensions of 50 randomly selected reproductive structures were measured and recorded using light microscopy with complimentary Axiovision® 3.1 software and HRc Axiocam digital camera (Axioskop, Carl Zeiss, München, Germany).

2.2.2 DNA sequence comparisons

Clarified V8 broth was seeded with small mycelial plugs of isolates derived from single hyphal tips. Broth cultures were incubated at room temperature. Mycelium of the isolates was harvested, freeze-dried and stored at–20 °C until isolation of DNA. Lyophilised mycelium was pulverised using a mortar and pestle and 10–20 ng was transferred to Eppendorf tubes. Extracted DNA was isolated using the modified phenol-chloroform extraction method as described by Al-Samarrai & Schmid (2000). DNA (5 µl) was run on 1 % agarose gel, stained with 2 µl of ethidium bromide and visualised by UV

illumination. The concentration of the DNA was estimated using 1 kb molecular weight marker. The remaining DNA was suspended in sabax water and stored at –20 °C.

To confirm the identity of *Phytophthora* isolates used in this study, the Internal Transcriber Spacer (ITS) regions of the rDNA were amplified and sequenced using ITS 4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) and ITS 6 (5′GAA GGT GAA GTC TAA CAA GG 3′) primers as described by Cooke & Duncan 1997. Each PCR reaction mixture (50 µl) consisted of 50-90 ng of DNA template, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM of dNTP's (Fermentas, UAB, Luthuania). PCR amplification conditions were 1 cycle at 96 °C for 2 min (initial denaturation), followed by 35 denaturation cycles at 96 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension 72 °C for 1 min, and final cycle at 72 °C for 10 minutes. Resulting amplified PCR products were separated on 1, 5 % agarose gel in a 1 × Tris–acetate / EDTA buffer. The PCR products were purified with the aid of a purification kit (Roche Diagnostics) and used as for sequencing reactions on an ABI automated 3100 DNA sequencer.(Perkin Elmer, Norwalk, CON). The same ITS primers used for the amplification reactions were used for the sequencing reaction conducted using the Big Dye Terminator Cycle sequencing reaction kit (Perkin Elmer, Applied Biosystems).

Raw sequence data was trimmed and aligned by using Sequencher software (ver. 4.5 Gene Codes, USA) and optimized by automatic alignment using MUSCLE software (Edgar 2004). A BLAST search of the GenBank database was conduced using the resulting consensus sequences and sdata for other *Phytophthora* spp. was added. Sequence data were analyzed using the maximum parsimony (MP) method in PAUP software version 4. The ends of aligned sequences were trimmed and uninformative characters removed. The most parsimonious phylogenetic trees were generated using the heuristic search with random stepwise addition of 100 replicates with tree bisection-reconnection branch-swapping option on and the steepest-descent option disabled. Maxtrees and branches of zero length were collapsed and the most parsimonious trees saved. Strict consensus and 50 % majority-rule trees were computed using *Pythium irregulare* (AY907917) as an out group. The confidence intervals of the branch nodes for the consensus tree were evaluated by bootstrap analysis of 1000 replications (Felsenstein 1985)

2.3 Mating type determination

The mating studies were determined using a method described by Erwin & Ribeiro (1996). All isolates used for the mating tests were cultured in 65 mm Petri dishes containing carrot agar (Brasier & Kirk 2004). Known A_1 (CMW21989, CMW21920) and A_2 (CMW21993; CMW21959) mating isolates of *P. nicotianae* and *P. cinnamomi* respectively, were used as testers. A mycelial plug (5 mm in diam) cut from the edges of actively growing colonies of known mating type isolates of *P. nicotianae* and *P. cinnamomi* were paired side by side with 30 *P. nicotianae* and 10 *P. cinnamomi* unknown isolates

respectively. All isolates were paired in all possible combinations, including positive and negative controls. Thereafter, Petri dishes were sealed with parafilm and incubated in the dark at room temperature for 15–30 days and examined for the production of sexual using a stereomicroscope (× 100 magnification). A polycarbonate membrane technique of Ko (1978), was also used to determine which isolates produced gametangia in compatible crosses. A sterile polycarbonate membrane (47 mm diam, 0.2µm pore size; Millipore) was sandwiched between a two mycelium plugs (10 mm) paired with one another. The cultures were incubated in the dark, at room temperature for 15–30 days and examined for oogonia or antheridia formation.

2.4 Pathogenicity tests

A subset of thirty *P. nicotianae* and ten *P. cinnamomi* isolates collected from various areas were used to inoculate one-year-old *E. smithii* trees in the field. All isolates were inoculated into *E. smithii* saplings in glasshouse pilot test prior to use in these experiments to ensure that they had not lost pathogenicity. The pathogenicity trial was located in a commercial stand at Sutton (29° 58´S, 30° 08´E), near Ixopo in the KwaZulu -Natal Province of South Africa. A complete random block design was used for field trial. Mean lesion lengths (mm) developed after 6 weeeks artificial inoculation of one-year-old *E. smithii* trees in the field were used to rank the aggressiveness of the *Phytophthora* isolates. A modification of the wounded bark inoculation technique described by Dixon *et al.* (1984) was used to inoculate side branches, roughly at the middle of each branch. A sterile blade was used to cut a flap (dimensions of 2 × 1 cm) through the outer bark. A 4 mm disc diameter mycelium plug from week-old cultures of *P. nicotianae* or *P. cinnamomi* grown on PDA was inserted under the flap and covered with masking tape. In total, 410 side branches (10 replicate trees per isolate) including controls were inoculated with each isolate of *P. nicotianae* and *P. cinnamomi*. Control side branches were inoculated with sterile agar plugs only.

