

# Effect on nursery and field performance of *Pinus patula* seedlings after inoculation with *Fusarium circinatum*

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*Fusarium circinatum* is an important fungal pathogen of *Pinus* species. In South Africa, it is the most significant pathogen of *Pinus patula* seedlings in forestry nurseries where it presents a substantial constraint to productivity and can continue to cause mortality in-field for up to two years after establishment. This study describes the results from two trials where *P. patula* seedlings were inoculated with *F. circinatum* to determine the impact of the pathogen on nursery and field performance. Seedlings were also subjected to water stress treatments to ascertain whether this would trigger the onset of disease symptoms. Inoculum load and timing of inoculation had significant effects on seedling survival in both the nursery and field. High inoculum concentrations caused greater levels of mortality and, where seedlings were inoculated at a young age, they showed higher levels of susceptibility to *F. circinatum*. Temporary water-stress in the nursery produced smaller plants and improved in-field survival, but this treatment did not trigger higher mortality in inoculated treatments. On the other hand, transplant stress was a major contributor to the higher levels of mortality observed in inoculated treatments. Overall, these studies confirmed that infection in the nursery leads to the disease problems observed during early plant establishment in the field.

**Keywords:** *Fusarium circinatum*, inoculation, nursery-to-field, *Pinus patula*, seedlings, survival

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## Introduction

*Fusarium circinatum* is the most significant fungal pathogen of *Pinus* spp. in South Africa and it has a severe impact on nursery production, particularly that of *P. patula* (Wingfield et al. 2008; Mitchell et al. 2011). Symptoms of infection by *F. circinatum* include shoot-tip die-back, discoloration of the roots and root collar region, and root rot, which ultimately causes seedling mortality (Wingfield et al. 2008; Mitchell et al. 2011). *Fusarium circinatum* is also associated with high levels of mortality in young *P. patula* trees in southern Africa, up to two years after establishment, causing serious losses, reduced stand density and variation in tree growth (Crous 2005; Mitchell et al. 2011). The disease known as pitch canker, typified by stem cankers and branch die-back in mature trees, is also found in South Africa, although this first appeared at least 15 years after the discovery of *F. circinatum* in nurseries (Coutinho et al. 2007).

*Fusarium circinatum* reproduces primarily by means of asexual conidia (Dwinell et al. 1985), which can be airborne, water-borne and soil-borne or carried by insects. Anecdotal evidence suggests that, in South Africa, infection of seedlings in nurseries is the source of the inoculum for trees that die in the field during the early stages of growth (Mitchell et al. 2011). It is postulated that in the nursery environment, conidia may easily be dispersed through the nursery

irrigation system, causing spores to become deposited on healthy seedlings, nursery growing media, seedling containers and on nursery structures. Under appropriate environmental conditions these spores can then germinate and infect the host species (James et al. 1991). While various reports suggest that physical damage to the plant is required for pathogen ingress (Dwinell et al. 1985; Gordon et al. 2001; Gordon 2006), it is believed that infection can also occur in the absence of physical wounding. This could occur through the predisposition of succulent shoot growth (Mitchell et al. 2011) or through the root tip regions (meristematic zone or in the elongation zone) as observed for *F. oxysporum* (Rodríguez-Gálves and Mendgen 1995), where root exudates may stimulate germination of conidia and hyphal growth (Deacon and Donaldson 1993). This phenomenon has been widely reported for various hosts of other pathogenic *Fusarium* spp. (Christou and Snyder 1962; Griffin 1969) where the root apex has been shown to be the site of infection (Locke and Colhoun 1977).

It is the interaction of numerous environmental factors and cultural practices that potentially influences seedling root colonisation by *Fusarium* spp. However, in apparently 'asymptomatic plants' the pathogen can be present but not detectable (James et al. 1991; Swett and Gordon

2011). A stress event, such as physical or physiological damage to the plant may then trigger the pathogen to manifest itself. While plant resources can be targeted towards overcoming stresses, the pathogen can become more active and begin to penetrate root cells (causing root die-back). It would then advance to the root collar region, where the typical symptoms associated with *F. circinatum* in the nursery become evident (CL Swett, Department of Plant Pathology, University of California, Davis, pers. comm., 2012). Following this argument, it is believed that seemingly healthy plants are being dispatched to the field, only to succumb to infection by *F. circinatum* at a later stage.

*Fusarium circinatum* poses a substantial risk to the continued deployment of *P. patula* in South Africa as there are limited prospects for the eradication of established infections (Mitchell et al. 2012). Despite the requirement for strict phytosanitary measures in nurseries, the pathogen continues to cause losses. It is, therefore, imperative to understand the importance of inoculum levels and their impact on infection. It is equally important to understand more regarding the susceptibility of seedlings to infection during their development in the nursery. Given this concern, relevant industry and academic stakeholders established the South African Pitch Canker Control Programme in January 2010 to address the on-going threat of *F. circinatum*. As part of this programme, two trials were implemented to determine the role of the nurseries in translocating inoculum to the field, which results in substantial post-planting mortality. The aim of this study was thus to establish if exposure of *P. patula* seedlings to *F. circinatum* could be quantitatively associated with mortality experienced in the plantation, post-planting, and in this context also to determine whether physiological plant stress, quantity or timing of inoculum exposure in the nursery could influence disease-related nursery and/or post-planting mortality. In addition, the potential for cross-contamination or secondary infection of non-inoculated seedlings was also explored.

## Materials and methods

### Plant materials

Using a commercial *P. patula* seedlot (PP66317) two trials were implemented. The seedlot was shown to be free of *F. circinatum* as determined by direct plating of seeds on to peptone PCNB agar (Leslie and Summerell 2006). *Fusarium*-like cultures were treated as described below in the nursery and field trial measurements section. Seeds were hand-sown into steam-sterilised Unigro 98 trays (U98, Dela-Plast, Pretoria, 90 ml cavity volume) containing composted pine bark medium (12 mm). This medium was shown to be free of *F. circinatum* by direct plating of bark samples onto peptone PCNB agar (Leslie and Summerell 2006). Seedlings for both trials were raised at the University of KwaZulu-Natal (UKZN) Plant Pathology facilities (greenhouse and shadehouse), which represented a *F. circinatum*-free environment. For Trial A, the commercial control seedlings were raised at a Sappi seedling nursery, where levels of *F. circinatum* typical of a commercial production facility were expected to occur. For Trial B, control seedlings were raised at the Institute for Commercial Forestry Research (ICFR) nursery.

## Trial A: Spore load study

### Nursery treatments

Treatments included inoculation of healthy *P. patula* seedlings, at 3.5 months, with spores of a single isolate of *F. circinatum* (FCC 3579), selected for its virulence (Porter et al. 2009) and prepared according to the protocol used by Porter (2010). Two spore levels were tested, representing a high spore load (2 000 spores U98 tray<sup>-1</sup> = 425 spores m<sup>-2</sup>) and a low spore load (1 000 spores U98 tray<sup>-1</sup> = 212 spores m<sup>-2</sup>). However, very low seedling mortality observed in the first month following inoculation prompted a second inoculation. This took place 42 d after the first inoculation for both spore load treatments, when seedlings were five months old.

