Chapter Three

Role of *Phytophthora* root disease in the poor establishment of *Eucalyptus smithii* in South Africa

ABSTRACT

Eucalyptus smithii is one of the important commercial tree species cultivated in higher altitude summer rainfall regions of South Africa, but is notorious for high seedling mortality during establishment. The aim of this study was investigate the effects of site preparation and role of root disease in the poor establishment of *E. smithii*. Five study sites of standard quadrat design (8 × 12 × 4 trees per quadrat, 4 replications per study site) were established in two commercial *E. smithii* stands near Ixopo in the KwaZulu-Natal Province. Seedling mortality was monitored and recorded over 12 months following establishment. Soil samples were taken from each study site and diseased seedlings examined to determine the cause of death. Seedling mortality rates varied significantly between and within the sites throughout the monitoring period. Three *Phytophthora* spp. were detected from re-established stands and included *P. nicotianae*, *P. boehmeriae,* and the recently described *P. frigida*. None of these *Phytophthora* spp. were recovered from the grassland site. However, 20 % seedling mortality was recorded at this *Phytophthora*-free site and mortality did not differ significantly from the three re-established stands investigated. The highest mortality (47 %) was found on one re-established site that was later replanted with alternative species. The death of seedlings, together with the absence of *Phytophthora* spp. on the grassland site highlights the involvement of factors other than pathogens in the poor establishment of *E. smithii* on the stands investigated.

Key words: *Eucalyptus* establishment, seedling mortality, *Phytophthora* dieback

1 Introduction

Eucalyptus spp. are planted extensively in South Africa for the production of pulp and paper and various forest products (Owen & van der Zel 2000). Improved varieties and clones of *E. grandis* are the most commonly planted exotic tree species (Schönau 1994). Over the past few decades, *Eucalyptus* fibre plantations have expanded towards marginal areas that are inordinately cold and dry for *E. grandis* (Darrow 1996; Louw 2006). This has prompted the South African forestry industry to introduce alternative species to increase the pulp and fibre supply under such environmental conditions (Clarke 1995).

Eucalyptus smithii is one of the newly introduced species that has become an economically important and is planted on a moderate scale in the colder, higher altitude areas of South Africa (Herbert 2000). It is usually plated in the Mpumalanga and KwaZulu-Natal provinces of South Africa on sites with altitude above 1150 m, rainfall less than 900 mm and soil depth greater than 600 mm (Swain & Gardner 2003). This species is drought tolerant, grows rapidly and has superior pulping qualities compared to other commercially grown *Eucalyptus* spp. (Clarke *et al*. 1999). *Eucalyptus smithii* is notorious for high levels of post-establishment mortality that often leads to poor stocking (Swain *et al.* 2000). However, even with poor stocking, biomass production over a rotation is still high compared to other species (Clarke 1999). The post-planting mortality of *E. smithii* seedlings is most severe during the first year of establishment, but subsides as the trees mature (Swain *et al*. 2000).

The majority of plantations in South Africa are re-established on sites previously planted to either to eucalypt, wattle or pine and various silvicultural methods are used to promote survival and growth of the seedling (Smith *et al*. 2001). Poor establishment of eucalypts is often attributed to a number of factors such as poor seedling quality, damage during planting and suboptimal planting conditions, which often increase the vulnerability of seedlings to insect pests and soil borne pathogens (Caulfield *et al*. 1992). *E. smithii* is known for poor survival, particularly when planted on re-established sites (Jarvel 1998). The focus of previous studies was mainly on seedling quality and planting practice to improve post-planting survival and growth of *E. smithii* (Zwolinski & Bayley 2001). However, very little has been published on the effects of site treatment in predisposing *E. smithii* seedlings to soil-borne root pathogens. Linde *et al*. (1994) reported that *E. smithii* seedlings planted on water gaining sites are more susceptible to root and collar rot disease mainly caused by *P. cinnamomi*. Site treatment during establishment is important for the survival of eucalypt transplants (Pallett & Sale 2004). The aim of this study was investigate the effects of site preparation and the incidence of root disease caused *Phytophthora* spp. on establishment of *E. smithii*. Accurate identification of *Phytophthora* spp.

involved in early death of *E. smithii* seedlings and understanding the underlying causes of seedling mortality are key in reducing the risk of *E. smithii* seedling mortality in the future.