Six weeks after the inoculation, labelled side branches were harvested and placed into plastic bags. These were transported to the laboratory for analysis. Lesion lengths extending above and below the inoculation points were measured. Lesion development in the inner bark was used as a measure of pathogenicity. Tissue cut from three randomly chosen side branches was plated onto selective media, as described previously, in order to ensure that the inoculated fungi could be re-isolated from the lesion and thus satisfying the Koch's postulates. The experiment was established in Summer (February) and repeated in Spring (October) to evaluate the influence of temperature on lesion development. Data were analysed using STATISTICA, a data analysis software system, version 6 (StatSoft Inc. 2001). Analysis of variance (ANOVA) was used to compare the pathogenicity between *P. nicotianae* and *P. cinnamomi* isolates used in this study. Pearson correlation coefficient *r* between the summer and spring trials was also calculated.

3. Results

3.1 Collection of samples

In total, 220 *Phytophthora* isolates were retrieved from 564 diseased trees (39 %) and only 48 isolates were obtained from the soil samples (15 %). In most cases, *Phytophthora* spp. were readily isolated from infected root collars compared to soil samples taken from the rhizosphere. The occurrence and distribution of *Phytophthora* spp. retrieved from soil and diseased plant samples varied from stand to stand. The pathogen population levels fluctuated considerably among the sampled stands throughout the duration of this study. The highest levels of tree decline and pathogen retrieval levels were observed during late summer (January and February) and early autumn months (March and April). A summary of the retrieval of *Phytophthora* spp. from different hosts is shown in Table 2.

3.2 Identification of isolates

3.2.1 Morphology

Phytophthora nicotianae and *P. cinnamomi* isolates typically produced stoloniferous and rosaceous (petaloid) growth pattern on V8 and carrot agar, respectively. However, some isolates displayed a variation in growth patterns. Some *P. nicotianae* and *P. cinnamomi* isolates did not produce any defined colony growth pattern on the media used. Measurements of asexual and sexual reproductive structures for *P. nicotianae* and *P. cinnamomi* were, therefore, also important (Table 3). *P. nicotianae* was the most common species retrieved from soil and diseased plant material collected from the sampling sites. The fungus was widely distributed with no obvious trend in geographic distribution observed. In total, 132 isolates (56 %) from both soil and diseased plant material were identified as *P. nicotianae*. Both mating types A1 and A2 of *P. nicotianae* were retrieved from both soil and diseased plant material. The A2 mating type (82 %) was more prevalent than the A1 mating type (18 %).

Phytophthora cinnamomi was the second most prevalent species retrieved from cold-tolerant eucalypts at the sites investigated. The percentage retrieval of *P. cinnamomi* from soil and diseased plant material was 24 % (54 isolates). *P. nicotianae* was more readily isolated from soil when compared to *P. cinnamomi.* However, at some sites both pathogens were isolated from dying plants. Of the *P. cinnamomi* isolates, 24 % were of mating type A1 and 76 % were of the A2 mating type. *Phytophthora boehmeriae* (4 %) and two newly described species, *P. alticola* (7 %), and *P. frigida* (9 %), were also retrieved occasionally.

3.2.2 DNA sequence comparisons

The amplification of the ITS region resulted in a single band of 900bp for all isolates examined. The final sequence data matrix consisted of 34 taxa with 865 characters of which 631 were excluded and 234 characters were included in the analysis. A significant phylogenetic signal of ($P < 0.01$, g1 = -0.68) was obtained after evaluating 1000 random trees. The heuristic search resulted in 2 most parsimonious trees (CI = 0.743, RI = 0.923, RC = 0.686, HI = 0.257), which differed only in the lengths of the branches.

The ITS rDNA sequence data set consisted of two major clades comprising ITS clade 1–10 as described by Cooke *et al*. (2000). The isolates sequenced in this study fell into two distinct groups together with authentic isolates of *P. nicotianae* and *P. cinnamomi.* The first clade consisted of the larger *Phytophthora* clade with species belonging to the ITS clades 1, 2, 6 and 7 (Cooke *et al*. 2000) and the second clade consisted of *P. boehmeriae* which belongs to the ITS 8 clade of (Cooke *et al*. 2000; Kroon *et al*. 2004). The *P. nicotianae* and *P. cinnamomi* isolates used in this study grouped in clade 1 and clade 7, respectively (Fig 3).

3.2 Pathogenicity tests

Discoloured lesions were observed on the green inoculated side branches above and below the points of inoculation after 6 weeks. When the bark was scrapped with a scalpel blade, distinct brown lesions were observed and whereas the control side branches did not display lesions (Fig 4). *P. cinnamomi* and *P. nicotianae* were re-isolated from up to 10 mm beyond the visible lesions when plated directly on selective culture medium but could not be reisolated from control branches.