Seedlings raised at the Sappi nursery represented the commercial control exposed to natural *F. circinatum* inoculum levels, while a non-inoculated set of seedlings represented the experimental control (Table 1). Seedlings inoculated with low or high spore levels were removed from the shadehouse, away from the control treatment (not inoculated), for application of the inoculum and thereafter returned to the shadehouse. Inoculation was carried out using a spray bottle, with the nozzle aimed at the root collar region of a group of four seedlings per application of inoculum, without any wounding of the plants.

Approximately one month after the final spore inoculation, when the seedlings were six months old, trays at the UKZN facilities were moved from the greenhouse to a shadehouse. At the same time, the 12 trays of seedlings that had been raised at the Sappi nursery were transferred to the UKZN facility for the implementation of a factorial water stress treatment (Table 1). Plants were routinely watered and allowed to acclimatise to the outside environment for 2.5 weeks prior to the application of the water stress treatment, in which water was withheld for 4 d.

### Nursery and field trial designs

The trial layout in the greenhouse consisted of a split-plot design with the stress treatment as the main plot and the three spore load treatments (control, low and high) as the subplots, represented by six replications. Each treatment within each replication was represented by one U98 tray (with 98 seedlings). The 12 U98 trays raised at the Sappi commercial nursery were laid out in a single block, raised in close proximity to commercially propagated *P. patula* seedlings.

When all the seedlings had been transferred to the shadehouse, the trial design accommodated the practicality of watering. The trial was laid out as a 2 × 4 factorial (2 stress treatments × 4 spore load treatments), resulting in a total of eight treatments in a split-plot design, replicated six times (Table 1), with each treatment represented by one U98 tray.

When seedlings were approximately seven months old, the field trial was implemented. Each of the eight nursery treatments were subjected to further varying stress levels at planting: water planting (2 litres per pit) of irrigated plugs; planting without water (dry planting); and planting with 2 litres of a 0.1% Benomyl solution (benzimidazole, 500 g kg<sup>-1</sup>). This introduced a further level to the factorial design and increased the number of treatments in the trial

**Table 1:** Comparison of treatment levels and trial details for the two *F. circinatum* inoculation trials implemented

Trial details	Trial A	Trial B
Sowing date	7 July 2010	28 July 2011
Spore load treatments <sup>a</sup>	High – 2,000 spores tray <sup>-1</sup> Low – 1,000 spores tray <sup>-1</sup> Control (not inoculated) Commercial control <sup>b</sup>	High – 500 spores tray <sup>-1</sup> Low – 125 spores tray <sup>-1</sup> Control (not inoculated) Isolated control <sup>c</sup>
Inoculation timing	<i>Repeat application:</i> 14 weeks – 21 October 2010 20 weeks – 3 December 2010	<i>Staggered application:</i> 9 weeks – 6 October 2011 16 weeks – 24 November 2011 24 weeks – 27 January 2012
Water stress treatments	Stress: 4 days; 28–31 January 2011 No stress	Stress: 6 days; 4–9 February 2012 No stress
Nursery trial design	Split-plot	Split-split plot
Nursery trial details	8 treatments, 4 replications, 1 U98 tray per treatment (98 plants tray <sup>-1</sup> )	16 treatments, 4 replications, 1 U98 tray per treatment (78 plants tray <sup>-1</sup> )
Field trial planting	22 February 2011	22 March 2012
Field treatments	Dry planting Water planting Benomyl planting	Water planting
Field trial design	Split-plot	Split-split plot
Field trial details	24 treatments, 6 replications, 14 tree row plots	16 treatments, 6 replications, 14 tree row plots

<sup>a</sup> Spore concentrations represent combined inoculum applications at 3.5 and 5 months for Trial A

<sup>b</sup> Seedlings propagated in a commercial Sappi Nursery and exposed to natural infection

<sup>c</sup> Seedlings propagated in a *F. circinatum*-free environment away from potential infection from the inoculated treatments

to 24 (Table 1). The field trial was also established as a split-plot design, with transplant stress level as the whole plot and the eight nursery treatments (spore load by nursery stress level) as the subplots. Since the aim was to only monitor early establishment, plots were established as 14-tree line plots. This required less space and ensured a better distribution of the treatments across the site, with six replications (Table 1).

The field trial, located in the KwaZulu-Natal Midlands (29°16'54.85" S, 30°11'49.80" E), was established on a prior *Eucalyptus macarthurii* site where potential risk of *F. circinatum* infection directly from the field was deemed to be low. The site was situated at 1 600 m above sea level (asl), with a mean annual temperature of 15.5 °C and mean annual precipitation of 1 102 mm.

### **Trial B: Inoculation timing study**

#### *Nursery treatments*

Treatments included inoculation of healthy *P. patula* seedlings on three separate occasions when seedlings were 9, 16 and 24 weeks old, respectively (Table 1). This trial utilised the same *F. circinatum* isolate and inoculation method as Trial A. However, the two spore levels tested were substantially lower than those used in Trial A. For Trial B, the high spore load treatment represented 500 spores U98 tray<sup>-1</sup> (106 spores m<sup>-2</sup>), whereas the low spore load treatment represented 125 spores U98 tray<sup>-1</sup> (26 spores m<sup>-2</sup>). Seedlings inoculated with high or low spore levels were removed from the shadehouse, away from the control treatment (not inoculated), prior to application of the inoculum and returned thereafter. A fourth treatment included raising seedlings at the ICFR nursery where *F. circinatum* is not present. This treatment (isolated control) served to identify potential cross-contamination

between inoculated and non-inoculated treatments in close proximity (Table 1).

One week after the final inoculation, when seedlings were approximately six months old, a factorial water stress treatment was applied (Table 1). After a final watering, water was withheld for 6 d (Table 1). Thereafter, water was reapplied to the stressed seedlings in order for them to recover.

#### *Nursery and field trial designs*

The trial layout in the shadehouse consisted of a split-plot design with the timing of inoculation (control [not inoculated], 9-, 16- and 24-week applications) as the main plot and spore concentration (low and high) as the subplots. This resulted in a total of eight treatments with eight replications (Table 1).

When the seedlings were about six months old, the trial design was modified to a split-split plot to accommodate the implementation of the water stress treatment. The stress treatment was the main plot, timing of inoculation the subplot and spore concentration represented the sub-subplot. The trial consisted of 16 treatments with four replications and each treatment plot consisted of one U98 tray (with 78 seedlings).

At eight months of age, asymptomatic seedlings from each of the 16 nursery treatments were established in a field trial, designed as a split-split plot. The stress treatment represented the main plot, spore concentration, the subplot and inoculation timing the sub-subplot. Seedlings were established as 14-tree line plots, replicated six times (Table 1). The trial site, previously planted to *P. elliotii*, located in the KwaZulu-Natal midlands (29°24'10.29" S, 30°17'47.39" E) at 1 164 m asl, has a mean annual temperature of 16.7 °C and a mean annual precipitation of 1 255.6 mm.

## **Nursery and field trial measurements**

### ***Nursery seedling survival and seedling assessment***

From the time of inoculation with *F. circinatum*, all symptomatic and dying seedlings removed (culled) from the trial were recorded. A final nursery seedling survival count was completed for both trials prior to planting in-field.

For both trials, all culled seedlings displaying any symptoms of disease were analysed for the presence of *F. circinatum* by culturing potentially diseased root tips on peptone PCNB agar (Leslie and Summerell 2006). Samples of fungal cultures that were *Fusarium*-like in appearance after incubation for 5–10 d at 25 °C were then transferred to Spezieller Nährstoffarmer agar (SNA) (Leslie and Summerell 2006) to enable identification based on morphological characteristics. Isolates were positively identified as *F. circinatum* when microscopic examination revealed that conidia were borne on false heads from polyphialides and that sterile coiled hyphae were present (Nirenberg and O'Donnell 1998).