2 Materials and Methods

2.1 Study area and experimental design

This study was conducted on two *Eucalyptus* plantations located in the midlands region of KwaZulu-Natal Province, one of the major forest plantation areas of South Africa. It is situated in the summer rainfall region characterized by cold and dry winters. To investigate the role of various site treatments on poor plantation survival of *E. smithii* seedlings, five experimental sites were set up within two newly established commercial plantations stands located at Sutton (29° 58′, 30° 08′E) and Lilydale (30° 18′S, 29° 49′E). These plantations are separated by a distance of approximately 40 km. For each study site and factor investigated, experiments were deliberately set up on high riks sites prone to flooding and waterlogging and conducive to *Phytophthora* growth and survival and low risk sites with well-drained soil.

Details of the experiment sites are summaried in Table 1, and a brief explanation of the various land preparation treatments referred to in this study is provided below. Treatments investigated in this study included, various clearing methods (soil ripping, hot or cool burn), previous crop (reestablishment sites or grassland), development of competing weeds and water drainage. Soil ripping refers to ripping with a single tine mounted on a tractor. The removal of plantation residues using fire prior to treatment application can be either be cool or hot burn. Cool burn refers to controlled burning of plantation residues aimed at stimulating the germination of forest trees and hot burn is uncontrolled burning which lead to soil water-repellency.

Standard silvicultural land treatments treatment procedures such as pitting, watering the pit, application of fertiliser (100g super phosphate) and weeding were followed as described by du Toit (1995). Seedlings were planted into the centre of each pit (depth of 15 cm) at a spacing of 2 × 3 m. However, the Lilydale site was a new forest land site established on grassland and had been subjected to a soil ripping as a pre-planting treatment. This study site comprised of four quadrats (96 trees / per quadrat planted at 2 × 4 m spacing) and was regarded as a control site, since it has not yet been forested and most likely the soil was free of *Phytophthora*.

Four study sites each with four quadrats (96 trees/quadrat: 384 trees/study site) were set up on newly established *E. smithii* compartments at Sutton within commercial compartments D7 and D9 (two study sites in each compartment) (Fig 1). Compartment D7 and D9 at Sutton were re-establishment stands and had been subjected to a slash and cool burn pre-planting treatment. Two sites were established in compartment D7 and were designated D7a and D7b, respectively. Study site D7a was established on a well-drained and relatively flat surface while D7b was established along a flood plain, considered favourable for *Phytophthora* spp. The remaining two sites, namely D9a and D9b, were established in compartment D9. Study site D9a was established on an area with no weeds while D9b had heavy weed cover (Fig 1).

2.2 Sampling, isolation, and assessment

Seedlings were planted in December 1999, 2 months before the first assessment was conducted. Assessments were undertaken during summer (January–February), autumn (March–April) and spring months (October–November) of 2000 and 2001. No assessments were done in winter since *Phytophthora* spp. are likely to be inactive as this is a summer rainfall region and winter conditions are very cold and dry.. Each quadrat was subdivided into eight blocks containing 12 seedlings to facilitate easy sampling and mapping of sites. In order to collect data on the distribution of diseased seedlings and seeding mortality, each block was assigned a letter (A–H) and the position each seedling was mapped using a ground-based identification method which entailed walking parallel transects in 2 m × 3 m grid system.

During each evaluation, the number and position of dead and replacement seedlings planted in the position of the dead seeding was recorded. All dying seedlings within quadrats were recorded, removed, and analysed to determine the cause of death. The number of dying seedlings from which *Phytophthora* spp. were recovered in each quadrat was also recorded. Segments of the diseased plant tissue and surface disinfested roots were plated directly on selective media for (NARPH) the isolation of *Phytophthora* (Difco, Corn Meal Agar (CMA), 17 g/l^{-1} amended with 50 $\mu g/ml^{-1}$ nystatin, 200 $\mu g\ ml^{-1}$ ampicillin, 10 $\mu g\ ml^{-1}$ rifampicin, 25 μgml^{-1} pentacloronitrobenzene (PCNB), and 50 $\mu g\ ml^{-1}$ hymexazol 3 hydroxy-5-methylisoxazole, Sigma-Aldrich, St. Louis] (Hüberli *et al*. 2000). Plates were then incubated at room temperature for a period 2–3 days.

Rhizosphere soil samples were collected from each block in each quadrat at all sites to test for the presence of *Phytophthora* spp. All samples were collected and tested during the spring and summer months when soil moisture and temperature were conducive for the isolation of *Phytophthora* spp. To determine the presence or absence of *Phytophthora* spp, four sub-samples of soil were randomly collected from each block and were mixed and pooled into one sample of approximately 250 g,

resulting in 32 soil samples per site. In total, 160 soil samples were collected from all quadrats during each visit and each soil sample was placed in a sealed plastic bag. Soil samples were baited using the host and citrus leaf pieces as described by Grimm & Alexander (1973). Baited leaf pieces were harvested and plated on to a modified selective media for *Phytophthora* as described above.

