



### Screening of *Eucalyptus smithii* half-sib families for tolerance to infection by *Phytophthora cinnamomi* and *P. nicotianae*

#### ABSTRACT

*Phytophthora* root and collar rot caused by *Phytophthora cinnamomi* and *P. nicotianae* is associated with severe disease and mortality of *Eucalyptus smithii* in forest nurseries and plantations in South Africa. Variation in mortality levels has been observed between *E. smithii* genotypes in three different breeding trials in Kwa-Zulu-Natal. Twelve half-sib *E. smithii* families were screened for tolerance using two rapid screening techniques. A large number of one-year-old seedlings were stem inoculated using pre-selected virulent isolates of *P. cinnamomi* and *P. nicotianae* to determine their susceptibility under greenhouse conditions. Total soluble phenolic compounds were extracted from young, fully expanded leaves of plants representing 12 half sib *E. smithii* families. Concentrations of the resulting crude extracts were analysed using High Performance Liquid Chromatography. *P. cinnamomi* resulted in rapid lesion development and produced significantly longer lesions relative to those inoculated with *P. nicotianae*. The disease severity ratings for the *E. smithii* families based on the inoculations correlated with field observations for susceptibility. The mean total soluble leaf phenolic concentration was found to be higher in tolerant than in susceptible *E. smithii* genotypes. Screening using inoculation and assessment of total soluble leaf phenolics may provide a rapid means to screen *E. smithii* for resistance to infection by *P. cinnamomi* and *P. nicotianae*

**Keywords:** *Phytophthora cinnamomi*; *P. nicotianae*; disease tolerance, stem inoculation

## 1 Introduction

*Eucalyptus smithii* was first introduced into South Africa during the early 1900s, chosen for its high leaf cineole oil content (Poynton 1979). It is tolerant to drought, snow and frost (Gardner & Swain 1996), grows vigorously, and has outstanding pulping properties in comparison to other eucalypts grown in South Africa (Clarke *et al.* 1997; 1999). As a result, *E. smithii* is an ideal species for establishment in frost and drought prone areas situated above an altitude of 1000 m (Darrow 1996; Swain & Gardner 2003).

*Eucalyptus smithii* is well known to suffer from high seedling mortality in nurseries and plantations sites, leading to poor establishment and stocking (Bayley & Snell 1997). Poor establishment of *E. smithii* in plantations is due to a number of factors including transplanting over aged seedlings, unsuitable planting sites and root and collar rot (Swain *et al.* 2000). Of these, root and collar rot disease is the single most limiting factor for large scale planting of *E. smithii* in South Africa (Herbert 1994). The introduced soil borne pathogen *P. cinnamomi* is a well known causal agent of root and collar rot disease of *E. smithii* (Linde *et al.* 1999). However, recently *P. alticola*, *P. frigida* and *P. nicotianae* have also been reported to be cause this disease of *E. smithii* (Maseko *et al.* 2001; 2007).

Breeding and selection for disease tolerant planting stock is an important part of an integrated strategy to reduce losses caused by fungal diseases of non-native trees in plantations in South Africa (Wingfield & Roux 2000; Wingfield *et al.* 2004). It also is the most economical and practical method for the long-term management of *Phytophthora* root and collar rot. Based on field reports, the susceptibility of *E. smithii* to *Phytophthora* spp. is worst during the first year of establishment, but subsides considerably as the trees mature (Bayley & Snell 1997, Jarvel 1998). Some individual trees within *E. smithii* half-sib families planted on sites with a history of *Phytophthora* root and collar rot survive despite exposure to *Phytophthora* inoculum. This suggests that there is some level of genetically controlled disease tolerance amongst the half- sib families. Tolerance to *P. cinnamomi* has been reported for some forest species such as *Pinus radiata*, *E. marginata* and *E. regnans* in Australia (Butcher *et al.* 1984; Harris *et al.* 1985; Stukely & Crane 1994). In contrast, there is limited information available regarding the susceptibility of plantation trees to *P. cinnamomi* in South Africa (Wolfaardt *et al.* 1997; Maseko 1999).