For a random subset of samples, the morphology-based identifications were confirmed using a *F. circinatum*-specific PCR-based method (Schweigkofler et al. 2004). Each 25 µl PCR mixture contained 10 µM of each of the primers CIRC1A and CIRC4A (Schweigkofler et al. 2004), 200 µM of each dNTP (dATP, dGTP, dCTP and dTTP), 2 mM MgCl<sub>2</sub> and reaction buffer (Supertherm, Southern Cross Biotechnology, Cape Town). A sterile needle was used to transfer a small amount of mycelium from cultures directly to the PCR mixture, which was then subjected to denaturation steps at 95 °C for 15 min and 80 °C for 2 min on a thermocycler. To each reaction mixture, 1 U of *Taq* DNA polymerase (Supertherm, Southern Cross) was then added, after which another incubation step at 80 °C for 2 min was performed. This was followed by 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 54 °C for 30 s and fragment elongation at 72 °C for 1 min, after which a final elongation at 72 °C for 5 min was performed. PCR amplicons were analysed using standard 2% agarose gel electrophoresis (Sambrook and Russell 2001) and GelRed™ (Biotium, Hayward, CA).

### ***Stomatal conductance***

In both trials, stomatal conductance (mmol m<sup>-2</sup> s<sup>-1</sup>) was monitored (between 12:00 and 14:00) on 5 six-month-old seedlings per treatment in two replications using a hand-held leaf porometer (Model SC-1; Decagon Devices, Inc., Pullman, WA) before and after the water stress treatments were implemented.

### ***Nursery growth measurements***

When seedlings were approximately eight months old, stem collar diameter and seedling height measurements were recorded for all seedlings established in the field trials.

### ***Pathogen isolation from asymptomatic plants***

Samples of asymptomatic seedlings from each treatment (20 for Trial A and 14 for Trial B) were examined for the presence of *F. circinatum* by culturing excised root and tissue samples from unwashed (non-sterile) seedlings on peptone PCNB agar (Leslie and Summerell 2006). Morphological identifications were conducted as previously

described and again a subset of positively identified samples were subjected to the *F. circinatum* diagnostic PCR to confirm the morphological diagnoses.

### ***Field spore load measurements (only for Trial A)***

Filter paper (Whatman no. 1, 70 mm diameter) spore traps were placed in-field one week prior to planting, and one week and one month after planting. Filter paper discs were located at 15 positions across the trial site, between each main plot and at one located 5 m from the edge of the trial. The paper discs were placed at two elevations, 20 cm and 50 cm from the ground (attached to poles), resulting in 32 samples per timing and removed after one week. Prior to placing the filter paper discs in-field, they were moistened with TE buffer (10 ml of 1 M Tris-Cl [pH 7.5] per litre, 2 ml of 500 mM EDTA [pH 8.0]). After remaining in the field for a week, the discs were collected and analysed using real time-PCR to detect and quantify the potential presence of *F. circinatum* in the field using the method described by Fourie et al. (2014).

### ***Field survival measurements***

In-field seedling survival was measured at one, three and six months after planting (but only six-month data are presented for Trial B). Dead seedlings were not replaced.

### ***Trial analyses***

Due to the unbalanced data arising from the morphological diagnoses of symptomatic and dying seedlings, this information was summarised for both trials. Data relating to nursery seedling survival, growth and contamination of asymptomatic seedlings were analysed using analysis of variance (ANOVA) (GenStat 13th Edition, VSN International, Hemel Hempstead). Percentage data were angular transformed prior to analysis. The appropriate least significant differences (LSD) at the 0.05% level were used to determine differences between treatment means.

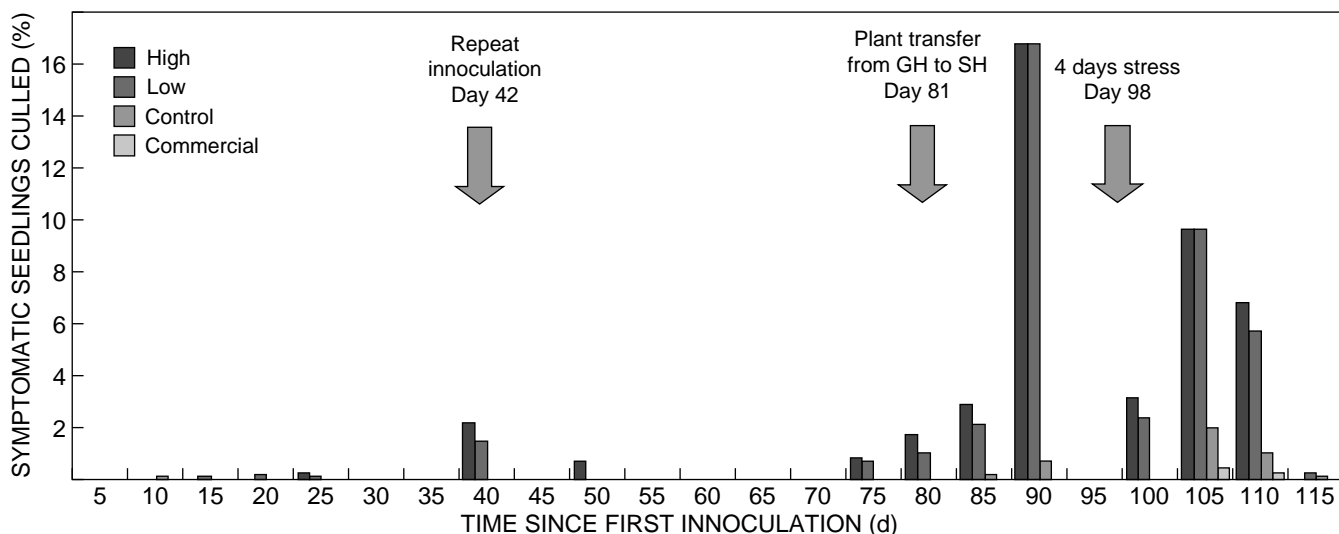
The field trials were also analysed using ANOVA (GenStat 13th Edition), and percentage data transformed using angular transformations, prior to analysis. In Trial A, replication was used as the blocking term and the interaction of field treatment (main plot) by nursery treatment (subplot) was analysed. Asymptomatic seedling infection levels in Trial A were also correlated with field survival at six months. In Trial B, replication was used as the blocking term and ANOVA (GenStat 13th Edition) used to determine the full interaction for stress level (main-plot) by spore load (subplot) by inoculation timing treatments (sub-subplot). Least significant differences (at the 0.05% level) were calculated to determine differences between treatment means.