All *Phytophthora* isolates inoculated on one-year old *E. smithii* branches in the field displayed a range in aggressiveness levels. However, side branches inoculated with *P. cinnamomi* isolates consistently had a rapid rate of lesion development and produced significantly ($P < 0.01$) longer lesions relative to those inoculated with *P. nicotianae*. The results of the mean lesion lengths produced by *P. cinnamomi* and *P. nicotianae* isolates are shown in Fig 5. *P. cinnamomi* isolates produced mean lesions length ranging between 19 mm to 59 mm in spring and between 33 mm and 77 mm in summer. The three most aggressive *P. cinnamomi* isolates on *E. smithii* were CMW21959, CMW21979, and CMW21983. A considerable variation in aggressiveness amongst the 30 *P. nicotianae* isolates was observed. Lesions produced by *P. nicotianae* isolates were between 7 mm to 23 mm long in spring and between 13 mm to 38 mm in summer. Isolates (CMW20326, CMW21972, and CMW22038 were the most aggressive *P. nicotianae* isolates.

The analysis of variance (ANOVA), revealed highly significant differences (P<.0001) in mean lesion lengths produced by the different *Phytophthora* isolates (Table 4). When the season and individual *Phytophthora* isolates were analysed as variables for the pathogenicity tests highly significant differences (F = 389.34, P <.0001, F = 58.37, P <.0001) were obtained. However, the correlation coefficient (r = 0.74) obtained between the spring and summer data sets indicate presence of seasonal interaction. Thus, the ranking of the mean lesion length produced by the different *Phytophthora* isolates was not always the same during the different seasons as illustrated in a scattergram (Fig 6). The overall summer lesion least squares means mean was 17.5 cm, the standard error was 0.36, the confidence limits (0.95) was 16.76. For the Spring inoculation spring, the average lesion length was 27.3 cm, standard error of 0.34 and the confidence limits (0.95) of 26.6.

4. Discussion

The aim of this study was to verify the identity of the *Phytophthora* spp. responsible for recent outbreaks of root and collar rot on several cold-tolerant *Eucalyptus* plantations in the Mpumalanga and KwaZulu-Natal Provinces of South Africa. Results confirmed the presence of both *P. nicotianae* and *P. cinnamomi* on cold-tolerant *Eucalyptus* stands as previously reported by Linde *et al*. (1994). However, they indicated that *P. nicotianae* rather than *P. cinnamomi* is the most common pathogen associated with diseased plant samples and soil. The consistent isolation of *P. nicotianae* from soil and diseased plant material was unexpected since *P. cinnamomi* has typically been seen as the dominant species affecting cold- tolerant *Eucalyptus* spp. in South Africa.

Very little is known about the role of *P. nicotianae* in die-back of cold-tolerant *Eucalyptus* spp. This is despite the abundance of *P. nicotianae* in soil and diseased *Eucalyptus* observed in this study. *P. nicotianae* has a wide host range (Erwin & Riberio 1996) and is associated with black-butt disease of *A. mearnsii* in South Africa (Zeijlemaker 1971). Planting of *Eucalyptus* spp. on sites previously planted to *A. mearnsii* may explain the high levels of *P. nicotianae* found in this study.

The relatively low level of recovery of *Phytophthora* spp., especially *P. cinnamomi* observed in this study, has also been found in *Eucalyptus* forest soils in Australia. (Marks *et al*. 1975, Weste & Ruppin 1977; Davison & Tay 2005). However, the consistent association of *P. cinnamomi* and *P. nicotianae* with infected root collars provides good anecdotal evidence that they are responsible for the root and root collar disease problem observed in plantations.

The morphology and ITS sequence data results confirmed the identity of both *P. nicotianae* and *P. cinnamomi* as well as *P. boehmeriae* and two newly named species (Maseko *et al*. 2007). These results are similar to those of an earlier report (Linde *et al.* 1994). Results of this study, strongly suggest that

through the routine use of DNA sequencing techniques, new records and possibly new *Phytophthora* spp. are likely to be encountered. Furthermore, the results revealed that the A2 mating type is the dominant form for both *P. nicotianae* and *P. cinnamomi. This* suggests that both pathogens predominantly reproduce asexually as reported in a previous study (Linde *et al*. 1999a)

Pathogenicity tests clearly illustrated that both *P. nicotianae* and *P. cinnamomi* are capable of causing disease in *Eucalyptus*. In a previous study (Linde *et al*. 1994), the pathogenicity of *P. nicotianae* and *P. cinnamomi* on *E. grandis* was demonstrated. The small numbers of *P. nicotianae* isolates included in that study were more aggressive than *P. cinnamomi*. In contrast, pathogenicity tests in the present study indicate that *P. cinnamomi* isolates are more virulent than those of *P. nicotianae* on *E. smithii.* In this study, highly significant differences in mean lesion length were found for different *P. nicotianae* and *P. cinnamomi* isolates inoculated onto *E. smithii*. Such variable levels of pathogenicity amongst isolates have previously been noted for *P. nicotianae* and *P. cinnamomi* (Linde *et al*. 1999b).