Genetic improvement programs for *E. smithii* and development of rapid and reliable disease screening methods against root pathogens are two strategies that can be used to achieve higher yields in intensively managed forest plantations. The stem inoculation method has been used successfully for selecting dieback resistant *E. marginata* and *P. radiata* seedlings in Australia (Butcher *et al.* 1984; Stukely & Crane 1994). This method is relatively simple and cost effective to screen large number of genotypes. In South Africa this method was successfully used to screen *E. fraxinoides* and *Pinus*

hybrids under field conditions (Wolfaardt *et al.* 1997; Roux *et al.* 2007). In this study, large numbers of half-sib seedlings of *E. smithii* families were screened for susceptibility to *P. cinnamomi* and *P. nicotianae*. This was achieved by using stem inoculation technique. Results were further correlated with the total leaf phenolic content of the inoculated families. The results obtained were then compared with the available field data relating to susceptibility of *E. smithii* to root rot.

## 2 Materials and Methods

### 2.1 Plant material

Twelve half-sib *E. smithii* families were used in this study and these represented a portion of the progeny selected from a collection of 50 open-pollinated parent trees that had been established in a provenance trial. Three trial sites were established on stands with a history of *Phytophthora* root rot. These trial sites were located at Gourock (30° 08'S, 27° 30'E), Palerang (30° 40'S, 29° 24'E) and Mullon (29° 47'S, 29° 54'E) in Kwa-Zulu-Natal Province.

Twelve half-sib *E. smithii* families were selected to represent the six most tolerant and susceptible families based on their performance in the provenance trials. Seeds were sown in seedling trays (340 × 340 mm; consisting of 49 cavities with 80 ml vol) containing composted bark medium. Seedlings were re-potted into 2 L black plastic pots containing a river sand potting mix. Seedlings were grown in a shade house, watered daily and fertilised weekly. During this period, the seedlings were pruned periodically to retain a single stems of 5-10 mm in diameter. A month prior to the commencement of the inoculation experiment, seedlings were moved to a greenhouse with an ambient temperature (25 ± 5 °C) in order to acclimatize them to these conditions.

### 2.2 Inoculum preparation

Two of the single most aggressive isolates of *P. cinnamomi* (CMW19408, isolated from *E. fraxinoides*) and *P. nicotianae* (CMW19444, isolated from *E. smithii*) were selected based on previous results from an inoculation trial on 1-year-old *E. smithii* (Maseko unpublished data). The inoculum for the trial was prepared by cutting mycelium-covered plugs from the edges of an actively growing colony and transferring them to Petri dishes (90 mm) containing half strength Potato Dextrose Agar (Biolab Agar; 20 g<sup>-1</sup> Biolab, Johannesburg). Plates were sealed with cling plastic wrap and incubated for 5 days in the dark at 25 °C.

### 2.3 Inoculation procedure

Inoculations were first conducted in October 2001 and these were repeated in September 2002. Individual trees representing the twelve half-sib *E. smithii* families were inoculated using a 4 mm

(diam) cork borer to remove a piece of bark more or less at the midpoint of the stems. A 4 mm<sup>2</sup> agar disc colonised by one of the two *Phytophthora* species was inserted into the wound and sealed with Parafilm (M, Pechiney Plastic Packaging) to restrict desiccation. Control seedlings were inoculated with sterile agar disks.

In total, 1920 half-sib seedlings were used for the experiment and there were 40 replicates per plant family. Due to uneven growth of seedlings the stem diameters and heights of the inoculated seedlings were measured. Each family was then divided into two random blocks consisting of 20 thicker (10 mm, diam) and taller (90–110 cm) and 20 thinner (8 mm, diam) and shorter (65–80 cm) stems. Each block included two control plants for each half-sib family. All of the *E. smithii* families were arranged using a completely randomised design in a greenhouse. After 3 weeks, the bark around the points of inoculation was carefully scraped with a scalpel blade to expose the wound. Lesion lengths were measured above and below the inoculation points on the surface of the stems. Small pieces of the host tissue cut from the margins of lesions were plated onto selective medium to re-isolate the pathogens used for the inoculations. The identity of the recovered *P. cinnamomi* and *P. nicotianae* isolates was confirmed using light microscopy.