## **Results**

### ***Trial A: Spore load study***

#### ***Seedling infection/contamination in the nursery***

Inoculation of the seedlings with *F. circinatum* produced symptomatic plants approximately 40 d after spore application (Figure 1). Typical symptoms such as tip die-back and discolouration of the needles were observed as reported by Viljoen et al. (1994). After the plants were relocated to the shadehouse, the percentage of symptomatic seedlings



**Figure 1:** Percentage of symptomatic seedlings per spore load treatment in Trial A. Symptomatic seedlings were culled after the first *F. circinatum* inoculation. Arrows indicate: Day 42, the day of the second inoculation; Day 81, when seedlings were relocated from the greenhouse (GH) to the shadehouse (SH); and Day 98, when the water stress treatment was imposed for 4 d

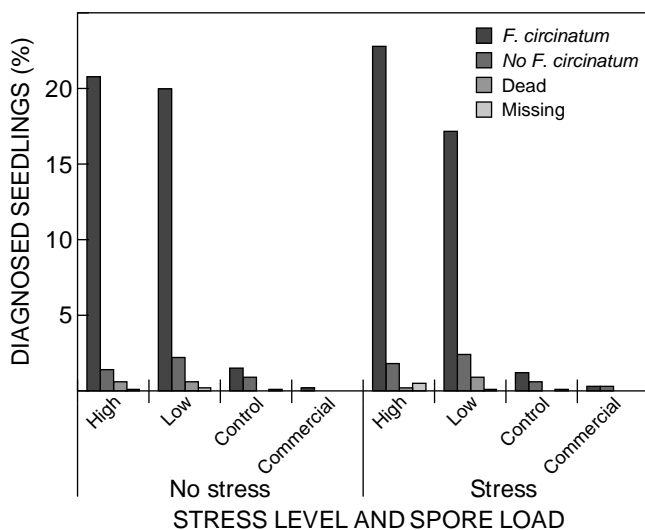
observed increased exponentially in both inoculated treatments (Figure 1). This coincided with the change of environment, from the moist and humid greenhouse to the drier, warmer exterior conditions and higher light intensity of the shadehouse, which could have been the main factor contributing to the induction of disease symptoms.

At the end of the nursery phase, *F. circinatum* could be isolated from 84% of the 994 symptomatic seedlings culled from the experiment (Figure 2). Most *F. circinatum*-infected seedlings were from treatments that had been inoculated with the pathogen (Figure 2). There was very little difference in seedling mortality between the high and low spore load applications with an average of 20% mortality in these treatments ascribed to *F. circinatum*, irrespective of the imposed water stress (Figure 2).

Results of isolation from asymptomatic seedlings assessed for the presence of *F. circinatum* showed that approximately 90% of the seedlings inoculated with *F. circinatum* were contaminated or infected. This was irrespective of whether a high or low inoculum level had been used (Figure 3). The control seedlings (not inoculated but raised in proximity to the inoculated treatments in the trial) showed a statistically significant ( $p < 0.001$ ) lower level of contamination, but still had *F. circinatum* infection levels of approximately 60%. In contrast, the commercially propagated seedlings that had been grown for five months at a separate location from the trial, in a nursery known to harbour the pathogen, showed almost 50% contamination (Figure 3).

#### Seedling survival in the nursery

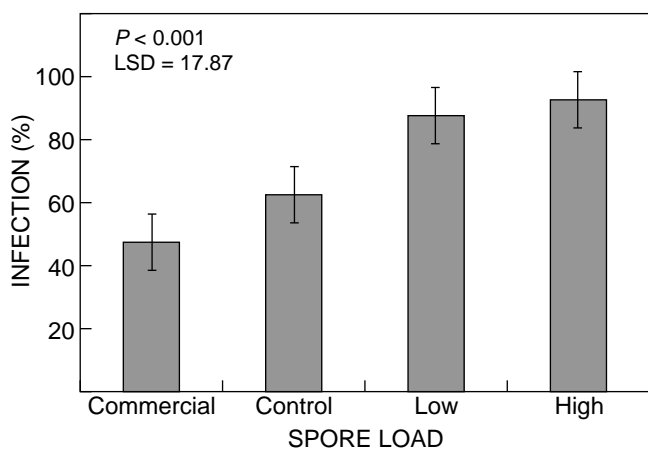
Mean seedling survival at the end of the nursery phase (approximately 230 d after sowing) was 78%, with the commercially propagated seedlings performing best with 98% and 99% survival for the stressed and non-stressed treatments, respectively (Figure 4). This was followed closely by the control treatment (not inoculated), while the high and low spore load treatments both incurred significantly



**Figure 2:** Diagnostic results of all symptomatic/dying seedlings analysed in Trial A during the nursery phase. Control = not inoculated; Commercial = seedlings propagated in the Sappi nursery; Low = 1 000 spores tray<sup>-1</sup>; High = 2 000 spores tray<sup>-1</sup>

( $p < 0.001$ ) higher levels of mortality compared to the control or commercially propagated seedlings (Figure 4).

Despite recording a significant difference ( $p < 0.001$ ) in the stomatal conductance of stressed and non-stressed treatments at the end of the water stress period (data not shown), there were no significant ( $p = 0.489$ ) stress effects on seedling survival in the nursery. Similarly, the interaction between the stress treatment and spore load was not significant ( $p = 0.673$ , Figure 4). Regardless of the observation of wilting seedlings in the stressed treatment, these plants recovered rapidly, suggesting that the imposed water stress was not a major trigger for the pathogen in this trial.



**Figure 3:** Confirmed infection/contamination by *F. circinatum* of randomly selected asymptomatic seedlings from each of the spore load treatments in Trial A. Control = not inoculated; Commercial = seedlings propagated in the Sappi nursery; Low = 1 000 spores tray<sup>-1</sup>; High = 2 000 spores tray<sup>-1</sup>. Error bars represent the SE of the difference

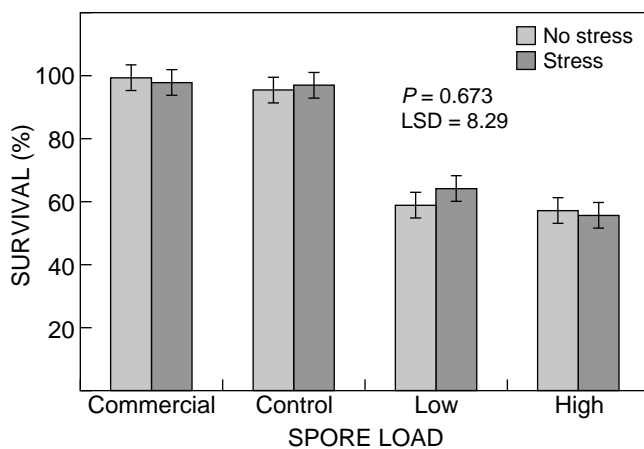
#### Seedling growth measurements in the nursery

The commercially propagated seedlings received more regular fertigation, producing taller ( $p < 0.001$ ) plants than those in the other treatments, particularly those that had been inoculated. Plants in the control treatment (not inoculated) were also taller than the inoculated seedlings, suggesting that the inoculated seedlings may have diverted resources into plant defence, rather than into growth or that the infection reduced the capacity for plants to grow (results not shown). There were, however, no significant differences ( $p = 0.113$ ) in root collar diameter (RCD) between the different spore load treatments.

Plants subjected to the water-stress treatment were significantly ( $p < 0.001$ ) smaller in height and in RCD compared to non-stressed seedlings. There were significant interactions between the spore and stress level for both height ( $p < 0.001$ ) and RCD ( $p = 0.003$ ) measurements, but this was mainly as a result of the commercially propagated seedlings that were water-stressed, being larger than the non-stressed plants. The reverse trend was seen for all the treatments that had been raised in the UKZN shadehouse (results not shown).