Likewise, variation in pathogenicity amongst different Australian isolates of *P. cinnamomi* inoculated onto *E. marginata* has been reported (Dudzinski *et al*. 1993). Season had a significant effect on the pathogen retrieval levels as well as on the development of lesion caused by the different *Phytophthora* isolates. Lesions produced by the different *Phytophthora* isolates were generally larger in the summer trial. The levels of tree decline pathogen retrieval levels were late summer and early autumn months.

Results of this study indicate that *Phytophthora* spp. are prevalent and important pathogens on cold-tolerant *Eucalyptus* spp. in South Africa. *Phytophthora nicotianae* is the most frequently recovered species and has the widest distribution. It was retrieved from declining trees and rhizosphere soil, which supports our hypothesis, that this pathogen rather than *P. cinnamomi* is the key causal agent of recent disease outbreaks. Thus, *P. nicotianae* should also be included in future disease-screening assays of cold-tolerant *Eucalyptus* seedlings in South Africa

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Table. 1 Isolates examined and description of the sites sampled for the presence of *Phytophthora* spp.

Isolate Number	Species identity	Host	Mating Type	Origin	Latitude	Longitude
CMW21959	*P. cinnamomi*	*E.nitens*	A2	Hlelo	30° 65´S	26° 09´ E
CMW21960	*P. cinnamomi*	*E. fastigata*	A1	Lothair	30° 44´ S	26° 28´ E
CMW21968	*P. cinnamomi*	*E .smithii*	A2	Shafton	30° 13´S	29° 19´ E
CMW21976	*P. cinnamomi*	*E .smithii*	A2	Hodgsons	30° 40´S	29° 24´E
CMW21920	*P. cinnamomi*	*E .smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW21979	*P. cinnamomi*	*E .smithii*	A2	Epson	29° 47´S	29° 54´E
CMW21982	*P. cinnamomi*	*E. nitens*	A1	Lothair	30° 44´ S	26° 28´ E
CMW21983	*P. cinnamomi*	*E .smithii*	A2	Demagtenburg	30° 20´S	29° 33´E
CMW21984	*P. cinnamomi*	*E .smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22003	*P. cinnamomi*	*E. smithii*	A2	Spitzkop	30° 76´S	27° 41´E
CMW20203	*P. nicotianae*	*E. smithii*	A2	Enon	30° 24´S	29° 82´E
CMW20204	*P. nicotianae*	*E. smithii*	A2	Hlelo	30° 65´S	26° 09´ E
CMW20205	*P. nicotianae*	*E. smithii*	A2	Glenbain	30° 03´S	30° 00´E
CMW20297	*P. nicotianae*	*E. smithii*	A2	Shafton	30° 13´S	29° 19´ E
CMW20326	*P. nicotianae*	*E. smithii*	A2	Hodgsons	30° 40´S	29° 24´E
CMW21965	*P. nicotianae*	*E. dunnii*	A2	Pinewoods	30° 10´S	29° 64´E
CMW21970	*P. nicotianae*	*E. macarthurii*	A2	Demagtenburg	30° 20´S	29° 33´E
CMW21972	*P. nicotianae*	*E. smithii*	A2	Ingwe	30° 18´S	29° 49´E
CMW21989	*P. nicotianae*	*E. smithii*	A1	Hodgsons	30° 40´S	29° 24´E
CMW19442	*P. nicotianae*	*E. smithii*	A2	Spitzkop	30° 76´S	27° 41´E
CMW19443	*P. nicotianae*	*E. smithii*	A2	Shafton	30° 13´S	29° 19´ E
CMW21993	*P. nicotianae*	*E. smithii*	A2	Hodgsons	30° 40´S	29° 24´E
CMW22000	*P. nicotianae*	*E. smithii*	A2	Hodgsons	30° 40´S	29° 24´E
CMW22001	*P. nicotianae*	*E. smithii*	A1	Hodgsons	30° 40´S	29° 24´E
CMW22004	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22009	*P. nicotianae*	*E. macarthurii*	A2	Demagtenburg	30° 20´S	29° 33´E
CMW22010	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22011	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22016	*P. nicotianae*	*E. dunnii*	A2	Winterton	29° 48´S	29° 03´E
CMW22020	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22022	*P. nicotianae*	*E. macarthurii*	A2	Winterton	29° 48´S	29° 03´E

Table. 1 Cont. Isolates examined and description of the sites sampled for the presence of *Phytophthora* spp.