Phytophthora isolates retrieved from the soil and from diseased plant material were sub- cultured on to clarified V8 juice agar for further identification and maintenance. Sporulation of *Phytophthora* isolates was induced by mineral salt solution (Erwin & Riberio 1996). Isolates were stored in sterile distilled water at room temperature (Ko 2003). All isolates obtained in this study were deposited in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

2.3 Analysis of results

The severity of the disease within each quadrat, measured in terms of seedling mortality or survival, was recorded, at least twice per season, during the 12 months after establishment. The total number of dead seedlings was used as a measure of the rate of mortality over time. The mortality of the seedlings was expressed as the percentage of dead seedlings at the end of each sampling period, relative to the number of seedlings at the beginning of sampling period.. A generalized linear model was used to analyse the relationship between seedling mortality with different variables including study sites, season, presence, or absence of *Phytophthora* spp. in diseased seedlings and rhizosphere soil. All statistical analyses were conducted using the SAS/STAT® software, version 8.02 (SAS Institute, Inc., Cary, North Carolina).

2.4 Identification of *Phytophthora* isolates

Phytophthora isolates isolated retrieved from roots and infected collars from symptomatic seedlings collected from the study sites were identified with the aid of keys in Stamps *et al*. (1990). Characteristics used to group isolates were the sporangia, growth patterns in culture, morphology of the hyphae oogonia and antheridia. The identity of three representative isolates for each species were confirmed using comparisons of ITS sequences as described previously (Maseko *et al*. 2007)

3 Results

3.1 Seedling mortality

Result of analysis of variance (ANOVA) showed highly significant differences in mortality levels on all study sites ($P < 0.001$, $F = 7.9$, $R^2 = 0.56$). Highly significant differences in seeding mortality were also found between study sites ($P < 0.001$, $F = 149.32$), within the different quadrats in sites ($P < 0.001$, $F = 4.34$) and between quadrats over time ($P< 0.01$, $F = 1.5$). A summary of the result analysis is presented in Table 2. First-year seedling mortality data for all study sites is illustrated in Fig 2. It is evident from the graph that seedling mortality rates varied between and within the sites throughout the monitoring period. The highest seedling mortality was recorded at site D9b in Sutton. Seedling mortality occurred shortly after planting and it progressed gradually during the first growing season.

At the end of the monitoring period the total cumulative mean mortality on all five study sites was 24 %. However, the total cumulative mean seedling mortality on four of the five study sites was below 20 % (D7a = 16 %, D7b = 18 %, D9a = 14 %, Lilydale = 19 %). The exception was site D9b at Sutton, which had total cumulative seedling mortality of 47 %.

3.2 Seedling mortality due to *Phytophthora*

Due to the challenge associated with distinguishing between seedling mortality caused by *Phytophthora* and other causes. Only if *Phytophthora* was re-isolated from the infected seedling was death attributed to *Phytophthora* being the main cause of seeding death. The mean percentage recovery of *Phytophthora* spp. from diseased seedlings was lowest (29 %) during the drier autumn month of April and highest during the wet summer month of January (58 %). A significant difference ($P < 0.01$) in the recovery of *Phytophthora* from diseased plants was observed within and between the four study sites as well as during the different sampling periods. The highest pathogen recovery and seedling mortality was recorded on theD9b study site. The recovery of *Phytophthora* spp. from study sites, D7a, D7b, D9a, and D9b is shown in Fig 3. Dying seedlings appeared withered especially during autumn months (March and April) when soil conditions where dry. The mean monthly rainfall data from the weather station, Ixopo located near the experimental site is illustrated in Fig 4. All diseased seedlings assayed had noticeable lesions on the upper root, collar, and the lower stem regions. In general, disease symptoms were observed either on single seedlings scattered on each quadrat or in small patches in areas prone to water-logging. No seedling mortality was attributed to *Phytophthora* from Lilydale (control site) since *Phytophthora* spp. were could not be retrieved from either soil or seedlings. Instead, seedling damage due to cutworms was observed at the Lilydale study sites.