#### Field spore load

Filter paper spore traps were placed in-field prior to planting and after seedling establishment to assess the *F. circinatum* spore load *in situ*. Spore traps placed in-field one month after planting were lost due to inclement weather conditions and were therefore not analysed. Results of the real-time PCR analysis of the traps collected before planting indicated that aerial inoculum of *F. circinatum* was not present at the site, previously planted to *E. macarthurii*. However, a week after trial planting, *F. circinatum* was detected on 9.4% of the traps, albeit at low levels (i.e.  $38 \times 10^1$  spores m<sup>-2</sup>). The latter were primarily for the traps placed closest to the ground, suggesting the introduction of the spores from the planted seedlings.



**Figure 4:** Nursery survival in Trial A of seedlings per spore load treatment and water stress level, when seedlings were seven months old. Control = not inoculated; Commercial = seedlings propagated in the Sappi nursery; Low = 1 000 spores tray<sup>-1</sup>; High = 2 000 spores tray<sup>-1</sup>. Error bars represent the SE

#### Seedling survival in the field

All seedlings established in the field trial were asymptomatic at time of planting, irrespective of the nursery treatment. From the earliest field survival measurements, results showed significant ( $p < 0.001$ ) effects as a result of the water stress treatment implemented in the nursery and similarly significant ( $p < 0.001$ ) responses to the spore load treatment. These responses remained evident six months after planting (Table 2). Seedlings that were water-stressed in the nursery tended to show better in-field survival (Table 2).

The two inoculated treatments (high or low spore load) had lower survival ( $p < 0.001$ ) than the non-inoculated treatments (control or commercially propagated), with the gap widening over time (Table 2). The low survival of seedlings that were not inoculated (control) was likely due to secondary infection as a result of their proximity to inoculated and commercially propagated seedling treatments in the shadehouse. It is possible that control seedlings could have become infected in-field through airborne dispersal of spores as suggested by the results from the in-field spore traps. However, it is more likely that these seedlings were already infected/contaminated in the nursery as indicated by the results from the analyses of asymptomatic seedlings (Figure 3). Field planting treatments also showed significant effects within the first six months, with better initial survival in seedlings that received the Benomyl treatment (Table 2); although this was no longer evident post six months (results not shown). No differences were observed between the dry- and water-planted treatments. This was most likely due to a major downpour on the day of planting, which effectively nullified the dry planting treatment (Table 2).

There were no significant treatment interactions for field survival within the first six months of growth. This suggests that the imposed nursery stress did not trigger increased mortality of inoculated seedlings. The larger effect was seen in the difference between one-month and three-month

**Table 2:** Field survival results from one to six months after planting in Trial A. Effects significant at the 0.05% level are highlighted in bold. Values denoted by the same letter are not significantly different. Commercial = seedlings propagated in the Sappi nursery; Control = not inoculated; High = 2 000 spores tray<sup>-1</sup>; Low = 1 000 spores tray<sup>-1</sup>; LSD = least significant difference; SE = standard error of the difference

Treatment	Survival (%) <sup>1</sup>		
	One month	Three months	Six months
<b>Field treatment</b>			
Benomyl	84.08 <sup>a</sup>	50.00 <sup>a</sup>	43.60 <sup>a</sup>
Dry planting	81.10 <sup>b</sup>	44.35 <sup>b</sup>	36.90 <sup>b</sup>
Water planting	83.63 <sup>a</sup>	43.75 <sup>b</sup>	37.35 <sup>b</sup>
SE	0.930	2.558	2.481
LSD	1.863	5.122	4.968
<i>p</i> -value	<b>0.020</b>	0.065	<b>0.039</b>
<b>Nursery stress</b>			
Water stress	85.62 <sup>a</sup>	48.31 <sup>a</sup>	42.66 <sup>a</sup>
No stress	80.26 <sup>b</sup>	43.75 <sup>b</sup>	35.91 <sup>b</sup>
SE	1.592	1.923	1.874
LSD	2.806	3.389	3.304
<i>p</i> -value	<b>&lt;0.001</b>	<b>0.018</b>	<b>&lt;0.001</b>
<b>Spore load</b>			
High	70.24 <sup>c</sup>	22.62 <sup>c</sup>	17.46 <sup>c</sup>
Low	72.76 <sup>c</sup>	23.41 <sup>c</sup>	17.06 <sup>c</sup>
Control	91.07 <sup>b</sup>	56.35 <sup>b</sup>	46.23 <sup>b</sup>
Commercial	97.82 <sup>a</sup>	81.75 <sup>a</sup>	76.39 <sup>a</sup>
SE	2.252	2.719	2.651
LSD	3.969	4.793	4.672
<i>p</i> -value	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>

<sup>1</sup> Data were angular transformed prior to ANOVA, but percentage data is presented in the table

survival for the various spore load treatments, rather than between stressed and non-stressed seedlings at any time (Table 2). Transplant shock was likely the larger contributor to the mortality of inoculated seedlings.

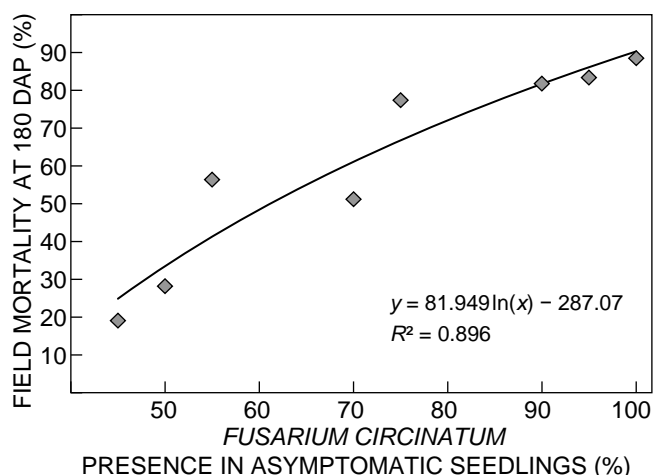
The presence of *F. circinatum* on the roots of asymptomatic seedlings in the nursery, in both inoculated (high and low spore load) and non-inoculated treatments (control and commercially propagated plants) that had either been water stressed or not showed a high ( $R^2 = 0.896$ ) and significant ( $p = 0.05$ ) correlation with field mortality at six months (Figure 5). Field mortality increased with an increase in the level of contaminated seedlings at planting (Figure 5).

### Trial B: Inoculation timing study

Following Trial A, the second trial was used to distinguish effects of using lower inoculum levels and also investigate the timing of inoculation, to determine whether seedling susceptibility varied over time. Furthermore, since the stress results obtained in Trial A were inconclusive, this treatment was again incorporated in Trial B, but for a slightly more extended period, to determine whether stress could act as a trigger of pathogenicity.