Isolate Number	Species identity	Host	Mating Type	Origin	Latitude	Longitude
CMW22037	*P. nicotianae*	*E. smithii*	A1	Sutton	29° 58´S	30° 08´E
CMW22038	*P. nicotianae*	*E. smithii*	A2	Ingwe	30° 18´S	29° 49´E
CMW22039	*P. nicotianae*	*E. smithii*	A2	Geelhoutboom	30° 14´S	29° 50´E
CMW22040	*P. nicotianae*	*E. smithii*	A2	Geelhoutboom	30° 14´S	29° 50´E
CMW22041	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22042	*P. nicotianae*	*E. dunnii*	A2	Tamboekiesbult	30° 52´S	27° 47´ E
CMW22043	*P. nicotianae*	*E. smithii*	A2	Ingwe	30° 18´S	29° 49´E
CMW22044	*P. nicotianae*	*E. dunnii*	A2	Tamboekiesbult	30° 52´S	27° 47´ E
CMW22045	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E

Table 2. Isolation frequency of *Phytophthora* sp from cold-tolerant *Eucalyptus* stands surveyed in this study

Species	Host species	No. of isolates	No. of symptomatic trees	No. of soil samples	Total retrieval frequency (%)
P. cinnamomi	*E. dunnii, E. elata, E. fastigata, E. nitens, E. smithii*	54	172	115	24
P. nicotianae	*E. dunnii, E. elata, E. fastigata, E. nitens, E. smithii*	123	280	152	56
P. boehmeriae	*E. macarthurii, E. smithiii*	8	24	15	4
P. frigida	*E. dunnii, E. smithii*	20	47	20	9
P. alticola.	*E. badjensis, E. dunnii, E. macarthurii*	15	41	18	7
Total		**220**	**564**	**320**	**100**

Table 3. Measurements for the asexual and sexual structures of *Phytophthora* spp. isolated from *Eucalyptus* spp. in South Africa

Identity	Culture No.	Sporangia Measurements L×B (µm)	Oogonium (µm)	Oospore (µm)	Antheridia (µm)	Chlamydospore (µm)
P. nicotianae	CMW21970	(33–)35(–40)× (22–)30 (–33)	(23–)20 (–25)	(20–)22(–26)	(13–)19(–25)×(10–)15(–24)	(20–)22(–34)
P. nicotianae	CMW21993	(35–)48(–60)× (28–)36 (–55)	(18–)20 (–22)	(15–)18(–20)	(13–)19(–25)×(10–)15(–24)	(25–)30(–45)
P. nicotianae	CMW22000	(40–)49(–74)× (30–)35 (–50)	(16–)18 (–26)	(14–)16(–20)	(13–)19(–25)×(10–)15(–24)	(24–)28(–32)
P. nicotianae	CMW22001	(30–)38 (–43)× (25–)32 (–58)	(24–)20(–28)	(18–)20(–24)	(13–)19(–25)×(10–)15(–24)	(18–)20(–28)
P. nicotianae	CMW22009	(42–)52(–62)× (36–)42 (–47)	(19–)21(–25)	(16–)22(–28)	(13–)19(–25)×(10–)15(–24)	(28–)34(–45)
P. nicotianae	CMW22016	(25–)45(–60)× (19–)29 (–37)	(19.8–)20(–23)	(15–)18(–20)	(13–)19(–25)×(10–)15(–24)	(26–)30(–44)
P. cinnamomi	CMW21960	(55–)75(–95)× (35–)41 (–45)	(22–)34(–40)	(18–)28(–34)	(12–)16(–24)×(10–)14(–18)	(25–)42(–54)
P. cinnamomi	CMW21979	(55–)76(–100)× (30–)40 (–56)	(25)30(–35)	(22–)26(–28)	(14–)16(–26)×(12–)12(–16)	(34–)40(–52)
P. cinnamomi	CMW21959	(45–)67(–95)× (30–)38 (–49)	(32)40(–48)	(18–)24 (–34)	(16–)20(–30)×(14–)16(–22)	(31–)37(–50)
P. cinnamomi	CMW21985	(49–)58(–66)× (39–)48 (–57)	(25–)34(–50)	(15–)30(–49)	(13–)19(–25)×(10–)15(–24)	(26–)38(–55)
P. cinnamomi	CMW21977	(40–)51(–64)× (26–)32 (–40)	(30–)40(–50)	(23–)36(–48)	(15–)18(–24)×(13–)16(–22)	(26–)38(–55)
P. cinnamomi	CMW21983	(35–)65(–80)× (24–)30 (–39)	(23–)36(–45)	(20–)32(–40)	(15–)20(–28)×(13–)17(–20)	(27–)44(–56)

Table. 4. Summary of the data analysis of the lesion lengths caused by *P. nicotianae* and *P. cinnamomi* in summer and spring when inoculated into *Eucalyptus smithii*

Variable	df	SS	MS	F Value	Pr > F	R2	Coeff Var	Root MSE
Model	79	140102.9	1773.5	37.67	<.0001	0.81	30.3	6.861840
Error	682	32111.9	47.08					
Corrected	761	172214.8						
Season	1	18458.44	18458.4410	392.03	<.0001			
Isolate	39	110303.40	2828.2923	60.07	<.0001			
Season × isolate	39	110303.40	290.7971	6.18	<.0001			

Fig 1 – Disease symptoms associated with *Phytophthora* infection on cold tolerant *Eucalyptus* spp. A. *Phytophthora* dieback in an affected stand, B. Canker at the base of the tree showing kino exudations, C. Lesion on wood following removal of the bark. Photo courtesy of J Roux, FABI, UP