#### 2.4 Statistical analysis

Analysis of variance of resulting mean lesion lengths measured after 3 weeks was computed and compared between sources of variance (family genotypes and pathogen) and co variance (height and diameter) using the generalized linear model (GLM) procedure and ANOVA. Data sets were checked for normal distribution and equal variance and transformed using log transformation. Results of the September and October trials were analysed separately. Analyses of all data were performed using the SAS/STAT® Software, Version 8.02 (SAS Institute, Inc., Cary, North Carolina).

#### 2.5 Extraction and determination of total phenolics

Young and fully expanded leaves (3-5 per seedling) were excised from 10 randomly selected seedlings representing the 12 half-sib families used in the inoculation trials. These thus represented six families known to be susceptible to *P. cinnamomi* and six considered more resistant to the pathogen. Pooled leaf samples from each genotype were washed; air-dried and sealed in plastic bags, stored at -70°C and lyophilized in a freeze dryer 48 hours before use. Leaves were crushed into a fine powder using a mortar and pestle and passed through a fine steel sieve (20 mesh screen). The homogenised fine leaf powder was weighed and 0.05g was transferred into 1.5 ml Eppendorf tubes. The experiment was done in triplicate for each of the plants representing the 12 half-sib families.

Total phenolics were extracted using a modification of the method described by Cork & Krockenberger (1991). Phenolics were first extracted by adding 1 ml methanol-acetone-water (7:7:1

v/v) solvent solution to each tube. The mixture was incubated overnight on a rotary shaker (set at 200 rpm, 25 °C). The tubes were then centrifuged at 10 000 rpm for 1 min and the supernatant was collected and transferred into new 1.5 ml Eppendorf tubes. The pellets were sequentially extracted three times using the same volume of methanol-acetone-water (7:7:1 v/v) followed by a 30 min incubation on a rotary shaker (200 rpm, 25 °C). The resulting supernatants were mixed and concentrated to 1 ml under vacuum. Deionized water was added to the supernatant and the tubes were centrifuged at 10 000 rpm for 1 min. This step was repeated three times to remove chlorophyll and the resulting upper phase supernatant was adjusted to 1 ml for further analysis using High Performance Liquid Chromatography (HPLC) and the results were analyzed statistically.

In total, 20 µl of plant extract from each of the ten plants representing the 12 half-sib families was injected into the HPLC. The HPLC gradient elution was performed using a linear gradient of 10% acetonitrile in water for 1 minute. The concentration of acetonitrile was increased linearly to 55% acetonitrile over 15 minutes and the flow rate of 2 ml/min maintained. The chromatographic apparatus consisted of three phase high-pressure solvent delivery pumps (Varian, Model 9012). Ferrulic (FA) and p caumaric (PCA) acids were also used for comparative purposes.

### 3 Results

#### 3.1 Greenhouse trials

*E. smithii* plants inoculated with *P. cinnamomi* and *P. nicotianae* developed necrotic lesions within three weeks and control plants showed a wound response only (Fig 1). There were significant differences in the response of the plants representing the 12 half-sib *E. smithii* families to the different *Phytophthora* spp. Lesions produced by *P. cinnamomi* were significantly longer ( $P < 0.001$ ) and developed more rapidly than those inoculated with *P. nicotianae* in both trials. When these results were presented graphically, the mean lesion lengths for each family showed a range of disease tolerance to both pathogens (Fig 2). Three half-sib families (SN21, SN30 and SN37) consistently developed longer lesions (> 25 mm) when inoculated with both *P. cinnamomi* and *P. nicotianae* and were considered susceptible. Families (SN17, SN45 and SN47) produced shortest mean lesion lengths (8 – 11 mm) and were considered disease tolerant.

Analysis of variance showed significant main effects of half-sib families ( $P < 0.001$ ), the pathogens ( $P < 0.0001$ ) and the interaction ( $P < 0.001$ ) between these factors (Table 1). The data revealed significant effects ( $P < 0.05$ ) relating to stem diameter and height as covariate factors. In both trials, significant differences in lesion length were found among individual seedlings and between the half-sib families following inoculation with *P. cinnamomi* and *P. nicotianae*. The mean lesion lengths induced by isolates of *P. cinnamomi* ( $r^2 = 0.9983$ ,  $P < 0.0001$ ) and *P. nicotianae* ( $r^2 = 0.9965$ ,  $P < 0.0001$ ) were significant and showed a strong positive correlation for lesions length for both trials (Fig 3).