#### Seedling infection in the nursery

The use of lower inoculum loads in Trial B resulted in a much lower frequency of symptomatic seedlings in the nursery (Figure 6), compared with Trial A (Figure 1). A total of 271 wilting or dying seedlings were removed from the



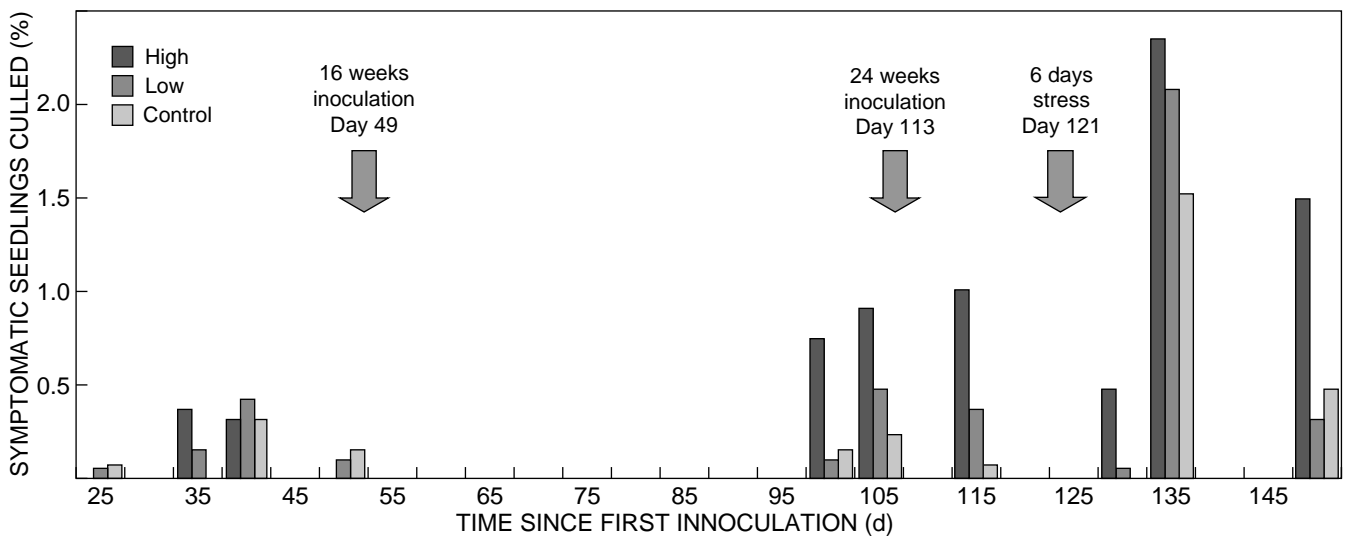
**Figure 5:** Correlation of the presence of *F. circinatum* in asymptomatic seedlings with six-month field mortality in Trial A. DAP = Days after planting

trial over a four-month period for diagnostic analysis. Results revealed that the majority of samples did not test positive for *F. circinatum* with only 31% showing presence of the pathogen (Figure 7). The lower frequency of recovery of the pathogen was likely a result of the much lower inoculum levels applied. Furthermore, the symptomatic seedlings culled between 25 and 50 d after the first inoculation were not infected with *F. circinatum*, suggesting other causes for this early-observed mortality (Figure 6). The lag in symptom development corresponded with that observed in Trial A (Figure 1). Closer examination of seedlings testing positive for the presence of *F. circinatum* revealed that the largest proportion of infection (31%) consisted mainly of seedlings that were inoculated early during their development (at nine weeks of age) and particularly those that had been exposed to the water stress treatment (Figure 7).

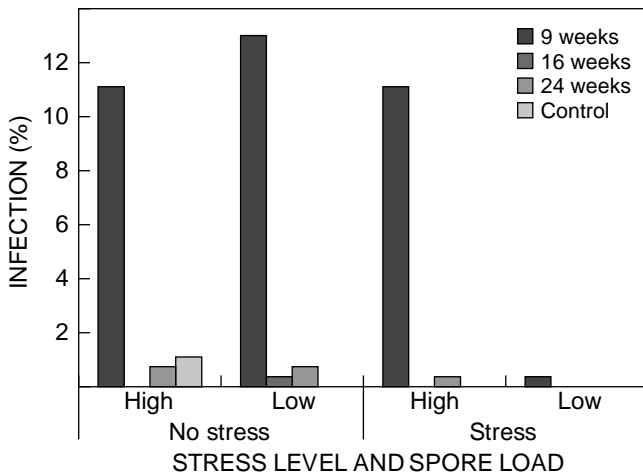
Similarly to Trial A, a sample of asymptomatic seedlings from each treatment was examined for the presence of *F. circinatum*. Results did not reveal any significant trends (possibly due to the small sample size), but higher levels of contamination were observed particularly in seedlings inoculated at nine weeks (Figure 8). The non-inoculated control also demonstrated high levels of infection, due to its proximity to inoculated treatments, mimicking the responses observed in Trial A.

#### Seedling survival in the nursery

Mean seedling survival at the end of the nursery phase (approximately 230 d after sowing) was above 90%. Despite high survival, significant effects ( $p < 0.001$ ) were observed for the spore load treatment (Figure 9). Seedlings inoculated with the high spore load (500 spores tray<sup>-1</sup>) had poorer survival than the low spore load treatment (125 spores tray<sup>-1</sup>), suggesting that the lower level of applied inoculum in Trial B was sufficient to enable an observable difference in the seedling survival response to inoculum exposure. Seedlings inoculated at nine weeks old showed the poorest ( $p < 0.001$ ) survival in the nursery (Figure 9). Seedlings raised in an isolated environment



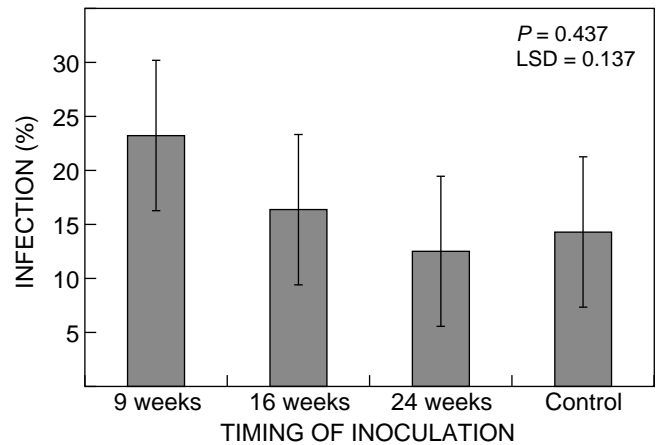
**Figure 6:** Percentage of symptomatic seedlings per spore load treatment in Trial B. Symptomatic seedlings were culled after the first *F. circinatum* inoculation when seedlings were nine weeks old. Arrows indicate subsequent inoculations on Day 49, when seedlings were 16 weeks old and Day 113, when seedlings were 24 weeks old. Day 121 represents the initiation of the 6-day water stress period



**Figure 7:** Confirmed infection/contamination by *F. circinatum* of symptomatic/dying seedlings in Trial B diagnosed during the nursery phase. Control = not inoculated Low = 125 spores tray<sup>-1</sup>; High = 500 spores tray<sup>-1</sup>

(Isolated control), away from the trial and from any potential secondary infection, had the best survival, while there were no differences in survival between seedlings inoculated at 16 and 24 weeks and the control (raised in close proximity to the trial, Figure 9).