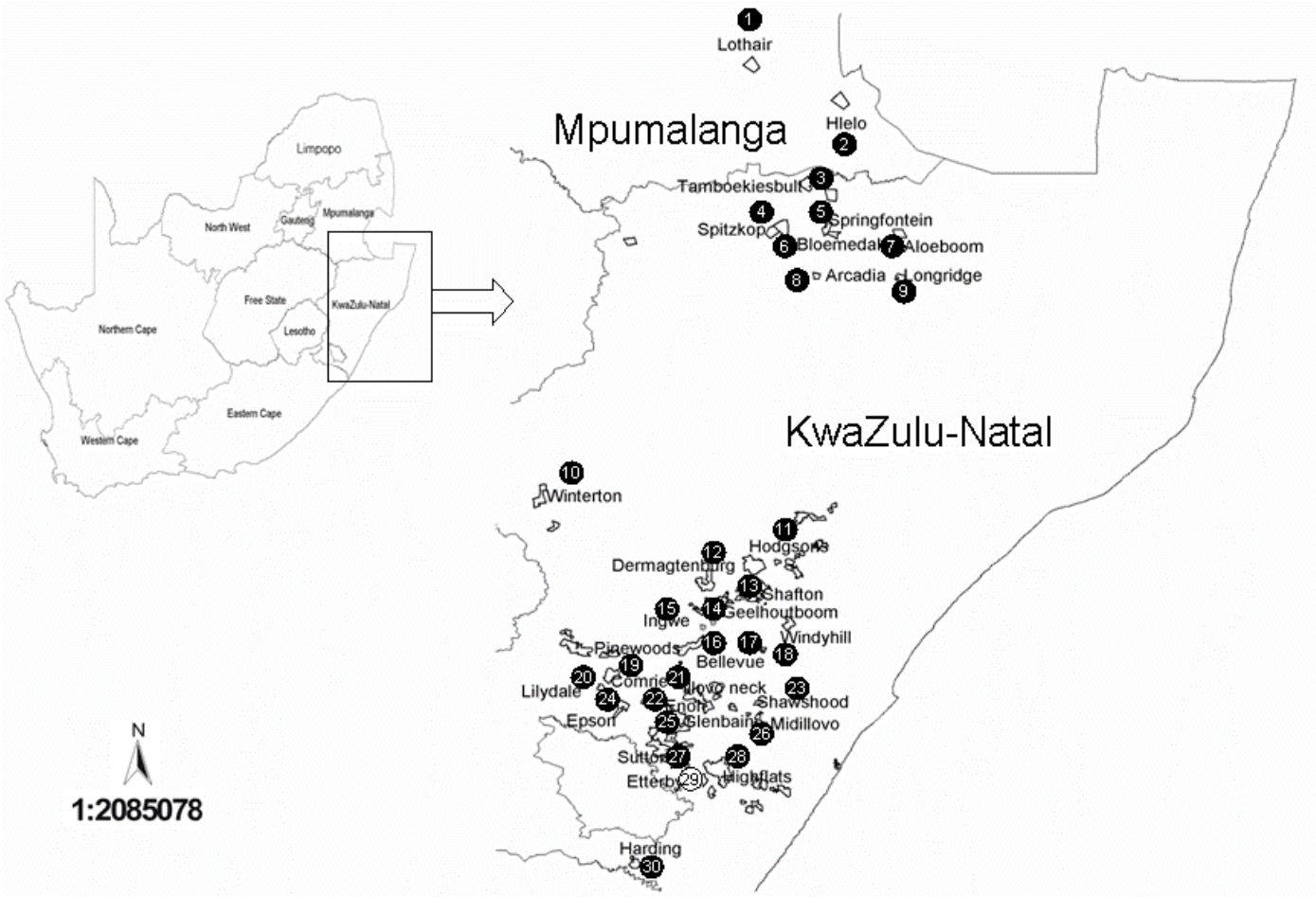

Fig 2 – Map showing the location and distribution of the 31 *Eucalyptus* stands sampled for the presence of *Phytophthora* spp. Insert map showing the location of the Mpumalanga and KwaZulu-Natal Provinces within the Republic of South Africa, where samples were taken.