### 3.2 Comparison between field survival and green house inoculations data

Survival of *E. smithii* families planted on *Phytophthora*-infested sites ranged between 41 % and 86 % in three trial sites (Jones unpublished data). Based on field survival data, six half-sib families (SN8, SN21, SN24, SN26, SN30 and SN37), were considered susceptible while six half-sib families (SN2, SN6, SN14, SN17 SN45 and SN47) were considered tolerant (Table 2). These field survival results were used to distinguish between susceptible and tolerant families. The results of the greenhouse inoculation trials indicated that only three of 12 families (SN17, SN45 and SN47) had a significantly higher level of tolerance to *P. cinnamomi* and *P. nicotianae*. Half-sib families SN21, SN30 and SN37 that were highly susceptible in the field were also highly susceptible in the greenhouse inoculation tests.

### 3.3 Total phenolic content

The HPLC chromatograms of crude leaf extracts for plants representing the 12 half-sib *E. smithii* families had similar patterns with one major peak and five minor peaks. Results showed that the mean phenolic content in the leaves for each of the twelve half-sib *E. smithii* families varied significantly ( $P < 0.05$ ) (Fig 4). The highest, medium and lowest mean leaf tannin content for the 12 half-sib *E. smithii* families ranged between 345 – 360 mg<sup>-g</sup> dry wt, 200 – 298 mg<sup>-g</sup> dry wt and 148 – 180 mg<sup>-g</sup> dry wt, respectively. The results show that the three families, SN17, SN45 and SN47, which had low percentage field mortality and produced significantly shorter lesion in the greenhouse trial had higher leaf phenolic concentrations.

## 4 Discussion

Results of this study showed that it was possible to use rapid screening techniques to obtain a statistically reliable estimate of susceptibility and tolerance of *E. smithii* breeding stock to *P. cinnamomi* and *P. nicotianae*. The two techniques employed are relatively simple and cost effective and could be used to screen large numbers of genotypes relatively rapidly. Three tolerant and three susceptible *E. smithii* half-sib families were identified using the stem inoculation technique. In addition, we found that field tolerant genotypes had higher concentrations of leaf phenolic compounds than susceptible genotypes. Results obtained using the two techniques compared well and reflected field performance relating to *Phytophthora* root rot.

The *Phytophthora cinnamomi* isolates used in this study consistently produced significantly larger lesions than those of *P. nicotianae* in both trials. Mean lesion lengths that developed on the different host genotype covered showed a range of disease tolerance and susceptibility to both pathogens, which

indicates genetic variation among the half-sib families. This genetic variation to *P. cinnamomi* is consistent with results of similar studies on *P. radiata* and *E. marginata* (Butcher *et al.* 1984; Stukely & Crane, 1994). Our study further showed a strong positive correlation between lesion length for both pathogens in two different trials. However, the family ranking in the two trials was not always consistent in both trials this was probably due to low levels of within family genetic variation.

Results of this study, demonstrated that susceptibility of *E. smithii* to *P. cinnamomi* and *P. nicotianae* is transmitted consistently to the progeny of parent plants. In this regard, greenhouse screening using stem inoculation validated the field performance of these genotypes. Results of this study are consistent with those of similar studies conducted in Australia where stem inoculation provides a reliable method to screen a large number seedlings (Stukely *et al.* 2007). Furthermore they confirm that mean lesion length can be useful in assessing susceptibility amongst half-sib families.

Total leaf phenolic content varied considerably within each of the 12 half-sib families investigated in this study. Our results showed that the phenolic compounds are present in greater amounts in field tolerant than in susceptible genotypes of *E. smithii*. These results also support the fact that phenolics play a role in disease tolerance (Hahlbrock & Scheel 1989). A similar technique has been used to screen cacao genotypes for resistant to *P. palmivora* (Okey *et al.* 1997; Tahi *et al.* 2000) in the past and it could provide a general view of the likely susceptibility of *E. smithii* planting stock to infection by *P. cinnamomi*.