The water stress treatment also had significant effects ( $p < 0.001$ ) on seedling survival in the nursery. The stressed seedlings exhibited lower survival (Figure 9) and manifested browning of needles after water was withheld for 6 days. Prior to the water stress treatment, stomatal conductance measurements for the stressed and non-stressed treatments were similar (Figure 10). However, while the non-stressed treatments showed stable stomatal conductance during the imposed stress period, the



**Figure 8:** Confirmed infection/contamination by *F. circinatum* of randomly selected asymptomatic seedlings from each spore inoculation timing treatment in Trial B. Control = not inoculated. Error bars represent the SE

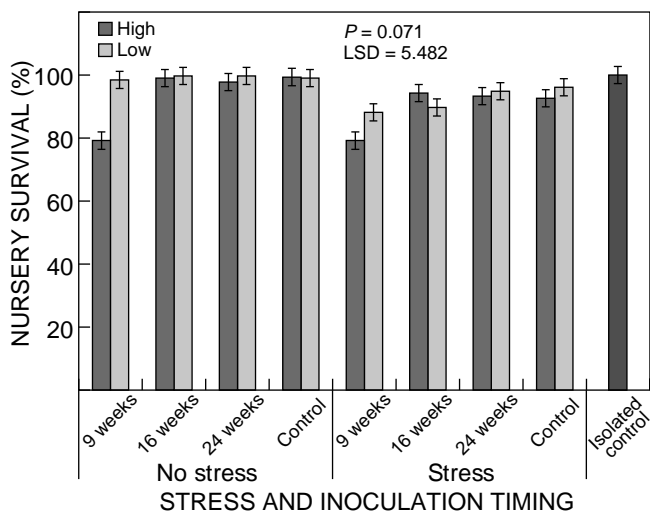
treatments exposed to the water stress experienced rapid stomatal closure and a gradual recovery after re-watering (Figure 10). There was, however, no significant interaction for nursery seedling survival between the water stress and spore load treatments ( $p = 0.130$ , results not shown).

There was a significant treatment interaction ( $p < 0.001$ ) between inoculum load and inoculation timing (Figure 11). This was driven by the poorer survival of the seedlings inoculated at 9 weeks with the high spore load, suggesting not only the importance of spore load, but also that younger seedlings may be more susceptible to the pathogen.

#### Seedling growth measurements in the nursery

Seedlings inoculated with the low spore load tended to be shorter ( $p = 0.021$ ) than those treated with the high





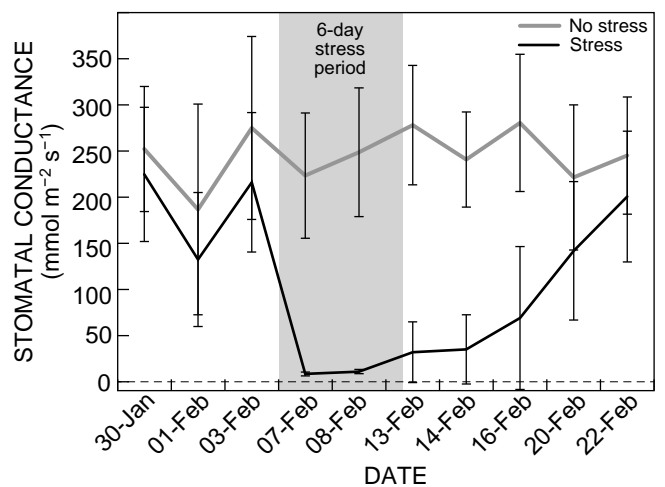
**Figure 9:** Nursery survival in Trial B of seedlings inoculated with high or low levels of *F. circinatum* inoculum at various times during their development in the nursery and after exposure to a water stress treatment for 6 days. Control = not inoculated; Isolated control = not inoculated treatment propagated separately from the trial. Error bars represent the SE

spore load, and stressed seedlings were also smaller than non-stressed seedlings ( $p = 0.008$ ). There was a significant interaction between spore load and timing of inoculation ( $p < 0.001$ ), driven by poor growth of seedlings that received later applications of inoculum. However, this was only observed for the low spore load treatment. In terms of RCD measurements, stressed plants were smaller although the later seedlings were inoculated, the larger their RCD tended to be, particularly for those treated with the high spore load. This suggests that early-inoculated seedlings (nine weeks) may have needed to divert resources into plant defence mechanisms rather than into growth.

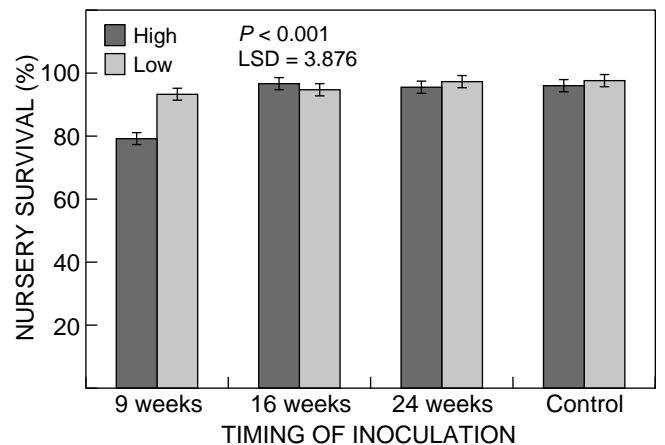
#### Seedling survival in the field

Field survival results at 6 months showed that spore load effects were no longer significant ( $p = 0.229$ ) compared to observations in the nursery (Figure 9). As in Trial A, the stressed seedlings resulted in better survival ( $p = 0.051$ ) than the non-stressed seedlings. There were no significant interactions between the stress treatment and the level of inoculum applied or with timing of inoculation, suggesting that the nursery stress event was not the trigger for further symptom expression in-field. The nursery stress treatment may have had more of a morphological than physiological effect on seedling survival, with shorter seedlings from the stress treatment surviving better under the field conditions.

Timing of nursery inoculation had a highly significant effect on field survival. Young seedlings inoculated at nine weeks of age experienced the highest mortality ( $p < 0.001$ ), particularly those that received the high spore load ( $p < 0.001$ , Figure 12), while all later inoculations (at 16 and 24 weeks or 2–4 months later) and the control treatments showed similar responses (Figure 12).



**Figure 10:** Stomatal conductance of seedlings in Trial B exposed to a 6-day water stress treatment in the nursery. Error bars represent the SE



**Figure 11:** Nursery survival in Trial B of seedlings inoculated with high and low levels of *F. circinatum* inoculum at different times during their development in the nursery. Control = not inoculated; Low = 125 spores tray<sup>-1</sup>; High = 500 spores tray<sup>-1</sup>. Error bars represent the SE

## Discussion

Attempts to mimic the nursery and post-plant mortality caused by natural infection of *P. patula* seedlings by *F. circinatum* in the nursery were effective. Seedlings, propagated from *F. circinatum*-free seed, were successfully infected or contaminated without the deliberate creation of any wounds. Such infections could occur through natural cracks in the roots of seedlings or possibly via apices of young roots as suggested by Hart and Endo (1981).