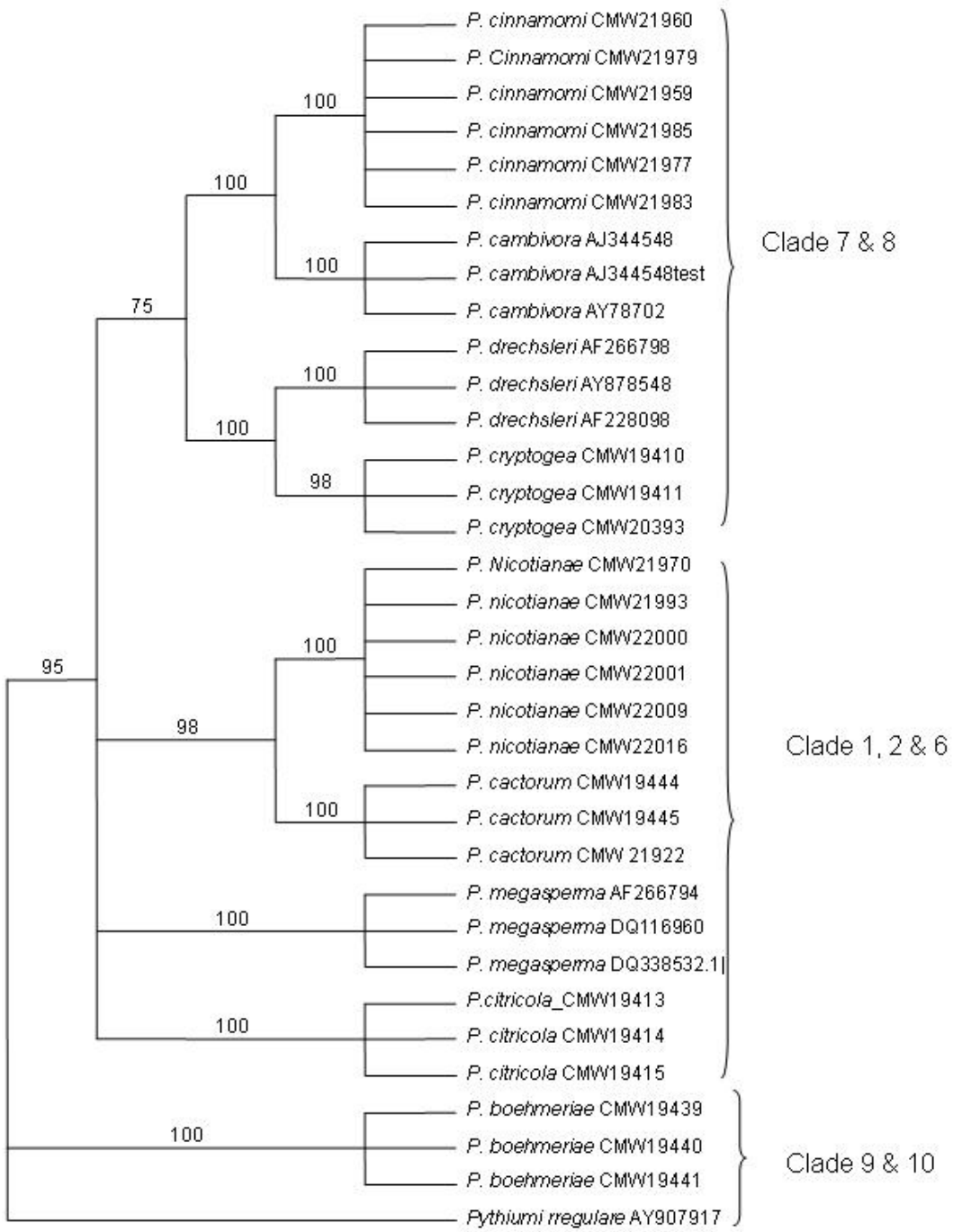

Fig – 3 ITS cladogram of *Phytophthora* spp. known to infect *Eucalyptus* spp. One of four most parsimonious trees (length=444, CI=0.743, RI=0.923, HI=0.257) from heuristic searches in PAUP (ver 4.0b1. Bootstrap support values of 1000 replicates

Fig – 4 Response of year old side branches of *Eucalyptus smithii* in the inoculated with *Phytophthora cinnamomi* and *Phytophthora nicotianae* isolates