Breeding and selection for disease tolerant planting stock is part of an integrated disease management strategy to manage root and collar rot of cold tolerant eucalypts in South Africa. However, root and collar rot disease predominantly caused by *P. cinnamomi* and *P. nicotianae* remains the single most common disease problem amongst cold tolerant eucalypts, including *E. smithii*. The development of genetically tolerant planting material is the most sustainable long-term solution to minimize yield loss due to *Phytophthora* root and collar in nursery and plantations. This study provides promising results using two screening methods that could be useful in assessing the susceptibility and tolerance of a large number of half-sib *E. smithii* families to root and collar rot prior to large scale planting.

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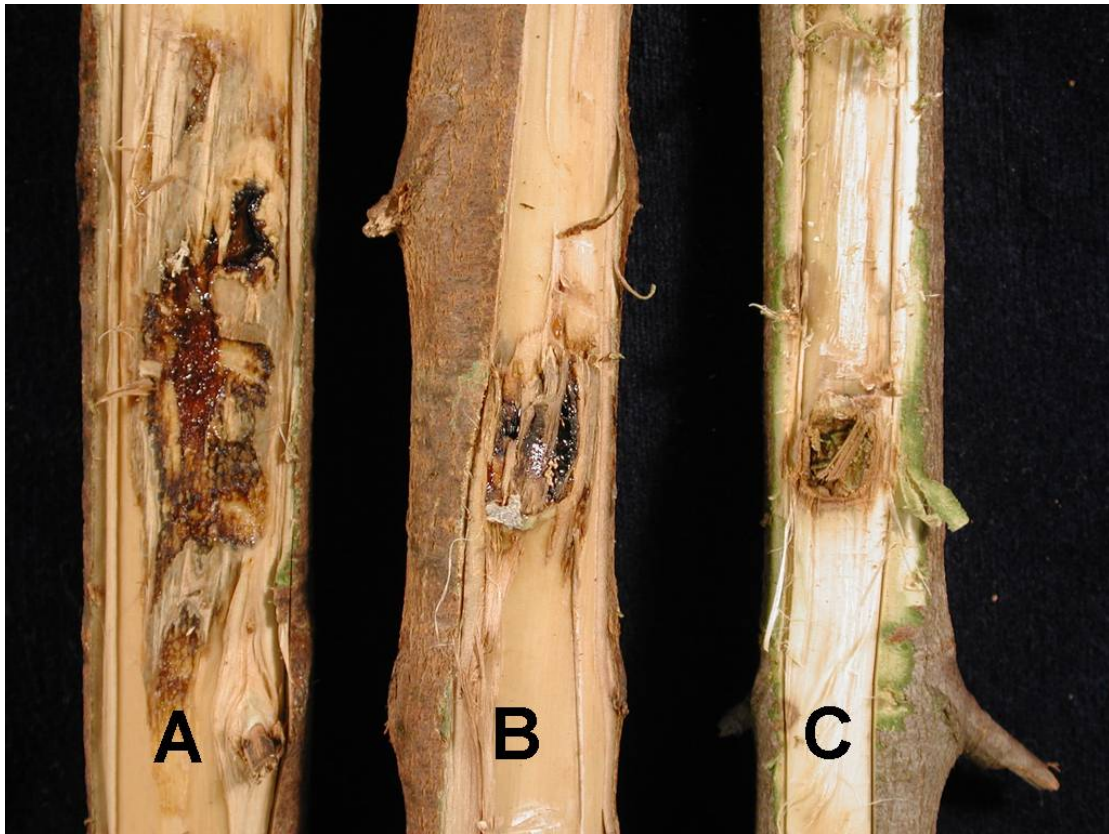


**Table 1. Summary of the analysis of variance of mean lesion length (mm) between and within the 12 half-sib families in response to inoculation with virulent isolates of *P. nicotianae* and *P. cinnamomi***