3.3 Recovery of *Phytophthora* spp. from soil

A total of 800 soil samples were collected from all experimental sites for the duration of this study. *Phytophthora* spp. were only retrieved from 9 % (71 out of 800) of the soil samples assayed. A summary of the results for isolation of *Phytophthora* spp. from rhizosphere soil is given in Table. 3. *Phytophthora* spp. were isolated only from re-established sites (D7a, D71b, D9a and D9b) and not from the grassland site. Isolation of *Phytophthora* spp. varied from site to site during the monitoring period and it had a similar pattern with the mean monthly rainfall in the area (Fig 4). The highest recovery of *Phytophthora* spp. was recorded during the warm, rainy months from October to January and the lowest during February to April. Significant differences in the recovery rate of *Phytophthora* spp. were found for different sites (F-ratio 3.27, $P < 0.05$). Study site D9a had significantly higher pathogen levels in the soil relative to the other re-establishment sites investigated.

3.4 Identification of *Phytophthora* isolates

Three *Phytophthora* spp., namely *P. nicotianae*, *P. boehmeriae* and *P. frigida* were retrieved from the soil samples. *Phytophthora nicotianae* and the newly described *P. frigida* were isolated most frequently and were recovered from all re-establishment sites. *Phytophthora boehmeriae* was recovered only occasionally and only from quadrat D7 b (Table 3). The identity of the *P. nicotianae*, *P. boehmeriae* and *P. frigida* was determined based on morphological characteristics and further confirmed using DNA sequence comparisons.

4 Discussion

In general, the results of this study indicate that the diffeent land preparation treatments on re-established and on sites that have not yet been forested have little effect on the final survival of *E.smithii* in the field. However, the effect of the land preparation treatment was only significant ($P<$ 0.01) at only one of the reestablsihement site. In particular, study D9b had a highest mortality levels and *Phytophthora* species is most likely playing an important role but other factors such as cut worm, poor drainage contribute to seedting mortality. Site D9a appeared to be the best with lower mortality but *Phytophthora* was reisolated from diseased plant material and sol. Not significant difference in mortality levels was found at site D7a and D7b but a proposition of death due to *Phytophthora* was recorded. The Lilyadale site had a modate level of mortality. This was enexpected and interesting as was considered a disease free site and control. Quite a high proposion of the trees that died has cutworm damage and this togheter with normal transport, stock seedlings mortality gave a higher that expected numbers of deaths.

In view of the outcome of Koch's postulates Results of this study show that *Phytophthora* spp. are involved in the death of *E. smithii* seedlings but that a proportion of seedling death is caused by other agents. *Eucalyptus smithii* is well-known susceptible afforestation species with specific planting requirements (Swain *et al*. 2000). Unsuitable planting sites results in poor establishment and this is often aggravated by the susceptibility of the species to the *Phytophthora* root and collar rot (Wingfield & Roux 2000).

Previous reports attribute poor survival of *E. smithii* to various factors including seedling age, land-use history of regeneration sites, planting methods and *Phytophthora* root and collar rot Bayley & Snell (1997) and Jarvel 1998 and Herbert 2000 reported *E. smithii* seedling mortality levels ranging from between 31 % (survival 69.3 %) and 41 % (survival 59.5 %). In this study, the total mortality assessed after a period of 12 months was below 20% at four sites and 43 % on one other site. No significant difference in seedling mortality was found amongst the four sites (D7a D7b, D9a, Lilydale), which were subjected different pre-planting treatments. This observation indicates that pre-planting treatments and land-use history of a site did not contribute to the poor survival of *E. smithii* seedlings. Results obtained in this study indicate that seedling mortality due to undetermined causes, but propably including transplant shock, J rooting, wounding and localized drought occurs shortly after planting and gradually increase up to 20 %. Similarly, Bayley & Snell (1997) reported that most seedling mortality occurred within the first month after planting and the seedling age was found to be the main course of seedling failure.

In this study, recovery of *Phytophthora* spp.from diseased seedlings followed a clear seasonal pattern. The recovery of *Phytophthora* spp. from diseased seedlings was low (29 %) during Autumn and high during summer (58 %). In addition, significant differences ($P < 0.01$) in the recovery of *Phytophthora* spp. from diseased plants were observed within, between the four reforestation study sites (D7a, D7b, D9a, and D9b), and during the different sampling periods. The high level of recovery of *Phytophthora* spp. from diseased plants and high seedling mortality recorded at the D9b study site, suggest that root rot is a key factor contributing to seedling death in that situation.