The experimentally imposed exposure to *F. circinatum* in the nursery resulted in the planting of asymptomatic seedlings associated with *F. circinatum* and in their post-plant mortality due to disease. The quantitative relationship found between infection of seedlings and subsequent mortality, and the transitory nature of airborne

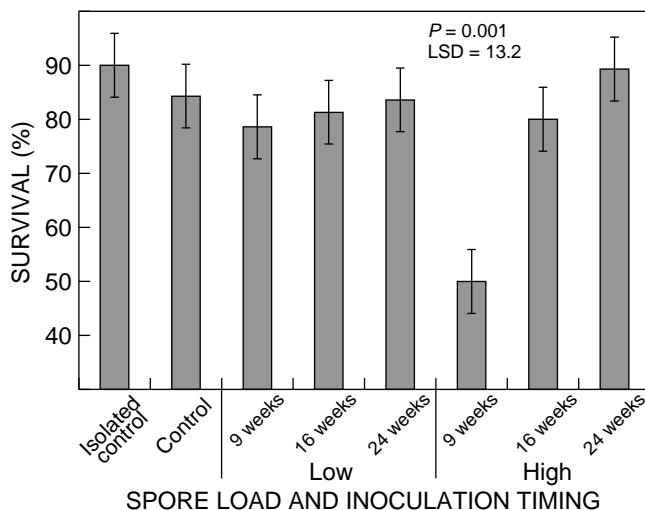
spores in the field, supports this link as the main cause of post-planting mortality. Results of analyses conducted on non-sterilised tissue samples, suggest that healthy-looking seedlings can either be contaminated (harbouring the pathogen externally) or be infected (harbouring the pathogen internally), or both. This may also explain why, along with genotypic differences between the seedlings (Gordon et al. 1998; Storer et al. 1999), symptoms of *F. circinatum* infection are not uniformly manifested.

This study has also shown that there was potential for secondary infection or contamination from inoculated to non-inoculated treatments due to their proximity in the nursery. Spores from an inoculated treatment can be dispersed either as airborne spores (Fourie et al. 2014) or through the irrigation system (van Wyk et al. 2012). This would explain the high proportion of asymptomatic seedlings contaminated/infected with *F. circinatum* in the non-inoculated treatments.

Almost half as many asymptomatic plants in the non-inoculated treatments (control and commercially propagated plants) tested positive for the presence of *F. circinatum*. However, the observed nursery mortality of these seedlings was significantly lower than that seen in inoculated seedlings. This suggests that the secondary infection of non-inoculated treatments may have occurred later (post-inoculation) and that it had not yet resulted in expression of nursery disease symptoms. In contrast, no *Fusarium*-like symptoms or seedling mortality was observed in the isolated, non-inoculated control plants raised separately from the trial in a *Fusarium*-free environment. This highlights the importance of early and regular culling of symptomatic seedlings in the nursery to limit the spread of the disease. In this regard, Fourie et al. (2014) showed that nursery sanitation practices are crucial to lowering airborne inoculum of *F. circinatum*.

In Trial A, no differences were observed in nursery and post-plant mortality as a result of spore concentrations tested. This is probably because these concentrations (1 000 and 2 000 spores tray<sup>-1</sup>) were inordinately similar to distinguish differences in the proportion of disease-like symptoms in the inoculated treatments (Gordon et al. 1998, Hammerbacher 2006). However, the amended spore levels (125 and 500 spores tray<sup>-1</sup>) tested in Trial B confirmed previous reports that inoculum concentration can significantly influence the development of *Fusarium*-induced symptoms (Elmer and Lacy 1987; Salgado and Schwartz 1993; Rogdríguez-Gálvez and Mendgen 1995; Hammerbacher et al. 2009) and, furthermore, enabled discrimination of the treatment effects. Even in mature trees, different inoculum loads of *F. circinatum* have been associated with different levels of disease symptoms and with various degrees of infection by the pathogen (Garbelotto et al. 2008). In the present study, the lowest levels of exposure resulted in mortality most similar to operational experience, while high levels of inoculum resulted in nursery and post-planting mortality, much higher than generally observed operationally.

With regards to timing of inoculation, disease severity increased with an increase in the exposure to the pathogen. Similar results have been reported for celery seedlings of various ages, exposed to *F. oxysporum* (Hart and Endo



**Figure 12:** Field survival in Trial B at 6 months showing the interaction of spore load and timing of inoculation. Control = not inoculated; Isolated control = not inoculated treatment propagated separately from the trial; Low = 125 spores tray<sup>-1</sup>; High = 500 spores tray<sup>-1</sup>. Error bars represent the SE

1981). Seedling exposure to *F. circinatum* inoculum, earlier in the nursery production cycle, resulted in higher levels of nursery and post-planting mortality. It is well known that seasonal variation can affect incidence and severity of plant infection and disease by altering the vulnerability of the host and the virulence of the pathogen (Roden and Ingles 2009). For *F. circinatum*, in particular, a cool, humid environment is known to be favourable for sporulation (Schweigkofler et al. 2004).

Seedlings inoculated at nine weeks (in mid-summer) would have had actively growing roots spreading out into the contaminated medium of the seedling plug. Particularly under the high spore load treatment, they would have had a greater chance of being infected. Furthermore, the soft, juvenile tissues of these seedlings could have been more susceptible to infection by the pathogen than older, more lignified seedlings. In addition, young seedlings do not have fully developed natural defence mechanisms (Dick and Simpson 2003), again making them more susceptible to a range of fungal pathogens. Thus, exposure of such young seedlings to infection may make them more prone to mortality at a later stage.

Plant roots differ in their susceptibility to infection by soil-borne pathogens (Bruehl 1986; Curl and Truelove 1986). Excluding root wounds and regions where lateral roots emerge, older and upper portions of the root system are often able to resist infections due to secondary cell wall thickening (Bruehl 1986). Pathogens are able to invade the 1–2 mm zone of elongation (behind the root apex) of healthy young roots (Baluska et al. 2001; Gunawardena and Hawes 2002), which is the site for the release of root exudates that may enhance spore germination (Rovira 1969; Curl and Truelove 1986). Early inoculation of the root collar region (as conducted in the present study) could easily target newly developing roots close to the surface of the growth medium. In contrast, later inoculation of older

seedlings would potentially target a much larger proportion of older, more lignified roots compared to the youngest, lower segment of the root system (Atzmon et al. 1994; Morris et al. 2014) at the base of the container.

Physiological plant stress in the nursery, induced by water shortage, did not contribute to post-planting mortality. It did, however, result in slightly shorter seedlings that tended to survive better in-field, possibly due to a hardening effect that enabled stressed seedlings to acclimatise better to field conditions. This is not an uncommon event, since exposure of conifer seedlings to drought stress has been shown to increase their subsequent drought resistance (Zwiazek and Blake 1989; Kaushal and Aussenc 1989; van den Driessche 1990). Stomata may also respond more sensitively to subsequent drought episodes (Roberts and Dumbroff 1986; Zwiazek and Blake 1989) or osmotic adjustment may occur resulting in increased resistance (Ritchie and Roden 1985). Our attempts to mitigate stress at planting by implementation of various field treatments either had a transitory response as previously reported for Benomyl (Mitchell et al. 2004; Crous 2005) or did not reduce post-planting mortality. Instead, it is likely that more persistent 'transplant stress' contributed to increased mortality of inoculated seedling treatments, particularly in Trial A, where higher inoculum levels were applied. Damage to the root system at planting, low root volume, poor root-to-soil contact and often high transpiration rates can result in seedling stress at planting (Sands 1984; Rietveld 1989; Haase and Rose 1993). The additional stress of plant defence against a pathogen under these conditions may cause seedlings to succumb, particularly since the pathogen is able to affect the root system, causing reduced root growth and or root dieback (Viljoen et al. 1994).

Viewed collectively, the results of this study suggest that the nursery environment could easily contribute to the perpetuation of the pathogen and to its implicit dissemination via seemingly asymptomatic seedlings in the field. Here, the shock associated with the transplantation of seedlings may act as the primary trigger for the onset of post-plant disease-related mortality. Future studies should focus on methods to allow early detection and validation of asymptomatic infected or contaminated seedlings in the nursery, in order to avoid their dispatch to the field.

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