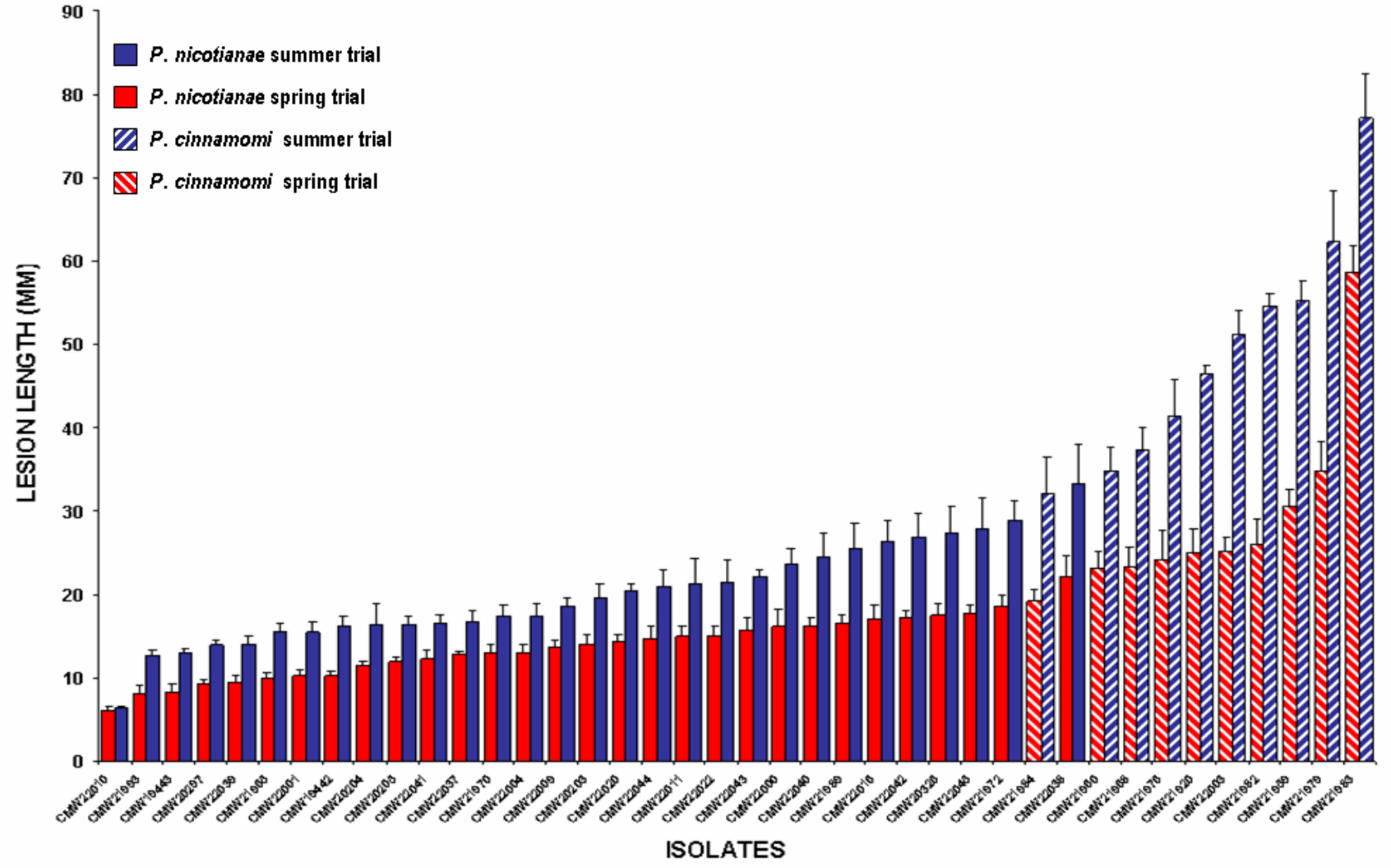

Fig 5– Ranked mean lesion length (mm) on year old side branches of *E. smithii* inoculated with 10 *P. cinnamomi* and 30 *P. nicotianae* isolates for six weeks in spring and summer trials. Error bars represent ± standard error.

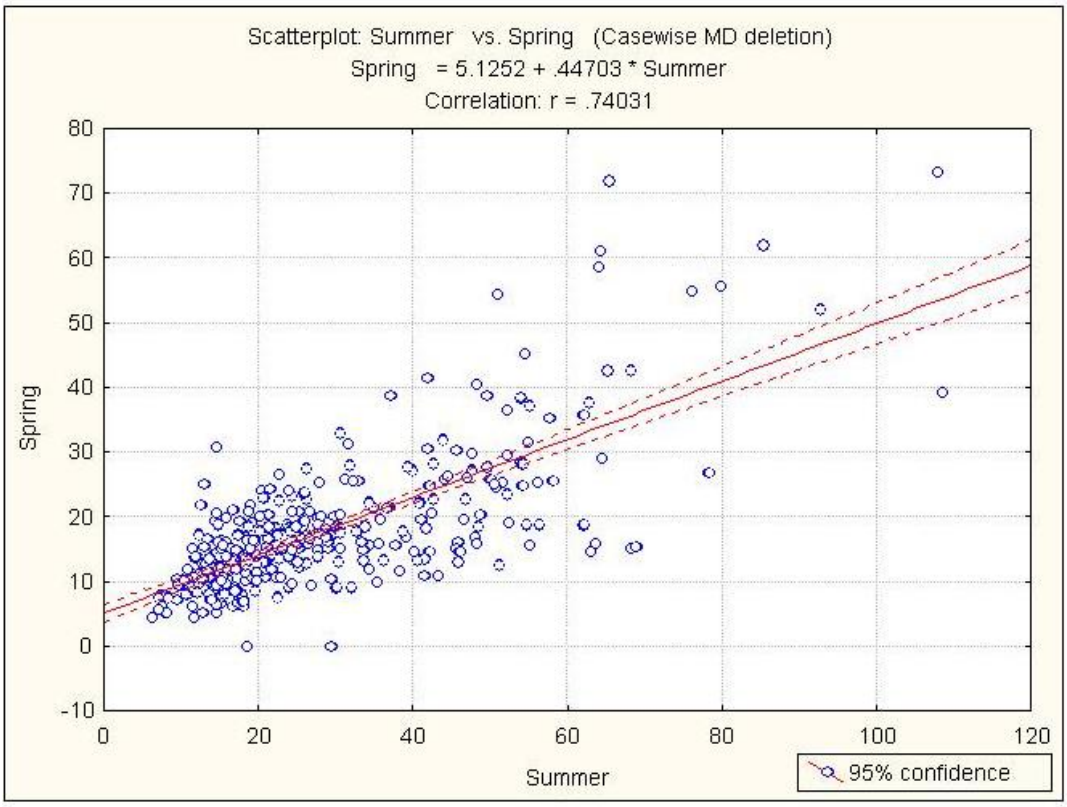

Fig 6–. Scattergram showing the correlation of the mean lesion length produced by the different *Phytophthora* isolates during the spring and summer.