Seedling death was more apparent during February and March when soil conditions where dry. Previous studies have shown that drought stress enhances the development of *Phytophthora* within the infected eucalypts tissue is enhanced (Cahill *et al*. 1985; Shearer & Tippett 1989). In South Africa, dieback of *E. smithii* associated with *Phytophthora* spp. is prevalent on plantation sites prone to both waterlogging and drought stress (Herbert 2000). It is known that soil moisture also plays an important role in the development of *Phytophthora* diseases, because zoospores require free water and warm soil conditions for dispersal (Erwin & Ribeiro 1996). Thus, it is likely that the seedlings were infected

spring and summer, when there were favourable temperature and moisture in the soil for infection and that the symptoms were observed somewhat later. Seedling mortality on the control grassland site could not be attributed to *Phytophthora* as these pathogens were not retrieved from either soil or dying seedlings.

Poor recovery of *Phytophthora* sp from the soil in re-establishment sites observed in this study is consistent with results of previous studies (Erwin & Ribeiro 1996; McDougall *et al*. 2002). Recovery of *Phytophthora* spp. improved when diseased plant material was plated directly into selective media. This observation suggests strongly that plant tissue most probably acts as a reservoir for *Phytophthora* inoculum. Observations from this study also indicate that *Phytophthora* spp. have an uneven distribution within the quadrats that tested positive for the presence of this pathogen. This observation is consistent with other reports showing uneven distribution of *Phytophthora* inoculum in soil (Marks *et al*. 1975; Shearer & Shea 1987).

Phytophthora nicotianae, *P. boehmeriae* and *Phytophthora frigida* were retrieved from the soil and diseased plant samples from re-establishment sites. These results suggest that more than one *Phytophthora* sp. is responsible for the *Phytophthora*-related deaths of *E. smithii* seedlings at the sites investigated. *Phytophthora cinnamomi* was not retrieved from any of the study sites although it has previously been reported as the most common pathogen associated with root and collar rot of cold tolerant eucalypts in South Africa (Lundquist & Baxter 1985). *Phytophthora nicotianae* and *P. boehmeriae* have also been reported as pathogens of *Eucalyptus* spp (Sankaran *et al*. 1995; Linde *et al*. 1994). Pathogenicity tests conducted in South Africa have shown that *P. nicotianae* and *P. boehmeriae* are pathogenic to *Acacia mearnsii* and *Eucalyptus* spp. (Linde *et al*. 1994, Maseko *et al*. 2001; Roux & Wingfield 1997). The role of *Phytophthora frigida* as a pathogen requires further investigation. In conclusion, five study sites were established on newly established *E. smithii* stands to investigate the effects of site preparation and role of root disease in the poor establishment of *E. smithii*. Our results indicate that a number of factors contribute to the poor establishment of *E. smithii* and at least three *Phytophthora* spp. are involved in the early death of seedlings.

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Table 1 Details of compartments used in this study.

Plantation compartment details						Land preparation Method		
Plantation Name	Stand No (Size)	Quadrat designation	Species	Previous crop	Month of establishment	Clearing method	Site problems Recorded	*Rating
Sutton	D7 (33.6 ha)	D7a	*E. smithii*	Pine	Jan 2000	slash & burn	None	Low Risk
Sutton	D7 (33.6 ha)	D7b	*E. smithii*	Pine	Jan 2000	slash & burn	Flood plain	High Risk
Sutton	D9 (36 ha)	D9a	*E. smithii*	Pine	Nov 1999	slash & burn	None	Low Risk
Sutton	D9 (36 ha)	D9b	*E. smithii*	Pine	Jan 2000	slash & burn	Weeds & Cutworms	High Risk
Lilydale	Lilydale	Control	*E. smithii*	Grass	Nov 1999	soil ripping	None	Low Risk

*Rating based on results reported by Jarvel, 1998

Table 2 Summary of the generalized linear model analysis for mortality

Variable	***df***	***SS***	***MS***	***F Value***	***Pr > F***	***R^2***	***Coeff Var***	***Root MSE***	***Mortality Mean***
Model	99	156904.63	1584.9	7.86	0.0001	0.6	60.5	14.2	23.5
Error	620	124986.0	201.6						
Corrected Total	719	281890.67							
			Summary of mortality analysis between study sites quadrats and time						
Site	4	120402.6	30100.7	149.3	0.0001				
Quadrat (site)	15	13125.68	875.04	4.34	0.0001				
Time (site ×quadrat)	80	23376.3	292.2	1.45	0.0092				

Table 3: Summary of the results for the recovery of *Phytophthora* spp. from rhizosphere soil