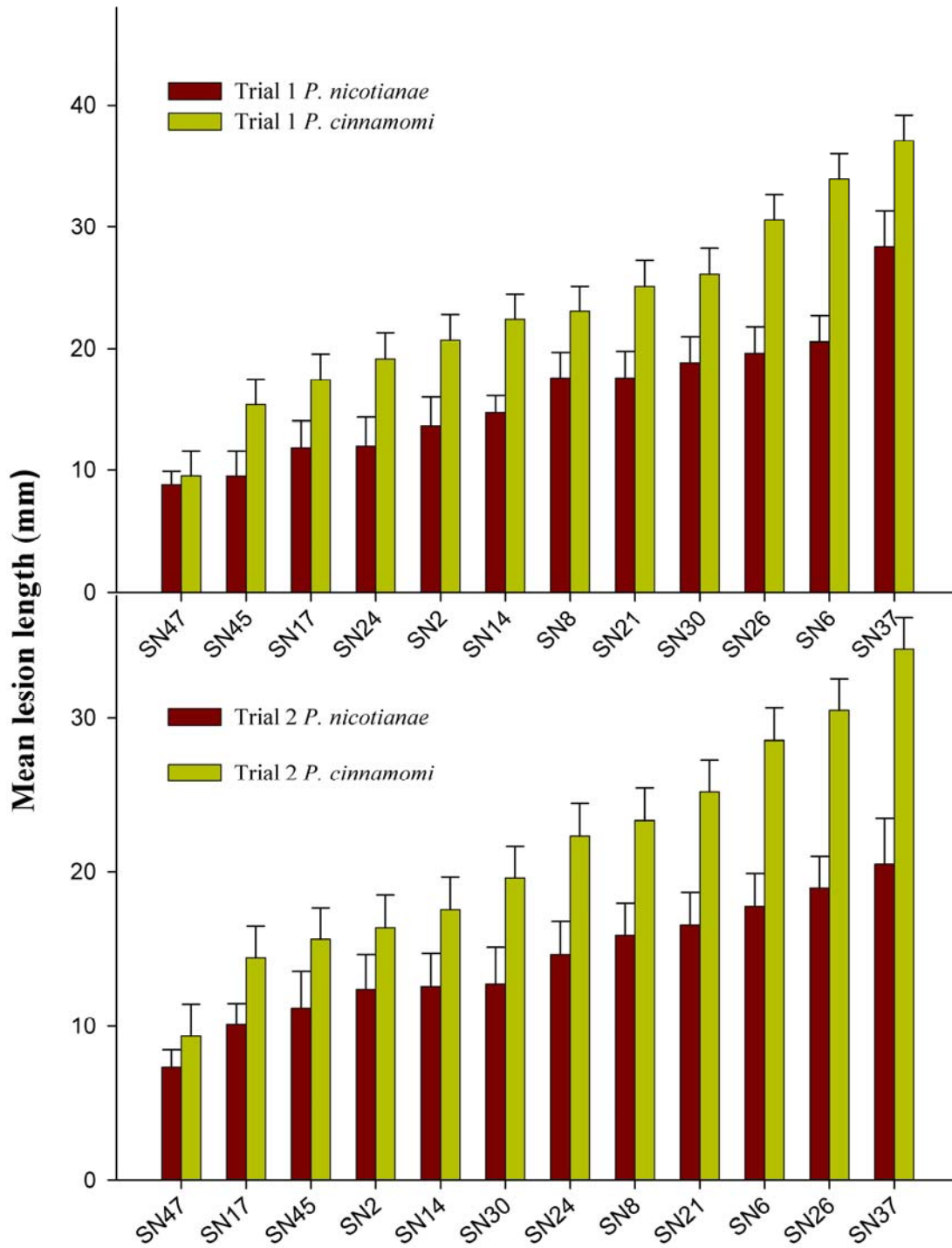
Effect	Lesion length		
	DF	F Value	Prob > F
Family	11	24.64	< 0.001
Pathogen	1	55.47	< 0.001
Family* Pathogen	11	3.54	< 0.001
Height	1	0.55	< 0.04
Diameter	1	5.76	< 0.05

**Table 2. Summary of field survival and total phenolic leaf content of the 12 half-sib *E. smithii* families investigated in this study**