Site	Mean percentage recovery of *Phytophthora* spp. from soil (N =32)					Total mean frequency of recovery (%) of *Phytophthora* spp. from soil sampling (160 samples)	*Phytophthora* spp. recovered
	February	March	April	October	January		
D7a	9.4	6.3	3.1	13	19	10	*P. nicotianae, P. frigida*
D7b	13	9.4	3.1	9.4	22	11	*P. nicotianae, P. boehmeriae*
D9a	9.4	6.3	3.1	6.3	16	8	*P. nicotianae, P. frigida*
D9b	16	9.4	6.3	16	28	15	*P. nicotianae. P. frigida*
Lilydale	0	0	0	0	0		None

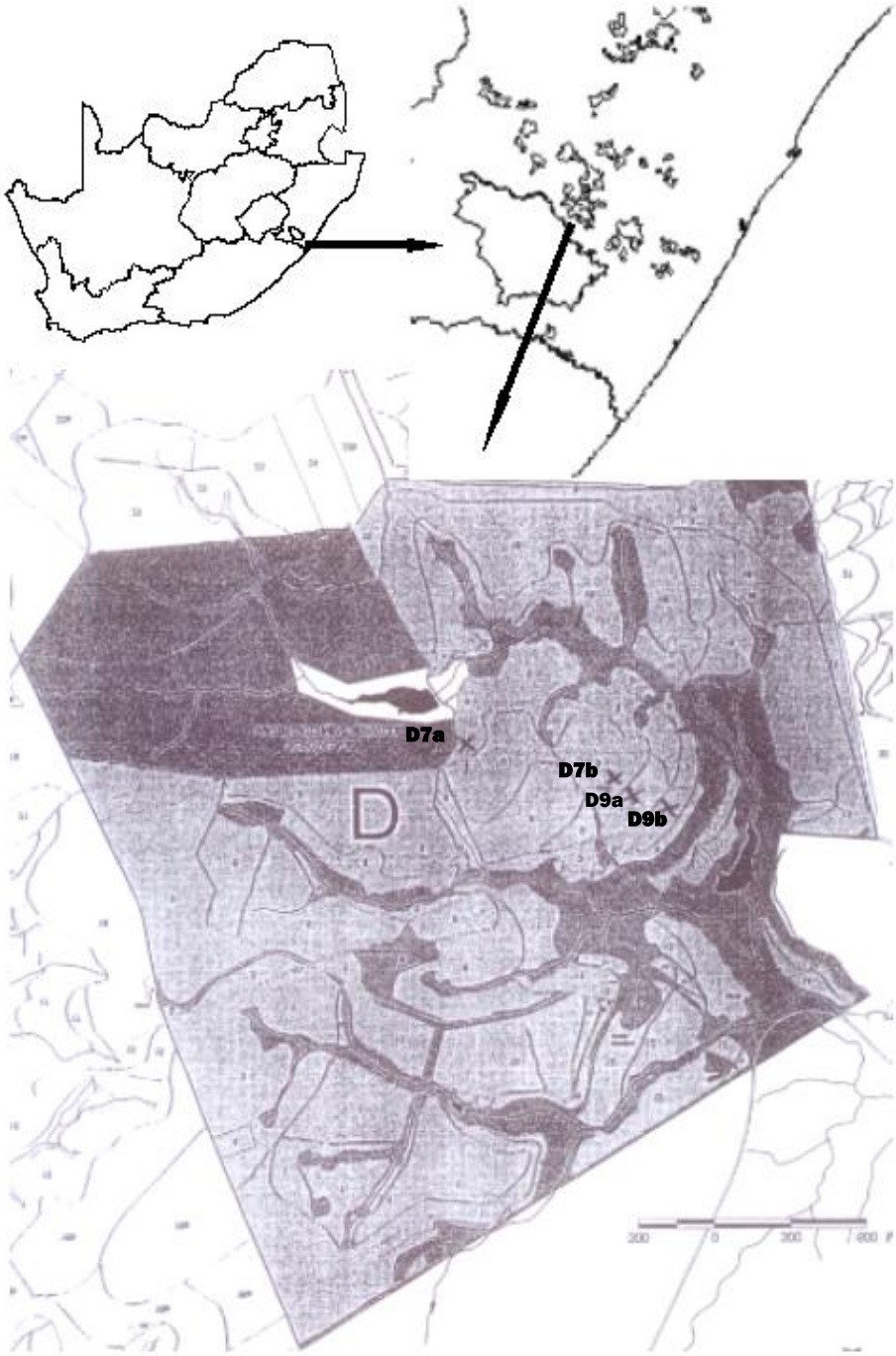

Fig 1 – Map of showing location of the experimental plot at Sutton plantation, in the KwaZulu-Natal Province of South Africa. The crosses (×) on the map indicate the location of four quadrats (D7a, D7b, D9a and D9b).

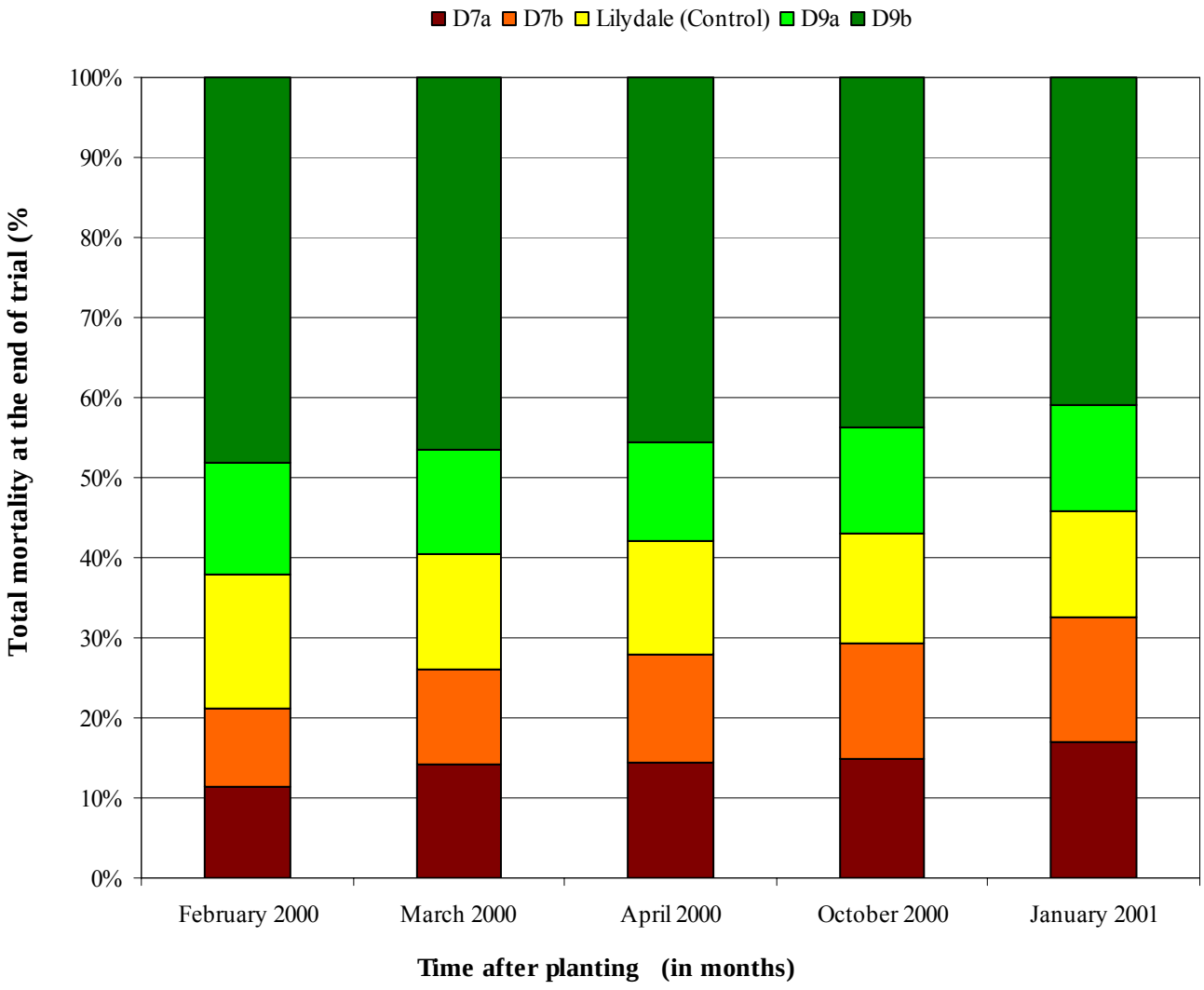

Fig 2 –Mortality levels (%) of *E.smithii* seedlings at the end of trial

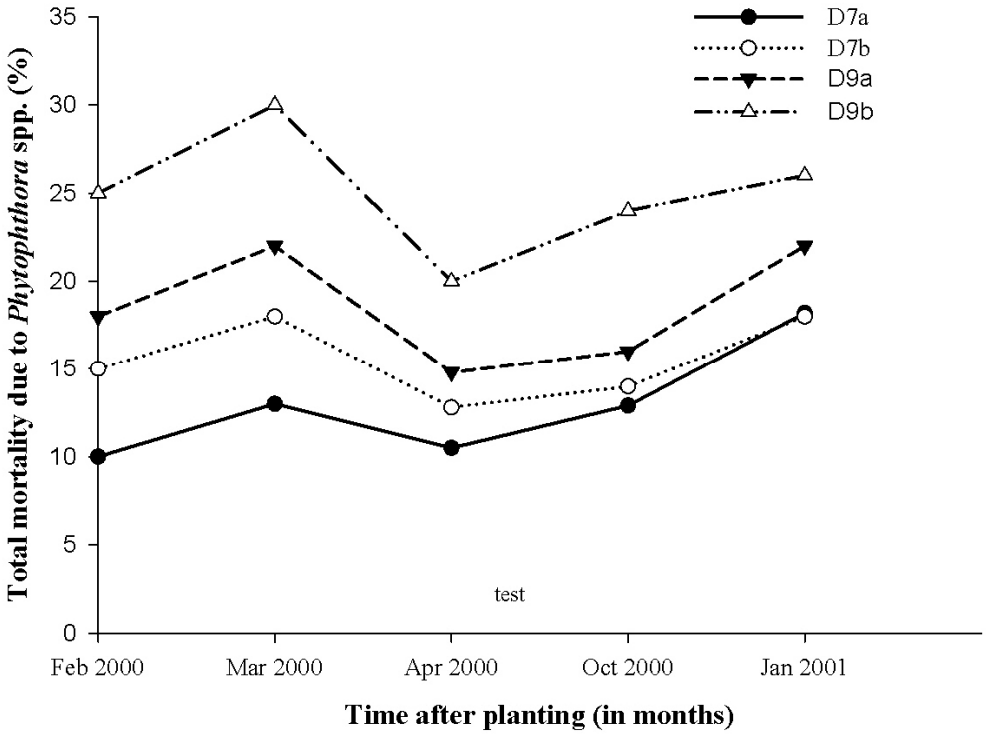

Fig 3 –Total mortality levels of *E.smithii* seedlings associated with *Phytophthora* dieback at the end of trial

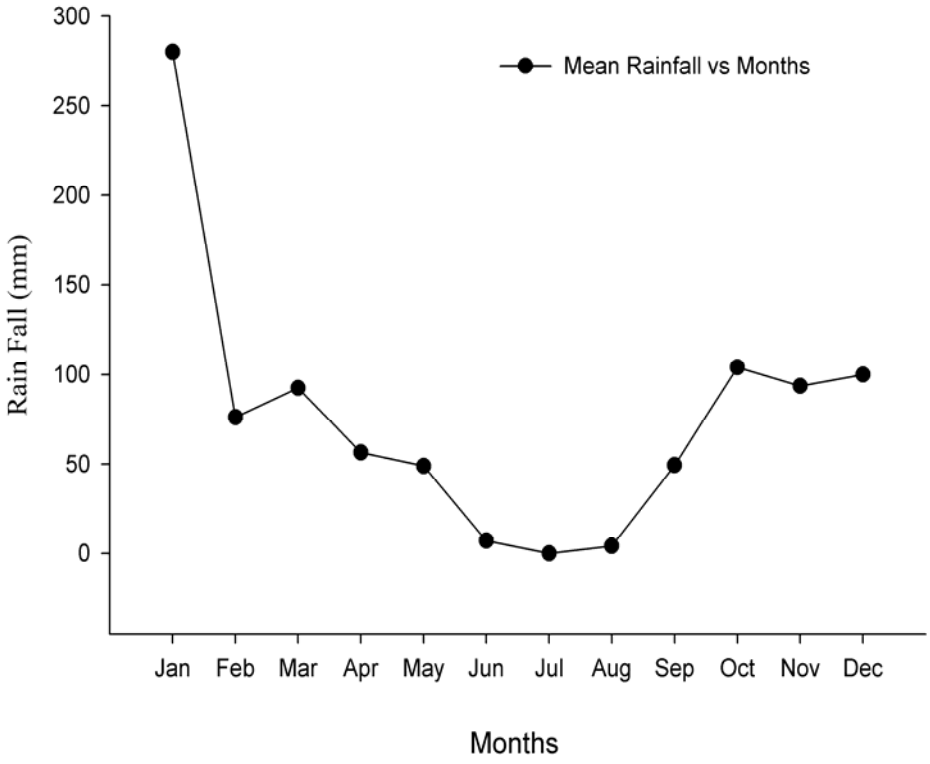

Fig. 4 Mean Monthly Rain (mm) Data for station [0210099 2] - IXOPO