Family	Survival %	Field ranking	Glasshouse ranking	Phenolic ranking
SN30	41 %	Susceptible	Moderately Tolerant	high
SN37	46 %	Susceptible	Susceptible	low
SN21	47 %	Susceptible	Susceptible	low
SN24	47 %	Susceptible	Susceptible	low
SN26	47 %	Susceptible	Moderately Tolerant	low
SN8	48 %	Susceptible	Moderately Tolerant	medium
SN14	80 %	Tolerant	Moderately Tolerant	medium
SN17	81 %	Tolerant	Highly Tolerant	high
SN47	81 %	Tolerant	Resistant	high
SN45	84 %	Tolerant	Highly Tolerant	high
SN2	85 %	Tolerant	Moderately Tolerant	medium
SN6	86 %	Tolerant	Moderately Tolerant	medium



**Fig. 1** Lesions produced on *E. smithii* three weeks following stem inoculation with virulent strains of *P. cinnamomi* and *P. nicotianae*. (A) lesion caused by *P. cinnamomi* lesion, (B) = lesion caused by *P. nicotianae*, (C) = Control.



***E. smithii* families**

**Fig. 2** Mean lesion lengths (mm) in stems of 12 half-sib *E. smithii* families measured three weeks after inoculation with virulent isolates of *P. cinnamomi* and *P. nicotianae* in two trials. Bars indicate standard errors

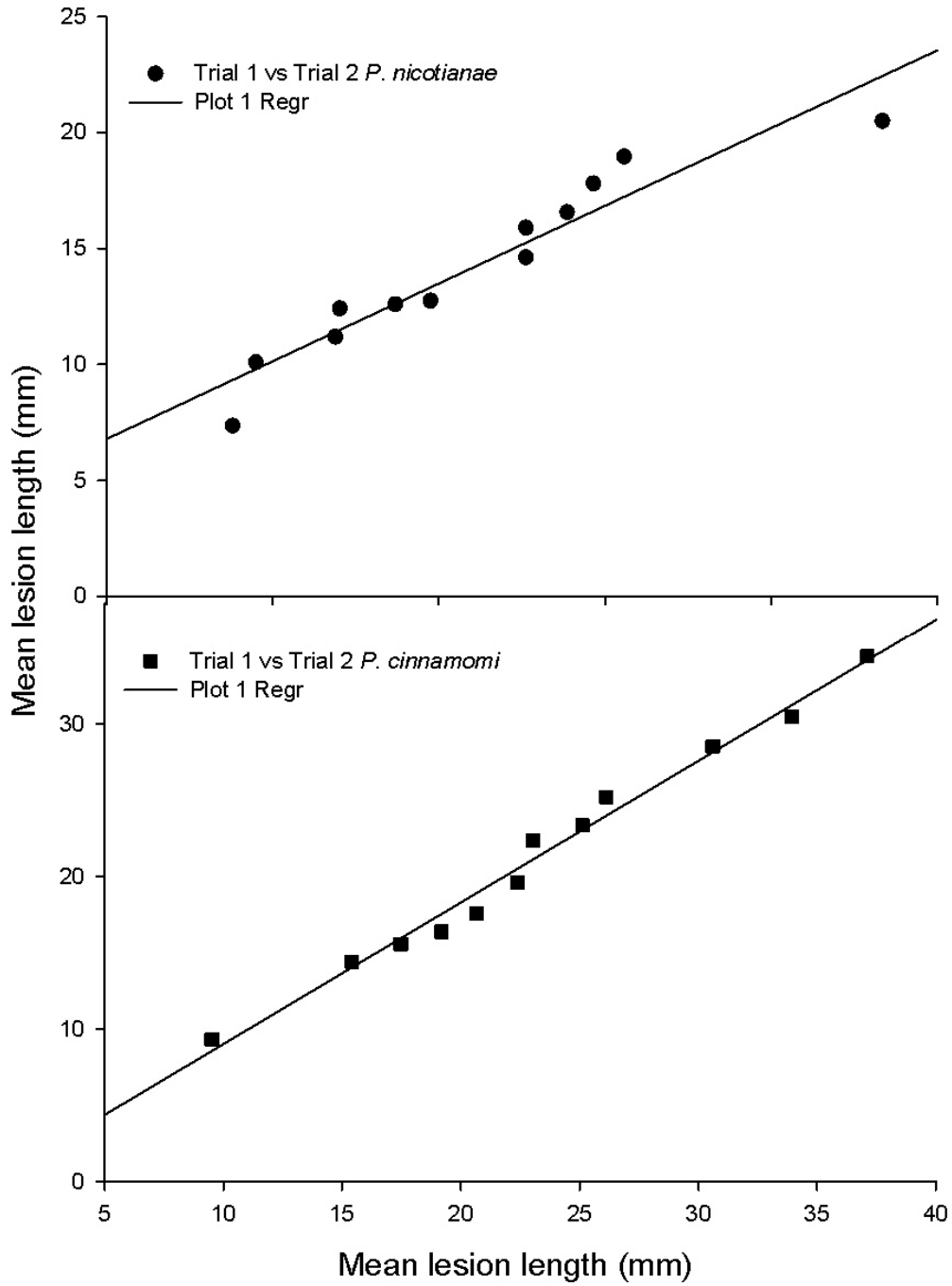
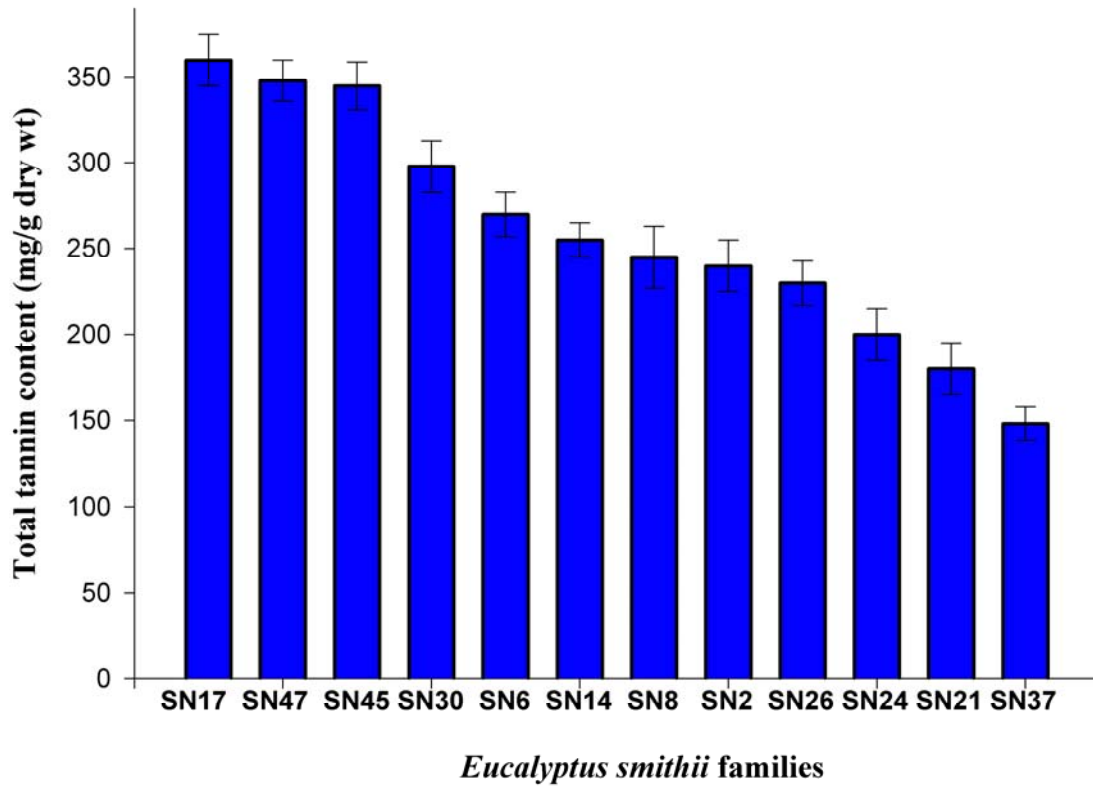


Fig. 3 Scatter plot illustrating the positive correlation between measurement of lesions lengths produced by virulent isolate of *P. cinnamomi* and *P. nicotianae* in two trials.



**Fig. 4** Mean total phenolic leaf content expressed as mg tannic ac./g DW of 12 half-sib *E. smithii* families. Error bars represent  $\pm$  standard error